Universidad de Navarra

Facultad de Farmacia y Nutrición

Origanum vulgare L. spp. *vulgare*: Chemical characterisation, pharmacological screening and design of new pharmaceutical forms

María Pilar de Torre Fernández

Pamplona, 30th August 2021

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Trabajo presentado por **María Pilar de Torre Fernández** para obtener el grado de doctor en Farmacia

Fdo. María Pilar de Torre Fernández

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Dña. Mª Isabel Calvo Martínez, Catedrática de Farmacología del Departamento de Tecnología y Química Farmacéuticas de la Universidad de Navarra, y **Dña. Rita Yolanda Cavero Remón**, Profesora Titular del Departamento de Biología Ambiental de la Universidad de Navarra, certifican que:

El presente trabajo titulado: **"*Origanum vulgare* L. spp. *vulgare*: Chemical characterisation, pharmacological screening and design of new pharmaceutical forms"**, presentado por **María Pilar de Torre Fernández** para optar al grado de doctor, ha sido realizado bajo su dirección y, una vez revisado, no encuentran objeciones para que sea presentado a su lectura y defensa.

Fdo: Dra. M.I. Calvo Martínez

Fdo: Dra. R.Y. Cavero Remón

Pamplona, 30 de Agosto de 202

“A man might find for a moment that he was unable to work, but that's exactly the right time to remember his past accomplishments and to consider that later on, when the obstacles has been removed, he's bound to work all the harder and more efficiently.”

— **Franz Kafka, The Metamorphosis**

A todos aquellos que me han ayudado a superar cada obstáculo, en especial Maribel Calvo, José Luis Vizmanos y mi familia.

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Gracias, perdón y te quiero. Son tres sentimientos que nos cuesta expresar muchas veces y que tendemos a guardarlos dentro.

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Todo comenzó al terminar mis estudios de Grado de Farmacia cuando mi directora de Trabajo de Fin de Grado (Maribel Calvo) me ofreció la oportunidad de continuar los estudios llevados a cabo. Hasta entonces no contemplaba la posibilidad de dedicarme a la investigación, pero el campo en el que iba a trabajar (Plantas Medicinales) siempre ha estado presente en mi familia, infancia y entre mis inquietudes. Todo lo que escriba aquí es poco, Maribel. Para ti es mi primer *gracias* y mi primer *perdón* por ser el conejo de Alicia en el País de las Maravillas; siempre te llevaré en el corazón.

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Aquellos que me conocen sabrán cuán grande era mi vocación por ser farmacéutica – viene de familia – y cuánto me gustaba investigar en el laboratorio. Siempre fui una persona curiosa con ganas de superarse. Sigo siendo un poco gafotas. Sin ir más lejos en mi segundo año de carrera quise determinar por HPLC los valores de cafeína de diferentes productos comercializados; David Lucio sigue acordándose de ello y de ahí surgió una amistad. Gracias por todo el apoyo, David.

Lo que pasa que a veces tener tanta curiosidad y querer hacer tantas cosas hace que te puedas despistar. Menos mal que tengo a personas como Ana Brotons a mi lado para poner los pies en la Tierra, no dispersarme y seguir el camino por suelo firme. Aunque piense que hago más caso a las palabras de otras personas, immer bist du für ein sehr gutes und nachahmenswertes Beispiel, Danke!

Tenía claro que quería ser farmacéutica y disfruto de mi trabajo tanto como he disfrutado estos cuatro años. Sin embargo, si hoy en día ejerzo como tal es gracias a Carmen San Martin. Gracias por el apoyo, por centrarme y ayudarme durante todo este trayecto, sino ahora podría estar diseñando edificios y no cumpliendo mi sueño de niña de ser farmacéutica.

He pasado tanto tiempo diciendo que estaba haciendo una Tesis Doctoral, algo que tenía que explicar en la mayoría de los casos hasta en varios idiomas, que se me hace raro cerrar esta etapa de mi vida de 3 años más el año de Covid–19. Como para todos, este último año no ha sido nada fácil. Todos hemos tenido nuestros problemas y seguro que nos ha cambiado la vida. Pero, ¿sabéis qué? Gracias a mi crecimiento personal durante la Tesis, creo que he podido afrontar esta pandemia mejor de lo que lo hubiera hecho la Maripili de 2017. La Investigación te ayuda a superar traumas como el del fracaso, a saber organizarte, a ver más allá, a sacar partido a algo que habías dado por perdido... Así que aquí va otro *gracias*, desde el corazón y con un *perdón* por mis cambios de humor, para todos los que han estado conmigo en mi día a día estos tres años, en especial a: Hugo, Gwen, Cristina y Silvia. Puede que para vosotros haya sido una doctoranda más, que a veces estaba y otras no, pero sin vosotros la mitad de los resultados no se habrían conseguido – en parte porque algunos métodos los hemos puesto a punto juntos.

No puedo nombrar a todo el mundo, porque entonces presentaría un trabajo de 600 páginas y no quiero aburrir al lector. Son muchas las personas que han pasado por mi vida desde que esta aventura comenzó. Algunas de ellas ya no siguen en mi vida por el motivo que sea, pero también hay para vosotros porque me acompañasteis en el camino: merci, thank you, obrigada, danke, grazie, gracias. Otras aparecieron una vez puesto en marcha el tren, gracias por no bajaros en marcha y *perdón* por estas últimas semanas de nervios y agobio. Ojitos verdes, pronto seré libre: gracias, perdón y te quiero.

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He estado guardando el último GRACIAS para una persona a la que considero *mi padrino* de la Tesis: Jose Luis Vizmanos. No solo me abriste la puerta al WormLabWorld, facilitándome y enseñándome el fascinante mundo de los *C. elegans* – junto con Cristina y Ana – sino que, aunque no lo creas, has sido de las personas que más me ha ayudado a crecer y superar los obstáculos de la vida con otra filosofía. Ahora empiezo a ver los colores a través del prisma.

¡Muchas gracias a todos!

INDEX

ABBREVIATIONS – ABREVIATURAS

A

AAI: Antioxidant Activity Index

AAS: Acetylsalicilic acid

Abs: Absorbance

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

ABTS•: Free radical of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

AChE: Acetylcholinesterase enzyme

AD: Alzheimer's disease

ADN: Ácido desoxirribonucleico, DNA in English (Desoxyrribonucleic Acid)

ASP: Aspirin

ATCC: American Type Culture Collection

ATCh: Acetylcholine

AUC: Area Under Curve

B

BHT: Butylated hydroxytoluene

BSA: Bovine Serum Albumin

C

C. *albicans*: *Candida albicans*

CGC: *Caenorhabditis* Genetics Center

CN: Código Nacional

COX: Cyclooxygenase

CVD: Cardiovascular Disease

D

DE: Desviación estándard

DHBA: Dihydroxybenzoic acid

DM1 or DT1: Diabetes Mellitus type I

DM2 or DT2: Diabetes Mellitus type II

DNEM: Dulbecco's Modified Eagle's medium

DPPH: 2,2–diphenyl–1–picrylhydrazyl

DPPH•·: Free radical of 2,2–diphenyl–1–picrylhydrazyl

DTNB: Thiol reagent 5,5′–dithiobis–2–nitrobenzoic

E

EC_{50}: 50 % Effective Concentration

E. coli OP50: *Escherichia coli* OP50 (food source *C. elegans*)

EMA: European Medicines Agency

EtOH: Ethanol

F

FRAP: Ferric Reducing Antioxidant Power

FTIR: Fourier Transform Infrared

G

GA: Gallic acid

H

H. pylori: *Helicobacter pylori*

HbA1c: glycosylated haemoglobin

HDL–c: High Density Lipoprotein Cholesterol

HMG–CoA reductase or HMGCR: 3–hydroxy–3–methyl–glutaryl–coenzyme A reductase

HMPC: Herbal Medicinal Products Commitee

^{1}H–NMR: Proton nuclear magnetic resonance

HPLC–DAD: High Performance Liquid Chromatography with Diodo Array Detector

HTA: Arterial Hypertension

I

IL–1B: Interleuquina (Interleukin) 1B – and so for rest of IL–x

IC_{50}: 50 % Inhibition Concentration

IR spectroscopy: Infrarred spectroscopy

L

L1: First Larva stage, *C. elegans*

L2: Second Larva stage, *C. elegans*

L3: Third Larva stage, *C. elegans*

L4: Fourth Larva stage, *C. elegans*

LB: Lysogeny Broth

LC–MS: Liquid Chromatography–mass spectrometry

LDL–c: Low Density Lipoprotein Cholesterol

LPS: Lipopolisacáridos, lipopolisacharides

M

MIC: Minimum Inhibitory Concentration

MP: Mobile phase

MTT: 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide

N

NADP: Nicotinamide Adenine Dinucleotide Phosphate

NADPH: β–Nicotinamide adenine dinucleotide 2′– hosphate reduced tetrasodium salt hydrate

NGM: Nematode Growth Medium

NP: Natural Products

NSAID: Non–steroidal anti–inflammatory medicines

O

OV: *Origanum vulgare* L. subsp. *vulgare*

OV1: Aqueous cold maceration extract (water, 4 ºC)

OV2: Aqueous hot maceration extract (water, 100 ºC)

OV3: Hydroalcoholic cold maceration extract (ethanol 50%, 4 ºC)

OV4: Hydroalcoholic hot maceration extract (ethanol 50%, 80 ºC)

OV5: Alcoholic cold maceration extract (ethanol, 4 ºC)

OV6: Alcoholic hot maceration extract (ethanol, 80 ºC)

OVx–Int: Intestinal absorbable fraction of the correspondant extract (x ϵ {1–6})

OV–E: OV3 crude extract. OV–E refers to OV3 before digestion and non–formulated to differentiate since OV–P and OV–C are also OV3, but formulated.

OV–P: OV3 formulated as dry powder. In bioactivity assays, OV–P refers to intestinal absorbable fraction

OV–C: OV3 formulated as capsule. . In bioactivity assays, OV–C refers to intestinal absorbable fraction

ORAC: Oxygen radical absorbance capacity

P

p–NPG: 4–Nitrophenyl–α–*D*–glucopyranoside

PBS: Phosphate buffered saline

PBST: Phosphate Buffer Saline with Tween 20

R

RA: Rosmarinic acid

RBC: Red Blood Cells

RDE: Relación droga–extracto

Rf: Retention factor in TLC

ROS: Reactive Oxygen Species (Especies Reactivas de Oxígeno)

S

SD: Standard Deviation

SP: Stationary phase

T

TE: Trolox –6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid

TLC: Thin Layer Chromatography

TNB: 5–thio–2–nitrobenzoic acid

TRAP: Total Reactive Antioxidant Potential

TPC: Total Phenolic Compounds (Folin–Ciocalteau assay)

U

UV: Ultraviolet

ABSTRACT

BASIS: *Origanum vulgare* L. is a widely used Medicinal Plant that, unlike *O. dictamus* L. and *O. majorana*, its safety and efficacy has not been underwritten by international organisms such as EMA, ESCOP and OMS, due to the lack of enough chemical, pharmacological and toxicological data. The aim of this study is to select the best extract of this plant for pharmacological use based on different assays carried out *in vitro* and *in vivo*.

MATERIAL AND METHODS: Six extracts of flowered aerial parts of *O. vulgare* L. ssp. *vulgare* were prepared through maceration by combinating two parameters of extraction, polarity of solvent and temperature of extraction. The extracts were chemically characterised and quantified through several chromatographic techniques (TLC, HPLC–DAD and LC–MS), together with determination of total phenolic compounds by spectrophotometry. Three of the extracts were submitted into a gastrointestinal digestion process *in vitro*.

Pharmacological assays were performed *in vitro* with the extracts and their digestive fractions in order to determine spectrophotometrically their antioxidant (against DPPH and ABTS• free radicals), hypoglycaemic (inhibition of *α–glucosidase*), hypolipidemiant activity (inhibition of HMG–CoA reductase), anti–acetylcholinesterase and anti–inflammatory activities (albumin denaturation and membrane stabilisation of red blood cells). Cytotoxicity was tested in Caco–2, HepG–2 and A375 cell lines through MTT assay.

C. elegans was chosen for the accomplishment of *in vivo* pharmacological assays of aqueous extracts testing antioxidant activity against DPPH free radical, 24 h survival rate, ROS accumulation, hypoglycaemic activity and lifespan. Two simple oral pharmaceutical forms were designed with OV3: hard gelatin capsules and lyophilised powder to be resuspended in water. Quality control assays of both formulations were undertaken by following the guidelines established by Real Farmacopea Española. Both formulations underwent an *in vitro* gastrointestinal digestion process, after which the *in vitro* and *in vivo* activities were tested. Lastly, four potential formulations were designed for cutaneous use, two with OV2 (cream and stick) and other two with OV3 (body milk and hydroalcoholic gel). Correspondant quality control assays were performed as defined in Real Farmacopea Española.

RESULTS AND CONCLUSION: The six extracts revealed diferent yield of extraction and chemical profile. Six type of chemical compounds were detected, dihydroxibenzoic acids, dihydroxycinnamic acids, flavonoids, syringic acids and salvianolic acids. Chemical composition of OV1 was different to the rest, being 2,5–dihydroxybenzoic acid the predominant compound. Rosmarinic acid was the main component in the rest of the extracts. From a pharmacological point of view, results were also dissimilar, despite the fact that the six extracts were considered to be very strong antioxidants and non–toxic for the cell lines tested. OV1 was the only extract with a significant hypoglycaemic activity *in vitro* that was confirmed *in vivo*, where it also exhibited an *anti–aging* effect. OV2 demonstrated to be a very versatile and powerful potential oral treatment for age–related pathologies. Besides, topically administrated, this extract could be an effective remedy for blows and so, stick and creams were suggested as formulation. OV3 was also proposed for topical administration, in this case as venotonic remedy in the form of a gel or lotion. Finally, according to low activity obtained, the extracts OV4, OV5 and OV6 would not be recommended for therapeutic use in humans.

RESUMEN

ESTADO DEL ARTE: *Origanum vulgare* L. es una especie muy utilizada en la medicinal tradicional pero todavía, a diferencia de *O. dictamus* L. y *O. majorana* L., su seguridad y eficacia no ha sido avalada por organismos internaciones como EMA, ESCOP y OMS por no disponer de suficientes datos químicos, farmacológicos y toxicológicos.

MATERIAL Y MÉTODOS: Se prepararon seis extractos por maceración combinando dos parámetros de extracción: polaridad del disolvente y temperatura de extracción. Dichos extractos se caracterizaron y cuantificaron mediantes diferentes técnicas cromatográficas (TLC, HPLC–DAD y LC–MS), junto con la determinación espectrofotométrica para polifenoles totales. Tres de los extractos se sometieron a un proceso de digestión gastrointestinal *in vitro*.

Se realizaron ensayos farmacológicos *in vitro* con los extractos y los productos de su digestión, determinando espectrofotométricamente la actividad antioxidante (frente a los radicales libres DPPH y ABTS•), hipoglucemiante (inhibición de α–glucosidasa), hipolipemiante (inhibición de HMG–CoA reductasa), anti–acetilcolinesterasa y antiinflamatoria (desnaturalización de albúmina y estabilización de la membrana de eritrocitos). La citotoxicidad se ensayó frente a las líneas celulares Caco–2, HepG–2 y A375 por el ensayo del MTT.

Para la realización de los ensayos farmacológicos *in vivo* de los extractos acuosos se empleó el modelo *C. elegans*, donde se ensayó la actividad antioxidante frente al radical DPPH, supervivencia a las 24 h, acumulación de ROS, actividad hipoglucemiante y esperanza de vida. Se diseñaron dos formulaciones galénicas orales con OV3: cápsulas de gelatina dura y polvo liofilizado para reconstituir. El control de calidad de ambas formulaciones se realizó siguiendo los ensayos establecidos en la Real Farmacopea Española. Ambas formulaciones se sometieron a un proceso de digestión gastrointestinal *in vitro* y se ensayaron actividades *in vitro* e *in vivo*. Por último, se diseñaron cuatro posibles formulaciones tópicas, dos con OV2 (crema y stick) y otras dos con OV3 (loción y gel hidroalcohólico). Los ensayos de control de calidad pertinentes se realizaron de acuerdo con lo establecido por la Real Farmacopea Española.

RESULTADOS Y DISCUSIÓN: Los seis extractos presentaron diferente rendimiento de extracción y perfil químico. Se detectaron seis tipos de compuestos químicos, ácidos dihidroxibenzoicos, ácidos dihidroxicinámicos, flavonoides, ácidos siríngicos y ácidos salvianólicos. OV1 presentó una composición química diferente al resto, siendo el ácido 2,5–dihidroxibenzoico el compuesto mayoritario. En el resto de extractos el componente

principal fue el ácido rosmarínico. Desde el punto de vista farmacológico, los seis extractos también mostraron resultados dispares, aunque todos ellos fueron considerados antioxidantes muy potentes y no tóxicos para las líneas celulares utilizadas. OV1 fue el único capaz de mostrar una actividad hipoglucemiante relevante *in vitro* que fue confirmada *in vivo*, donde también mostró un efecto antienvejecimiento. OV2 demostró ser un posible tratamiento oral muy versátil y potente para las enfermedades relacionadas con la edad. Además, administrado de manera tópica, este extracto podría ser un remedido eficaz para los golpes y por ello, se sugiere su furmulación en stick o crema. Por otro lado, OV3, utilizado en las formulaciones orales, preservó mejor la actividad antioxidante tras la digestión en forma de cápsula y la actividad antidiabética en forma de polvo para disolver en agua. OV3 también se propone para su administración tópica, en este caso como remedio venotónico en forma de gel o loción. Por último, debido a la baja actividad resultante, los extractos OV4, OV5 y OV6 no parecen recomendables para su terapéutico en humanos.

INTRODUCTION

Origanum vulgare L. subsp. *vulgare*

1 Taxonomía y distribución geográfica

El género *Origanum* está incluido en el clado de las Eudicotiledóneas, subclado Superastéridas, en concreto en Astéridas y dentro de éstas, en el subgrupo Lámidas, Orden *Lamiales* y familia *Lamiaceae* [1]. Está representado por unas 40 especies de distribución circunmediterránea o macaronésica, llegando a alcanzar Asia (isla de Formosa). Su mayor diversidad se encuentra en el este de la región mediterránea [2].

En la Península Ibérica están documentadas tres especies: *Origanum majorana* L. originaria de Chipre y localidades próximas a la Península Anatólica, cultivada y asilvestrada en numerosas regiones mediterráneas; *O. compactum* Benth distribuida en el sur de España y el norte de Marruecos; y *O. vulgare* L., ampliamente distribuida y cultivada en toda España, gran parte de Europa, región macaronésica, Asia y noroeste de África. Dentro de *O. vulgare* se distinguen dos subespecies (**Figura 1**): *O. vulgare* L. subsp. *virens* (Hoffmanns. & Link) Bonnier & Layens distribuida por casi toda la península Ibérica y *O. vulgare* L. subsp. *vulgare* que crece en matorrales y claros de bosque atlántico, que es la subespecie más frecuente en el Norte de España, desde Cantabria hasta Huesca [2].

Figura 1. Distribución geográfica de *Origanum vulgare* subsp. *virens* (izquierda) y O. *vulgare* subsp. *vulgare* [3] (derecha).

2 Descripción botánica

Origanum vulgare L. subsp. *vulgare* es una planta aromática sufruticosa de 30 – 70 cm con aspecto herbáceo y cepa leñosa rizomatosa (**Figura 2**). Los tallos, erguidos, presentan en las caras opuestas y alternas de cada entrenudo, pelos dispersos y retrorsos. Las hojas, opuestas y decusadas, son ovadas o elípticas, ciliadas y pelosas y con glándulas verdes en el haz y glaucas en el envés. Las flores se reúnen en

verticilastros agregados en panículas de hasta 30 cm. y éstos están protegidos por brácteas púrpuras de 3,5 x 2,8 mm. El cáliz presenta glándulas esferoidales brillantes, es de color púrpura, de forma tubular y de 2 – 2,8 mm y terminado en cinco dientes de 0,8 mm. La corola también de color púrpura, es bilabiada y de 0,5 mm de longitud. El labio superior consta de 2 lóbulos de 1 – 1.5 mm y el labio inferior, de tres lóbulos de 1,5 – 1,8 mm siendo el central mayor. El androceo está formado por cuatro estambres, los dos más internos más cortos y con las anteras convergentes y rosas. El fruto es un tetraesquizocarpo castaño oscuro, con mericarpios subesféricos de 1 x 0,8 mm.

Figura 2. *Origanum vulgare* subsp. *vulgare*. De izquierda a derecha: detalle del tallo, hojas e inflorescencias.

3 Composición química

Las partes aéreas de *O. vulgare* contienen un número elevado de compuestos activos. Los compuestos fitoquímicos más importantes presentes en esta planta se pueden agrupar (en función de sus propiedades hidrofílicas o hidrofóbicas) en dos categorías: aceites esenciales y compuestos fenólicos [4].

3.1 Aceites esenciales

Origanum vulgare y otras especies del mismo género presentan un alto contenido en aceites esenciales volátiles [5] que contribuyen al aroma y sabor de la planta. Los aceites esenciales de orégano son muy variados estructuralmente y se pueden clasificar en los siguientes grupos:

1. **Monoterpenos**: estructuras C–10 (**Figura 3**).

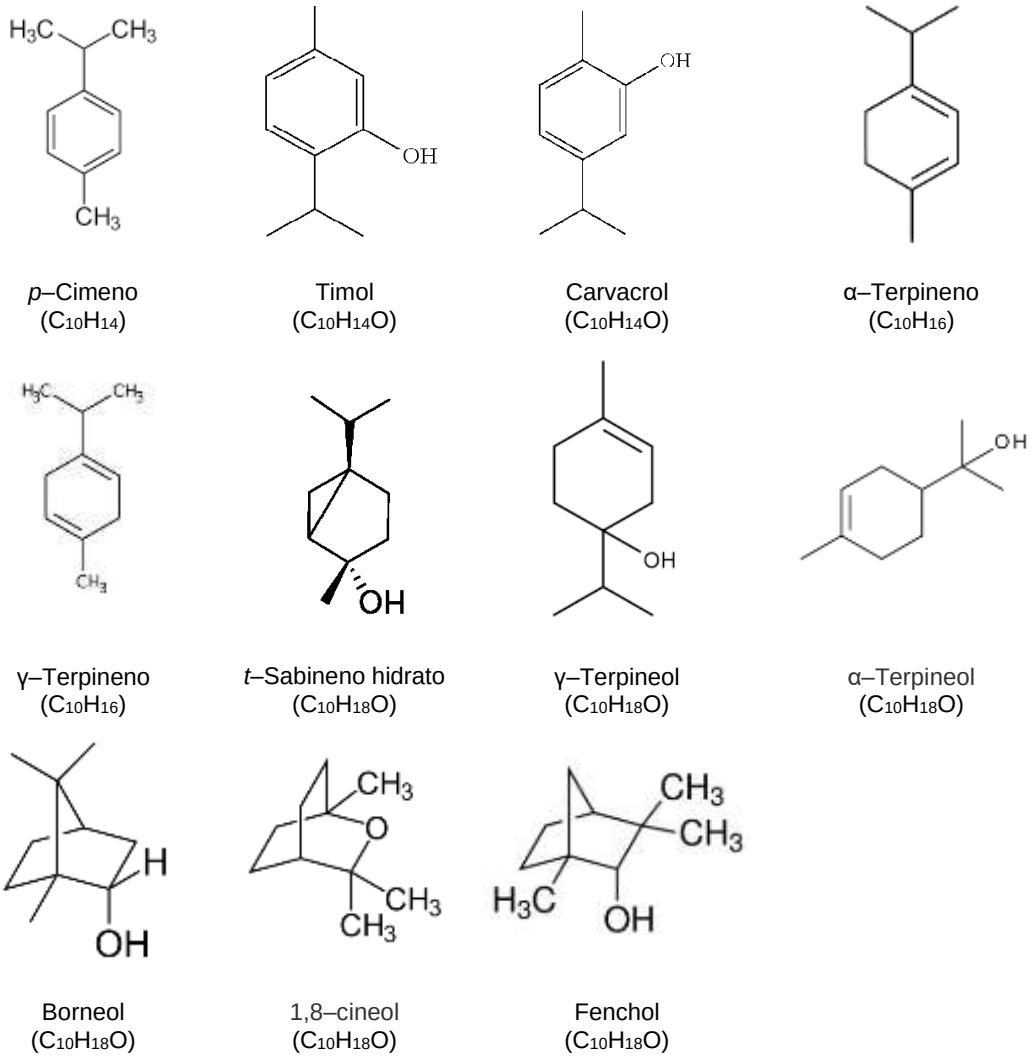
Estos compuestos se pueden clasificar en función de su aromaticidad y de su grado de oxidación. En función de si tienen átomos de oxígeno o no en su estructura se pueden encontrar de distintos tipos (**Figura 3**):

– monoterpenos aromáticos no oxigenados: *p*–cimeno [6]

– monoterpenos aromáticos oxigenados: timol y carvacrol [7]

– monoterpenos alifáticos no oxigenados: α–terpineno, γ–terpineno [7]

– monoterpenos alifáticos oxigenados: α–terpineol, borneol, 1,8–cineol [8], fenchol [7], γ–terpineol, trans–sabinero hidrato [6].



p–Cimeno ($C_{10}H_{14}$) | Timol ($C_{10}H_{14}O$) | Carvacrol ($C_{10}H_{14}O$) | α–Terpineno ($C_{10}H_{16}$)

γ–Terpineno ($C_{10}H_{16}$) | *t*–Sabineno hidrato ($C_{10}H_{18}O$) | γ–Terpineol ($C_{10}H_{18}O$) | α–Terpineol ($C_{10}H_{18}O$)

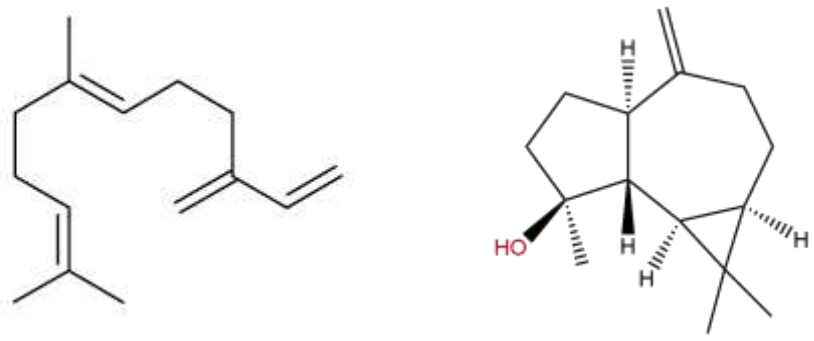
Borneol ($C_{10}H_{18}O$) | 1,8–cineol ($C_{10}H_{18}O$) | Fenchol ($C_{10}H_{18}O$)

Figura 3. Monoterpenos oxigenados y no oxigenados presentes en *O. vulgare*.

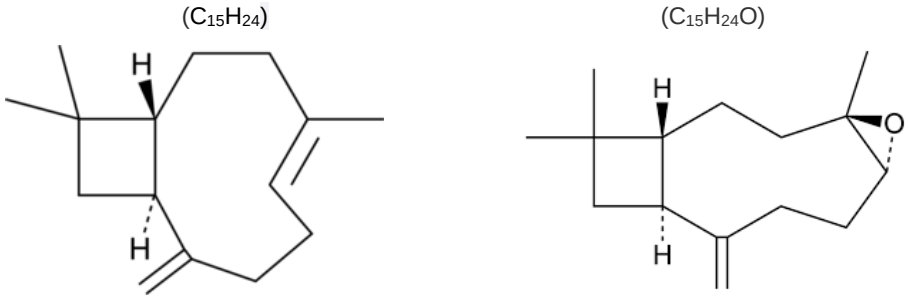
2. **Sesquiterpenos**: estructuras C–15 (**Figura 4**).

– sesquiterpenos no oxigenados: espatulenol [8], β–cariofileno y β–farneseno [6]

– sesquiterpenos oxigenados: β–cariofileno óxido [6]



β–Farneseno | Espatulenol



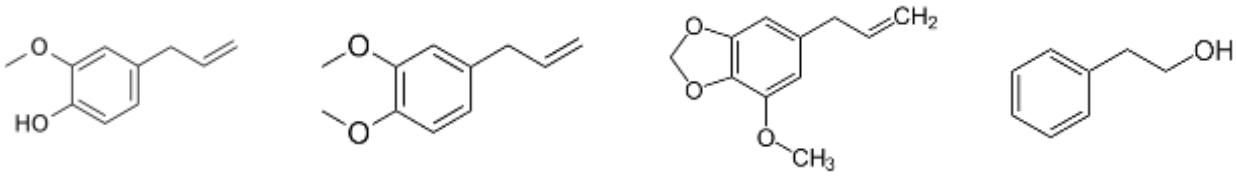
β–Cariofileno
($C_{15}H_{24}$)

β–Cariofileno óxido
($C_{15}H_{24}O$)

Figura 4. Sesquiterpenos alifáticos oxigenados y no oxigenados presentes en *O. vulgare*.

3. Fenilpropanoides y feniletanoides: estructuras C6–C3 y C6–C2 (**Figura 5**).

Los fenilpropanoides son moléculas naturales que provienen de la ruta del ácido sikímico con estructura C6–C3. Los compuestos presentes en el orégano son metileugenol, miristicina [6] y eugenol [9]. Los fenieltanoides con estructua C6–C2 son poco frecuentes y únicamente se ha descrito el alcohol fenetílico [9]. Sus estructuras químicas se indican en la **Figura 5**.



Eugenol
($C_{10}H_{12}O_2$)

Metileugenol
($C_{11}H_{14}O_2$)

Miristicina
($C_{11}H_{12}O_3$)

Alcohol fenetílico
($C_8H_{10}O$)

Figura 5. Feniletanoides y fenilpropanoides presentes en *O. vulgare*.

Existen descritos más de 60 aceites esenciales diferentes en *O. vulgare*. Entre los numerosos estudios, muchos autores han intentado establecer una correlación entre la actividad farmacológica de los aceites esenciales y su estructura química, otros han intentado buscar sinergismos de acción entre ellos, pero sin llegar a conclusiones claras. Lo único en lo que están de acuerdo la mayoría de autores es en la relación directa de estos compuestos con su actividad antimicrobiana [10].

3.2 Compuestos fenólicos

Aunque generalmente las investigaciones se han centrado en el estudio de los aceites esenciales [11], estos solo representan uno de los principales grupos presentes en el orégano.

Los flavonoides y los ácidos fenólicos son los principales grupos de compuestos fenólicos presentes en orégano [12]. Ambos tipos de compuestos se han estudiado por su posible potencial terapéutico, principalmente como agentes antioxidantes [13]. Se caracterizan por tener, al menos, un anillo aromático con uno o más grupos hidroxilos sustituidos. Son compuestos con una gran variabilidad estructural que se pueden clasificar según el número y la disposición de sus átomos de carbono en: flavonoides y no flavonoides y, generalmente, se encuentran conjugados con azúcares y ácidos orgánicos [14].

1. Flavonoides

Todos los flavonoides derivan de los aminoácidos aromáticos, fenilalanina y tirosina y son compuestos C_{15} dispuestos en tres anillos (C_6–C_3–C_6) que se designan como A, B y C [15]. Su estructura varía en función de la estructura del anillo C y el grado de sustitución en los diferentes anillos: grupos hidroxilos, prenilos, glicosilación, etc. que modifican la molécula primaria [16]. Estas modificaciones pueden alterar su solubilidad en agua, lo que puede afectar directamente a su bioaccesibilidad [17]. Los tipos más frecuentes son flavonoles, flavonas, flavan–3–oles, antocianinas, flavanonas e isoflavonas (**Figura 6**).

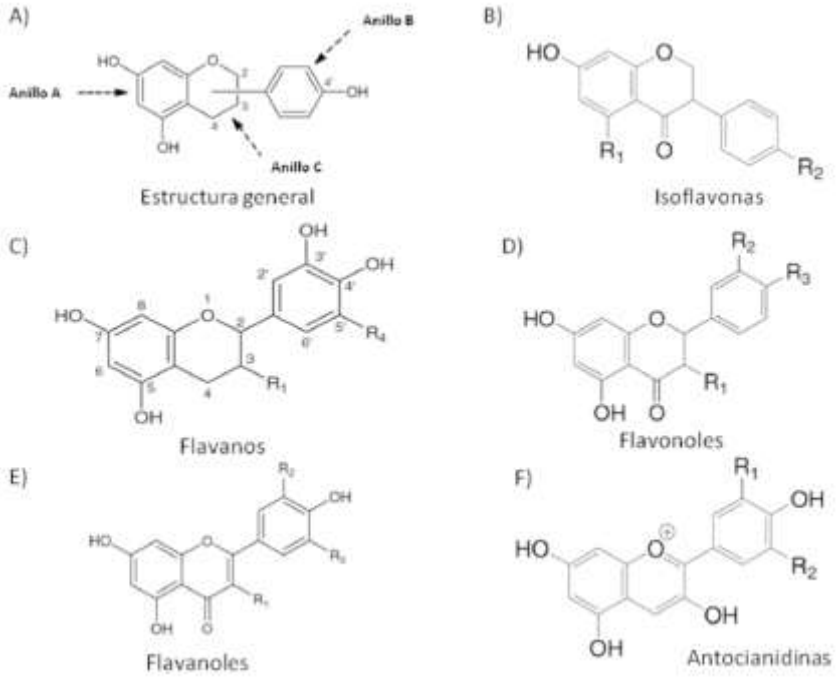


Figura 6. Estructura básica de los diferentes tipos de flavonoides A) Flavonas. B) Isoflavonas. C) Flavanos. D) Flavonoles. E) Flavanoles. F) Antocianidinas [18].

Las flavonas son los flavonoides más abundantes presentes en las especies de orégano, seguido de los flavonoles y flavanonas (**Figura 6**) [15,19]. Los flavonoides (**Figura 7**) descritos son:

- Flavonas: apigenina, luteolina, escutelareína y sus derivados glicosilados.
- Flavonoles: quercetina y sus derivados quercitrina, isoquercitrina e hiperósido.

- Flavanonas: naringenina y naringina.

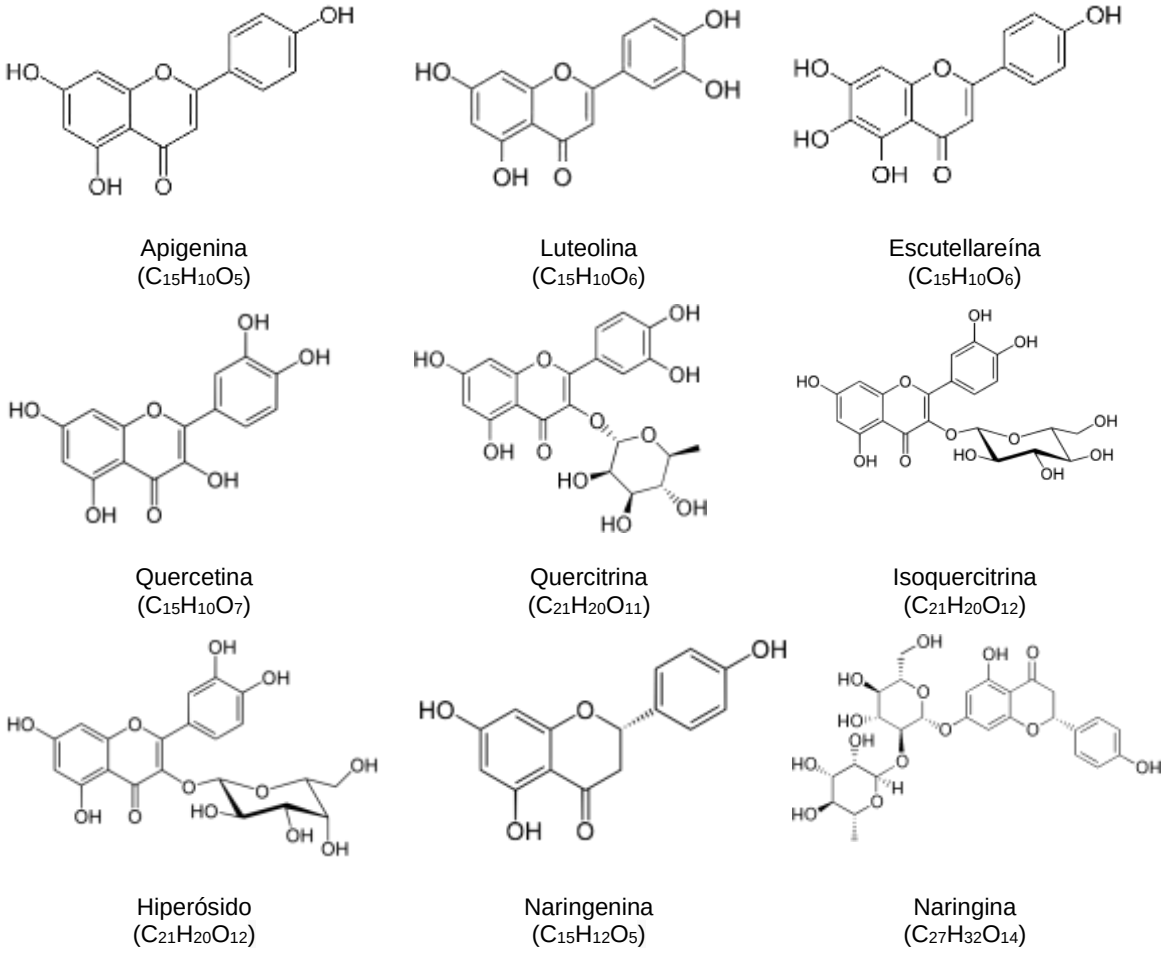


Figura 7. Principales flavonoides descritos en *O.vulgare*.

2. Compuestos fenólicos de tipo no flavonoide

Los compuestos fenólicos de tipo no flavonoide están compuestos por ácidos fenólicos, ácidos hidroxicinámicos, ácidos hidroxibenzoicos y estilbenos, principalmente (**Figura 8**).

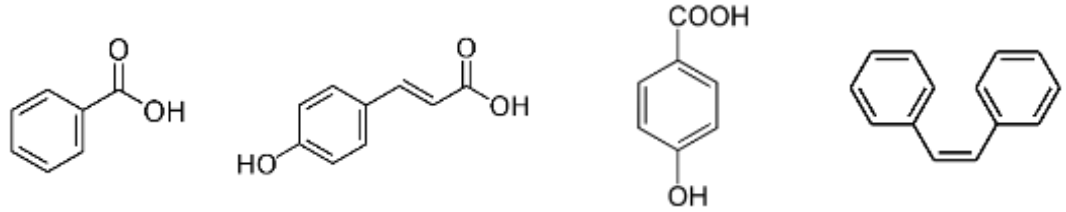


Figura 8. Estructura básica de los diferentes fenoles de tipo no flavonoide

Los ácidos hidroxizcinámicos, formados por un anillo aromático y una cadena de tres carbonos (C6–C3) y los ácidos fenólicos (C6–C1) son los que aparecen más frecuentemente en *O. vulgare* [20]. En la naturaleza, suelen estar asociados con otros

compuestos como el ácido clorogénico, que es el vínculo entre el ácido cafeico y el ácido quínico. En la **Figura 9** se indican los ácidos descritos en esta especie [19].

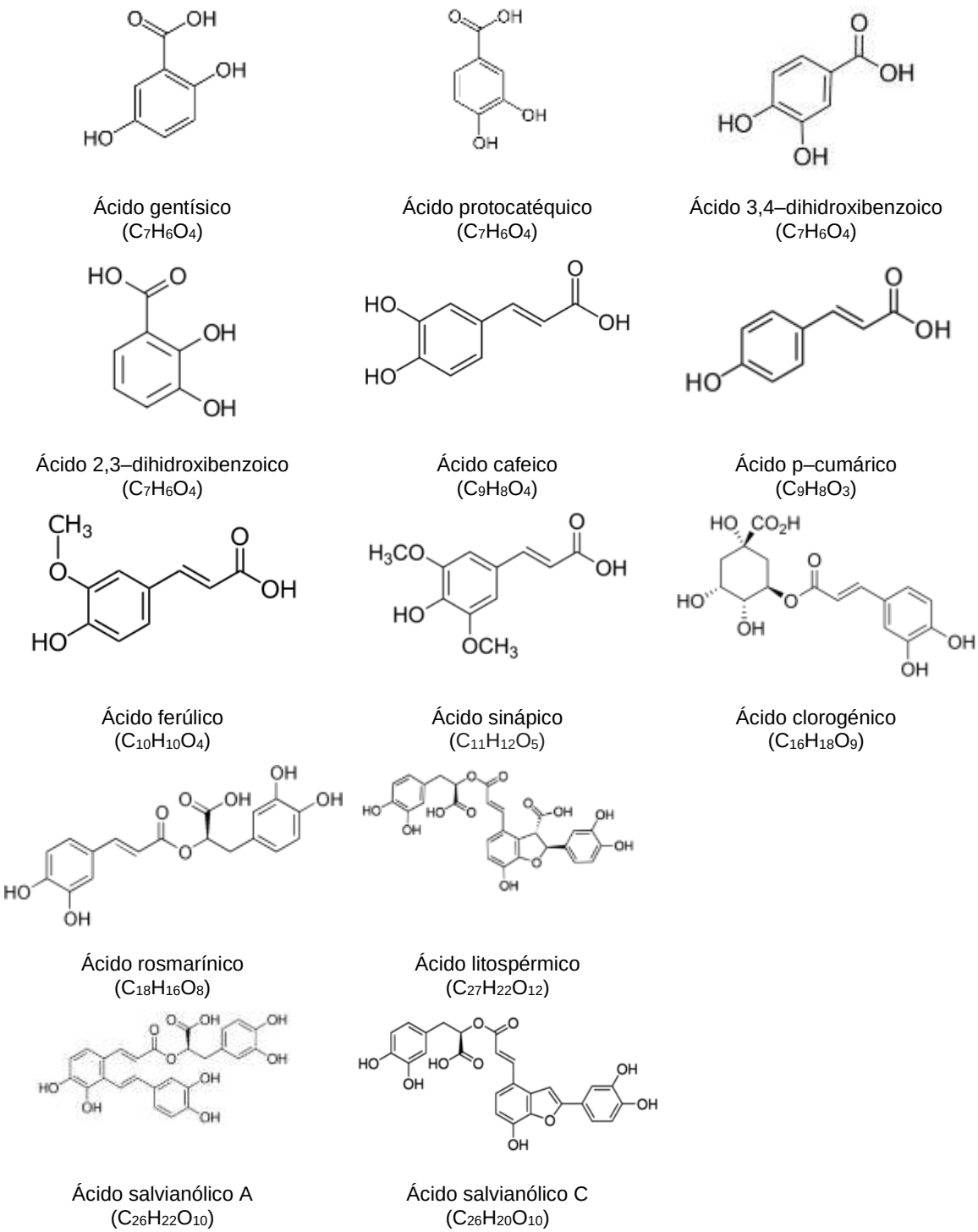
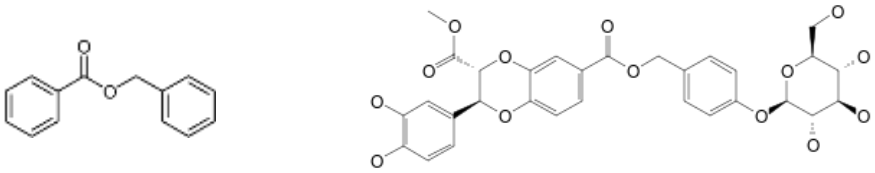


Figura 9. Principales compuestos fenólicos no flavonoides descritos en *O. vulgare*.

En la **Figura 10** se indica la estructura del origalignanol, benzolignano también presente en el orégano [21].



Benzolignano

Origalignanol
($C_{27}H_{24}O_{12}$)

Figura 10. Estructura de origalignanol, benzolignano presente en *O. vulgare*.

El contenido y la distribución de estos compuestos en el orégano pueden variar según las condiciones de cultivo y tiempo de cosecha así como otros factores geográficos y ambientales [22]. De hecho, algunos autores postulan que el perfil fitoquímico de cada orégano se puede utilizar para diferenciar entre quimiotipos dentro de la misma especie [23]. Además, dicho contenido también puede variar según la etapa vegetativa de la planta [24,25].

Los compuestos fenólicos, incluidos los flavonoides y los ácidos fenólicos, son responsables de su actividad antioxidante [26]. Además, estos antioxidantes fenólicos poseen otras actividades biológicas, como por ejemplo antiulcerosas, antiinflamatorias, antidiabéticas, antivirales, citotóxicas y antitumorales [27], y están fuertemente asociadas con los efectos terapéuticos de la planta.

4 Uso tradicional

Las especies de *Origanum* se han utilizado durante miles de años como especia y en etnomedicina [28]. Desde el punto de vista etnofarmacológico, es una planta conocida en todo el mundo que se utiliza principalmente contra los trastornos bronquiales, como antiséptica y para el tratamiento de resfriados. También está descrita su acción digestiva en casos de indigestión, molestias estomacales y como carminativo [29]. Entre otros usos tradicionales cabe destacar su uso como antiséptico de vías urinarias, en la caries dental, dolores de muelas y en la artritis reumatoide [30], en casos de niveles anormales de glucosa en sangre o leucemia [31,32], y como antiparasitario y antihelmíntico [33].

Varias especies de orégano. también se utilizan como potentes desinfectantes y agentes aromatizantes en perfumes y jabones [34]. Como hierba culinaria se utiliza para aromatizar productos alimenticios y bebidas alcohólicas [35].

5 Actividad farmacológica

El reino vegetal ofrece un enorme potencial para el descubrimiento de nuevos fármacos para el tratamiento y la prevención de enfermedades. A lo largo de la historia, las plantas se han utilizado como alimento, especias y medicamentos. Se administraban en forma de tinturas, infusiones, cataplasmas, polvos y otras formulaciones sencillas para el tratamiento de todo tipo de dolencias. En la historia más reciente, su uso como medicamentos evolucionó gracias a la obtención de compuestos activos químicamente puros, como por ejemplo la morfina del opio a principios del siglo XIX [36]. Desde entonces, los productos naturales han sido el origen del descubrimiento de muchos compuestos químicos empleados actualmente en terapéutica. En este sentido, actualmente existen numerosas líneas de investigación enfocadas a detectar productos naturales de origen vegetal con espectros de actividad interesantes desde el punto de vista farmacológico [37].

Las aplicaciones farmacéuticas de las plantas aromáticas se atribuyen en parte a los aceites esenciales [38]. Los aceites esenciales son compuestos volátiles, naturales y complejos caracterizados por un fuerte olor y formados por las plantas aromáticas como metabolitos secundarios. Estas sustancias pueden estar constituidas por aproximadamente 20 a 60 componentes a concentraciones bastante diferentes. Por lo tanto, los efectos biológicos de los aceites esenciales pueden ser el resultado de un sinergismo de todas las moléculas o reflejar solo los de las moléculas principales presentes en los niveles más altos [39]. Hay estudios en los que el componente principal del aceite esencial es más activo por sí solo que cuando estaba presente en el aceite esencial, lo que sugiere que también puede haber antagonismos entre los componentes del aceite [40], mientras que en otros estudios ocurre lo contrario, se detecta un sinergismo de acción entre los distintos compuestos presentes en el mismo.

El orégano, su aceite esencial y el carvacrol, fenol monoterpenoide mayoritario, se han estudiado como agentes antioxidantes, antibacterianos, antifúngicos, anticancerígenos y antiinflamatorios [29,41,42]. A continuación, se muestran algunos de los estudios más significativos.

5.1 Actividad anticancerígena, antiproliferativa y citotóxica

Entre las diversas y bien reconocidas propiedades de *O. vulgare*, su actividad anticancerígena puede considerarse la menos estudiada. El informe de la Organización Mundial de la Salud estima la existencia de 10.000.000 casos de cáncer por año en todo el mundo y 6.000.000 casos de muertes, siendo la segunda causa principal de muerte en

los países desarrollados [43]. A pesar de que muchos estudios establecen la eficacia de *O. vulgare* frente a esta enfermedad en modelos preclínicos de cáncer con resultados interesantes, hasta la fecha no se ha diseñado ningún ensayo clínico para investigar su potencial anticancerígeno en humanos.

Existen al menos 250.000 especies de plantas vasculares en todo el mundo, de las cuales se ha descubierto que más de 1.000 poseen propiedades anticancerígenas. De hecho, las plantas medicinales se consideran como una fuente importante de nuevos compuestos con este espectro de actividad. Se estima que alrededor del 60 % de los fármacos antitumorales y antiinfecciosos, ya comercializados o en ensayo clínico, son de origen natural. Algunas de estas especies de plantas, incluidas *Taxus baccata* L., *Podophyllum peltatum* L., *Camptotecha acuminata* Decne. y *Vinca rosea* (L.) G. Don, tienen una actividad anticancerígena bien reconocida en el cáncer de mama, y varios compuestos puros aislados y sus derivados semisintéticos están comercializados como parte de su tratamiento [44,45].

En este sentido, las investigaciones con orégano han demostrado que los compuestos fenólicos presentes en él ofrecen buenos resultados para prevenir la mutagénesis y la carcinogénesis, abriendo nuevas líneas de investigación como alternativa a esta patología. Sin embargo, el número de estudios disponibles hasta el momento son limitados [46].

El cáncer de colon es una de las principales neoplasias en los países desarrollados. La dieta y sus componentes juegan un papel importante en su etiología. En un estudio, Savini *et al.* [46] analizaron el efecto de los extractos etanólicos de *O. vulgare* (300 y 500 µg/mL) sobre el equilibrio redox, la proliferación celular y la muerte celular en células Caco–2 de adenocarcinoma de colon durante 12, 24, 48 y 72 h. Los resultados obtenidos mostraron que el extracto de orégano produce un efecto citotóxico dosis y tiempo–dependiente, así como un aumento de la apoptosis celular mediada por las caspasas iniciadoras y efectoras, el factor de activación de proteasa apoptótica 1 y la liberación de citocromo c [46]. Por ello, los autores concluyeron que las cantidades de orégano aportadas en la dieta podían ejercer efectos proapoptóticos selectivos para las células cancerosas. También observaron que el extracto etanólico completo presentaba mayor actividad citotóxica que un compuesto aislado del mismo.

También se ha investigado la actividad del orégano frente a las líneas celulares HT–29 (adenocarcinoma de colon humano) y MCF–7 (adenocarcinoma humano de mama) [47] observándose un efecto citotóxico más fuerte (mediante el ensayo de sulforodamina B) del 4–terpineol, el principal componente del aceite esencial obtenido por hidrodestilación

de *O. vulgare*, en las células HT–29 en comparación con las células MCF–7. Las concentraciones más efectivas para HT–29 y MCF–7 fueron 50 mg/mL a las 72 h, conduciendo una inhibición del crecimiento celular de 60,8 y 48,9 %, respectivamente para ambas líneas celulares.

Al–Kalaldeh *et al.* [48] estudiaron la actividad antiproliferativa del aceite volátil hidrodestilado de *O. vulgare*, así como extractos etanólicos y acuosos contra el adenocarcinoma de mama (también en la línea celular MCF–7). Ninguno de los dos extractos analizados ni el aceite esencial a 50 µg/mL durante 72 h mostraron actividad antiproliferativa. El análisis fitoquímico del aceite esencial mostró contenidos destacables en alcoholes monoterpenoides, que representan más del 60 % del aceite volátil, siendo el hidrato de trans–sabineno (27 %) y el 4–terpineol (19 %) los mayoritarios.

En otro estudio, se examinó un extracto metanólico de *O. vulgare* para determinar la actividad citotóxica en células HCT–116 (cáncer de colon) y MDA–MB–231 (cáncer de mama hormono–independiente) [49]. Mediante el ensayo MTT, los autores encontraron que los valores de IC_{50}, después de 24 y 72 h, eran 140,77 ± 2,13 µg/mL (HCT–116 a las 24 h), 109,51 ± 1,28 µg/mL (HCT–116 a las 72 h) y 231,46 ± 1,15 µg/mL (MDA–MB–231 a las 24 h), 506,31 ± 4,13 µg/mL (MDA–MB–231 a las 72 h).

Balusamy *et al.* [50] evaluaron los efectos apoptóticos del aceite esencial de orégano frente a líneas celulares de cáncer de estómago humano. El aceite esencial alteró las características de formación de colonias de las células cancerosas, la capacidad de migración de las células cancerosas y su proliferación.

Otro trabajo examinó la citotoxicidad *in vitro* del extracto de acetona de *O. vulgare* en células HeLa mediante el ensayo MTT, obteniendo un valor de IC_{50} = 126,3 ± 1,00 µg/mL [51]. Además, con distintos tipos de tinción (eosina y hematoxilina, naranja de acridina y yoduro de propidio [51]) los autores sugirieron que este extracto podría inducir la muerte celular por apoptosis a través de cambios morfológicos. En este sentido, otros autores han investigado la actividad anticancerosa de *O*. *vulgare* L. subsp. *viridae* (Boiss.) Hayek empleando diferentes disolventes de extracción: agua, etanol, metanol, acetato de etilo, hexano [52]. Utilizaron células HeLa (cáncer cérvico–uterino) y encontraron una actividad antiproliferativa similar en todos los extractos (excepto el de hexano) a concentraciones de 50 a 250 µg/mL, resultados que están en consonancia con el trabajo anterior. Los autores atribuyeron este efecto al ácido rosmarínico, compuesto fenólico más abundante en los cuatro extractos.

Marrelli *et al.* [53] analizaron trece extractos hidroalcohólicos de especies de plantas del sur de Italia, entre las que figuraba *O. vulgare*, frente a las líneas celulares MCF–7, HepG–2

(cáncer hepático) y LoVo (cáncer colorrectal) a una concentración de 100 µg/mL durante 24 h. Los autores encontraron una inhibición de la proliferación celular en un 47, 67, 39 %, respectivamente. Además, las células HepG–2, en un rango de concentración de 2,5 – 100 µg/mL, mostraron un valor de IC_{50} = 32,59 µg/mL 48 h después del tratamiento.

El análisis MTT de infusiones de varias plantas medicinales, entre las que figuraba *O. vulgare*, en dos líneas celulares: HT–29 (cáncer de colon humano) y PC3 (cáncer de próstata humana) [54] encontró una fuerte inhibición del crecimiento en ambas con un efecto tanto tiempo–dependiente (24 y 72 h) como dosis–dependiente (0, 2, 6 mg/mL) y una disminución de IL–8 (marcador antiinflamatorio).

Otro trabajo investigó *O. vulgare* en células MCF–7, HeLa, CEM (leucemia linfoblástica aguda T), A–549 (epitelial basal alveolar humano carcinómico), MDA (adenocarcinoma de mama humano) y Caco–2 (adenocarcinoma colorrectal epitelial) [55]. Mediante el ensayo MTT, los autores evaluaron los efectos sobre la supervivencia de las células cancerosas expuestas a 72 h de incubación y obtuvieron valores de IC_{50} que oscilaban entre 29,1 y 109,7 µg/mL. Con estos resultados los autores sugirieron que la modulación del estado redox podría afectar la viabilidad de todas las líneas celulares de cáncer analizadas, aunque en particular A–549, CEM y HeLa. Esto puede indicar una correlación entre las propiedades antioxidantes y anticancerígenas de esta planta.

En los últimos años se están produciendo avances muy importantes en el campo de la medicina gracias a la nanotecnología, aplicable tanto en el diagnóstico y detección de patologías como en la administración de fármacos [56]. Esta tecnología combina métodos físicos y químicos para la síntesis de nanopartículas. Dado que las reacciones químicas emplean y liberan productos tóxicos [57], existe una necesidad cada vez mayor de buscar materiales limpios, no tóxicos, biocompatibles y respetuosos con el medio ambiente. Se han realizado importantes intentos para sintetizar nanopartículas de plata, oro, platino, paladio, etc. utilizando productos de origen natural [58]. De todas ellas, las nanopartículas de plata son las que más se están desarrollando porque parecen ser las menos tóxicas para el cuerpo humano a concentraciones bajas [59]. Sankar *et al.* [60] utilizaron extracto acuoso de hojas de *O. vulgare* para sintetizar nanopartículas de plata mediante la reducción de una solución de nitrato de plata 1 mM. Las nanopartículas formadas eran estables (–26 ± 0,77 mV) a temperatura ambiente y mostraron una notable actividad citotóxica *in vitro* dosis–dependiente frente a la línea celular A–549 de cáncer de pulmón humano con un 50 % de mortalidad a 100 µg/mL. Los autores concluyeron que las nanopartículas podrían ser una alternativa eficaz para atacar las células cancerosas, aunque se necesitan más estudios para traducir este enfoque en la práctica clínica.

También se ha usado la misma línea celular A–549 para comprobar *in vivo* las propiedades citotóxicas y antiinflamatorias del aceite esencial de *O. vulgare* ssp. *hirtum*, constatándose que disminuye significativamente la proliferación celular después de 24 h de incubación. Este análisis ha mostrado también una reducción en el índice mitótico y el número de aberraciones cromosómicas (sin modificar el número de fases celulares), lo que sugiere que podría ejercer no solo una actividad anticancerígena, sino también quimiopreventiva [61].

Otros autores han estudiado el efecto del extracto etanólico de orégano (con concentraciones entre 0 y 250 µg/mL) frente a las mismas células A–549 [62]. Los resultados han mostrado una disminución de la viabilidad celular (IC_{50} = 14 µg/mL) de forma dosis–dependiente. Los resultados de los análisis con los componentes aislados o sus combinaciones demostraron que la viabilidad celular con timol, carvacrol, *p*–cimeno o 1–octacosanol solo disminuía de forma concentración–dependiente, siendo el timol el compuesto más citotóxico. La combinación de los cuatro compuestos a concentraciones equimolares presentes en el extracto de orégano fue menos citotóxica que el propio extracto. Por todo ello, concluyeron que la citotoxicidad del extracto de orégano se debe principalmente a la presencia de carvacrol y timol. El *p*–cimeno y el 1–octacosanol, aunque son citotóxicos a concentraciones elevadas, no parecen contribuir a la citotoxicidad de la mezcla, probablemente por su baja potencia y muy baja disponibilidad en la mezcla (rango nM). De hecho, parece que podrían existir otros componentes del extracto no medidos y, en concentraciones más bajas, que podrían tener mayor potencia y desempeñar un papel en los efectos generales de los extractos. No se han analizado los mecanismos por los cuales el timol y el carvacrol provocan la muerte celular en líneas celulares de mamíferos, pero los resultados de un estudio *in vitro* [63] permiten establecer que el carvacrol es un inhibidor muy potente del crecimiento celular en la línea celular A–549 ya que provoca una disminución concentración–dependiente en el número de células, la degeneración de la morfología celular y una disminución en la cantidad total de proteínas. El timol induce la muerte celular en el osteosarcoma y los astrocitos humanos y puede conducir a apoptosis a través de las vías mitocondriales [30,64]. Aunque se está investigando si los efectos sinérgicos del carvacrol y el timol con respecto a la viabilidad celular están relacionados con una combinación de sus mecanismos, es evidente a partir de los resultados del estudio de Coccimiglio *et al.* [62], que la coincubación de carvacrol y timol aumenta la captación del timol más citotóxico y aumenta la citotoxicidad de la mezcla. Esto puede ser debido a que el carvacrol es ligeramente más lipófilo y con mayor coeficiente de partición que el timol [65], y por tanto, el carvacrol se reparte más en la membrana citoplasmática provocando una mayor permeabilidad [66].

También se ha analizado la capacidad de los extractos acuosos y etanólicos de hojas *O. vulgare* (y otras plantas) para modular la proliferación, diferenciación y citotoxicidad celular en células HL–60 y U–937 (leucemia mieloide aguda) [67]. Se han observado efectos antiproliferativos de ambos extractos (IC_{50} = 38,1 ± 1,9 µg/mL a las 72 h para las células HL–60). Sin embargo, en las células U–937 sólo se observó una citotoxicidad modesta o nula y una inducción limitada de apoptosis.

Finalmente, los seres humanos a lo largo de la vida están expuestos a radiaciones ionizantes a través de agentes externos e internos. Respecto a los agentes internos, en el organismo se acumulan sustancias radiactivas provocando daños en órganos críticos. La radiación ionizante, al pasar por los tejidos vivos, genera radicales libres que interaccionan con el ADN y pueden provocar mutagénesis y carcinogénesis. Una de las principales fuentes de radiación interna es el uso de radiofármacos empleados a menudo para diagnóstico y con fines terapéuticos. Estos radiofármacos contienen al menos un radionúclido y un ligando no radiactivo. Entre los radionúclidos terapéuticos, el yodo radiactivo (^{131}I) es uno de los que más se emplea para el tratamiento de pacientes con enfermedades de la tiroides como hipertiroidismo (reducir el tamaño de la glándula tiroides) o con cáncer de tiroides diferenciado (eliminar células remanentes después de la tiroidectomía). Sin embargo, hay una preocupación persistente sobre la posible inducción de un segundo tumor y el daño genético generado tras este tipo de terapia. En este sentido, un estudio realizado por Arami *et al.* [68], examinó el efecto radioprotector del extracto etanólico 75 % v/v de *O. vulgare* (12.5, 25, 50 y 100 lg/mL) contra la genotoxicidad inducida por ^{131}I en linfocitos humanos. Las tres dosis más altas redujeron notablemente la incidencia de micronúcleos en los linfocitos humanos. El máximo efecto protector y la máxima disminución en la frecuencia de micronúcleos se observaron con la dosis más alta, con una reducción del 70 % ($p < 0,001$). Estos resultados indican el posible papel protector del extracto de orégano frente al daño genético inducido por la administración de radiofármacos.

A partir de esta descripción general de la actividad anticancerosa *in vitro* de *O*. *vulgare*, se percibe una imagen muy compleja. Incluso en los estudios que utilizan las mismas líneas celulares, como HeLa, MCF–7 y Caco–2, los materiales vegetales de partida (tejidos/órganos vegetales utilizados) son bastante diferentes, así como los métodos de extracción y las técnicas de destilación. Algunos estudios utilizan extractos de etanol crudo [60,61], otros extractos acuosos [48], otros analizan ambos [48,52] o el aceite esencial [47,61]. No menos importante, la diversidad fitoquímica depende tanto de factores endógenos de las plantas (composición genética) como de las variables ambientales (clima, agua, microorganismos presentes, etc.), origen geográfico y condiciones de crecimiento [69,70].

Así, algunos autores han indicado que los efectos de *O*. *vulgare* se deben al ácido rosmarínico [52], pero otros al 4–terpineol [47]. Esto aumenta la dificultad en la comparación de los estudios publicados. No obstante, parece demostrado un cierto efecto anticancerígeno de *O. vulgare* que podrían justificar su uso en modelos experimentales superiores.

De hecho, además de los experimentos *in vitro*, esta especie vegetal también se ha ensayado en modelos animales. En un estudio realizado por Srihari *et al.* [71] se determinó que la suplementación dietética con extracto acuoso de *O. vulgare* puede ofrecer protección frente al cáncer de colon. Para ello observaron el efecto de *O. vulgare* a distintas concentraciones (20, 40 y 60 ppm) sobre la peroxidación lipídica a través de un modelo de carcinogénesis de colon de rata inducido por 1,2–dimetilhidrazina (DMH). El examen de colon *post mortem* y bioquímico de ratas con cáncer de colon mostró que el suplemento dietético continuo con orégano (40 ppm) tenía un papel modulador sobre la peroxidación de lípidos tisulares, sugiriendo una posible propiedad anticancerígena.

Un estudio más reciente ha evaluado el efecto quimiopreventivo de la administración de orégano liofilizado junto con la dieta (0.3 y 3 %) en ensayos *in vitro* (frente a la línea celular MCF–7 de adenocarcinoma humano de mama) e *in vivo* en un modelo de ratones con cáncer de mama inducido por N–nitroso–N–metilurea administrada por vía intraperitoneal [72]. Tras 14 semanas se evaluó en la autopsia el tamaño del tumor y los cambios macroscópicos en hígado, bazo, riñón, estómago, intestino y pulmón. Los resultados revelaron una reducción significativa en la tasa de carcinomas mamarios poco o bien diferenciados, una reducción de la frecuencia de tumores (55,5 %) y una disminución del volumen del tumor (44,5 %) y de incidencia de tumores (44 %) en comparación con los animales control a dosis bajas. A dosis altas, se prolongó la latencia del tumor en 12,5 días y se observó un aumento de la expresión de caspasa–3 en las células del carcinoma. Además, los estudios *in vitro* mostraron resultados contrarios a los publicados previamente [48] observando que la administración de orégano disminuía la supervivencia y la proliferación de las células MCF–7. Este hecho demuestra que la composición química del material vegetal está directamente relacionada con la actividad farmacológica, parece que el aceite esencial hidrodestilado no presenta actividad y, en cambio, el orégano liofilizado y suspendido en el medio mostraría resultados esperanzadores frente a esta patología.

También se ha analizado el efecto de dosis bajas del aceite esencial de *O. vulgare* en ratones al los que se indujo una neoplasia maligna trasplantable (carcinoma de pulmón de Lewis) por vía intramuscular [73]. La administración de aceite esencial (0,15 µg/mL) en agua de bebida durante 3 meses provocó una disminución del tamaño del tumor en 1,5

veces y también una disminución de la peroxidación de lípidos en los tejidos hepáticos (hasta 36 %).

En conclusión, a pesar de los datos preclínicos positivos sobre el uso de *O*. *vulgare*, especialmente en modelos de cáncer de mama y colon, aún falta un análisis concreto y en profundidad de la eficacia anticancerosa de esta planta en humanos. Sería de esperar que, en el futuro, esta laguna se pudiera subsanar con prontitud.

5.2 Actividad antiinflamatoria

La inflamación es una respuesta biológica compleja del cuerpo a estímulos dañinos, como alérgenos, patógenos o sustancias irritantes y se considera una reacción fisiológica que involucra células inmunes, vasos sanguíneos y muchos mediadores clave [74]. Una respuesta inflamatoria alterada se relaciona frecuentemente con un gran número de enfermedades, como por ejemplo, el síndrome metabólico, ciertas disfunciones cardiovasculares, trastornos autoinmunes y cáncer. Se han estudiado muchos compuestos derivados de plantas por sus efectos antiinflamatorios que pueden estar relacionados con las vías de señalización NF–κB, JAK–STAT, vías apoptóticas u otras redes celulares y biomoleculares diferentes [75]. A continuación, se exponen los resultados más significativos encontrados de la relación entre *O*. *vulgare* y la inflamación.

En un estudio preliminar se analizó su eficacia sobre la aldosa reductasa (implicada en las complicaciones secundarias de la diabetes) y sobre la lipoxigenasa de soja (un modelo de la cascada del ácido araquidónico y la inflamación) [76]. El extracto metanólico y acuoso poseían actividad inhibidora sobre la lipoxigenasa, mayor en las muestras a concentraciones más bajas (10 µg/mL) que altas (100 µg/mL), sugiriendo que estos resultados deberían confirmarse en modelos celulares y animales más complejos que estos ensayos bioquímicos.

Se ha analizado también la combinación de aceite esencial de orégano y tomillo en un modelo de inflamación intestinal, donde la colitis se indujo por ácido trinitrobencenosulfónico (TNBS) [77]. El tratamiento de ratones con aceites esenciales mezclados con la dieta tuvo como resultado una disminución en la tasa de mortalidad del 53 al 33 %, una ganancia significativa de peso corporal y una reducción significativa del daño macroscópico de los tejidos del colon. Además, la administración combinada de 0,2 % de aceite esencial de tomillo y 0,1 % de orégano disminuyó los niveles de citoquinas proinflamatorias como IL–1β, IL–6, GM–CSF y α–TNF. Estos efectos podrían atribuirse a la inhibición de NF–κB como factor clave regulador de la respuesta inflamatoria y de las citoquinas proinflamatorias [78,79]. Un estudio de la barrera intestinal en un modelo de cerdo

sin inducir colitis después del tratamiento con aceite esencial de *O. vulgare* alimentando los animales durante 28 días (25 mg/kg de aceite esencial) mostró un aumento de la altura de las vellosidades en el yeyuno y la expresión de zonula occludens–1 (o uniones estrechas, localizada en las células del epitelio y endotelio que crea una barrera impermeable que impide el libre flujo de sustancias entre células) y occludina (enzima que regula la formación, el mantenimiento y la función de las uniones estrechas). Además, se observó una disminución de los niveles de endotoxinas en el suero y un aumento en el yeyuno de las citoquinas pro inflamatorias [78–82]. Todo esto indicaría que la administración del aceite esencial de *O. vulgare* en la dieta podría promover la integridad de la barrera intestinal en los cerdos, probablemente modulando las bacterias intestinales y el estado inmunológico.

Además, el análisis de extractos de *O. vulgare* mediante fluido supercrítico en la línea celular THP–1 humana activada (macrófagos activados con lipoproteínas oxidadas de baja densidad) [80], modelo experimental *in vitro* de aterosclerosis, dio como resultado una disminución de la liberación de citoquinas pro–inflamatorias (α–TNF, IL–1 e IL–6) y una mayor secreción de citoquinas antiinflamatorias.

También se han analizado los efectos sinérgicos, aditivos o antagonistas del orégano, el tomillo y el café en la línea celular monocítica U–937 transfectada con un indicador del gen de luciferasa con 3 sitios de unión a NF–κB) [78]. Las células se trataron con lipopolisacáridos (LPS) y extractos de plantas y se evaluó el factor de transcripción NF–kB (implicado en el estrés celular, respuestas inmunes e inflamatorias). El orégano inhibió la activación de la NF–kB–luciferasa en las células U–937 de forma dosis–dependiente y, cuando se combinó con tomillo o café, indujo una mayor actividad inhibidora, con sinergia para todas las combinaciones ensayadas. Estos mismos autores habían analizado de manera previa sus efectos (asociados con café, tomillo, clavo y nuez) en la vía NF–κB en ratones [79].

La actividad antiinflamatoria de *O. vulgare* se ha estudiado tanto en modelos celulares como animales. Gunawardena *et al*. [81] probaron más de 100 plantas alimenticias comunes (incluido el orégano) y hongos mediante bioensayos celulares *in vitro*, en concreto, la activación de micro glía murina N11 y macrófagos RAW 264.7 y la secreción de moléculas inflamatorias como el α–TNF y el radical libre óxido nítrico (NO). *O*. *vulgare* mostró ser una de las plantas más activas, presentando valores de IC_{50} por debajo de 0,1 y 0,5 mg/mL en modelos de microglía y macrófagos, respectivamente.

Grondona *et al*. [61] además del potencial antiproliferativo, tal y como se ha comentado anteriormente, también analizaron el efecto antiinflamatorio del aceite esencial de *O.*

vulgare en un modelo de inflamación de las vías respiratorias de ratón inducida por la administración intranasal de LPS. El aceite esencial provocó una fuerte actividad quimiotáctica con un incremento de las células polimorfonucleadas (PMN) y una disminución de los macrófagos alveolares, sin modificar el peso relativo del órgano (pulmón). De manera similar a otros estudios [78–80], los niveles de α–TNF y citoquinas proinflamatorias también disminuyeron.

Asimismo, Carrasco *et al*. [82] encontraron una notable actividad inhibidora de *O. vulgare* y *Thymbra capitata* (L.) Cav. sobre la lipoxigenasa y la acetilcolinesterasa. Atribuyeron esta actividad al carvacrol, el principal compuesto en ambos aceites esenciales.

El aceite esencial de orégano está ganando popularidad y actualmente existen en el mercado muchos productos formulados con él para el cuidado de la piel. Sin embargo, son escasos los estudios sobre sus efectos en las células de la piel humana. Un estudio ha evaluado la actividad biológica del aceite esencial con un alto contenido de carvacrol en un sistema BioMAP HDF_3CGF, línea celular de fibroblastos dérmicos humanos, diseñado para modelar la inflamación crónica y la fibrosis de una manera robusta y reproducible [83]. El estudio proporciona evidencia importante que respalda las actividades antiinflamatorias, remodeladoras de tejidos y anticancerígenas del aceite esencial ya que ha mostrado una actividad antiproliferativa significativa e inhibición de los biomarcadores inflamatorios y de remodelación tisular de forma concentración dependiente, incluidos proteína quimioatrayente de monocitos 1 (MCP–1), molécula de adhesión celular vascular 1 (VCAM–1), molécula de adhesión celular intracelular 1 (ICAM–1), proteína 10 inducida por interferón gamma (IP–10), quimioatrayente alfa de células T inducible por interferón (I–TAC) y monoquina inducida por (MIG). También se ha observado una disminución de los biomarcadores relacionados con la remodelación tisular de manera concentración dependiente, colágeno I, colágeno III, receptor del factor de crecimiento epidérmico (EGRF), metaloproteinasa de matriz 1 (MMP–1), inhibidor del activador del plasminógeno 1(PAI–1) e inhibidor tisular de metaloproteinasa (TIMP1 y TIMP2). Un biomarcador inmunomodulador, el factor estimulante de colonias de macrófagos (M–CSF) también parece fuertemente inhibido por el tratamiento con el aceite esencial. El análisis de la expresión génica de todo el genoma demostró que el aceite esencial ejercía un fuerte y variado impacto en muchos genes y vías de señalización, muchos de los cuales están directamente involucrados en los procesos de inflamación, remodelación de tejidos y procesos de señalización del cáncer. Los resultados obtenidos por estos autores concuerdan en gran medida con los estudios que indican el potencial antiinflamatorio, cicatrizante y anticanceroso del aceite esencial de orégano [84].

5.3 Actividad antimicrobiana, antifúngica y antiparasitaria

Los aceites esenciales y sus compuestos, debido a su carácter hidrófobo, presentan un gran potencial para aplicaciones farmacológicas como agentes antimicrobianos [84,85] aunque sus mecanismos de acción no se han estudiado con gran detalle. De hecho, dado que los aceites esenciales son mezclas complejas de varios compuestos, su actividad antimicrobiana probablemente no sea atribuible a un mecanismo específico [86,87]. En las células eucariotas, los aceites esenciales pueden provocar la despolarización de las membranas mitocondriales al disminuir el potencial de membrana. También cambian la fluidez de las membranas, que se vuelven anormalmente permeables dando como resultado la fuga de radicales, citocromo C, iones calcio y proteínas, como en el caso del estrés oxidativo y el fallo bioenergético. La permeabilización de las membranas mitocondriales externas e internas conduce a la muerte celular por apoptosis y necrosis [88,89]. Así, la apoptosis inducida por timol en células cancerosas se asoció a la vía mitocondrial [64,90].

Se ha estudiado ampliamente la eficacia de diferentes extractos y aceites esenciales de *O. vulgare* frente a bacterias patógenas. La infección por *Helicobacter pylori*, bacteria gram negativa, es una de las infecciones humanas más prevalentes afectando aproximadamente al 40 % de la población, aunque en poblaciones con nivel socioeconómico más bajo y peores condiciones sanitarias llega a ser del 80 %. *H. pylori* causa infecciones en el estómago y provoca una respuesta inflamatoria que conduce a gastritis crónica, úlceras gastroduodenales y complicaciones mayores como al cáncer de estómago y linfoma MALT gástrico [91]. Estas bacterias están dotadas de flagelos que le facilitan penetrar por el moco gástrico hasta alcanzar las mucosas y producir la infección. El medio gástrico es ideal para ella por ofrecer un medio microaerófilo fundamental para su supervivencia. Debido a su movilidad, junto a la virulencia de algunas cepas, el tratamiento de la infección por *H. pylori* se realiza de manera combinada con al menos dos antibióticos diferentes a la vez para evitar el desarrollo de resistencias a uno de ellos y garantizar su erradicación. Sin embargo, las terapias antibióticas son caras y se detectan cada vez más resistencias [92]. Por este motivo, existen numerosas estudios realizados con plantas medicinales y sus derivados en los que se analiza su posible acción frente a *H. pylori* (cepas ATCC 43504, originadas a partir de muestras gástricas humanas). En un estudio realizado por Chun *et al.* [93] se analizaron las propiedades antimicrobianas de extractos alcohólicos y acuosos de clones de orégano con alto contenido fenólico y ácido rosmarínico frente a *H. pylori* utilizando el método de difusión en agar. En base a los resultados obtenidos, los autores sugirieron que las propiedades físico–químicas de los ácidos fenólicos con estructuras C_6–C_1–COOH y C_6–C_3–COOH

desempeñaban un papel fundamental en la inhibición de su crecimiento y que dicha actividad antimicrobiana estaba correlacionada con la alta actividad fenólica y antioxidante de los extractos.

Las nanopartículas de planta con extracto acuoso de orégano, además de la actividad citotóxica citada anteriormente [60], también han mostrado una potente actividad antimicrobiana con respecto al cloranfenicol frente a varias bacterias patógenas humanas como *Aeromona hydrophilla*, *Bacillus sps, Escherichia coli*, *Klebsiella sps*., *Salmonella paratyphi*, *Shigella dysentriae y S. sonnie*.

Coccimiglio *et al.* [62] también describieron la actividad antimicrobiana del extracto etanólico de las hojas de *O. vulgare* frente a las cepas bacterianas Gram negativas y Gram positivas: *Acinetobacter baumannii*, *A. lwoffii*, *Bacillus subtilis, Bordetella bronchiseptica*, *Burkholderia cenocepacia*, *E. coli*, *Moraxella catarrhalis*, *Pseudomona aeruginosa*, y *Staphylococcus aureus*. Los mejores resultados se encontraron frente a los patógenos Gran negativos *P. aeruginosa* y *B. cenocepacia*. Estos son bacilos oportunistas que se encuentran comúnmente en el medio y que generan infecciones en pacientes que padecen enfermedades pulmonares como la fibrosis quística [94,95]. La actividad se atribuye principalmente al alto contenido en carvacrol y timol, resultado que apoya trabajos anteriores [96] que atribuyen la actividad al aceite esencial de orégano y sus componentes activos (carvacrol y timol) y a su interferencia con el gradiente de pH y la permeabilidad de la membrana. Un estudio que examinó las actividades antibacterianas separadas y combinadas de los principales componentes químicos del orégano y otras especias (eugenol, cinamaldehído, timol y carvacrol) mostró que cada componente posee propiedades antibacterianas y que actúan sinérgicamente [97].

Debido a que la actividad antibacteriana del orégano se atribuye principalmente al carvacrol y timol, componentes mayoritarios del aceite esencial [33], existen numerosos estudios con éste en lugar de con las hojas o con sus extractos. Además, la aparición de cepas resistentes está aumentado a un ritmo alarmante [98], lo que justifica la necesidad de identificar y desarrollar nuevos y potentes agentes antibacterianos de amplio espectro.

Del mismo modo, varios estudios respaldan la actividad antimicrobiana del aceite esencial de orégano frente a varios tipos de hongos patógenos como *Aspergillus flavus* (MIC = 10 µg/mL), *Bacillus subtilis* (0,125 µg/mL), *Candida albicans* (10–20 µg/mL), *Enterobacter cloacae* 0.5 µg/mL, *Escherichia coli* 0.5–1.2 µg/mL, *Listeria monocytogenes* (0,2 – 2,36 µg/mL), *Mycobacterium avium* (72,0 µg/mL), *Pseudomonas aeruginosa (*1,5 µg/mL), *Salmonella typhimurium* (0,1 – 3,1 µg/mL), *S. enteritidis* (0,5 µg/mL) y *Staphylococcus aureus* (0,25 – 3,2 µg/mL) [99–101] .

Teixeira *et al.* [7] utilizaron el aceite esencial para tratar un conjunto de cinco bacterias: *Salmonella thyphimurium*, *Escherichia coli*, *Listeria monocytogenes*, *Shewanella putrefaciens* y *Pseudomonas putida*. Los resultados obtenidos mostraron la inhibición de su crecimiento, particularmente de *L. monocytogenes*, con valores de MIC = 0,4 – 4,5 mg/mL.

El aceite esencial inhibe también el crecimiento de *Enterococcus faecalis* (MIC = 8 mg/mL), un microorganismo comensal del tracto intestinal humano. La actividad antimicrobiana se correlacionó con la tasa de liberación de componentes celulares (6,96 – 7,35 % según la concentración de aceite de 7 – 9 mg/mL) y las leves hendiduras en las superficies de las células tratadas [102].

El aceite esencial también ha presentado resultados positivos frente a algunas cepas de bacterias Gram positivas *Bacillus cereus* y *B. subtilis* (MIC = 50 µg/mL), que causan trastornos respiratorios, gastrointestinales, cutáneos y urinarios, aunque en un rango inferior al de la gentamicina (1,56 µg/mL) empleada como compuesto de referencia [103]. Sin embargo, en otro ensayo realizado con *B. cereus,* este mostró una susceptibilidad similar a gentamicina [104] Los mismos autores también confirmaron la inhibición del crecimiento de *S. aureus*, *E. coli* y *Pseudomonas aeruginosa*. Miller *et al.* [105] obtuvieron valores incluso más bajos (0,31 µg/mL) frente a *S. aureus*, *E. coli* y *Streptococcus mutans*; y Assiri *et al.* [106] frente a *S. enteriditis*, *S. aureus*, *E. coli* y *L. monocytogenes*.

En otro estudio se analizó la actividad antimicrobiana de veinte muestras de aceite esencial de diferentes plantas medicinales. El más activo frente a *Salmonella enterica* y *Listeria monocytogenes* fue el de *O. vulgare* con valores de MIC = 0.6 – 1.2 µL/mL. El mismo aceite esencial a concentraciones más bajas, mostró efectos bactericidas frente a *S. enterica* serotipo e*nteriditis* y *L. monocytogenes* [107].

El estudio de Fournomiti *et al*. [108] con el aceite esencial de plantas cultivadas de orégano, salvia y tomillo en distintas condiciones (irrigadas y no irrigadas) frente a cepas clínicas aisladas de *Escherichia coli*, *Klebsiella oxytoca* y *K. pneumonia* mostró que el orrganismo más sensible fue *K. oxytoca* (MIC = 0,9 µg/mL), seguido de *K. pneumonia* (73,5 µg/mL) y *E. coli* (219,9 µg/mL), sin diferencias significativas entre ambos tratamientos.

Los aceites esenciales de diferentes biotipos de *O. vulgare* recolectados en diferentes etapas de crecimiento también se ha mostrado significativamente activo contra *Staphylococcus epidermidis* (MIC = 3,12 – 25 µg/mL). Estos resultados on comparables, en algunos casos, con la del cloranfenicol (3,12 µg/mL) [109]. De manera similar, Yaldiz *et al*. [110] observaron una mayor actividad del aceite esencial de orégano que de otras plantas aromáticas contra *S. epidermidis*, *S. aureus*, *E. faecalis* y *E. coli*.

El complejo *Burkholderia cepacia* (CBC, o BCC por sus siglas en inglés) son un grupo de bacterias Gram negativas (no fermentadoras), aerobias y productoras de catalasa; compuestas por al menos nueve especies diferentes: *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. vietnamiensis*, *B. stabilis*, *B. ambifaria*, *B. dolosa*, *B. anthina*, y *B. pyrrocinia*. *B. cepacia* es un importante patógeno de humanos causante frecuentemente de neumonía en pacientes con enfermedades debilitantes pulmonares como la fibrosis quística o inmunocomprometidos como la enfermedad granulomatosa crónica [111]. Maida *et al.* [112] analizaron la actividad del aceite esencial contra el complejo *B. cepacia*. Todas las cepas de complejo probadas, incluidas aquellas con alta resistencia a ciprofloxacino, fueron extremadamente sensibles. Estos hallazgos fueron confirmados por Pesavento *et al*. [113], así como para otras bacterias multirresistentes responsables de infecciones respiratorias en la fibrosis quística, como *Stenotrophomonas maltophilia*, *P. aeruginosa*, *S. aureus*, y *Achromobacter xylosoxidans*. De manera similar, tres especies de *Clostridium* (*C. perfrigens*, *C. intestinale* y *C. ramosum*) mostraron también una alta susceptibilidad (MIC = 2,2 – 6,13 µL/ml) [114]. Entre cinco aceites esenciales diferentes, la muestra de *O. vulgare* mostró la mayor actividad antimicrobiana (MIC = 256 – 512 µg/mL) frente a estreptococos faríngeos aislados [115].

En el caso de la actividad antifúngica, Manohar *et al*. [116] detectaron la ausencia de hongos en un riñón infectado por *Candida albicans* tras el tratamiento eficaz con 8,6 mg de aceite de orégano en 100 µL de aceite de oliva/kg de peso corporal. Se han analizado las propiedades antifúngicas del aceite esencial de orégano tanto *in vitro* como *in vivo* frente a *C. albicans*, empleando como controles positivos nistatina y anfotericina B [116]. Los resultados han mostrado que éste, a una concentración de 0,25 mg/mL, inhibe completamente su crecimiento en cultivos *in vitro* de forma dosis–dependiente. Además, se han observado inhibiciones del crecimiento del 75 % y > 50 % a concentraciones de 0,125 y 0,0625 mg/mL, respectivamente. También se examinó la eficacia terapéutica del aceite esencial en un modelo experimental de candidiasis sistémica murina, empleando ratones infectados con *C. albicans* a los que se administraron cantidades variables de aceite de orégano en un volumen final de 0,1 mL, empleando aceite de oliva como vehículo. La administración diaria de 8,6 mg de aceite en 100 µl de aceite de oliva/kg de peso corporal durante 30 días mostró una supervivencia del 80 % sin carga renal de *C. albicans* en comparación con el grupo de ratones alimentados con solo aceite de oliva, que murieron en 10 días. Se obtuvieron resultados similares con carvacrol aislado, aunque los ratones alimentados con aceite exhibieron una apariencia física mejor en comparación con los tratados con carvacrol.

En un estudio realizado por Adams *et al*. [117] se examinaron las actividades antifúngicas *in vitro* del aceite esencial crudo de orégano frente a cuatro levaduras patógenas humanas (*C. albicans*, *Cryptococcus albidus*, *C. neoformans* y *Rhodotorula rubrum*) en pacientes inmunodeprimidos. El aceite esencial demostró eficacia tanto tiempo como concentración–dependiente frente a las cuatro especies, con una concentración mínima inhibitoria de 200 µg/mL en todas ellas.

La equinococosis humana es una zoonosis (es decir, una enfermedad transmitida al ser humano por los animales) provocada por tenias parásitas del género *Echinococcus*. Los seres humanos se infectan al ingerir huevos de parásitos presentes en los alimentos, el agua o el suelo contaminados o por contacto directo con animales que actúan como hospedadores. Las dos formas más importantes de la enfermedad en el ser humano son la equinococosis quística o hidatidosis provocada por *E. granulosus* y la equinococosis alveolar provocada causada por la infestación por *E. multilocularis*. Se estima que, en cualquier momento, hay más de 1 millón de personas afectadas por equinococosis. En la equinococosis quística se forman quistes dañinos de crecimiento lento normalmente en el hígado, seguido de los pulmones y otros órganos (bazo, cerebro, corazón, riñones, ojos, músculos, huesos), donde a menudo pasan inadvertidos durante años. Los síntomas inespecíficos son anorexia, pérdida de peso y debilidad. Cuando el quiste se encuentra en el hígado (equinococosis hepática) los síntomas incluyen hepatomegalia, dolor epigástrico en el lado derecho, náuseas y vómitos. Si el quiste se encuentra en los pulmones, los síntomas suelen ser una tos crónica, dolor torácico, hemoptisis y disnea. La rotura del quiste y la liberación de su contenido líquido pueden provocar reacciones alérgicas e incluso la muerte por shock anafiláctico, así como infecciones secundarias múltiples por la propagación de las protoescólices (forma larvaria). El tratamiento de la equinococosis, tanto en su forma quística como la alveolar, suele ser caro y complicado y en ocasiones requiere una intervención quirúrgica de envergadura y/o una terapia farmacológica prolongada [118]. Pensel *et al.* [119] determinaron el efecto antihelmíntico *in vitro* del aceite esencial de *Thymus vulgaris* y *O. vulgare* frente a protoescólices (estado larvario) y quistes de *E. granulosus*. Los aceites esenciales tuvieron un efecto tiempo–dependiente provocando la pérdida completa de la viabilidad de las larvas después de 60 – 72 días de incubación. Este mismo efecto también se detecto en las fases quísticas del helminto.

5.4 Actividad antioxidante

En las últimas décadas, existe también una gran necesidad de encontrar plantas medicinales como fuente alternativa de compuestos antioxidantes. Los productos

naturales que se extraen de plantas medicinales y otros vegetales poseen propiedades antioxidantes importantes con una gran aplicación para el desarrollo de nuevos fármacos [120].

Cuando los sistemas de desintoxicación endógenos celulares ya no son capaces de eliminar las especies reactivas de oxígeno (ROS) surge el estrés oxidativo. Esta condición puede dañar macromoléculas como lípidos, proteínas y ácidos nucleicos, provocando daños irreversibles y muerte celular. Hay varios trastornos crónico–degenerativos que están correlacionados con el estrés oxidativo, y, en consecuencia, con enfermedades inflamatorias, cardiovasculares y neurodegenerativas [121]. En este escenario, el uso de antioxidantes naturales puede representar una estrategia válida para mejorar el conjunto de defensas antioxidantes endógenas del organismo [122,123].

El cribado de las propiedades antioxidantes de las plantas y sus derivados requiere métodos apropiados que aborden tanto el mecanismo como la cinética de la reacción. Un método ideal para determinar la actividad antioxidante debe cumplir con los siguientes requisitos: sencillez, claro mecanismo de reacción, reactivos e instrumentación fácilmente disponibles, buena reproducibilidad y servir tanto para moléculas hidrófilas como hidrófobas. Dado que no existe un método con todas estas características, generalmente se suelen emplear varios métodos para obtener la máxima información posible [124]. El método más empleado es el de la captación del radical libre 2.2–difenil–1–picrylhydrazilo [125], aunque existen otros como el método de tiocianato, β–caroteno, ORAC, TRAP, FRAP que también se suelen emplear según la naturaleza de la muestra a analizar [124].

Las propiedades antioxidantes de *O. vulgare* y sus derivados han sido objeto de numerosas estudios. En los numerosos ensayos realizados, el aceite esencial de orégano muestra un ligero poder antirradicalario [7,102]. Los resultados de los ensayos de actividad antioxidante por el método de reducción del hierro férrico (FRAP) han confirmado una actividad antioxidante débil–media [7,107,110].

Sin embargo, los extractos acuosos, etanólicos, de acetato de etilo, acetona y éter dietílico de *O. vulgare* han mostrado una actividad antioxidante frente al radical libre DPPH de moderada a alta, con valores de IC_{50} = 34,5 – 86 µg/mL, aunque al menos siete veces más baja que los del ácido ascórbico (5,25 µg/mL). De entre ellos, el extracto etanólico parace el más activo, con un índice de actividad antioxidante (AAI) de 1,16 [126].

También se han evaluado varios extractos de agua de *O. vulgare* obtenidos por decocción e infusión junto con un extracto hidroalcohólico. Las muestras de infusión y decocción mostraron un valor de IC_{50} = 142,43 ± 10,30 y 132,93 ± 6,61 µg/mL, respectivamente en el ensayo de DPPH•. La decocción presentó mejores valores en el ensayo de β–caroteno

(115,69 ± 16,34 µg/mL frente a 262,30 ± 2,58 µg/mL) e inhibición de la peroxidación lipídica (8,73 ± 0,55 µg/mL frente a 22,75 ± 0,54 µg/mL). Ambas preparaciones fueron más antioxidantes que el extracto hidroalcohólico en todos los ensayos [127].

En otro estudio se analizó la actividad antioxidante del extracto acuoso obtenido por infusión de *O. vulgare* bajo tres procesos diferentes, a 85 ºC, a temperatura ambiente y a temperatura ambiente con baño de agua por ultrasonidos, con o sin extracción previa con éter de petróleo [128]. Los métodos empleados fueron el ensayo ABTS•• y DPPH•. Los resultados mostraron una correlación lineal positiva entre el contenido fenólico total y la actividad antioxidante en las infusiones estudiadas y, que el procedimiento de extracción influye significativamente en el contenido fenólico total y en la actividad antioxidante. Se observó que la mayor extracción de compuestos antioxidantes se produce a mayor temperatura, de manera similar al empleo de ultrasonidos. La preextracción con éter de petróleo no parecía influir en la actividad antioxidante final.

A la vista de los resultados expuestos anteriormente, la actividad antioxidante de un extracto puede verse afectada significativamente por el método de extracción y de ensayo utilizado, así como por la complejidad fitoquímica de los mismos, ya que contienen compuestos con diferentes grupos funcionales, polaridad y comportamiento químico.

5.5 Actividad antidiabética

La diabetes *mellitus* es una enfermedad crónica que aparece cuando el páncreas no secreta suficiente insulina o cuando el organismo no utiliza eficazmente la insulina que produce. La insulina es una hormona que regula la concentración de glucosa en la sangre, es decir, la glucemia. Entre 2000 y 2016, se registró un incremento del 5 % en la mortalidad prematura por diabetes. Se estima que en 2019 la diabetes fue la causa directa de 1,5 millones de defunciones a nivel mundial [129].

En la diabetes tipo 1 (DT1), el cuerpo no produce insulina. En la diabetes tipo 2 (DT2), la más común, el cuerpo no produce o no usa la insulina de manera adecuada. Sin suficiente insulina, la glucosa permanece en la sangre. Con el tiempo, el exceso de glucosa en la sangre puede provocar daños oculares, renales y nerviosos. La diabetes también puede causar enfermedades cardíacas, derrames cerebrales e, incluso, la necesidad de amputar un miembro. Las mujeres embarazadas también pueden desarrollar diabetes, llamada diabetes gestacional.

La DT1 es una enfermedad autoinmune que se desarrolla como consecuencia de la muerte de las células β pancreáticas inducida por mediadores proinflamatorios. En este sentido y dado que *O. vulgare* posee actividad antiinflamatoria, se ha estudiado su acción

en esta patología en un modelo de ratón inducido por múltiples dosis bajas de estreptozotocina [130]. Los beneficios del extracto metanólico de *O. vulgare* (administrado mediante inyecciones intraperitoneales a una dosis de 5 mg/kg por día durante 10 días) sobre la diabetes fueron mediados por el aumento de la capacidad antioxidante endógena, suprimiendo la respuesta inmune pro–inflamatoria relacionada con las células T, bloqueando la apoptosis celular y preservando la secreción normal de insulina. La determinación de la composición celular (citometría de flujo) y la producción de citoquinas (ELISA) se realizó el día 12 después de la inducción de la diabetes. En este caso, el tratamiento con el extracto preservó significativamente la morfología de los islotes pancreáticos y redujo el efecto modulador de los macrófagos pro–inflamatorios. Sin embargo, los autores de este estudio no observaron ningún efecto con el extracto acuoso.

5.6 Actividad cicatrizante

Las heridas son uno de los principales problemas relacionados con la salud en todo el mundo. Se producen por daños físicos, lesiones químicas e infecciones microbianas patógenas, lo que conduce a la pérdida de la continuidad celular y funcional de los tejidos vivos y una necesidad de una cicatrización.

Como se ha mencionado anteriormente, Sankar *et al.* [60] demostraron la capacidad antibacteriana y anticancerígena de las nanopartículas de plata formuladas con *O. vulgare*. Siguiendo con esta línea de investigación, en otro trabajo han estudiado su potencial actividad en la cicatrización de heridas evaluando el cierre de la herida, la histopatología y el perfil de proteínas, obteniendo resultados muy prometedores [131].

5.7 Actividad hepatoprotectora

Una lesión hepática conduce a una alteración de las funciones metabólicas del organismo. Los productos de origen vegetal se consideran tratamientos eficaces y seguros para los problemas de hepatotoxicidad aunque existe muy poca bibliografía al respecto.

Sikander *et al.* [56] estudiaron la actividad protectora de un extracto acuoso de *O. vulgare* frente a una hepatotoxicidad inducida por tetracloruro de carbono en ratas normales y hepatotóxicas. Para evaluar dicha actividad, los animales se dividieron en seis grupos: grupo de control, grupo tratado con *O. vulgare*, grupo tratado con tetracloruro de carbono (CCl_4; 2 mL/kg de peso corporal) y tres grupos de tratamiento que recibieron CCl_4 y extracto en dosis de 50, 100, 150 mg/kg de peso corporal por vía oral durante 15 días. Se midieron los valores de alanina aminotransferasa (ALT), fosfatasa alcalina (ALP) y aspartato aminotransferasa (AST) en suero y peróxido de lípidos (LPO), GST, CAT, SOD,

GPx, GR y GSH en tejido hepático. Los análisis bioquímicas se complementaron con un examen histopatológico de secciones de hígado de los animales. La administración de CCl_4 provocó alteraciones histológicas y bioquímicas características de daño hepático en comparación con el grupo control, sin embargo, la administración del extracto provocó una protección significativa contra la hepatotoxicidad inducida por CCl_4 de forma dosis–dependiente, siendo la dosis más alta la que presentó mayor actividad.

Habibi *et al.* [132] evaluaron también los efectos de *O. vulgare* a 50, 100, 200 y 400 mg/kg en este caso frente a la toxicidad hepática causada por ciclofosfamida (200 mg/kg, via intraperitoneal) en ratones. El pre–tratamiento con *O. vulgare* (400 mg/kg) restauró claramente los marcadores hepáticos séricos (ALP, ALT y ALT), hecho que fue posteriormente confirmado por el examen histológico de los tejidos hepáticos mostrando el efecto hepatoprotector del orégano [133].

6 Ensayos clínicos

Sin embargo, y a pesar de tos los estudios anteriores, existen pocos ensayos clínicos con *O. vulgare* L. y algunos de ellos, aunque terminados, no han publicado ningún resultado. La mayoría parecen centrados en el tratamiento de la rinosinusitis por su actividad antimicrobiana, y en afecciones dermatológicas y de estrés oxidativo.

La rinosinusitis es una inflamación de las fosas nasales y de los senos paranasales caracterizada por el bloqueo, la obstrucción y/o la congestión nasal que se suma a una secreción nasal o rinorrea que puede drenar por la parte anterior o posterior de la nariz. A estos síntomas se pueden añadir la presencia de dolor o sensación de presión facial y la pérdida parcial o total del sentido del olfato. Se trata principalmente con antibióticos y esteroides, aunque sus resultados no suelen ser muy satisfactorios. Existen varios ensayos clínicos que estudian el efecto de *O. vulgare* frente a esta patología.

Madani *et al*. [134] demostraron la eficacia de un extracto hidroalcohólico de *O. vulgare* para aliviar la mayoría de los síntomas de la rinosinusitis. Para este estudio clínico aleatorizado doble ciego y controlado con placebo, los pacientes se dividieron en dos grupos (de 32 pacientes en cada uno), uno tratado y otro control según la edad, el sexo y la cronicidad de la enfermedad. Aproximadamente entre el 15,6 y el 25 % de los pacientes de ambos grupos tenían antecedentes de cirugía de senos nasales. La posología para el grupo tratado fue realizar inhalaciones con 5 mL de un extracto hidroalcohólico 75 % en agua hirviendo durante 15 minutos, tres veces al día durante dos semanas. Todos los síntomas, incluidos dolor de cabeza, congestión nasal, dolor en los senos nasales, secreción nasal

posterior, dolor ocular, purulencia en la cavidad nasal y tos, se redujeron significativamente. Ningún paciente informó de efectos secundarios.

En otro estudio clínico aleatorizado y a doble ciego, realizado en 2008, se analizó el tratamiento de la rinosinusitis aguda con aceites esenciales de plantas aromáticas. Su objetivo era demostrar si se producía un alivio en la obstrucción nasal dentro de los 20 minutos posteriores a la primera administración del tratamiento con el aerosol en pacientes con rinosinusitis crónica. Para ello, se comparó la eficacia del aerosol que contiene una mezcla al 1 % de aceites esenciales aromáticos (eucalipto, menta, orégano y romeros) frente al placebo (aceite esencial de limón) en el tratamiento de pacientes con rinosinusitis aguda. El estudio, que empleó 14 pacientes, finalizó en mayo de 2008 pero los resultados no se han publicado [135].

Un estudio clínico reciente [136] frente a esta afección compara la actividad de aerosoles nasales que contienen aceite esencial de orégano, fluticasona (grupo de control) o aceite de sésamo (grupo placebo) durante 4 semanas en pacientes con rinosinusitis crónica. Participaron 75 pacientes adultos con rinosinusitis. Los resultados de este estudio demuestran que el aceite de orégano produce beneficios clínicamente significativos respecto a la fluticasona y el aceite de sésamo para pacientes con rinosinusitis sin pólipos nasales.

La cicatrización de heridas es un proceso dinámico y complejo que afectado por la hidratación de los tejidos, la presencia de bacterias, la inflamación y otras variables. Tal y como se ha comentado anteriormente, el orégano tiene potentes propiedades antibacterianas, antifúngicas, antioxidantes y antiinflamatorias aunque los estudios clínicos para probar su eficacia son insuficientes. Un estudio clínico realizado por Ragi *et al.* [137] ensayó la eficacia de una pomada de extracto de orégano al 3 % en la cicatrización de heridas. Se aleatorizaron 40 pacientes que se sometieron a una escisión quirúrgica inicial. A los 12 días se tomaron muestras para cultivo y se evaluó el estado de las cicatrices a los 12, 45 y 90 días. El grupo tratado con la pomada de orégano presentó una menor contaminación bacteriana frente a *Staphlococcus aureus* (19 % positivos) frente al grupo control con vaselina (41 % positivos). Además, un paciente del grupo de crema de orégano desarrolló celulitis en comparación con tres pacientes del grupo de vaselina. El grupo de orégano tuvo una mejora estadísticamente significativa en el color de la cicatriz, la pigmentación y la flexibilidad. La pomada de extracto de orégano disminuyó la contaminación bacteriana y la posterior infección en las heridas posquirúrgicas.

La dermatitis atópica (eccema) es un trastorno que provoca enrojecimiento de la piel y picazón. Es frecuente en niños, pero puede manifestarse a cualquier edad. La dermatitis

atópica es duradera (crónica) y suele exacerbarse periódicamente. Actualmente no existe un tratamiento para esta patología, sin embargo, los tratamientos y las medidas de cuidado personal pueden aliviar la picazón y prevenir nuevos brotes. Es útil evitar los jabones fuertes, humectar la piel de forma regular y aplicar cremas específicas. Un estudio clínico, doble ciego, aleatorizado y controlado, realizado en 2014 evaluó y comparó la eficacia de una pomada formulada con un extracto acuoso de orégano al 3 % frente a otra pomada de hidrocortisona al 1 %, para el tratamiento de la inflamación asociada con la dermatitis atópica leve a moderada en pacientes pediátricos (2 – 17 años) [138]. La duración del estudio fue de un mes pero, de nuevo, los resultados no se han publicado hasta el momento.

El acné es una enfermedad frecuente de la adolescencia caracterizada por lesiones inflamatorias y no inflamatorias cuyo tratamiento tópico presenta muy a menudo fenómenos adversos como irritación o resistencia a los antibióticos que reducen el cumplimiento del tratamiento por parte del paciente. Mazzarello *et al.* [139] compararon un producto comercial (Acnatac® gel, MEDA Pharma GmbH & Co KG) a base de clindamicina–tretinoína (CTG) con una formulación galénica que contiene aceites esenciales de *Myrtus communis* L. y *Origanum vulgare* y tretinoína (MOTC) para evaluar su efecto anti–acné. Sesenta voluntarios se dividieron aleatoriamente en un grupo A (MOTC) y un grupo B (CTG) como control positivo. La efectividad se evaluó con análisis cutáneos no invasivos y recuentos del número de lesiones a los 15 y 30 días. En ambos grupos se detectó un empeoramiento por la pérdida de agua transepidérmica provocada por tretinoína. El grupo A (MOTC) mejoró el eritema papular y redujo las erupciones en la piel sana a partir de los 15 días de tratamiento.

En toda célula normal existe un equilibrio entre la acción de las sustancias prooxidantes u oxidantes, y las antioxidantes. El cuerpo humano posee agentes antioxidantes endógenos como las enzimas oxidorreductasas, y agentes reductores, como el glutatión, capaces de neutralizar los agentes oxidantes que se van produciendo continuamente. La exposición prolongada a altos niveles de estrés oxidativo está relacionada con el desarrollo o aceleración de varias disfunciones y enfermedades, como la enfermedad de Parkinson, Alzheimer, esclerosis múltiple, esclerosis lateral amiotrófica, patologías cardiovasculares, inflamación, diabetes tipo II, cánceres de mama, colon y próstata, pancreatitis y enfermedades hepáticas, entre otras. Estas patologías incluyen entre sus causas o consecuencias un desequilibrio a favor de la actividad de los agentes prooxidantes sobre las células con respecto a la respuesta antioxidante de estas. El desequilibrio ocurre cuando la concentración de antioxidantes disminuye en el organismo como consecuencia de una alteración funcional de las enzimas involucradas en la

respuesta antioxidante, pero también por el incremento en la producción de prooxidantes (compuestos químicos capaces de generar ROS) por diversas razones. En este sentido, y tal y como se ha comentado anteriormente, se ha demostrado que el orégano posee capacidad antioxidante en varios modelos *in vitr*o y numerosos estudios siguieren que podría ser potencialmente beneficioso para la salud humana, aunque faltan estudios en humanos. El objetivo de un estudio clínico publicado en 2006 [140] fue analizar la bioaccesibilidad y los efectos de la suplementación con extracto de *O. vulgare* sobre los lípidos séricos y la peroxidación de lípidos en hombres no fumadores sanos. Para ello se administró a 45 pacientes una suplementación diaria de zumo de mango y naranja (placebo), o zumo de mango y naranja enriquecido con 300 mg/d ó 600 mg/d de compuestos fenólicos procedentes de un extracto de orégano durante 4 semanas. La excreción de compuestos fenólicos aumentó notablemente en el grupo con la suplementación más alta de compuestos fenólicos en comparación con el grupo de placebo, pero no se observaron cambios significativos en los parámetros de seguridad, lípidos séricos o biomarcadores de peroxidación lipídica.

La disfunción endotelial (deterioro de las funciones normales del endotelio vascular) se produce por situaciones vasoconstrictoras, incremento de sustancias procoagulantes y proinflamatorias. Se realizó un estudio clínico con 224 participantes cuyo objetivo general era investigar los efectos del consumo diario de especias y plantas durante cuatro semanas consecutivas en la función endotelial y los factores de riesgo cardiovascular de hombres y mujeres posmenopáusicas. Se dividieron en cinco grupos de tratamiento (2,8 g/cápsula de canela, orégano, jengibre, romero y pimienta negra respectivamente) y un grupo control. A todos ellos se les midió, a la semana 0 y 4, los cambios en el tono vascular, el nivel circulante de lipoproteínas–lípidos plasmáticos, estrés oxidativo, activación endotelial y marcadores inflamatorios. El estudio finalizó en junio de 2012 pero los resultados no se han publicado [141].

En otro ensayo clínico cruzado, controlado y aleatorio, de tres períodos se estudió los efectos posprandiales de una comida rica en grasas con especias sobre la función endotelial, los lípidos/lipoproteínas, la función inmunológica y los marcadores plasmáticos de antioxidantes y estrés oxidativo. También se analizó el perfil metabolómico. En orden aleatorio, los participantes consumieron una comida rica en grasas (1000 kcal, 45 g de grasa), una comida rica en grasas con 2 g de una mezcla de especias (pimienta negra, albahaca, laurel, canela, cilantro, comino, jengibre, orégano, perejil, romero, pimiento rojo, cúrcuma y tomillo) o una comida rica en grasas con 6 g de especias. Entre cada tratamiento hubo un período de descanso de al menos 3 días. El estudio planteaba la hipótesis de que el consumo de una comida rica en grasas con especias atenuaría el

deterioro endotelial posprandial y los niveles de triglicéridos de forma dosis–dependiente en comparación con una comida rica en grasas. El estudio finalizó en mayo de 2018 pero los resultados tampoco se han publicado [142].

La halitosis es una enfermedad desagradable que afecta a personas de todas las edades y que puede tener un impacto negativo en su vida social. Un ensayo realizado por Ali y Mohammed [143] ha evaluado la eficacia de un enjuague bucal que contiene aceite esencial de orégano en la reducción de la halitosis oral en comparación con la clorhexidina y el agua destilada. Se diseñó un ensayo clínico paralelo, aleatorizado y doble ciego, en 54 estudiantes de odontología con halitosis oral. Fueron asignados al azar en tres grupos (orégano, clorhexidina y placebo) y se les indicó que se enjuagaran con 15 mL del enjuague bucal asignado dos veces al día durante siete días. La halitosis se midió utilizando dos métodos organolépticos y la prueba BANA (N–benzoil–DL–arginina–naftilamida) al inicio del estudio y después de los siete días. Los resultados mostraron que el enjuague bucal de aceite esencial de orégano fue eficaz para reducir la halitosis oral. Se encontró que tiene una eficacia similar a la clorhexidina en la reducción del mal olor oral sin efectos secundarios. Los grupos de clorhexidina y orégano mostraron una reducción significativa de las puntuaciones organolépticas de la lengua y el hilo dental y las puntuaciones de las pruebas BANA. No hubo diferencias significativas en todas las mediciones entre el grupo de clorhexidina y orégano.

La obesidad y el sobrepeso son un problema crítico de salud pública. En todo el mundo se han utilizado muchos productos naturales para el tratamiento de la obesidad y la pérdida de peso. Parece que el uso de suplementos naturales, basados en plantas medicinales puede ofrecer garantías de éxito. Siguiendo esta línea, se diseñó un ensayo clínico aleatorizado doble ciego para comparar el efecto de un suplemento herbal con cuatro plantas, *Origanum vulgare*, *Carum carvi*, *Trachyspermum copticum* y *Ruta graveolen*, basado en la medicina tradicional frente a un placebo junto con una dieta de adelgazamiento [144]. Los participantes fueron 64 sujetos obesos con un IMC > 30 y un rango de edad de 20 a 50 años, repartidos en dos grupos, placebo (*n* = 33) y tratados (*n* = 31). El período de estudio fue de ocho semanas y, tras la administración de tres cápsulas/día, se analizaron los índices antropométricos, la ingesta dietética y los parámetros bioquímicos. Los resultados indicaron que el peso corporal, el IMC y la composición de la grasa corporal en el grupo de tratamiento fue significativamente menor que el del control. Además, hubo una reducción significativa del perfil lipídico en el grupo de suplementos a base de hierbas, en comparación con el placebo.

AIMS OF THE STUDY

Origanum vulgare L. is a medicinal plant widely used in Traditional Medicine, which unfortunately its safetiness and efficacy has not been guaranteed yet by international orgaisms such as EMA (European Medicines Agency) or ESCOP (European Scientific Cooperative On Phytotherapy). Therefore, the main aim of this project is to deepen the phytochemistry and pharmacological knowledge of this species, as well as to design pharmaceutical forms that allow therapeutic application.

The current work has been divided into four specific objetives:

- Chemically characterize the polar extracts of the aerial parts of *O. vulgare* L. and establish their oral bioaccesibility after an *in vitro* grastrointestinal digestion process.
- Perform an *in vitro* pharmacological screening of *O. vulgare* L. extracts and/or their digests to determine bioactivities related to metabolic syndrome, as well as cytotoxicity assays.
- Perform an *in vivo* a pharmacological screening of *O. vulgare* L. extracts in the *C.elegans* animal model in order to establish a relationship with the results obtained *in vitro.* Performt a pharmacological screening in the *C. elegans in vivo* model for age–related pathologies.
- Design phytotherapeutic products in diverse pharmaceutical forms for oral and topical use, besed on phytochemical and pharmacological results obtained in the previous points.

OBJETIVOS DEL ESTUDIO

Origanum vulgare L. es una planta medicinal ampliamente utilizada en medicina tradicional pero que, hasta el momento no tiene avalada su seguridad y eficacia por organismos internacionales como la EMA (Agencia Europea del Medicamento) o la ESCOP (*European Scientific Cooperative On Phytotherapy*). Por ello, el objetivo principal de este proyecto es profundizar en el conocimiento fitoquímico y farmacológico de esta especie, así como diseñar formulaciones galénicas que permitan una aplicación terapéutica.

Este trabajo se ha dividido en cuatro objetivos concretos:

- Caracterizar químicamente los extractos polares de las partes aéreas de *O. vulgare* L y establecer su bioaccesibilidad oral tras un proceso de digestión gastrointestinal *in vitro*.
- Realizar un cribado farmacológico *in vitro* de los extractos de *O. vulgare* y/o sus productos de digestion para determinar bioactividades relacionadas con el síndrome metabólico, así como ensayos de citotoxicidad.
- Realizar un cribado farmacológico *in vivo* en el modelo animal *C. elegans* con el fin de establer una relación con los resultados obtenidos *in vitro,* y para patologías relacionadas con la edad.
- Diseñar productos fitoterápicos en varias formas farmacéuticas para uso oral y tópico en base a los resultados fitoquímicos y farmacológicos obtenidos en los capítulos anteriores.

EXPERIMENTAL DESIGN

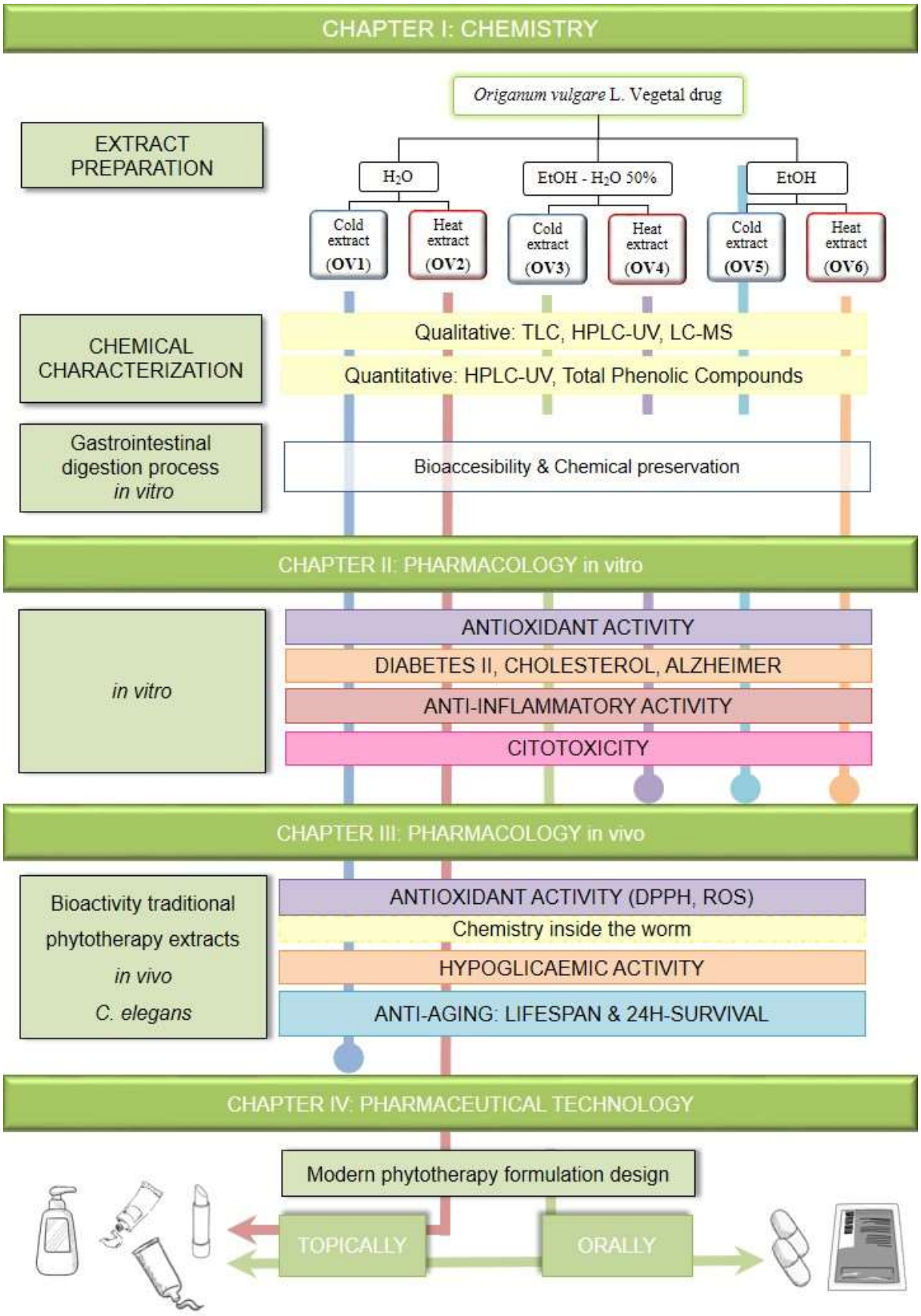


Table 0 /Tabla 0. Summary of the use of the extracts on each Chapter, assay by assay. Gray coloration indicates that the sample was tested for the indicated activity. *Resumen del uso de los extractos en cada capítulo, ensayo por ensayo. La coloración gris indica que la muestra se analizó para la actividad indicada*.

			OV1	OV2	OV3	OV4	OV5	OV6				BHT	RA	
Chapter I	**Chemical characterisation** *Análisis químico*	TLC												
		HPLC–DAD												
		LC–MS												
		TPC / *Polifenoles totales*												
	***In vitro* gastrointestinal digestion** *Digestión gastrointestinal* in vitro								OV1–Int	OV2–Int	OV6–Int			RA–Int
		TLC												
		HPLC–DAD												
Chapter II	**Pharmacological assays *in vitro*** *Ensayos farmacológicos* in vitro	Antioxidant / *Antioxidante* (DPPH•)												
		Antioxidant / *Antioxidante* (ABTS•)												
		Hypoglycaemic / *Hipoglucemiante*												
		Hypolipidemiant / *Hipolipemiante*												
		Anti–acetylcholinesterase / *Acetilcolinesterasa*												
		Albumin denaturation / Desnaturalización albúmina												
		RBC stabilisation membrane / *Estabilización* membrana eritrocito												
	Cytotoxicity assays *Ensayos de citotoxicidad*	Caco–2 celline												
		HepG–2 celline												
		A375 celline												
Chapter III	**Pharmacological assays *in vivo* (*C. elegans*)** *Ensayos farmacológicos* in vivo (*C. elegans*)	Antibiogram / *Antibiograma*												
		Pharyngeal pumping rate / *Bombeo faríngeo*												
		Growth / *Crecimiento*												
		Antioxidant / *Antioxidante* (DPPH•)												
		24h survival												
		ROS Accumulation / *Acumulación ROS*												
		Hypoglycemiant / *Hipoglucemiante*												
		Lifespan												
Chapter IV	**Pharmaceutical form design** *Desarrollo galénico*	Oral formulation *Formulación oral*												
		Topical formulation *Formulación tópica*												

CHAPTER I:

CHEMICAL CHARACTERISATION AND ORAL BIOACCESIBILITY OF *Origanum vulgare* L. spp. *vulgare* FLOWERED AERIAL PART EXTRACTS

If you want different results, do not do the same things.

Albert Einstein.

1. PREPARATION OF THE EXTRACTS

Traditional phytotherapy, consisting of infusions and decoctions, is giving rise to modern and rational phytotherapy with new and more complex pharmaceutical forms [145,146], in which quality, safety and efficacy are fundamental parameters [147,148]. Some of these pharmaceutical forms are formulated with extracts and integrated in a matrix, with an appearance similar to that of a conventional medicine [149–151]. It is important to bear in mind that medicinal plants show a wide therapeutic window, according to preclinical and clinical studies [152]. However, some of them can also interact with other drugs, be toxic or be contraindicated for some patients [153,154].

Thus, the research with medicinal plants (pharmacognosy) must be taking into account some critical pre–steps:

1. The correct identification of the plant species.
2. The selection of the parts of the plant containing the active compounds.
3. The selection of the optimal method for extraction.

The word *pharmacognosy* comes either from the Greek (φάρμακον pharmakon (drug), and γνῶσις gnosis (knowledge)) or the Latin (verb cognosco (con, 'with', and gnōscō, 'know'), which means “to conceptualize” or “to recognize”. Therefore, identifying and knowing the plant under study through previous published works and literature can accelerate these steps.

The European Medicines Agency (EMA) [155] compiles monographs of some of the medicinal plants used in traditional medicine (Traditional use) and/or endorsed by clinical trials (Well–established use). These monographs contain information of the part of the plant used, the type of extract for a specific therapeutic use, the posology and method of administration and the contraindications, warnings and precautions of use, interactions, safety in fertility, pregnancy and lactation, undesirable effects, toxicology (overdose) and pharmaceutical properties (pharmacokinetics, pharmacodynamics and preclinical safety) [148]. These monographs are in continuous growing thanks to studies such as this one [156,157].

The study of medicinal plants requires the design of an optimal pre–extraction and extraction procedure for each plant, so that the bioactive compounds are preserved. Leaves, barks, roots, fruits and flowers can be either fresh or dried before the extraction process [158]. In addition, the size of the particles is also a decisive factor. As Thermo Fisher Scientific laboratories explain in their technical note 208, there are several variables to take into consideration in the preparation of a extract [159]:

Physical pre–extraction process

Drying is the most common way to preserve the medicinal plant, since the humidity of the plant material (water content) before the extraction process might affect the amount and chemical form of the extracted bioactive compounds.

On the other hand, the reduction of the particle size increases the contact surface between the sample and the extraction solvents. Sometimes, size is not only a surface contact issue, but can also be a relevant factor for pharmaceutical formulation [160].

Extraction parameters

- **Solvent**. Chemistry plays an important role in extraction. Each solvent dissolve the chemical compounds with similar polarity [161]. Mixing solvents with different polarities (ethanol–water, for example) may extract a wider range of compounds than a single polarity solvent. While polarity plays the main role, there are other secondary properties of the solvent that must be considered and that often do not directly affect the extraction *perse*, but do affect the process and future use of that extract: safety, economics (cost of production), boiling point, density and viscosity [161].

- **Temperature**. Temperature also plays an important role in the extraction and the speed of the process. As the temperature increases, the extraction takes less time to obtain a similar yield. The increase in temperature implies a reduction in the viscosity of the solvent and, therefore, an improvement in its ability to moisten the matrix and solubilize the phytocompounds [158]. This is the parameter that best differentiates cold maceration (low temperature) from hot maceration (high temperature, as infusion or decoction) [159]. Technically, the decoction is only suitable for hard plant materials such as roots. In practice, this process is based on leaving the raw material boiling in the solvent until it is cooked, while infusion consists of soaking the material in the solvent already boiled for a short period (10 – 15 min approximately). When an extract is prepared by hot maceration with reflux, the plant material is deposited at the bottom of the flask so that the solvent and the sample are in constant contact, and the reflux provides recovery of the evaporated solvent, reducing production costs and improving extraction performance [158,162].

- **Pressure**. According to Gay–Lussac's law [163], when volume is constant, pressure and temperature are indirectly proportional. In this sense, the pressure accelerates the extraction process, especially at large production scales such as those required at an industrial level. However, on a laboratory scale, most extracts are produced at atmospheric pressure, with other variables being modifiable.

- **Cycles**. One, two, three, four… how many times must the solvent be renovated? The introduction of a new solvent to the extraction favours the equilibrium of extraction, avoiding its saturation and the lost of bioactive compounds. In fact, when low temperature extractions are desired (< 75 ºC), multiple static cycles are required [162]. This concept might be easier to understand with the example of a home–prepared tea: once the properly prepared tea has been drunk, the teabag releases more tea by pouring more water. A commercial tea company published a diagram showing the components available in green tea according to the solvent and the number of infusions made (**Figure 11**) [164]. Considering that 65 % of compounds in a teabag are insoluble in water (raw fibres, lipids, chlorophyll, carotenoids, vitamin E and proteins, among others), only 35 % of compounds are hydro soluble (catechins, caffeine, polysaccharides, flavonoids, amino acids, saponins, vitamin C and vitamin B, among others). In the first infusion, 10 – 13 % of these soluble compounds are obtained, in the second an additional 7 – 10 %, in the third infusion 5 – 7 % and there may be up to an extra 5 % with additional infusions. Each infusion could be considered a new extraction cycle.

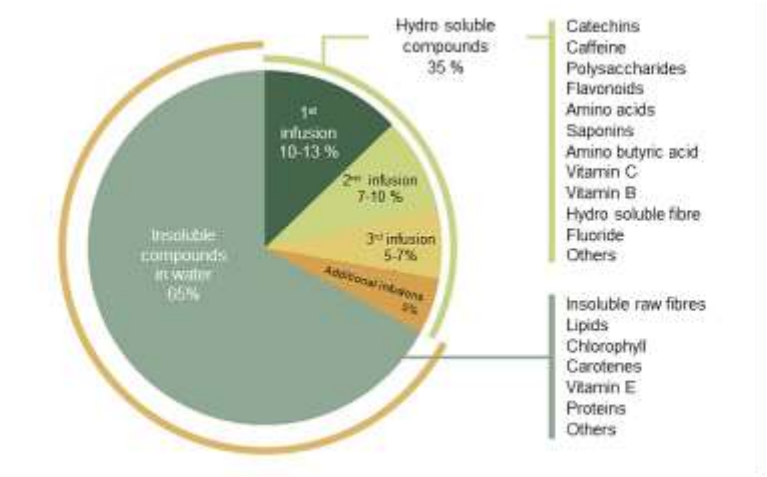


Figure 11. Green tea hydro soluble components available for extraction regarding solvent and extraction cycles [164].

- **Time**. Some compounds can be retained by the matrix within structures such as pores, so increasing the time can help with the extraction of those compounds [162]. Going back to the example of home–prepared tea, there is an indicated infusion time before drinking it. This time is necessary not only to extract the retained compounds, but also to extract them in sufficient quantity.

- **Agitation**. Movement is another factor to consider that is not explicitly mentioned on ThermoScientific list [162]. The agitation assists the release and extraction of bioactive

compounds. That is why the tea is usually moved, since it favours the circulation of solvent between the plant particles.

Generally, organic solvents tend to extract a higher amount of active biocompounds than water due to their polarity [165]. However, organic solvents such as methanol, ethanol or dimethyl sulfoxide, mostly selected as the first option, are toxic to the humans. Thus, the concentration of the extracts is usually followed by freeze–drying as a final step to evaporate the solvent, obtaining a lyophilized powder without residual solvents. On the other hand, water could be considered the best solvent both for the environment and for human health [165]. In addition, the properties of water can be modified by increasing the temperature: solubility, dielectric constant (reaching values similar to methanol), surface tension, viscosity, matrix permeation and an improvement in analyte diffusion [165].

In brief, the combination of these variables may lead to different technical requirements and different results.

1.1 MATERIAL AND METHODS

1.1.1 MATERIAL

Flowered aerial parts from *Origanum vulgare* L. spp. *vulgare* were collected in June 2017 in Santacara (Navarra, Spain) and dried at room temperature in the absence of light for five days. A voucher specimen (PAMP21629) was deposited in the herbarium of the Department of Environmental Biology at the School of Sciences, University of Navarra (Pamplona, Spain) after authentication by Dr. R.Y. Cavero.

1.1.2 METHODS

Regarding the aforementioned variables, for this work, the solvent and the temperature were the modified variables, with the pressure, time and agitation being similar for all the extracts. In this way, six extracts were prepared:

- Aqueous cold maceration (OV1): Water as solvent and low temperature (4 ºC).
- Aqueous hot maceration (OV2): Water as solvent and boiling temperature (100 ºC).
- Hydroalcoholic cold maceration (OV3): Ethanol–water 1:1 v/v. as solvent and low temperature (4 ºC).
- Hydroalcoholic hot maceration (OV4): Ethanol–water 1:1 v/v. as solvent and boiling temperature (80 ºC).
- Alcoholic cold maceration (OV5): Ethanol as solvent and low temperature (4 ºC).
- Alcoholic hot maceration (OV6): Ethanol as solvent and boiling temperature (80 ºC).

The extracts were prepared by the following steps:

All extracts were prepared from 10 g of dried flowered aerial parts of *O. vulgare* L. with 250 mL of solvent per cycle (four cycles in total). By mixing three different solvents (water, water–ethanol 1:1 v/v and ethanol) and two temperatures (cold and hot maceration). These extracts were prepared for human administration, so the possible toxicity of the solvent used was taken into account, for this reason etanol was used instead of methanol, the most common solvent in research [165].

The cold extracts (OV1, OV3 and OV5), after 24 h at 4 °C, were gravity filtered (**Figure 12**) and the solvent was removed at 40 °C in a rotary evaporator [132,166]. The temperature was fixed because some compounds could be chemically altered and destroyed [167]. This process was repeated four times with the same material to extract the highest amount of active compounds (**Figure 13, left**). Finally, the total volume was unified, lyophilized and stored at –20 °C. Lyophilisation improves the storage and further use of the extracts [158,159,168,169].

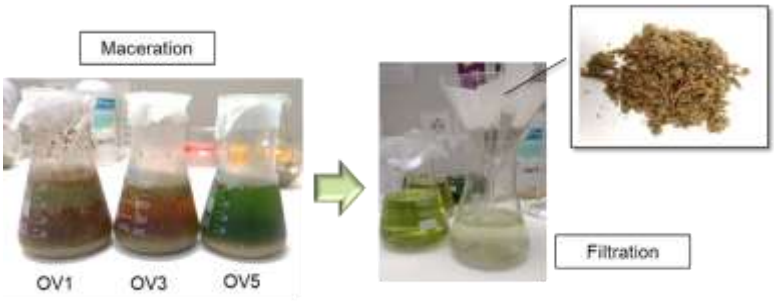


Figure 12. Photographs of maceration and filtration taken during the process. Left, maceration of the OV1, OV3 and OV5 extracts before filtration. Right, filtration and the remains reused three times more up to four cycles of extraction.

Hot extracts (OV2, OV4 and OV6) were obtained by immersing the same quantities of material and solvents in round–bottom flasks placed on a heater with a reflux system on top to minimize loses (**Figure 13, right**). This process was also repeated four times using the plant material after gravity filtration. The total volume was also concentrated with a rotary evaporator, lyophilized and stored at –20 °C.

After lyophilisation, the yield was calculated as a ratio that relates the quantity (in grams) of plant material needed to produce a unit mass (1 g for example) of lyophilized extract.

The yields of the processes were useful references for the industrial production and the subsequent reproducibility of the preparation of the extracts [152,170]:

$$Yield\ (\%) = \frac{Extract\ mass\ obtained\ (g)}{Initial\ vegetal\ mass\ (g)}\ x\ 100$$

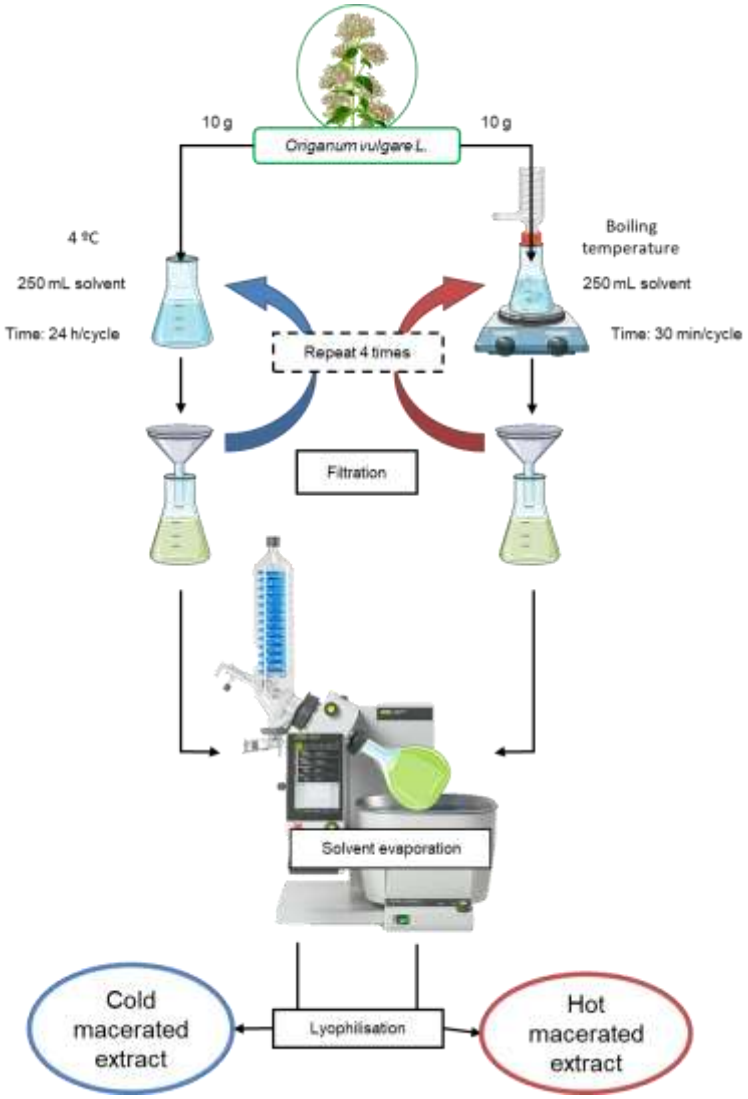


Figure 13. Schedule of extract preparation. Left, cold maceration (blue), corresponding to extracts OV1, OV3 and OV5. Right, hot maceration (red), corresponding to extracts OV2, OV4 and OV6.

1.2 RESULTS

As shown in **Figure 14**, the aqueous (OV1, OV2) and hydroalcoholic (OV3, OV4) extracts were brown in colour –regardless of the maceration temperature– and the ethanolic extracts (OV5, OV6) turned greenish due to the chlorophyll extraction. The colour trend of OV2 was similar to that of OV1, OV3 and OV4 (colour not shown because photograph was taken before processing was complete).

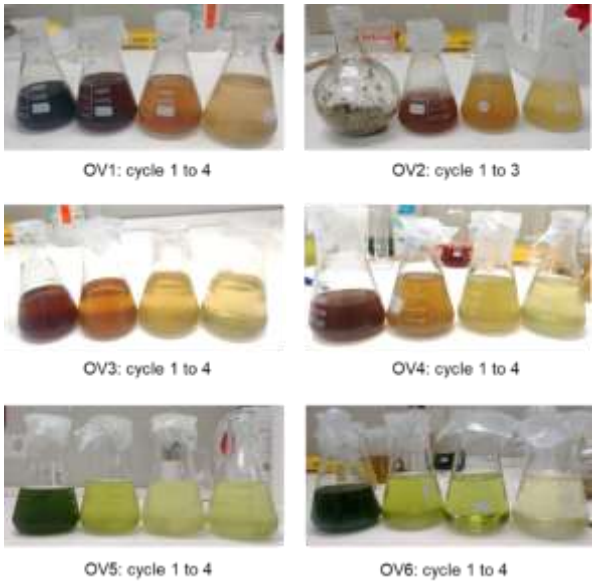


Figure 14. Photographs of the extracts obtained in each cycle.

Table 1 shows the yield of the extractions. According to the results, the most suitable solvents seem to be obtaining 50 % ethanol and water. In fact, the yield of the aqueous (OV1, OV2) and hydroalcoholic (OV3, OV4) extracts was approximately double that of the ethanolic (OV5, OV6) extracts. Regarding the ratio, at least 7 – 6 g of plant will be needed to obtain 1 g of lyophilized extract with ethanol (ratio 7 – 6:1, OV5 and OV6) *versus* 3 g of plant for aqueous and/or hydroalcoholic extracts (ratio 3:1, OV1, OV2, OV3 and OV4). These results are in line with the ratios of aqueous extracts prepared from wild growing *O. vulgare* L. in Serbia [126], 4:1 ratio for the aqueous extract, and better for ethanolic extract, 8:1 ratio [132] and 15:1 ratio [126].

Table 1. Yield results (in percentage) and ratio obtained from the prepared extracts (OV1–OV6) with the extraction characteristics (solvent, temperature), initial dry mass of drug (g) and lyophilised mass (g).

Name	Solvent	Temperature	Initial dry drug mass (g)	Lyophilised mass (g)	Yield (%)	Ratio (drug (g):extract (g))
OV1	Water	Cold	10.08	3.14	31.24	3:1
OV2		Heat	10.02	2.95	29.53	3:1
OV3	Ethanol – water 1:1 (v/v)	Cold	10.06	2.86	28.48	3:1
OV4		Heat	10.00	3.14	31.46	3:1
OV5	Ethanol	Cold	10.00	1.47	14.72	7:1
OV6		Heat	10.08	1.82	18.07	6:1

However, the polarity of the solvent can be crucial in the composition of extracts. Some potentially bioactive compounds might be more hydrophobic than others and therefore may not be present in high–yield extracts. For this reason, quantity is not the same as quality, so it is necessary to analyze the chemical profile and bioactivities to determine the optimal extraction method from a pharmaceutical point of view [16].

2. CHARACTERISATION OF THE EXTRACTS

In conventional medicine, the Active Pharmaceutical Ingredient is often a molecule resulting from a chemical reaction. For example, in industry, ibuprofen can be successfully obtained from isobutylbenzene and acetic anhydride as starting materials through a known organic chemical reaction called Friedel–Crafts acylation, a carbonyl reduction, a chloride substitution and the Grignard's reaction [171]. Finally, several techniques are used to determine if the process has been carried out correctly. According to the Pharmacopeia, infrared spectroscopy (IR spectroscopy), proton nuclear magnetic resonance (^{1}H–NMR) and melting point (MP) analysis are the most widely used.

When working with plants, even though nature of main compounds may be known from the literature (previous published botanical, chemical and pharmacological studies), it is important to bear in mind that plants are living organisms whose active principles are secondary metabolites with an important quantitative and qualitative variability depending on vegetative development, harvest season, climatic and soil conditions. In this sense, chemical characterization in plant extracts can be a long and difficult study, but it is certainly necessary.

Chromatography is a loanword from German to English (*Chromatographie*) whose etymological meaning comes from the Greek (*chroma* = colour and *graphein* = to write), which was first developed by Mikhail Tswett in 1903 by producing a colourful separation of plant pigments through a column of calcium carbonate [172]. Although chromatographic techniques have developed greatly since then, they are still useful in plant drug research and detection. In fact, in plant research, the observed chromatographic colour can be crucial in designing the fingerprint of chemical compounds.

Among all the chromatography techniques, in this work Thin Layer Chromatography (TLC), High Performance Liquid Chromatography with Diodo Array Detection (HPLC–DAD) and Liquid Chromatography–mass spectrometry (LC–MS) were used to identify compounds (qualitative analysis) and HPLC–DAD for the quantification of individual compounds. Besides, the chemical quantification was complemented with determination of total phenolic compounds by means of the Folin–Ciocalteu *in vitro* assay.

2.1 QUALITATIVE CHEMICAL CHARACTERISATION

2.1.1 THIN LAYER CHROMATOGRAPHY (TLC)

The chemical characterisation of an extract can be analysed through several complementary techniques. Thin Layer Chromatography (TLC) is generally the first approach to this characterisation, as it is a rapid technique that provides qualitative information on the main active compounds (similar to the fingerprint of an extract) and allows the detection of adulterations and substitutions.

2.1.1.1 MATERIAL AND METHOD

TLC is based on separation of compounds by polarity along a stationary phase.

- Stationary phase (SP): Silicagel 60 F_{254nm} with plastic base (Merck®)
- Mobile phases (MP): ethyl acetate:methanol:water (65:15:5 v/v/v) and ethyl acetate:glacial acetic acid:formic acid:water (100:11:11:26 v/v/v/v) in a Shandon chromatography chamber.
- Sample preparation: 200 µg of each extract was deposited on TLC plate. (concentration: 20 mg/mL; volume: 10 µL).
- Analysis conditions: The design of the layer is shown in **Figure 15**. TLC size (10 cm height x 7 cm wide); distance between samples (1 cm), TLC development (7 cm), concentration of sample (20 mg/mL), volume applied (10 µL), and mobile phase (A/M/A 65:15:5 v/v/v). Samples are deposited in spots on baseline (OV1, OV2, OV3, OV4, OV5 and OV6) with a distance of 7 cm until line on top. Analyte distance (A, A' in cm) would determine its Rf (retention factor).

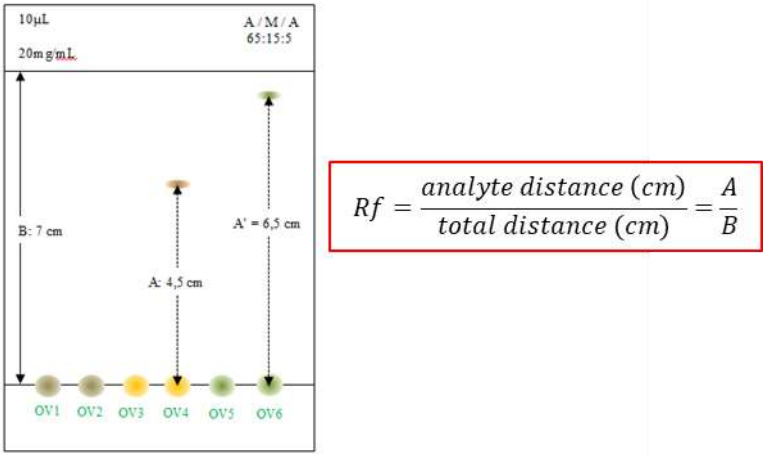


Figure 15. TLC plate design for OV1 – OV6 samples with formula of Rf on right.

After performing chromatography, the TLC plate was dryed at room temperature and visualised at visible – UV light (254 nm and 366 nm) after applying two reagents commonly used in Pharmacognosy [173], since many plant analytes react giving spots of representative colour.

- *Godin reagent*: composed by Solution I (vanillin 1 % ethanol) and Solution II (sulphuric acid 10 % ethanol (#V1104, Sigma–Aldrich Co., St. Louis, MO)) [174]. After applying Solution I followed by Solution II, the layer was heat to 100 °C until the coloration appeared.

- *Natural Products reagent*: solution of diphenylboric–2–aminoester acid (#D9754, Sigma–Aldrich Co., St. Louis, MO) 1 % in methanol. TLC plate was observed at 366 nm.

- Data process: The retention factor (R_f) was calculated as the length traveled by the analyte divided by the total distance travelled by the eluent (**Figure 15**).

2.1.1.2 RESULTS

Thin Layer Chromatography (TLC) is a simple and reproducible technique used as quick qualitative analysis of extracts and chemical compounds. Wagner and Bladt compiled a large number of photographed TLC of different medicinal plants in a atlas to aid in mobile phase decision and compounds identification (based on Rf calculated) [174].

Figure 16 shows the results obtained after separation of the extracts by TLC (ethyl acetate:methanol:water, 65:15:5 v/v/v) and detection with visible light, 254 and 366 nm and after spraying with Godin and Natural Products chemical reagents. Depending on the revealing reagent used, different compounds appear on TLC. Two different images of the visible detection are shown, one with the compounds labeled on the TLC – easier to identify by Rf – and the other (Visible (2) without labels) ito be able to make a better visual comparison with the 254 nm and 366 nm images. The Rf value of each spot was calculated (**Table 2**).

At the top of TLC (Rf = 0.90) a pink coloured spot (at 366 nm) was detected in the ethanolic extracts (OV5 and OV6). Wagner and Bladt [174] found similar fluorescent spots with high Rf (> 0.70) on TLC of methanolic extracts of *Hypericum perforatum* L. and identified them as chlorophylls. Chlorophylls are green pigments involved in photosynthesis located in the leaves of the plants. In this sense, it could be possible that these spots were chlorophylls because the flowering aerial parts of *O. vulgare* L. were the starting material.

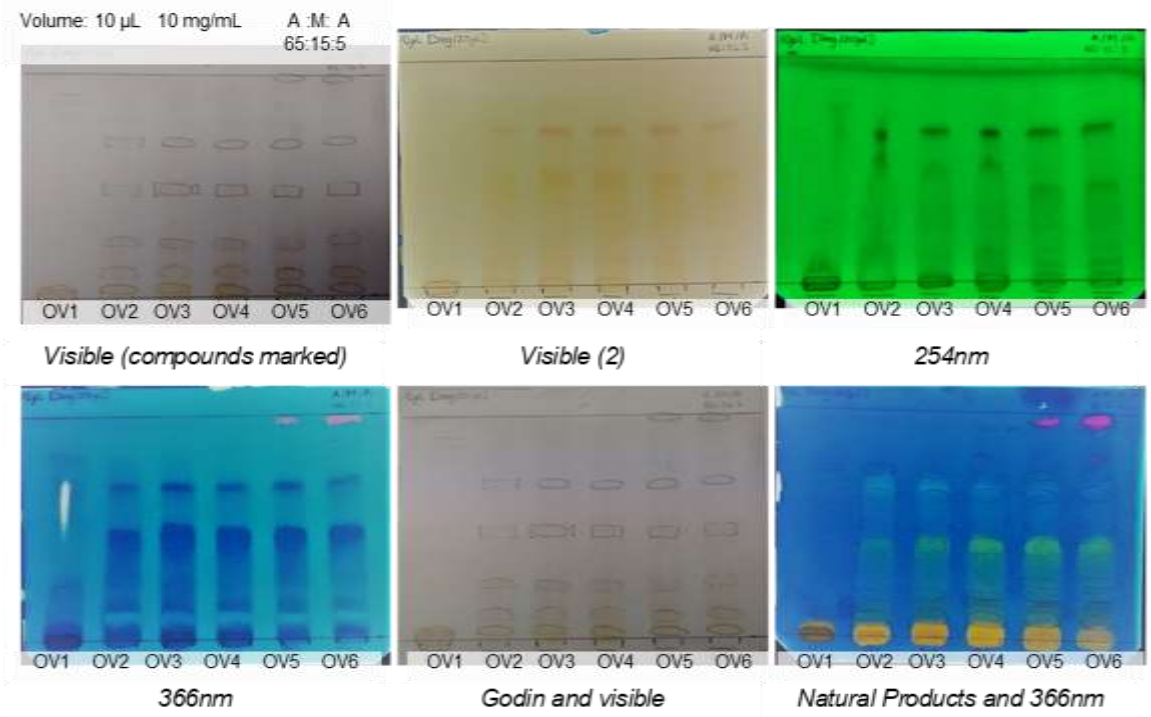


Figure 16. TLC of the six extracts (OV1–OV6) under lights of different wavelength (visible, 254 nm, 366 nm) and after revealing (Godin and NP). A:M:A: the mobile phase was ethyl acetate:methanol:water (65:15:5).

Table 2. Rf of spots detected by TLC. Each column represents a revealing technique used (Visible, under 254 nm, under 366 nm, after applying Godin and after applying NP under 366 nm).

Rf	Visible						254nm						366nm						Godin						NP (366nm)					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
0.90	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	+	+	–	–	–	–	+	+
0.70	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.55	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.40	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.25	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.00	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

OV1 (1); OV2 (2); OV3 (3), OV4 (4), OV5 (5); OV6 (6). Rf: Retention factor (+): very intense; (+): lightly intense; (–): non detectable. Note: In the table, colours have been added according to the colour observed in images in order to facilitate correlation (for example, pink square in table correspond to pink spot observed at NP at 366 nm on **Figure 16**).

A spots with Rf = 0.70, 0.55, 0.40 and 0.25 were also detected in all extracts except OV1. After revealing with NP reagent and observing at 366 nm, these spots turned blue, a characteristic colour of phenolic acids. The presence of green–blue spots corresponding to phenolic acids is in concordance with the chemical composition described for *O. vulgare* [13,19,127], where the most important phenolic compounds were 3,4–dihydroxybenzoic acid [21,175,176], rosmarinic acid [13,52,177] and caffeic acid [128,176,178].

TLC showed also a spot at the bottom of the plate (Rf = 0.00) in all six extracts, whose yellow colour was enhanced at 366 nm with the NP reagent. The yellow colour indicates

the presence of flavonoids [174], potentially bioactive compounds already described in *O. vulgare* L. [12,126,127,177,179]. In OV1 extract, a brown spot was also detected (Rf = 0.00), which could be tannins [174].

To confirm the chemical profile of extracts, complementary TLCs were prepared changing the mobile phase. According to Wagner and Bladt [174], ethyl acetate:glacial acetic acid:formic acid:water (100:11:11:26 v/v/v/v) is one of the best mobile phases to detect flavonoids and phenolic acids after NP treatment (**Figure 17**).

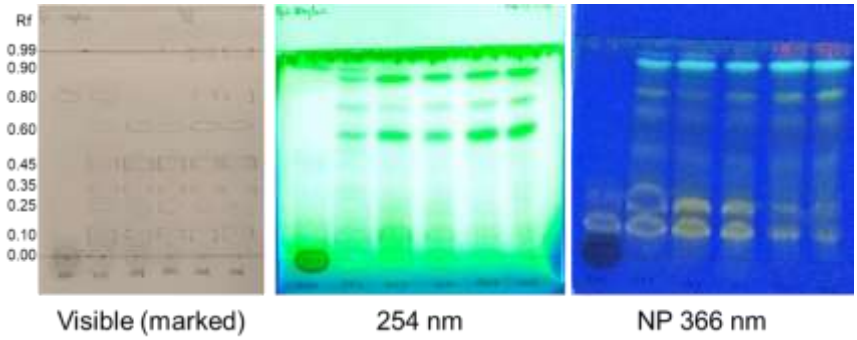


Figure 17. TLC of six extracts observed at 254 nm and revealed with NP reagent at 366 nm. Mobile phase: ethyl acetate:glacial acetic acid:formic acid:water (100:11:11:26 v/v/v/v).

At first sight, the separation of compounds was better than with the other mobile phase. The yellow spots at baseline on previous TLC were here separated into more than a single spot (**Table 3**).

Table 3. Spots observed in TLC classified by their Rf. Each column corresponds to each revealing method shown in Figure 17: Visual, under 254 nm and under 366 nm after revealing with NP solution.

Rf	Visible						254nm						NP(366nm)					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
0.99	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+	+
0.95	+	+	–	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.90	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.80	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.60	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.45	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.35	–	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+
0.25	–	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+
0.10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

OV1 (1); OV2 (2); OV3 (3), OV4 (4), OV5 (5); OV6 (6). Rf: Retention factor; (+): very intense; (+): lightly intense; (–): non detectable. Note: In the table, colours have been added according to the colour observed in images in order to facilitate correlation (for example, pink square in table correspond to pink spot observed at NP on top).

Different Rf values can be observed with this mobile phase. OV1 showed an intense brown spot at baseline (Rf = 0.00), absent in other extracts. The rest of the spots observed in all

the extracts confirm the presence of phenolic acids (in blue–green), flavonoids (in yellow) and chlorophylls (in pink).

TLC is a qualitative chromatographic technique in which neither the intensity of the bands should be used as a formal quantification nor the color given under certain conditions (reagent and observation wavelength) can be used for the identification of compounds beyond their chemical group (chlorophylls, flavonoids, phenolic acids ...). For this, it is necessary to apply complimentary techniques such as High Performance Liquid Chromatography (HPLC).

2.1.2 HPLC–DAD CHROMATOGRAPHY

TLC provides separation on a solid stationary phase and the compounds are seen in one layer. In the case of High Performance Liquid Chromatography (HPLC) the sample is pumped and separated through its passage through a column in which the different compounds are differentially adsorbed by their polarity. Identification can be done by attaching a detector to the end of the column. For example, the diode array detector (DAD) provides a UV–spectrum of each compound that allows its identification [180] and assignment to a specific chemical group [181].

2.1.2.1 MATERIAL AND METHOD

The chromatographic conditions used for the analysis of the six extracts were:

- Equipment: Waters 2996 Photodiode Array Detection as detector at 210–800 nm
- Stationary phase (SP): Nova–Pack® C18 (3.9 x 150 mm) (Column W10051M 007)
- Mobile phase (MP): a gradient with acetonitrole and water at pH = 2 (**Table 4**) Flow: 0.8 mL/min
- Temperature: column temperature 25.0 ± 5.0 °C
- Analyse time: 70 min
- Detection range: MaxPlot (190–600 nm)
- Sample preparation: 10 µL of sample at 20 mg/mL of each extract
- Data process: The peaks were identified both by their UV profile and their retention time [7,19,177]

Table 4. Gradient composition used as mobile phase in HPLC–DAD

Time (min)	Flow (mL/min)	% Acetonitrile	% Distilled water (pH 2)
0	0.8	5	95
10.50	0.8	5	95
20.00	0.8	10	90
35.00	0.8	20	80
45.00	0.8	40	60
50.00	0.8	80	20
55.00	0.8	5	95

2.1.2.2 RESULTS

Figure 18 shows the chromatogram of the six extracts (OV1–OV6) at MaxPlot to compare them before identification of the peaks.

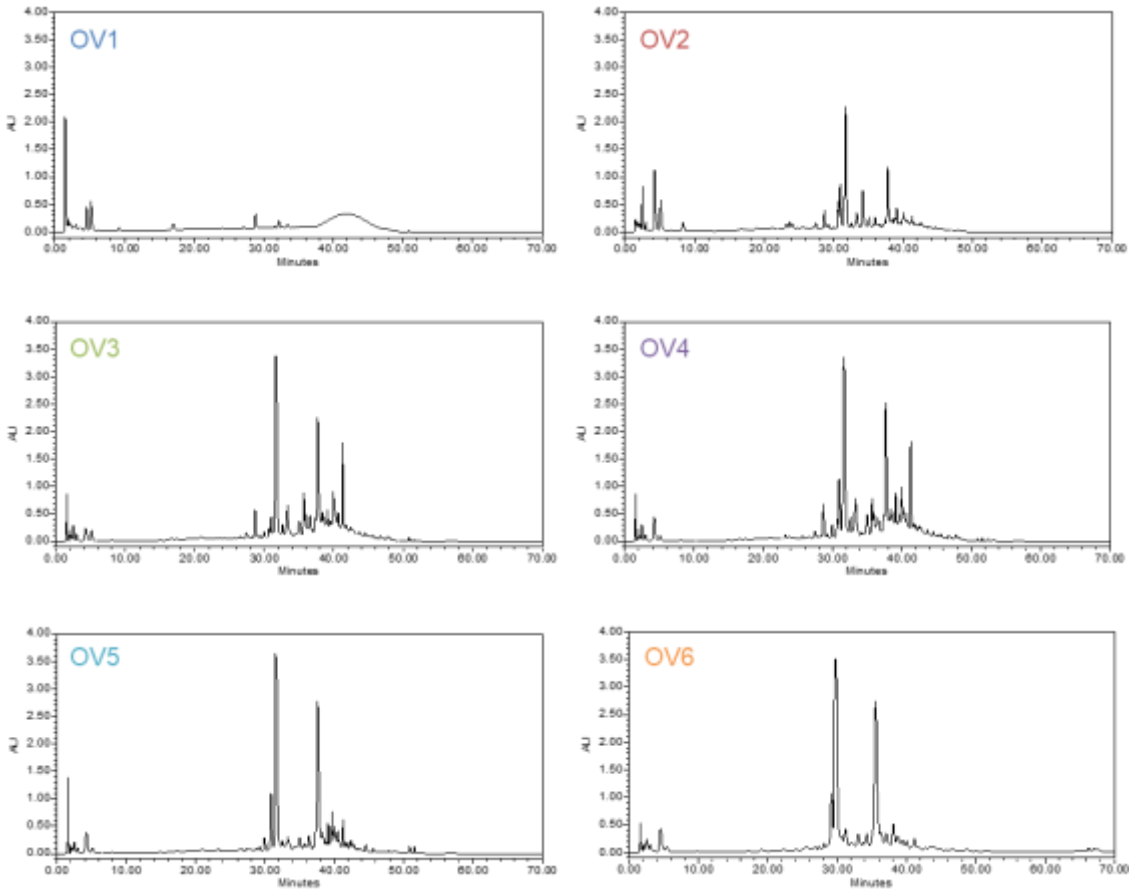
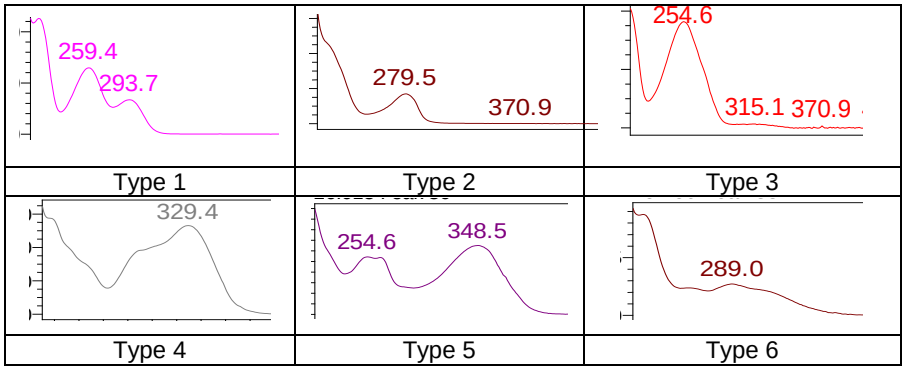


Figure 18. Chromatograms of the extracts (OV1–OV6) at MaxPlot**.** *x*–axis represents time (up to 70 min) and intensity of peaks (*y*–axis) was adjusted to 4.00 AUC for better comparison. AU: Area under curve (AUC).

The chromatograms showed visual differences between the extracts. OV1 showed less quantity and variety of compounds, something similar to what was previously observed in qualitative analysis by TLC. The OV2–OV6 extracts showed the same chromatographic profile with peaks in different proportions among them. The analysis of UV–spectrum of each peak showed six different types of chemical groups (**Table 5**).

Table 5. Types of UV–spectrum detected in chromatograms of *O.vulgare* extracts.



The 39 peaks detected in the extracts were grouped into the six groups based on their UV–spectrum: dihydroxybenzoic acids, syringic acids, essential oils, dihydroxycinnamic acids, flavonoids and salvianolic acids. **Table 6** compiles the results of the qualitative chemical fingerprint of HPLC–DAD. Minor peaks (low intensity) were excluded from the tables.

Table 6. Qualitative chemical characterisation by HPLC–DAD. For each peak – labeled with the peak number (First column)– the presence or absence in each extract, retention time, λ_{max} and type of compound is indicated. (X): presence of compound in extract; (–): absence of compound in extract. Sh:shoulder.

Peak	tR (min)	OV1	OV2	OV3	OV4	OV5	OV6	λ_{max} (nm)	Spectrum type	Type of compound
2	3.07	–	–	X	X	X	X	220.1, 249.9, 291.3	1	Dihydroxybenzoic acids
3	3.15	X	X	–	–	–	–	220.1, 251.1, 291.3	1	
6	5.60	X	X	X	X	X	X	220.2, 259.4, 293.7	1	
9	17.88	X	–	–	–	–	–	217.0, 259.4, 294.9	1	
21	29.88	–	X	X	X	X	X	217.0, 261.7, 294.9	1	
23	30.15	X	X	X	X	X	–	217.1, 261.7, 294.9	1	
1	2.56	–	X	X	X	X	X	215.5sh, 268.8	2	Syringic acids
4	4.63	–	X	X	X	X	X	220.5sh, 279.5	2	
5	4.19	X	–	–	–	–	–	221.7sh, 272.4	2	
10	19.07	–	–	–	–	X	X	213.4sh, 280.7	2	
11	22.63	–	X	X	X	–	–	220.3sh, 270.8	2	
13	24.22	–	X	X	X	–	–	220.5sh, 285.5	2	
18	28.76	–	X	–	X	–	X	220.5sh, 278.3	2	
27	32.90	–	–	X	X	X	X	220.5sh 284.3	2	
7	10.15	X	–	–	–	–	–	254.6	3	Essential oils
31	34.34	–	–	–	–	X	X	258.2	3	
8	17.50	–	X	X	X	X	X	325.1	4	Dihydroxycinnamic acids
28	33.28	X	–	X	X	–	–	316.3	4	
36	38.12	–	X	–	X	–	–	325.8	4	
37	38.21	–	–	–	–	X	X	324.6	4	
32	35.69	–	X	X	X	X	X	329.4	4	
12	22.76	X	–	–	–	–	–	267.6, 338.9	5	Flavonoids
17	27.50	X	X	X	X	X	X	260.5, 338.9	5	
20	29.54	X	–	–	–	–	–	266.5 366.6	5	
22	29.72	X	–	–	–	–	–	266.5 337.7	5	
25	31.24	–	–	–	–	X	X	268.8 340.1	5	
29	33.90	X	X	X	X	X	X	267.6 329.4	5	
30	34.07	X	X	X	X	–	–	267.6 330.6	5	
35	37.41	X		X				266.5, 337.7	5	
38	39.06	X	X	X	X			261.7, 340.1	5	
39	41.37	–	X	X	X	X	X	262.9, 318.7	5	
15	26.91	X	X	X	X	X	X	254.6 348.5	5	
16	27.08	X	X	X	X			254.6 348.5	5	
19	29.45		X	X	X	X	X	255.8 342.5	5	
24	31.48	X	X	X	X			251.1 340.1	5	
34	37.10	–	X		X		X	255.8 341.3	5	
14	25.76	–	X	X	X	X	X	289.0, 323.1sh	6	Salvianolic acids
26	33.26	–	X	–	–	–	–	284.2, 323.1sh	6	

The first type of peaks (λ_{max} = 220, 259.4, 293.7 nm), detected as a blue spot on TLC, correspond to dihydroxybenzoic acids, a type of phenolic acids already described in previous studies with *O. vulgare* [7,176]. One of them (peak **6**) was detected in all six extracts, while some others were present in only some of the extracts. Peak **2** was only detected in the ethanolic and hidroalcoholic extracts (OV3, OV4, OV5 and OV6) and peak **3** in the aqueous extracts (OV1 and OV2). Peak **9** was detected only in the extract with a different chemical profile (OV1). According to the retention time and the spectra (λ_{max} 225, 261, 294 nm), peak **23**, the highest of all, could be 3,4–dihydroxibenzoic acid, previously described in *O. vulgare* [182–184] and detected in OV1, OV2, OV3, OV4 and OV5. However, another technique will be needed to validate this identification.

The second type of UV peaks (λ_{max} = 220sh, 260–280 nm) corresponds to the syringic acids already described in *O. vulgare* [19,127,185,186]. Syringic acid is a phenolic compound, strictly named as 4–hydroxy–3,5–dimethoxybenzoic acid, synthetized from ferulic acid and caffeic acid by a series of enzymatic reactions in the shikimic acid pathway [186,187]. Thanks to its chemical structure, this cinnamic derivative has been shown to have a potentially antioxidant capacity [185,187,188]. Despite being a product of the shikimic acid pathway belonging to dyhydroxycinnamic acid derivatives, from the chemical point of view in this part of the work it was separated from other groups (type 4) by presenting a different UV–spectrum [187]. In total, eight derivatives of syringic acid were detected, of which peak **5** was only present in OV1, peak **18** in hot macerated extracts, peak **27** in ethanol and hydroacoholic extracts and peak **10** only in those extracted with ethanol (OV5 and OV6).

Type 3 of UV peaks (λ_{max} = 254 nm) could correspond to the typical essential oils of oregano: thymol and carvacrol [7,189]. Most of the published studies with oregano use essential oils as plant material, due to the important bioactivities of these essential oils [7,189–191]. Nevertheless, this group of compounds was only detected in OV1 (peak **7**) and in OV5 and OV6 (peak **31**), perhaps because they are volatile compounds.

In the group of dihidroxycinnamic acids (λ_{max} = 325–329 nm), the most important compounds were peak **8** and peak **32**, which could correspond to caffeic acid and rosmarinic acid, both previously described in *Lamiaceae* species [13,93,177,186,192]. These compounds could be related to the green–blue spot at Rf > 0.80 on TLC (mobile phase: ethyl acetate:glacial acetic acid:formic acid:water (100:11:11:26 v/v/v/v)) because of its absence in OV1.

Another chemical group present in oregano and *Lamiaceae* are flavonoids [193–195]. These secondary metabolites are generally present in glycosylated forms – with the main molecule attached to one or more sugars (glucose, galactose) [12,125,177,194,196]. In UV–

spectra (group type 5), these compounds are easily identified by two separated and characteristic shoulders. Fifteen flavonoids with two different types of spectra were detected in the six extracts. Peaks **15**, **16**, **19**, **24** and **34**, with λ_{max} = 254.6 and 348.5 nm, are characteristic of derivatives of luteolin [12,176], a type of flavonoid synthetized by oregano [177]. Peaks **12**, **17**, **20, 21**, **22**, **25**, **29, 30, 35, 38** and **39** could clearly corresponds to apigenin derivatives (λ_{max} = 267, 338 nm), whose presence in *O. vulgare* has already been reported [12,21,177,194,197].

Something similar happens with the last group of compounds (type 6: λmax = 289, 323sh nm), identified as salvianolic acids. These compounds have a complex chemical structure derived from rosmarinic acid, so from the chemical point of view in this part of the work they were separated from dihydroxicynamic acids (type 4) by presenting a different UV–spectrum. Two different salvianolic acids, peak **26** in OV2 and peak **14**, were detected in all extracts except OV1.This group of secondary metabolites was also previously found in oregano [7,21,175,198–200].

2.1.3 LC–MS: LIQUID CHROMATOGRAPHY WITH MASS ANALYSIS

After separating compounds from a sample by liquid chromatography (HPLC–DAD), highly sensitive instrumental analytical techniques, such as mass spectrometry (LC–MS), can be applied for the identification of individual compounds. This technology is based on the ionization of the separated compounds to obtain structural information [201]. A large number of secondary metabolites are glycosylated compounds attached to sugars and the fragmentation by LC–MS allows revealing the main structure and the compounds attached, making it a useful technique for phytochemical identification of compounds extracted from plants.

2.1.3.1 MATERIAL AND METHODS

This part of the work was carried out during an international research stay at University of Lisbon. Conditions of the method applied were: column Nova–Pack® C18 100 Å (150 x 2.1 mm, 1.7 µm) as Stationary phase (SP), a gradient with (A) distilled water (0.1 % formic acid) and acetonitrile (B) (0.1 % formic acid) (**Table 7**) as mobile phase (MP), temperature 25.0 ± 5.0 °C, flow 0.8 mL/min, and a 10 µL of sample (0.5 mg/mL in water) in a Ultimate 3000 RSLCnano system (Thermo Fischer Scientific, Idstein, Germany) interfaced with a quadrupole time–of–flight (QqToF) Impact II mass spectrometer equipped with an electrospray source (Bruker Daltonics, Bremen, Germany) [202].

Table 7. Gradient composition used as mobile phase in LC–MS.

Time (min)	Flow (mL/min)	% of distilled water (0.1 % formic acid)	% of acetonitrile
0	0.8	95	5
1.5	0.8	95	5
13	0.8	25	75
18	0.8	0	100
21	0.8	0	100
23	0.8	95	5
30	0.8	95	5

As in previous studies, high–resolution mass spectra were acquired in the electrospray ionization (ESI) positive/negative modes [202]. Optimized parameters were set as ion spray voltage, +4.5/−2.5 kV; end plate offset, 500 V, nebulizer gas (N2), 2.8 bars; dry gas (N2), 8 L/min; dry heater, 200 °C. Internal calibration was performed in High–Precision Calibration (HPC) mode with a solution of sodium formate 10 mM introduced into the ion source via a 20 µL loop at the beginning of each analysis using a six–port valve. Acquisition was performed in full–scan mode in the *m/z* 50–1300 range, and in a data–depending MS/MS mode with 3 Hz acquisition using a dynamic method with a fixed cycle time of 3 s. The duration of dynamic exclusion was 0.4 min.

The acquired data were processed by Data Analysis 4.1 software (Bruker Daltoniks, Bremen, Germany). The peaks were automatically numbered and the mass of the fragmentation was compared with the data obtained from the PubChem online database [203].

2.1.3.2 RESULTS

The six extracts were subjected separation and identification by LC–MS. The chromatograms obtained were calibrated with solvent but some peaks corresponding to the solvent (water) could not be eliminated from the chromatogram, although finally they were not taken into account for their identification.

Table 8 shows –extract by extract– the identification of the peaks that have been assigned by colours based on the chemical group of the compound identified in HPLC–UV: green for dihydroxycinnamic acids (caffeic acid, rosmarinic acid and derivatives), darker blue for those identified as syringic acid and derivatives, light blue for dihydroxybenzoic acid derivatives, orange for flavonoids (such as luteolin derivatives) and yellow for those identified as salvianolic acid derivatives.

Table 8. Qualitative chemical characterisation by LC–MS. For each peak – labeled with retention time (First column) – is indicated the presence or absence in each extract, molecular mass ($[M–H]^-$ (*m/z*)), fragment ions (*m/z*), name of compound, molecular formula and type of compound. (X): presence of compound in extract; (–): absence of compound in extract; Sh: shoulder.

tR (min)	OV1	OV2	OV3	OV4	OV5	OV6	$[M–H]^-$ (*m/z*)	Fragment ions (*m/z*)	Name	Formula	Type of compound
1.2	X						191.04	–	Quinic acid	$C_7H_{12}O_6$	
4.0	X						153.01	–	2,5–Dihydroxybenzoic acid	$C_7H_6O_4$	Dihydroxybenzoic acids (DHBAs)
6.5		X	X	X	X	X	153.01	–	3,4–Dihydroxybenzoic acid	$C_7H_6O_4$	
3.4			X	X	X	X	197.03	–	Syringic acid	$C_9H_{10}O_5$	Syringic acids
3.7		X	X	X	X	X	359.08	197.04	Syringic acid–4–beta–D–glucopyranoside	$C_{15}H_{20}O_{10}$	
0.9		X	X	X	X		341.07	179.03, 149.04, 96.95	Caffeic acid 4–alpha–D–glucopyranoside	$C_{15}H_{18}O_9$	Dihydroxycinnamic acids
1.1		X	X	X	X	X	179.05	–	Caffeic acid (3,4–Dihydroxycinnamic acid)	$C_9H_8O_4$	
1.1	X						367.09	191.04, 189.03, 165.03	3–O–Caffeoylquinic acid methyl ester	$C_{17}H_{20}O_9$	
1.3		X	X	X	X		353.10	191.01	Chlorogenic acid	$C_{16}H_{18}O_9$	
1.4						X	879.05	179.05, 96.95	Caffeic acid tetramer glucoside	$C_{42}H_{40}O_{21}$	
2.8		X					319.60	179.03, 137.02	4–O–(*p*–coumaroyl)shikimic acid	$C_{16}H_{16}O_7$	
7.3			X				167.06	123.04	Homogentisic acid	$C_8H_8O_4$	
7.4		X	X	X	X	X	359.06	–	Rosmarinic acid	$C_{18}H_{16}O_8$	
7.9			X	X	X	X	357.06	311.04, 193.04	Ferulic Acid (*p*–Coumaric Acid)	$C_{19}H_{18}O_7$	
2.4		X	X	X	X	X	609.17	301.80	Rutin	$C_{27}H_{30}O_{16}$	Flavonoids
2.5			X	X	X		629.13	315.06, 96.95	Isorhamnetin 3–(6''–galloylglucoside)	$C_{29}H_{26}O_{16}$	
5.6	X	X	X	X	X	X	387.15	445.11	Quercetin oxalate	$C_{17}H_8O_{11}$	
5.8	X	X	X	X	X	X	739.05	659.07, 285.03	Kaempferol–3–galactoside–6–rhamnoside–3–rhamnoside	$C_{33}H_{40}O_{19}$	
6.6	X		X	X	X	X	449.19	377.04, 287.05 153.01	Eriodictyol–7–*O*–glucoside	$C_{21}H_{22}O_{11}$	
8.0						X	653.14	343.07, 96.95	Syringetin 3–rutinoside	$C_{29}H_{34}O_{17}$	
9.2	X		X	X	X	X	447.05	327.21	Orientin (luteolin–8–*C*–glucoside)	$C_{21}H_{20}O_{11}$	
9.4			X	X	X		447.09	357.78, 327.22	Homoorientin (Luteolin–6–*C*–glucoside)	$C_{21}H_{20}O_{11}$	
6.3		X	X	X	X	X	537.09	358.06, 135.04	Salvianolic acid H	$C_{27}H_{22}O_{12}$	Salvianolic acids
6.8			X	X	X	X	717.12	553.08, 419.21, 358.06	Salvianolic acid B derivate	$C_{36}H_{30}O_{16}$	
7.1			X	X	X	X	717.12	421.1, 358.06	Salvianolic acid derivate	$C_{36}H_{30}O_{16}$	
7.3			X	X	X		987.22	451.11	Dimer of salvianolic acid	$C_{52}H_{44}O_{20}$	
7.6			X	X	X	X	717.12	553.08, 419.21, 358.06	Salvianolic acid B isomer I (F)	$C_{36}H_{30}O_{16}$	

The OV1 extract showed fewer detectable peaks than the rest of extracts. As mentioned above, dihydroxybenzoic acids (DHBAs), syringic acids, dihydroxicinnamic acids and salvianolic acids are obtained through the sikimic acid pathway in plants (**Figure 19**); but they were considered as different groups in the interpretation of the results of this study with *O. vulgare*.

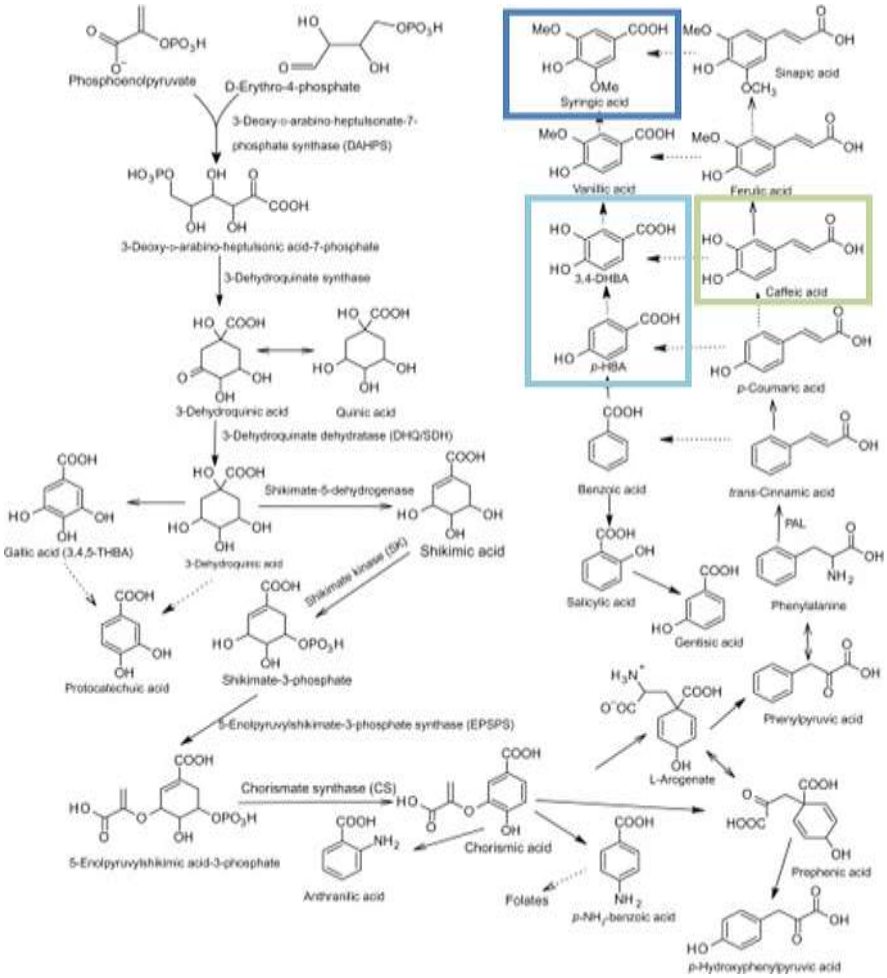


Figure 19. Shikimic acid pathway in plants. DHBA (in light blue), Caffeic acid (in light green) and Syringic acid (in marine blue) are products of this route [204].

The first type of compounds detected in LC–MS were caffeoylquinic acids, 3–*O*–caffeoylquinic acid methyl ester with a retention time of 1.1 min in OV1. At the same retention time, caffeic acid (peak **8**) was detected in OV2–OV6 and chlorogenic acid, a combination of caffeic acid and quinic acid, at 1.3 min in OV2–OV5 (**Figure 20**).

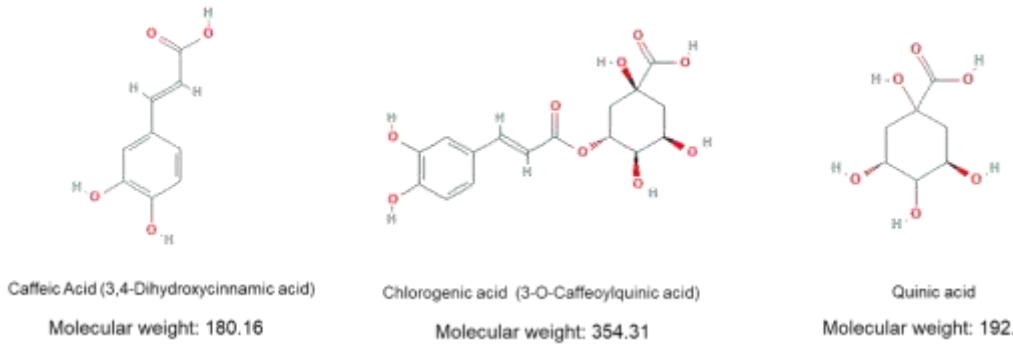


Figure 20. Chemical structures and molecular weight for caffeic acid, chlorogenic acid and quinic acid, drawn with LC–MS processing data software Compass Data Analysis (Bruker Daltonik GmbH).

OV6 also did not contained chlorogenic acid (1.3 min, *m/z* = 354.31), but this extract showed a more complex form of caffeic acid at 1.4 min (caffeic acid tetramer glucoside, *m/z* = 880.80). Another glycosylation of caffeic acid was also observed at 0.9 min in OV2, OV3, OV4 and OV5 (*m/z* = 342.30 with *m/z* fractionation = 179.03, 149.04, 96.95) (**Figure 21**).

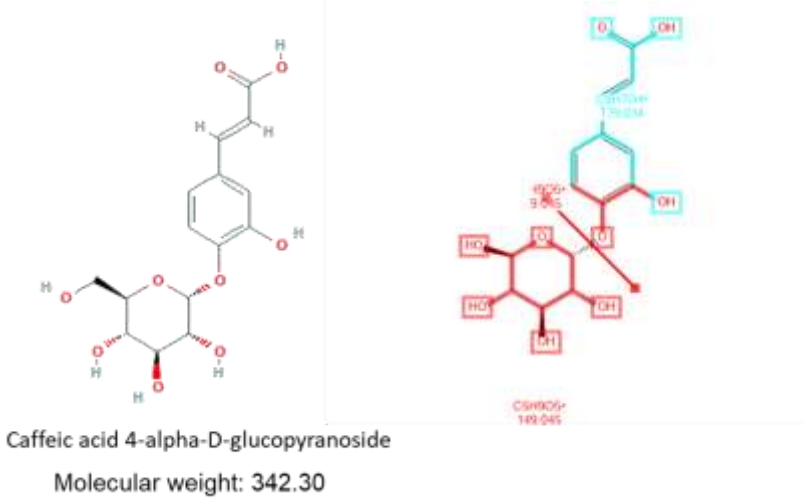


Figure 21. Chemical structures and fractioning of caffeic acid 4–alpha–D–glucopyranoside, drawn with LC–MS processing data software Compass Data Analysis. In blue, caffeic acid (*m/z* = 179.034 g/mol). In red, sugar (*m/z* = 149.045 g/mol).

The absence of these compounds in OV1 and OV6 could be related to a chemical transformation during preparation of extracts (effect of hot ethanol or a long time in water). Coffee is a chemically deeply studied plant whose major compounds are caffeolquinnic acids [205–207]. Some previous studies have shown chemical changes in the structure of these compounds by increasing the temperature and using organic solvents as ethanol [205,208,209]. In fact, chlorogenic acid has been shown to be formed water from trans–5–*O*–caffeoylquinic acid during the process of extraction with alcohol or a mixture alcohol [208]. Thus, the primary caffeoylquinic acid present in oregano could be the simplest form found in OV1 (3–*O*–caffeoylquinic acid and quinic acid) that were transformed into the chlorogenic acid present in the rest of the extracts. OV1 might be the poorest extract in terms of chemical composition, but also the most primary extract because the lack of temperature and organic solvents in its extraction process could be so harmless that it did not even extract some of main compounds.

Rosmarinic acid, whose name derives from *Rosmarinus officinalis* L. [210], has been identified as one of the most active compounds in several plants from *Lamiaceae* family, like rosemary and oregano [186,211]. Its identification in HPLC–DAD (**32**) and LC–MS (*m/z* = 359.06) leaves no doubt thanks to the extensive literature on this phytocompound [176,177,192,210,212].

The absence of rosmarinic acid (7.4 min, *m/z* = 360.30) in OV1 could also corroborate the hypothesis of the low capacity of cold aqueous maceration to extract caffeoylquinic acids, even after four days. Depending on plant chemistry and metabolism, rosmarinic acid is formed from an ester of caffeic acid and 3,4–dihydroxyphenyllactic acid (**Figure 22**) in the shikimic acid pathway. In this sense, the extraction process used to obtain OV1 was not effective for the main caffeoylquinic acid derivatives present in the plant raw material.

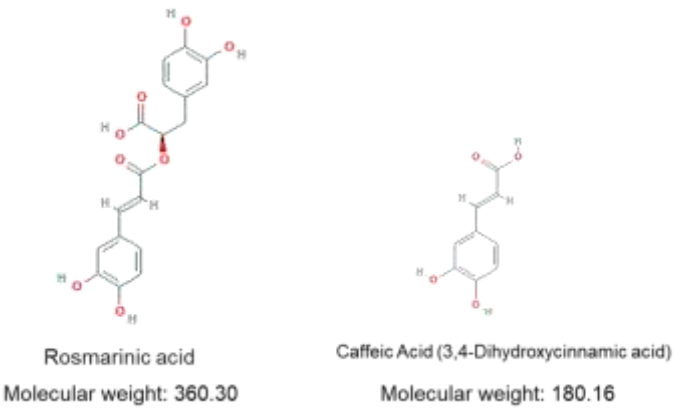


Figure 22. Chemical structures and molecular weight of Rosmarinic acid and Caffeic acid, drawn with LC–MS processing data software Compass Data Analysis.

Another type of compounds from the shikimic acid pathway (**Figure 22**) that can be result of the transformation of caffeic acid found in the extracts were dihydroxybenzoic acids. According to the retention times and fragmentation, two dyhydroxybenzoic acids were identified, 2,5–dihydroxybenzoic acid and 3,4–dihydroxybenzoic acid (**Figure 23**).

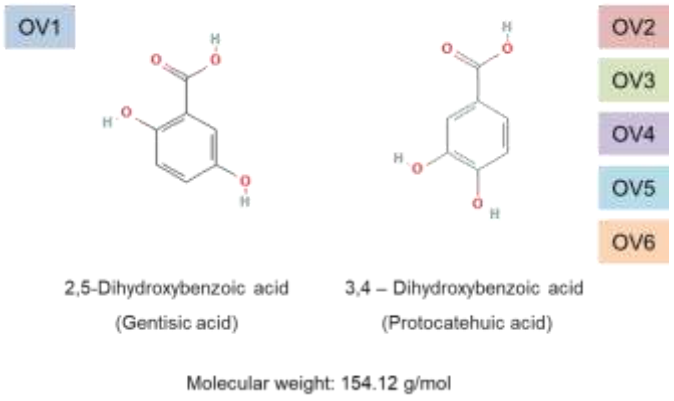


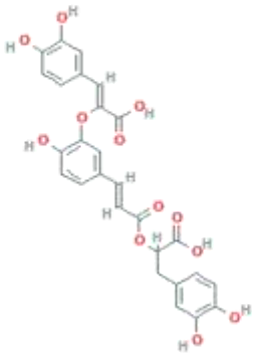
Figure 23. DHBA with same molecular weight found in the extracts. On left, 2,5–DHBA (Gentisic acid), present in OV1 (blue). On right, 3,4–DHBA (Protocatehuic acid) present in rest of extracts: OV2 (red), OV3 (green), OV4 (purple), OV5 (light blue) and OV6 (orange).

Again, OV1 showed a different chemical composition than the rest of the extracts. The positions of the two hydroxyl groups at C2 and C5 were favored in OV1, while the C3 and C4 positions were adopted in the DHBA form found in the rest of the extracts (**Figure 23**).

Both compounds showed the same *m/z* but different retention times (4.0 min and 6.5 min, respectively). This information is not enough to distinguish one from another. Final identification was carried out by co–injection of 3,4–DHBA standard (#D109800, Sigma–Aldrich Co., St. Louis, MO). Finally, peak **9** corresponds to 2,5–dihidroxybenzoic acid and peak **21** to 3,4–dihydroxibenzoic acid. The rest of the compounds detected as dihidroxybenzoic acids in HPLC–DAD characterization were not identified by LC–MS.

Nevertheless, compounds with type 2 spectra, syringic acids, on HPLC–DAD (λ_{max} = 220sh, 260 – 280 nm) were identified on LC–MS at 3.4 min and 3.7. In HPLC–DAD, practically no syringic acid and derivatives were found in OV1, only peak **5** without presence in the rest of extracts. Here, compounds identified as syringic acid (at 3.4 min, *m/z* 198.03) and a glycosylated variation, syringic acid–4–beta–D–glucopiranoside (at 3.7 min, *m/z* 360.31), were detected. Syringic acid corresponds to peak **27** in HPLC–DAD, and the glycosylated form may be either peak **4** or peak **1**.

The next group in the shikimic acid pathway were salvianolic acids (λ_{max} = 289, 323sh nm), previously reported in oregano [198]. From a chemical point of view, they could be considered a large and complex group (chemically related to rosmarinic acid, **Figure 24**) whose names are attributed with letters instead of radicals position: salvianolic acid A, B, E… In LC–MS, five salvianolic acids were identified. These compounds were absent in OV1 –as expected to be a rosmarinic derivate– and some of them in OV2. Peak **14** was detected in OV2–OV6, which could potentially be salvianolic acid H (at 6.3 min, *m/z* = 538.50). At 7.6 min, salvianolic acid B isomer I (F) was also identified.



Salvianolic acid H

3'-O-(8''-Z-caffeoyl) **rosmarinic acid**
(2Z)-2-{5-[(1E)-3-[1-carboxy-2-(3,4-dihydroxyphenyl)ethoxy]-3-oxoprop-1-en-1-yl]-2-hydroxyphenoxy}-3-(3,4-dihydroxyphenyl)prop-2-enoic acid

Molecular weight: 538.50

Figure 24. Salvianolic acid H structure, with molecular weight and chemical name given for better association to rosmarinic acid.

The last group were the flavonoids. Eight of fifteen flavonoids detected by HPLC–DAD were identified as rutin (peak **19**, *m/z* = 610.50) [192,213], isorhamnetin 3–(6–galloylglucoside) (*m/z* = 630.50) [214], quercetin oxalate (*m/z* = 388.20) [194], kaempferol–3–*O*–galactoside–6–*O*–rhamnoside–3–*O*–rhamnoside (*m/z* = 740.70) [215], eridictyol–7–*O*–glucoside (*m/z* = 450.40) [216,217], syringetin 3–rutinoside (*m/z* = 654.30) [214] and two glycosylates of luteolin: orientin (*m/z* = 448.40) [214] and homorientin (*m/z* = 448.40) [214]. Some of them were previously described in extracts of *O. vulgare* [12,21,127,179,194,211], being luteolin glycosylate one of the most common flavonoids [176,194].

Differences on chemical composition of the six extracts were also observed in LC–MS. Although the separation conditions of the compounds in liquid chromatography were different (the mobile phase in LC–MS was different from that used in HPLC–DAD and TLC) and their performance in different groups and equipment (LC–MS in Lisbon and HPLC–DAD in Pamplona), the chemical groups found were in agreement although the number of peaks observed were higher in HPLC–DAD. However, the objective of the chemical characterization of the six extracts lies more in the comparison among them, than in a deeper identification of compounds with a precise chemical structure. In further studies, with the extract that is more bioactive and of interest for possible clinical trials, a deeper chemical identification with more sensitive equipment would be recommended.

2.2 CHEMICAL QUANTIFICATION

2.2.1 TOTAL PHENOLIC COMPOUNDS *IN VITRO* TEST

As a complement to the qualitative chemical characterisation, the Folin–Ciocalteu assay [177] can be carried out to determine the Total Phenolic Compounds (TPC) of a sample. Phenolic compounds are secondary metabolites present in a wide range of medicinal plants with a chemical structure capable to act as an H donor, making them potentially antioxidant compounds [218]. In this analysis it will quantify the amount of TPC in terms of gallic acid in each of the extracts.

2.2.1.1 MATERIAL AND METHOD

- Experimental basis

Total phenolic compounds (TPC) were spectrophotometrically quantified following the Folin–Ciocalteu colorimetric method [177]. In this assay, phenolic compounds are oxidized in an alkaline medium by the Folin–Ciocalteu reagent (composed of a mixture of phosphowalframic acid, $W_{12}O_{40}H_3P$, and phospholimbidic acid, $H_3PMo_{12}O_{40}$) producing a

reduced mixture of blue oxides of tungsten and molybdenum (**Figure 25**) that can be quantified at 765 nm.

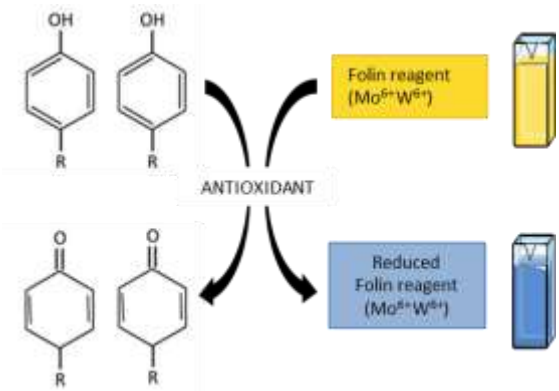


Figure 25. Chemical reaction and scheme of the Folin–Ciocalteu *in vitro* assay. A phenolic compound produces a change in coloration of Folin reagent (yellow) to blue, which can be measured by absorbance at 765 nm.

- Sample and reagent preparation

The extracts were dissolved in distilled water at 1 mg/mL. For the reaction, 15 µL of sample were mixed with 75 µL of Folin–Ciocalteu reagent (# 47641, Sigma–Aldrich Co., St. Louis, MO) allowing to react for 2 min. Distilled water was used as a blank sample.

Then, 225 µL of Na_2CO_3 and 1,185 µL of distilled water were added and, after shaking, the mixture was incubated at room temperature for 2 h.

- Analyse conditions and data process

In a 96–well plate, 300 µL of the solution were disposed per well, and the absorbance at 765 nm was monitored.The absorbance was transformed into µg of gallic acid per mg of lyophilized extract by extrapolation from a previously obtained calibration curve (y = 0.001 x + 0.0038, R^2 = 0.999, where y corresponds to absorbance and x to gallic acid concentration).

- Statistical analysis

This assay was performed in triplicate. Normality of results was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by a post hoc pairwise comparison test using Tukey's method (95 % CL) or post–estimation margins to check interaction between groups.

2.2.1.2 RESULTS

Previously on TLC (**Figure 17**), the results with NP at 366 nm showed blue and yellow spots in some of the extracts at different Rfs, which are characteristic for phenolic acids and flavonoids, both phenolic compounds. Here, the TPCs were determined for the six

extracts and the quantitative results were expressed in terms of gallic acid (µg per mg of lyophilised extract) (**Figure 26**).

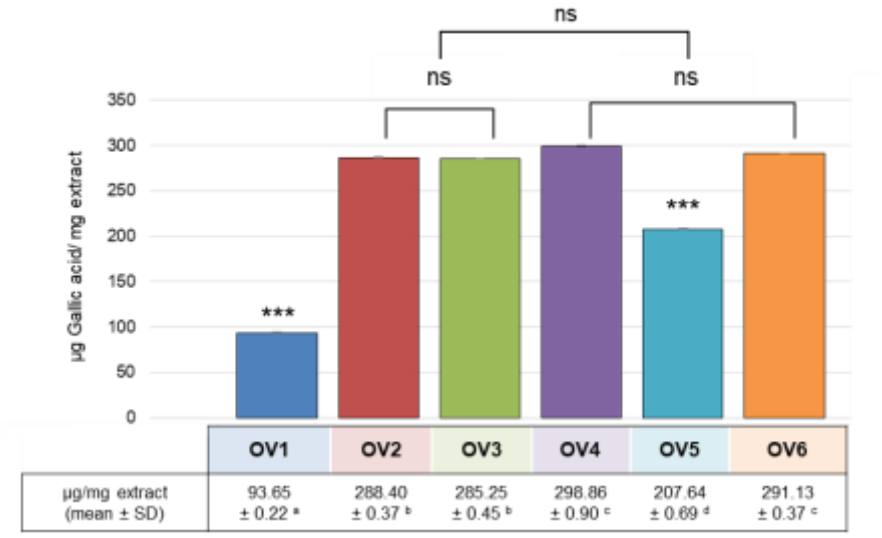


Figure 26. Total Phenolic Compounds (TPC) quantitative results of the six extracts expressed in terms of quantity of gallic acid (GA) per mg of extract (mean ± SD µg GA/mg extract). In graph, *ns* indicates non–statistical differences and ***, $p < 0.001$. In table below, values with different letters show significant differences ($p < 0.05$) and the same letter indicates that there are no significant differences ($p > 0.05$).

OV1 was the extract with the lowest amount of phenolic compounds, 93.65 ± 0.22 µg/mg ($p < 0.001$). This value correlates with the results observed on TLC where neither yellow nor blue spots were observed. OV5 showed 207.64 ± 0.69 µg /mg ($p < 0.001$). OV2 and OV3 had 286.40 ± 0.37 and 285.25 ± 0.45 µg/mg, respectively, with no statistically differences between them ($p = 0.971$). OV4 and OV6 were the extracts with a highest amount of phenolic compounds, 298.86 ± 0.90 and 291.13 ± 0.37 µg /mg, respectively) with $p = 0.686$ between them. Previous studies with oregano also determined TPC of the extracts [213,219], with results that, sometimes, were not similar to those obtained in this work. For example, a extract similar as OV6 was studied [177] and the TPC value was 315.82 ± 29.23 µg/mg, a result very similar to that obtained in this work. However, in a study with *O. vulgare* in Serbia [126] the water extract showed a higher TPC than the ethanol extract, results just opposite to those obtained here. To explain these differences, it is important to highlight that the chemical composition of an extract varies depending on the plant material, the growing conditions and the preparation. In that study [126], the extract preparation ratio was 4:1 for the aqueous extract, while OV1 was obtained at 3:1.

2.2.2 HPLC–DAD QUANTIFICATION

HPLC–DAD can be used for the identification of individual compounds or groups of compounds. The intensity of the peaks can also be measured through their area under the curve (AUC), providing at least semi–quantitative information.

2.2.2.1 MATERIAL AND METHODS

Semi–quantification was performed with the AUC of each peak to obtain the phenolic composition of each sample. To translate the AUCs into amounts of reference compounds, several standards were selected based on their presence in the extracts and calibration curves were constructed with standard samples (caffeic acid –#C0625–, rosmarinic acid –#R4033–, luteolin –#L9283– and 3,4–dihydroxybenzoic acid –# D109800–, all from Sigma–Aldrich Co., St. Louis, MO) at five different concentrations (**Table 9**). Thus, the AUCs of the main peaks of the extracts were expressed in terms of mg of the standard compound per 100 mg of extract by linear regression analysis. The reproducibility and complete validation of the process was previously performed in the department. All samples were injected in triplicate. Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA).

Table 9. Calibration curves of standard chemical compounds with the formula for extrapolation and correlation factor (R^2). The second column indicates compounds quantified in terms of the correspondent standard: luteolin for flavonoid derivatives, caffeic acid for dyhydroxycinnamic acid and salvianolic acid derivatives, rosmarinic acid for itself and 3,4–DHBA for hydroxybenzoic acid and syringic acid derivatives. x = AUC of the corresponding peak and y= mg compound/100 mg of dry extract.

Chemical group/compound		Type of compounds	Calibration curve	Correlation factor
Flavonoid	Luteolin	Flavonoid derivates	$y = 1.17 \cdot 10^8 x + 465{,}907$	R^2 = 0.9999
Phenolic acids	Caffeic acid	Dihydroxycinnamic acids Salvianolic acids	$y = 1.58 \cdot 10^8 x - 137{,}425$	R^2 = 0.9998
	Rosmarinic acid	Rosmarinic acid	$y = 3.84 \cdot 10^7 x + 88{,}581$	R^2 = 1.0000
	3,4–DHBA	Dihydroxybenzoic acids Syringic acids	$y = 4.35 \cdot 10^8 x - 57{,}118$	R^2 = 0.9988

2.2.2.2 RESULTS

This calculation was carried out by means of a previous categorization of the peaks according to the group to which they belong according to the UV spectrum. Only groups found in both HPLC–DAD and LC–MS were considered for quantification. For example, essential oils typical from oregano were not detected in LC–MS and showed low intensity peaks in HPLC–DAD so they were not considered in the chemical semi–quantification. Results are shown in **Tables 10** to **13**.

Flavonoids were all pooled together and expressed in terms of luteolin (**Table 10**). Characteristic signals of luteolin had previously been observed in UV spectra and 25 % of the compounds detected in LC–MS were also luteolin derivatives . Thus, the use of a single flavonoid for this quantification simplifies the analysis of the results and facilitates a standardization that can be useful for subsequent studies.

Table 10. Quantification of individual flavonoids in extracts (mg/100 mg dry extract).

Type	Peak	OV1	OV2	OV3	OV4	OV5	OV6
Flavonoids	**12**	0.15 ± 0.01	–	–	–	–	–
	15	1.42 ± 0.09	1.93 ± 0.05	1.99 ± 0.06	0.18 ± 0.05	0.08 ± 0.01	1.65 ± 0.02
	16	1.73 ± 0.11	4.22 ± 0.05	2.64 ± 0.03	0.89 ± 0.02	–	–
	17	0.24 ± 0.02	2.32 ± 0.03	1.80 ± 0.05	2.05 ± 0.02	0.26± 0.05	0.46 ± 0.02
	19	–	7.05 ± 0.02	9.35 ± 0.02	8.06 ± 0.03	0.36 ± 0.03	11.32 ± 0.11
	20	1.08 ± 0.05	–	–	–	–	–
	22	2.95 ± 0.06	–	–	–	–	–
	24	1.79 ± 0.05	2.72 ± 0.02	2.70 ± 0.03	1.97 ± 0.03		–
	25					5.68 ± 0.02	1.67 ± 0.03
	29	1.37 ± 0.03	4.20 ± 0.03	3.02 ± 0.02	3.57 ± 0.01	1.87 ± 0.02	5.54 ± 0.02
	30	1.00 ± 0.02	2.21 ± 0.01	7.09 ± 0.01	6.46 ± 0.04	–	–
	34	–	1.49 ± 0.08	–	3.29 ± 0.01	–	5.67 ± 0.02
	35	0.57 ± 0.01	–	2.68 ± 0.01	–	–	–
	38	1.23 ± 0.02	3.33 ± 0.10	3.41 ± 0.02	5.43 ± 0.01	–	–
	39	–	3.36 ± 0.02	1.08 ± 0.03	2.01 ± 0.02	7.31 ± 0.01	7.15 ± 0.04
Total		**13.53 ± 0.47**	**32.83 ± 0.41**	**37.76 ± 0.28**	**33.81 ± 0.24**	**15.56 ± 0.14**	**33.46 ± 0.26**

OV1 and OV5 were the extracts with the lowest flavonoid content (13.53 ± 0.47 and 15.56 ± 0.14 mg/100 mg, respectively). OV2, OV3, OV4 and OV6 showed a high concentration, 32.83 ± 0.41, 7.76 ± 0.28, 33.81 ± 0.24, and 3.46 ± 0.26 mg/100 mg, respectively. Rutin (peak **19**), absent in OV1, was the most abundant in OV2 (7.05 ± 0.02 mg), OV3 (9.35 ± 0.02), OV4 (8.06 ± 0.03 mg) and OV6 (11.32 ± 0.11 mg). Peak **39** was higher in OV5 (7.31 ± 0.01 mg) and OV6 (7.15 ± 0.04 mg) compared to OV2 (3.36 ± 0.02 mg), OV3 (31.08 ± 0.03 mg) and OV4 (2.01 ± 0.02 mg).

Table 11 shows the quantification of the compounds identified as hydroxycinnamic acid and salvianolic acid derivatives, quantified in terms of caffeic acid. OV5 (11.14 ± 0.15 mg) and OV4 (9.24 ± 0.11 mg) were the more abundant extracts in dihydroxycinnamic acids. This group was not abundant in OV1 (1.73 ± 0.08 mg), with 3–*O*–caffeoylquinic acid methyl ester (peak **28**) being the only quantifiable compound. OV3 and OV4 showed a similar amount of caffeic acid (peak **8**); 1.87 ± 0.14 mg and 1.67 ± 0.05 mg, respectively, as well as the ethanolic extracts (OV5 = 0.51 ± 0.05 and OV6 = 0.63 ± 0.06 mg).

Among salvianolic acids, salvianolic acid H (peak **14**) was the most important compound in OV3, OV4, OV5 and OV6 (3.35 ± 0.02 mg, 2.65 ± 0.07 mg, 3.13 ± 0.20 mg and 3.45 ± 0.14 mg, respectively). Peak **26** (no identified), only in OV2, showed the highest concentration of all (5.56 ± 0.09 mg).

Table 11. Quantification of dyhydroxycinnamic acids and salvianolic acid derivatives (mg/100 mg dry extract).

Type	Peak	OV1	OV2	OV3	OV4	OV5	OV6
Dihydroxycinnamic acids	8	–	1.07 ± 0.02	1.87 ± 0.14	1.67 ± 0.05	0.51 ± 0.05	0.63 ± 0.06
	28	1.73 ± 0.08	–	0.47 ± 0.05	0.45 ± 0.03	–	–
	36	–	3.35 ± 0.02	–	7.12 ± 0.03	–	–
	37	–	–	–	–	10.63 ± 0.10	1.32 ± 0.03
Total		**1.73 ± 0.08**	**4.42 ± 0.04**	**2.34 ± 0.19**	**9.24 ± 0.11**	**11.14 ± 0.15**	**1.95 ± 0.09**
Salvianolic acids	14	–	1.70 ± 0.08	3.35 ± 0.02	2.65 ± 0.07	3.13 ± 0.20	3.45 ± 0.14
	26	–	5.56 ± 0.09	–	–	–	–
Total		**–**	**7.26 ± 0.17**	**3.35 ± 0.02**	**2.65 ± 0.07**	**3.13 ± 0.20**	**3.45 ± 0.14**

Table 12 shows the amount of rosmarinic acid found in each extract (peak **32**). Despite being a derivate of hydroxycinnamic acid, it was treated individually due to its remarkable presence, which could be used as standardization of the *O. vulgare* extraction method.

In OV1, rosmarinic acid was not detected. The rest of extracts presented the same profile as the flavonoids, OV5 showed the lowest concentration (24.21 ± 1.08 mg) and the concentration in OV2, OV3, OV4 and OV6 was similar with values between 32.82 ± 0.02 mg and 36.13 ± 1.07 mg.

Table 12. Quantification of rosmarinic acid (mg/100 mg dry extract).

Type	Peak	OV1	OV2	OV3	OV4	OV5	OV6
Rosmarinic acid	32	–	35.58 ± 1.20	34.10 ± 0.04	32.82 ± 0.05	24.21 ± 1.08	36.13 ± 1.07
Total		**0.00 ± 0.00**	**35.58 ± 1.20**	**34.10 ± 0.04**	**32.82 ± 0.05**	**24.21 ± 1.08**	**36.13 ± 1.07**

Table 13 shows results of quantification of peaks identified as dyhydroxibenzoic acids and syringic acids, expressed as mg of 3,4–DHBA per 100 mg of extract. OV3 (21.72 ± 0.15mg) and OV4 (19.24 ± 0.15mg) were the more abundant extract in dihydroxybenzoic acids. 2,5–dihydroxibenzoic acid (peak **9**) was the most important compound in OV1 (6.81 ± 0.03 mg), 3,4–dihydroxibenzoic acid (peak **21**) in OV2, OV3, OV4 and OV6, and peak **23** (no identified) in OV5.

About syringic acids, the most abundant compound in all extracts (except OV1 and OV2) was syringic acid (peak **27**) with 7.85 ± 0.02 mg (OV3), 2.34 ± 0.01 mg (OV4), 1.36 mg ± 0.05 mg (OV5) and 3.01± 0.08 mg (OV6).

Table 13. Quantification of dihydroxibenzoics and syringic acids (mg/100 mg dry extract).

Type	Peak	OV1	OV2	OV3	OV4	OV5	OV6
Dyhydroxybenzoic acids	2	–	0.52 ± 0.01	0.92 ± 0.02	0.70 ± 0.02	0.75 ± 0.01	0.95 ± 0.01
	3	1.17 ± 0.02	1.05 ± 0.03	–	–	–	–
	6	0.32 ± 0.01	2.04 ± 0.01	1.57 ± 0.02	0.41 ± 0.08	0.45 ± 0.01	0.51 ± 0.05
	9	6.81 ± 0.03	–	–	–	–	–
	21	–	9.55 ± 0.01	16.96 ± 0.07	15.20 ± 0.03	0.17 ± 0.01	16.30 ± 0.08
	23	0.90 ± 0.05	3.04 ± 0.02	2.27 ± 0.04	2.93 ± 0.02	3.86 ± 0.01	
Total		**9,20 ± 0.11**	**16.20 ± 0.08**	**21.72 ± 0.15**	**19.24 ± 0.15**	**5.23 ± 0.04**	**17.76 ± 0.14**
Syringic acids	1	–	0.34 ± 0.01	1.19 ± 0.02	1.09 ± 0.02	0.48 ± 0.08	0.52 ± 0.03
	4	–	0.49 ± 0.02	0.65 ± 0.03	0.54 ± 0.05	0.62 ± 0.04	0.72 ± 0.02
	5	4.69 ± 0.05	–	–	–	–	–
	10	–	–	–		0.40 ± 0.07	0.65 ± 0.02
	11	–	1.44 ± 0.02	0.70 ± 0.02	0.51 ± 0.01	–	–
	13	–	1.84 ± 0.03	1.60 ± 0.02	0.49 ± 0.02	–	–
	18	–	1.09 ± 0.03		2.17 ± 0.11	–	1.92 ± 0.01
	27	–	–	7.85 ± 0.02	2.34 ± 0.01	1.36 ± 0.05	3.01 ± 0.08
Total		**4.69 ± 0.05**	**5.20 ± 0.11**	**11.99 ± 0.11**	**7.14 ± 0.22**	**2.86 ± 0.24**	**6.82 ± 0.16**

All these values are adequately correlated with the quantification results with the TPC analysis by Folin–Ciocalteu *in vitro* assay (**Table 14**).

Table 14. TPC from Folin–Ciocaltaeu and quantification by integration of main peaks of HPLC–DAD of the six extracts.

	OV1	OV2	OV3	OV4	OV5	OV6
TPC quantification (µg GA/mg extract)						
Total phenolic compounds	93.65±0.22	286.4±0.37	285.25±0.45	298.86±0.90	207.64±0.69	291.13±0.37
HPLC–DAD quantification (mg/100mg extract)						
Flavonoids	13.53±0.47	32.83±0.41	37.76±0.28	33.81±0.24	15.56±0.14	33.46±0.26
Dihydroxicynnamic acids	1.73±0.08	4.42±0.04	2.34±0.19	9.24±0.11	11.14±0.15	1.95±0.09
Rosmarinic acid	–	35.58±1.20	34.10±0.04	32.82±0.05	24.21±1.08	36.13±1.07
Salvianolic acids	–	7.26±0.17	3.35±0.02	2.65±0.07	3.13±0.20	3.45±0.14
Dihydroxibenzoic acids	9,20±0.11	16.20±0.08	21.72±0.15	19.24±0.15	5.23±0.04	17.76±0.14
Syringic acids	4.69±0.05	5.20±0.11	11.99±0.11	7.14±0.22	2.86±0.24	6.82±0.16
Total compounds	**29.15±0.71**	**101.49±1.84**	**111.26±0.79**	**104.9±0.84**	**62.13±1.84**	**99.61±1.86**

Rosmarinic acid, apigenin, luteolin and quercetin are the most recurrent compounds in this *Lamiaceae* species [29,52,128,156,177,195]. With increasing evidence on the biological activity from flavonoids and phenolic acids of oregano species, quantification of these compounds is important. Reports from different oregano species have shown that flavones are among the most abundant flavonoid subgroup followed by flavonols, flavanones and flavanols [4]. Besides, the most common phenolic acids in oregano are hydroxycinnamic acid and hydroxybenzoic acid derivatives [4,179] However, their content and distribution can vary depending on geographical, environmental growing factors and vegetative stage of the plant [24,93,109,156], showing a different chemical profile within the same species.

In previous studies, phenolic compounds were related to a relevant bioactivity of the extract as antioxidant because they could counteract free radical damage by providing hydrogen atoms or by donating electrons [220]. However, the amount of phenolic compounds in the crude extract could be directly associated with increased bioactivity? What would happen to the chemical compounds after a digestion process?

3. *IN VITRO* GASTROINTESTINAL DIGESTION

The main route of administration of medicinal plants is oral. Not only is it easier to administer, but also because a systemic effect is achieved by this route when the active ingredients reach the bloodstream. In the case of the pharmacological activities of oregano (antioxidant, antimicrobial of the upper respiratory tract, antispasmodic, hypoglycaemic…), some of them are directed to the digestive system itself or systemic effects are required. However, this route has some obstacles such as the acidic pH of the stomach or the breakdown of compounds by digestive enzymes. This means that the compounds

detected in crude extracts could be modified, inactivated or digested once they reach the intestine. In some cases, these biochemical transformations can also improve the activity of the extract.

3.1.1 MATERIAL AND METHODS

The gastrointestinal digestion process was based on Pastoriza *et al*. [221] with some modifications from Pinacho *et al*. [125].

- Experimental basis

The entire gastrointestinal digestion process takes place in three different steps: mouth, stomach and intestine. To simulate this process *in vitro*, all the conditions required for optimal digestion must be considered: pH, enzymes, temperature and duration of the step (**Table 15**).

Table 15. Conditions required to simulate the gastrointestinal digestion process *in vitro.*

Step	pH	Enzyme	Temperature	Duration
Mouth	6.5	Amylase	37 °C	10 min
Stomach	2.5	Pepsine	37 °C	120 min
Intestine	7.5	Pancreatine – Bilis	37 °C	120 min

Different conditions were established for each step. To adjust the pH, $NaHCO_3$ or HCl was added to the solutions to reach the optimum value after adding the enzymes. To simulate motility, the solutions were rotated at 22 rpm. The entire process, simulated in an incubator at 37.0 °C, is detailed below.

- Enzyme preparation

Enzymes can easily become dysfunctional, so some precautions were taken into account when working with them: they must be kept in the freezer, the solvents should be pre–chilled (2 – 8 °C) and they should be prepared fresh and keeping them on ice until use.
Enzyme secretion concentrations were selected according to a previous protocol [192] (**Figure 27**).

Figure 27. Enzymes and concentrations used in the gastrointestinal simulation process *in vitro.*

Amylase solution was prepared at 1.3 mg/mL in $CaCl_2$ 0.001 M and pepsine for the stomach at 160 mg/mL in HCl 0.1 M. The intestinal enzymes, pancreatin and bile, were prepared separately. After the bile solution was homogenised – sonication may be required –, pancreatin solution was added and mixed under agitation while cooling on ice.

- Sample preparation

OV1, Ov2 and OV6 were sujected to the gastrointestinal digestion process *in vitro*. The extract concentration used was 40 mg/mL, so that 500 mg of each extract was dissolved into 12.5 mL of deionized water. The assay was also performed without a sample (extract) as a control/blank for the process.

- Analyse conditions

The complete simulation process is summarized in **Figure 28**.

- Oral digestion (OD)

1. Add 125 µL of amylase solution to the sample solution (0.25 µL amylase/mg of extract).
2. Adjust to pH 6.5 with $NaHCO_3$ 1 M.
3. Introduce the tube to the incubator rotating it for 10 min.
4. Take out one of the samples and freeze immediately at –20 °C.

- Stomach digestion (SD)

1. Add 165 µL of pepsin solution to the sample solution (0.33 µL of enzyme/mg of initial extract).
2. Adjust to pH 2.5 with HCl 3 M.
3. Introduce the tube to the incubator rotating it for 120 min.
4. Take out one of the samples and freeze immediately at –20 °C.

- Intestinal digestion (ID):

1. Add 1,500 µL of the pancreatin–bile solution to the sample solution (3 µL of enzyme/mg of initial extract).
2. Adjust to pH 7.5 with $NaHCO_3$ 1 M.
3. Introduce the tube to the incubator rotating it for 120 min.
4. Take out the sample, measure final pH and pour the *chylo* into a centrifugation tube.
5. Centrifuge for 40 min at 4,000 rpm at 4 °C.
6. After centrifugation, soluble fraction (absorbable fraction) and sediment (non–absorbable fraction) must be separated and frozen (–20 °C) in different tubes.

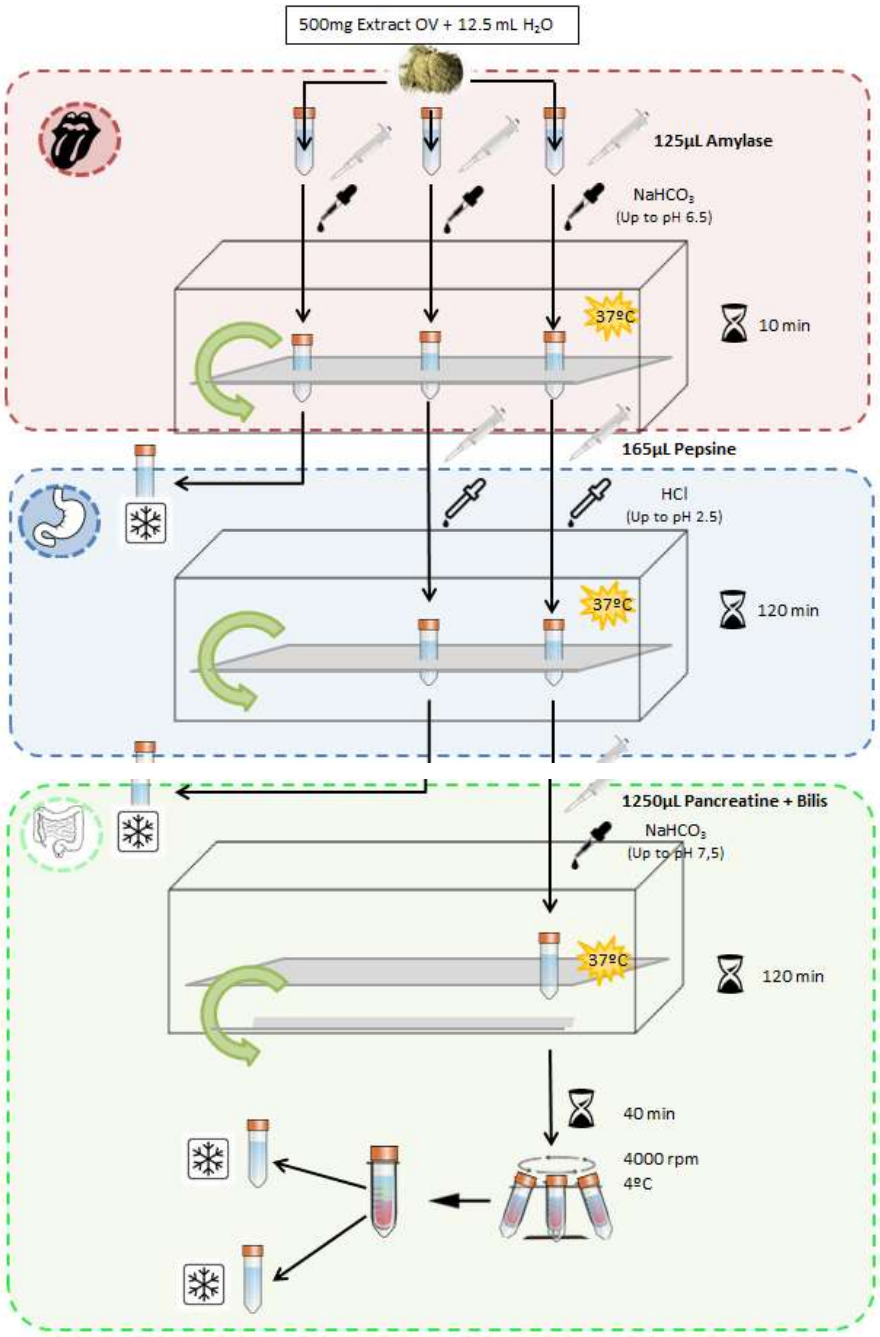


Figure 28. Scheme of the gastrointestinal simulation process *in vitro.* The three main steps of digestion are shown in different colours: red for digestion in the mouth, blue for the stomach, and green for the intestine.

- <u>Data analysis</u>

Bioaccesibility was expressed as a percentage by using the formula [192,222]:

$$Bioaccesibility\ \% = \frac{final\ concentration\left(\frac{mg}{mL}\right)}{initial\ concentration\ \left(\frac{mg}{mL}\right)}\ x\ 100$$

In which the final concentration is the concentration of the mixture after digestion (taking into account the enzymes and the solutions added to adjust pH in each step) and the initial concentration is the concentration of the sample prepared before this process (40 mg/mL).

3.1.2 RESULTS

In order to establish the stability and bioaccesibility of crude extracts and individual compound, several preliminary mathematical calculations are necessary.

The theorical final mass of the enzymes and the solutions used to adjust pH in each step are shown in **Table 16.** According to this calculation, in each digestion process there are 73.93 mg belonging to enzymes and their solvents.

Table 16. Mathematical calculations of the extra amount added for each in vitro digestion process to simulate physiological conditions. Shown by digestion step: enzyme and solvent in which it dissolves ($CaCl_2$ in mouth for amylase, HCl in stomach for pepsin and $NaHCO_3$ in intestine for pancreatine and bile).

Step	Enzyme solvent	Concentration	Volume	Mass added
MOUTH	Amylase	1.3 mg/mL	125 µL	0.1625 mg
	$CaCl_2$	0.001 M		0.0138 mg
STOMACH	Pepsin	160 mg/mL	165 µL	26.40 mg
	HCl	0.1 M		0.60159 mg
INTESTINE	Pancreatine	0.1 g / 25 mL	1250 µL	5.00 mg
	Bile	25 mg/mL		31.25 mg
	$NaHCO_3$	0.1 M		10.50 mg
SUM				**73.93 mg**

Furthermore, the volume required to adjust the pH in each step may vary depending on the chemical nature of the extract. **Table 17** shows the calculations and empirical data for the blank samples.

Table 17. Theoretical calculations from blank digestion compared to the final mass empirically obtained.

Step	Initial mass (mg)	Volume added to adjust pH (µL)	Final mass theory (mg)	Final mass empirical (mg)
MOUTH	0	25	2.28	–
STOMACH	0	400	73.03	–
INTESTINE Absorbable	0	0	119.78	< 0.01
INTESTINE Non–absorbable	0	–		**118.20**

During the process, no basification solution was needed between the gastric and intestinal steps because the enzymes (pancreatin and bile) proved to be sufficient to change the pH. By calculation, the final lyophilized mass obtained should have been 119.78 mg, divided between the absorbable and non–absorbable intestinal fraction. In practice, the results showed that 98.68 % of this mass (enzymes and solvents in solution) was non–absorbable (118.20 mg obtained as granules). The mass obtained in the absorbable fraction was insignificant (1.58 mg) compared to the non–absorbable one.

However, despite the fact that in practice all the mass added during the process was not within the potentially absorbable intestinal fraction, HPLC–DAD analysis was performed and no peak was detected. This result confirmed that neither the enzymes nor the pH adjustment solutions would interfere with the results of the post–digestion activity.

Table 18 shows the results of *in vitro* gastrointestinal digestion process for OV1, OV2 and OV6 extracts. Data shown correspond to the mean of the values, since the results were reproducible. OV1, OV2 and OV6 did not show losses in the first two steps of the digestion process, being their bioaccesibility 100 % for oral and stomach digestion.

Table 18. Digestion process for OV1, OV2 and OV6 with the three steps: mouth, stomach and intestine (with absorbable and non–absorbable (non–abs.) fractions).

	Step	Tampon Solution used	Vol. added (mL)	Mass adjustment (mg)	Enzyme mass (mg)	Total extra mass added (mg)	Initial mass (mg)	Final mass digested (mg)	Bioaccessibility (%)
OV1	Mouth	$NaHCO_3$ 1 M	0.5	42.00	0.18	42.18	501.0	543.3	100.00
	Stomach	HCl 3 M	0.6	65.63	27.20	92.83	500.1	590.8	100.00
	Intestine *Absorbable*	$NaHCO_3$ 1 M	1	84.01	73.93	157.94	500.2	465.0	**94.60 ± 0.93**
	Intestine *Non–abs.*							162.9	
OV2	Mouth	$NaHCO_3$ 1 M	2.3	19.32	0.18	19.5	500.5	517.9	100.00
	Stomach	HCl 3 M	0.4	4.37	27.20	31.57	501.1	525.0	100.00
	Intestine *Absorbable*	$NaHCO_3$ 1 M	3.65	30.65	73.93	104.58	500.3	474.0	**94.85 ± 0.81**
	Intestine *Non–abs.*							118.6	
OV6	Mouth	$NaHCO_3$ 1 M	2.5	21.00	0.18	21.18	496.2	514.0	100.00
	Stomach	HCl 3 M	0.24	2.63	27.20	29.83	501.5	527.7	100.00
	Intestine *Absorbable*	$NaHCO_3$ 1 M	1.6	191.54	73.93	265.47	500.0	402.4	**80.89 ± 0.58**
	Intestine *Non–abs.*							296.5	

The intestine is the main organ for absorbing nutrients. However, the calculated bioaccesibility might not represent the total amount absorbed, as only soluble compounds would be. Insoluble compounds would be eliminated by centrifugation. In this case, both OV1 (94.60 ± 0.93 %) and OV2 (94.85 ± 0.81 %) had potentially higher bioaccesibility than OV6 (80.89 ± 0.58 %), since in the intestine there is a greater amount of non–absorbable and potentially excreted compounds.

Finally, to identify potentially absorbable compounds, semi–quantification was performed by HPLC (*see Chapter I, chemical quantification*) of the three extracts in each digestion step (**Tables 19** to **21**).

Table 19. OV1 gastrointestinal digestion. Amount of each compound (mg standard per 100 mg dry extract) before and after digestion grouped by type of compound.

mg standard per 100 mg extract	Peak	OV1	Mouth	Stomach	Intestine	Intestinal bioaccesibility (%)
Dihydroxibenzoic acids	**3**	1.17 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.12 ± 0.01	10.25
	6	0.32 ± 0.01	0.33 ± 0.04	0.30 ± 0.02	0.63 ± 0.01	196.87
	9	6.81 ± 0.03	5.41 ± 0.02	5.15 ± 0.04	6.41 ± 0.01	94.12
	23	0.90 ± 0.05	0.04 ± 0.01	0.05 ± 0.01	0.50 ± 0.05	55.55
Total		**9.20 ± 0.11**	**5.79 ± 0.08**	**5.51 ± 0.08**	**7.66 ± 0.08**	**83.26**
Syringic acids	**5**	4.69 ± 0.05	1.28 ± 0.02	0.01 ± 0.01	0.36 ± 0.04	7.67
Total		**4.69 ± 0.05**	**1.28 ± 0.02**	**0.01 ± 0.01**	**0.36 ± 0.04**	**7.67**
Dihydroxycinnamic acids	**28**	1.73 ± 0.08	0.31 ± 0.02	0.55 ± 0.01	1.35 ± 0.03	78.03
Total		**1.73 ± 0.08**	**0.31 ± 0.02**	**0.55 ± 0.01**	**1.35 ± 0.03**	**78.03**
Flavonoids	**12**	0.15 ± 0.01	0.01 ± 0.01	1.70 ± 0.01	0.01 ± 0.01	6.67
	15	1.42 ± 0.09	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.70
	16	1.73 ± 0.11	0.01 ± 0.01	0.01 ± 0.01	0.05 ± 0.01	2.89
	17	0.24 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	29.17
	20	1.08 ± 0.05	1.01 ± 0.02	0.44 ± 0.02	1.50 ± 0.05	138.89
	22	2.95 ± 0.06	2.85 ± 0.01	0.07 ± 0.03	2.89 ± 0.07	97.97
	24	1.79 ± 0.05	1.51 ± 0.03	1.61 ± 0.09	1.53 ± 0.01	85.47
	25	–	–	0.10 ± 0.02	0.17 ± 0.01	new
	29	1.37 ± 0.03	0.37 ± 0.01	0.44 ± 0.01	1.60 ± 0.04	116.79
	30	1.00 ± 0.02	0.64 ± 0.02	0.11 ± 0.02	0.71 ± 0.02	71.00
	35	0.57 ± 0.01	0.46 ± 0.02	0.45 ± 0.02	0.50 ± 0.02	87.71
	38	1.23 ± 0.02	1.12 ± 0.03	0.01 ± 0.02	2.12 ± 0.03	172.36
Total		**13.53 ± 0.47**	**8.1 ± 0.19**	**5.03 ± 0.27**	**11.16 ± 0.29**	**82.48**

In general, OV1 showed a good preservation of the main compounds, with bioaccesibility per groups higher than 78 %, with the exception of syringic acids (7.67 %).

Among dihydroxybenzoic acids, 2,5–dihydroxibenzoic acid (peak **9**), the most important compound of this group showed 94.12 % of bioaccesibility. Peak **3** showed low bioaccesibility and peak **23** also, although to a lesser extent. On the contrary, peak **6** obtained 196.87 % of bioaccesibility, which could be explained as a degradation of peak **3** and peak **23** along gastrointestinal process producing another dyhydroxibenzoic acid (peak **6**).

The dihydroxicinnamic acids of OV1 presented 78.03 % of intestinal bioaccesibility. This type of compounds tend to undergo a greater number of chemical modifications that lead to a lower bioaccesibility after the digestion process [192] according to previous studies *in vitro* with coffee [206].

In the group of flavonoids, with intestinal bioaccesibility of 82.48 %, different results were observed between compounds and a new flavonoid (peak **25**) was detected. This compound was also detected in other extracts (OV5 and OV6) and it is possibly an apigenin glucoside [223–225]. Three flavonoids showed an intestinal bioaccesibility greater than 100 % (peaks **20**, **29** and **38**). The conservation of flavonoids after digestion can be related to the β–linkage between the carbohydrate and the aglycone, which cannot be hydrolysed by enzymes commonly used in the simulated digestion model. This same behaviour was observed in extracts of *Prunus spinosa* L.[125]. Furthermore, several studies

have shown that the degradation of flavonoid derivatives during the intestinal digestion process is different when they are incubated as pure compounds or as the same compounds in an extract. It has been suggested that interactions with other compounds in the extract matrix could influence and alter stability during digestion. In this sense, and considering the chemical complexity of the analysed extract, the obtained results can be easily explained.

OV2 followed the same trend as OV1 (**Table 20**). In general, there was a high conservation of the compounds since the detected peaks coincided and had a similar intensity.

Table 20. OV2 gastrointestinal digestion. Amount of each compound (mg standard per 100 mg dry extract) before and after digestion grouped per type of compound.

mg per 100 mg extract	Peak	OV2	Mouth	Stomach	Intestine	Intestinal bioaccesibility (%)
Dihydroxibenzoic acids	2	0.52 ± 0.01	0.49 ± 0.02	0.50 ± 0.02	0.51 ± 0.02	98.07
	3	1.05 ± 0.03	0.99 ± 0.02	1.02 ± 0.03	1.01 ± 0.09	96.19
	6	2.04 ± 0.01	2.04 ± 0.03	2.00 ± 0.03	2.04 ± 0.01	100
	21	9.55 ± 0.01	9.42 ± 0.02	9.22 ± 0.05	9.52 ± 0.02	99.68
	23	3.04 ± 0.02	1.72 ± 0.05	1.74 ± 0.03	2.88 ± 0.06	94.73
Total		**16.20 ± 0.08**	**14.66 ± 0.14**	**14.48 ± 0.16**	**15.96 ± 0.20**	**98.51**
Syringic acid derivates	1	0.34 ± 0.01	0.27 ± 0.01	0.26 ± 0.07	0.30 ± 0.02	88.23
	4	0.49 ± 0.02	0.53 ± 0.02	0.47 ± 0.02	0.53 ± 0.07	108.16
	11	1.44 ± 0.02	1.42 ± 0.08	1.42 ± 0.08	1.41 ± 0.02	97.92
	13	1.84 ± 0.03	1.55 ± 0.06	1.69 ± 0.03	1.78 ± 0.06	96.74
	18	1.09 ± 0.03	0.96 ± 0.02	1.17 ± 0.08	1.00 ± 0.06	91.74
Total		**5.20 ± 0.11**	**4.73 ± 0.19**	**5.01 ± 0.28**	**5.02 ± 0.23**	**96.53**
Dyhidroxycinnamic acids	8	1.07 ± 0.02	0.94 ± 0.01	1.03 ± 0.02	1.06 ± 0.03	99.06
	36	3.35 ± 0.02	0.31 ± 0.02	2.54 ± 0.03	1.35 ± 0.06	40.30
Total		**4.42 ± 0.04**	**1.25 ± 0.03**	**3.57 ± 0.05**	**2.41 ± 0.09**	**54.52**
Rosmarinic acid	32	31.83 ± 1.12	14.76 ± 1.09	14.50 ± 0.50	14.34 ± 0.19	45.05
Total		**31.83 ± 1.12**	**14.76 ± 1.09**	**14.50 ± 0.50**	**14.34 ± 0.19**	**45.05**
Flavonoids	15	1.93 ± 0.05	1.93 ± 0.04	1.93 ± 0.04	1.93 ± 0.02	100
	16	4.22 ± 0.05	3.14 ± 0.02	4.00 ± 0.05	4.05 ± 0.02	95.97
	17	2.32 ± 0.03	2.14 ± 0.06	2.26 ± 0.03	2.32 ± 0.03	100
	19	7.05 ± 0.02	6.94 ± 0.03	6.79 ± 0.07	6.79 ± 0.06	96.31
	24	2.72 ± 0.02	2.72 ± 0.05	2.59 ± 0.03	2.63 ± 0.05	96.69
	29	4.20 ± 0.03	0.53 ± 0.01	0.27 ± 0.02	0.41 ± 0.02	9.76
	30	2.21 ± 0.01	1.41 ± 0.02	2.21 ± 0.01	10.27 ± 0.03	464.70
	34	1.49 ± 0.08	1.46 ± 0.02	1.31 ± 0.10	2.32 ± 0.01	155.70
	38	3.33 ± 0.10	3.40 ± 0.05	2.84 ± 0.02	9.37 ± 0.02	281.38
	39	3.36 ± 0.02	3.40 ± 0.11	1.44 ± 0.02	4.69 ± 0.02	139.58
Total		**32.83 ± 0.41**	**27.07 ± 0.41**	**25.33 ± 0.39**	**44.75 ± 0.28**	**136.30**
Salvianolic acids	14	1.70 ± 0.08	1.28 ± 0.03	1.11 ± 0.06	1.25 ± 0.03	73.53
	26	5.56 ± 0.09	2.45 ± 0.05	0.62 ± 0.02	3.11 ± 0.02	55.94
Total		**7.26 ± 0.17**	**3.73 ± 0.08**	**1.73 ± 0.08**	**4.36 ± 0.05**	**60.05**

The 3,4–dihydroxibenzoic acid (peak **21**) was preserved from the digestion process, showing 99.68 % bioaccesibility. In addition, other compounds were found to be more abundant in the intestinal fraction: for example, the group of flavonoids with a total intestinal bioaccesibilityof 136.30 %, while dihydroxycinnamic acids, rosmarinic acid and

salvianolic acid were conserved at half (54.52 %, 45.05 % and 60.05 %, respectively). These results are in concordance with other studies in which rosmarinic acid was the phytochemical compound that underwent the most significant transformation during digestion [13,217].

In the same way, **Table 21** shows the results of individual compounds from OV6 extract.

Table 21. OV6 gastrointestinal digestion. Amount of each compound (mg standard per 100 mg dry extract) before and after digestion grouped per type of compound.

mg standard per 100 mg extract	Peak	OV6	Mouth	Stomach	Intestine	Intestinal bioaccesibility (%)
Dihydroxybenzoic acids	**2**	0.95 ± 0.01	0.48 ± 0.02	0.37 ± 0.03	0.35 ± 0.02	36.84
	6	0.51 ± 0.05	0.26 ± 0.01	0.19 ± 0.02	0.17 ± 0.01	33.33
	21	16.30 ± 0.08	16.30 ± 0.02	15.95 ± 0.03	16.30 ± 0.06	100.00
Total		**17.76 ± 0.14**	**17.04 ± 0.05**	**16.51 ± 0.08**	**16.82 ± 0.09**	**94.70**
Syringic acids	**1**	0.52 ± 0.03	0.47 ± 0.02	0.44 ± 0.02	0.44 ± 0.03	84.61
	10	0.65 ± 0.02	0.16 ± 0.02	0.13 ± 0.01	0.12 ± 0.03	18.46
	18	1.92 ± 0.01	0.08 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	2.08
	27	3.01 ± 0.08	2.96 ± 0.08	2.38 ± 0.02	1.49 ± 0.02	49.50
Total		**6.10 ± 0.14**	**3.67 ± 0.14**	**2.99 ±0.06**	**2.09 ±0.09**	**34.26**
Dihydroxycinnamic acids	**8**	0.63 ± 0.06	0.65 ± 0.02	0.36 ± 0.03	0.58 ± 0.03	92.06
	37	1.32 ± 0.03	1.21 ± 0.02	1.32 ± 0.02	1.32 ± 0.01	100
Total		**1.95 ± 0.09**	**1.86 ± 0.04**	**1.68 ± 0.05**	**1.9 ± 0.04**	**97.43**
Rosmarinic acid	**32**	36.13 ± 1.07	24.42 ± 0.08	22.22 ± 0.54	21.84 ± 0.89	60.45
Total		**36.13 ± 1.07**	**24.42 ± 0.08**	**22.22 ± 0.54**	**21.84 ± 0.89**	**60.45**
Flavonoids	**15**	1.65 ± 0.02	1.41 ± 0.02	1.30 ± 0.02	1.10 ± 0.02	66.67
	17	0.46 ± 0.02	0.21 ± 0.03	0.16 ± 0.02	0.18 ± 0.08	39.13
	19	11.32 ± 0.11	0.32 ± 0.06	3.98 ± 0.04	0.07 ± 0.01	0.62
	25	1.67 ± 0.03	0.48 ± 0.01	0.85 ± 0.02	0.53 ± 0.02	31.73
	29	5.54 ± 0.02	0.53 ± 0.05	0.49 ± 0.05	0.91 ± 0.02	16.42
	34	5.67 ± 0.02	5.08 ± 0.08	5.21 ± 0.02	4.99 ± 0.02	88.00
	39	7.15 ± 0.04	7.11 ± 0.01	7.04 ± 0.02	6.67 ± 0.01	93.29
Total		**33.46 ± 0.26**	**15.14 ± 0.31**	**19.03 ± 0.19**	**14.45 ± 0.18**	**43.18**
Salvianolic acids	**14**	3.45 ± 0.14	2.96 ± 0.02	2.90 ± 0.02	3.07 ± 0.01	88.98
Total		**3.45 ± 0.14**	**2.96 ± 0.02**	**2.90 ± 0.02**	**3.07 ± 0.01**	**88.98**

The crude extract OV6 had similar chemistry to OV2 (**Table 21**). The digestion process generated a lower bioaccesibility for the OV6 extract (80.89 %) than for the OV1 (94.60 %) and OV2 (94.85 %) extracts, so the OV6 compounds seemed to degrade more. Only 60.45 % of rosmarinic acid from the crude extract was detected in the absorbable intestinal fraction, although its intestinal bioaccesibility was higher than in OV2 (45.05 %) [177]. 3,4–dihydroxibenzoic acid (peak **21**) showed a maximum bioaccesibility after the digestion process (100 %) in this extract, with similar results to OV2 (99.68 %). According to these results and those observed in OV1, these compounds seem to be stable and resistant to the digestion process.

In syringic acids, the syringic acid (peak **27**) showed a low bioaccesibility (around 50 %). The flavonoids seemed to have the same profile as OV1 and OV2. Peak **19** (λ_{max} 255.8, 342.5 nm) showed the lowest bioaccesibility (0.62 %, with an amount found of 0.07 ± 0.01 mg out of 11.32 ± 0.11 mg per 100 mg from the initial extract). Other abundant flavonoids

such as peaks **34** and **39** showed higher bioaccesibility (88.00 % and 93.29 %, respectively).

In conclusion, the results are according to previous study [177], in which the main polyphenols of *Melissa officinalis*, *Lavandula latifolia* and *Origanum vulgare* extracts maintained high stability after *in vitro* gastrointestinal digestion.

Now the chemical profile of extracts prepared has been studied and characterised, in consonance with other studies with *O*. *vulgare* L. [7,13,226], would this preserved quantity still be bioactive for the pathologies of interest?

CHAPTER II:

STUDY OF *in vitro* PHARMACOLOGICAL ACTIVITIES OF *Origanum vulgare* L. ssp. *vulgare* FLOWERED AERIAL PARTS EXTRACTS

When the body starts to fail, medicine takes over.

Grey's anatomy.

Living organisms show very complete and complex functions that involve metabolism enzymatic reactions. Their proper functioning leads to a healthy state of homeostasis. However, when this balance is disturbed, pathologies appear. Some of them are easier to detect and their symptoms appear as a direct consequence of the problem that occurred. Unfortunately, some others are silent pathologies that occur asymptomatically as a consequence of more than one previously uncontrolled risk factor. The development of medicine has led to an increase in life expectancy in humans and, consequently, new pathologies related to aging have appeared in recent centuries.

Nowadays, in addition to cancer, resistance to antibiotics and the recent Covid–19, cardiovascular disease is the leading cause of mortality in Spain (31 %) and 80 % of that mortality could be avoided. In 2020 Spain directly dedicated €8,800 million to this mortality risk; €180 per Spanish [227].

Contrary to what its name suggests (*cardio* = heart), there are many risk factors that increase the probability of developing cardiovascular disease [228]:

- Related to the patient's behaviour: alcohol and tobacco consumption, sedentary lifestyle, unhealthy diet, stress and psychological factors
- Metabolic: Arterial Hypertension (HTA), hyperglycaemia (Diabetes Type II), hyperlipidaemia (High cholesterol) and overweight
- Other factors: age, sex and family background

According to the European guideline on cardiovascular prevention [228], cardiovascular risk increases with smoking (a modifiable factor), high blood cholesterol values, blood pressure (both partly modifiable parameters), sex (men have a higher risk than women of suffering a cardiovascular episode) and age. In this sense, glycaemia, glycosylated haemoglobin (HbA1C), cholesterol (Triglycerides, LDL–c and HDL–c), blood pressure (PAS, PAD and cardiac frequency) and weigh (BMI – Body Mass Index – and fat percentage) are some of the anthropometrical parameters accessible for detection and monitoring, even in the pharmacy. As medical advice, lifelong prevention efforts should be made: *the earlier to establish them, the better quality and longer life the patient might have.*

Recent studies have shown the benefits of phytomedicines in prevention and treatment of these diseases [228–231]. For example, among the bioactive compounds found in *Monascus* Fermented Rice (MFR), monacolin K is the most effective compound for lowering plasma cholesterol [230–232]. Red rice yeast, containing monacolin K, scientifically demonstrated to be such a potential natural remedy for therapeutically use through statin pathways [233]. Since 1979 [231], there is evidence that a metabolite of *Penicillium citrinum* (ML236B)

renamed as *lovastatin*, *mevinolin* and *mevacor*, isolated as a cholesterol synthesis inhibitor, had hypolipidemiant activity in several animal species [231,234].

Being a natural alternative to statins in early dyslipidaemia, red rice yeast with monakolin K as the main bioactive compound can be conveniently administrated in capsule form; for example those marketed by Arkopharma laboratories (Arkosterol ®) [235]. Besides, this laboratory also has a product for those patients in whom statins alone are not able to lower cholesterol values. This presentation incorporates Coenzime Q10 to potentiate the lipid–lowering effect, in some way thanks to its antioxidant properties that prevents the oxidation of high–density lipoprotein (HDL–c) [236].

When looking for a natural treatment, plant selection is the most important step in studies with plants. Hamburguer and Hostettmann published some useful guidelines [237]:

1. If the plant has a traditional use, there is a greater probability of having pharmacologically active compounds.
2. If there are previous published studies, there is a greater probability of having pharmaceutically active compounds.
3. If there is not much bibliographic information, the probability of finding new proprieties is greater.

The traditional uses of *O. vulgare* involve preparation as an infusion (tea) to treat digestive disorders (indigestion), headaches, sore throats or colds thanks to the antispasmodic, calmative, carminative, diaphoretic, expectorant, stomachic and tonic effects [238]. Topically, its oil is used in aromatherapy and perfumery [239]. Last but not least, the antiseptic activity has been known for centuries, but it was not until the beginning of XXth century that was scientifically characterize [216,238,240].

The target of this work is to find an *O. vulgare* L. extract capable of reducing lipids [241], helping with hyperglycaemia [242] and with antioxidant capacity [243] related to aging diseases [244] such as Alzheimer.

1 ANTIOXIDANT ACTIVITY

Following the perception of health as a balance of all the complex enzymatic reactions that take place in human organisms, diseases tend to appear when this homeostasis cannot last any longer; warning the patient that something is going wrong. That is where medicine can take over. Within normal and correct functionality of living organisms, there is a plethora of complex oxygen–dependent enzymatic reactions that provide the state of health [245].

Molecular oxygen (O_2, as it is commonly known) is involved in metabolic functions. However, it can also be present as short–lived highly reactive derivatives (Reactive Oxygen Species – ROS) as the result of these enzymatic reactions [244]. Superoxide (O_2•−), hydrogen peroxide (H_2O_2), and the hydroxyl radical (•OH) are some of these derivatives that can cause cell damage [246]. They can affect DNA and polyunsaturated fatty acids in membrane [245,247]. Organisms are prepared to counteract these damages through antioxidant defence systems. However, according to The Ageing and Free Radical Theory [244], the effectiveness of these protective systems tends to decrease with age, and the accumulation of these harmful molecules can create pathos in the body, developing diseases such as Alzheimer and Diabetes [246,248]. Most of the current research with natural products are focused on finding external co–adjuvants to counteract this oxidative damage, either from the point of view of prevention or treatment [249,250]. Compounds capable of counteracting this oxidative damage are called antioxidants [244]. As an exogenous aid to prevent damage to the body, these antioxidant compounds can reduce the formation of these free radicals or neutralize them [246,251]. Preclinical studies encompass this activity through both *in vitro* and *in vivo* models [243].

1.1 DPPH• *in vitro* ASSAY

Among *in vitro* assays, the DPPH•–based method is probably the most popular one due to its simplicity, speed, and low cost [243]. DPPH• (1,1–diphenyl–2–picrylhydrazyl) is a stable free radical that can be reduced by transferring a hydrogen from other compounds.

Since 1995, when Brand–Williams first published and discussed in depth the methodology [252], some variants have been developed. Depending on the equipment accessible to the researcher and the interest of the study, the reaction can be measured in different ways: kinetic conditions, DPPH• concentration, sample–reagent ratio…[252]. Nonetheless, the principle of the reaction is always the same [252]:

The reduction of DPPH• as indicated below is followed by monitoring the decrease in its absorbance at a characteristic wavelength during the reaction. In its radical form, DPPH• absorbs at 517 nm, but upon reduction by an antioxidant (AH) or a radical species (R•), the absorption disappears.

1.1.1 MATERIAL AND METHOD

- Experimental basis

Antioxidant activity is usually monitored using the scavenging effect of radicals on DPPH (#D9132, Sigma–Aldrich Co., St. Louis, MO) which changes from purple to yellow in the presence of an antioxidant compound. This change can be quantified by spectrophotometry at 517 nm (spectrophotometer UV PowerWave XS, BioTek Instruments, Inc., Winooski, VT, USA) [177,252] (**Figure 29**).

$$DPPH\bullet + AH \rightarrow DPPH{-}H + A\bullet$$

$$DPPH\bullet + R\bullet \rightarrow DPPH{-}R$$

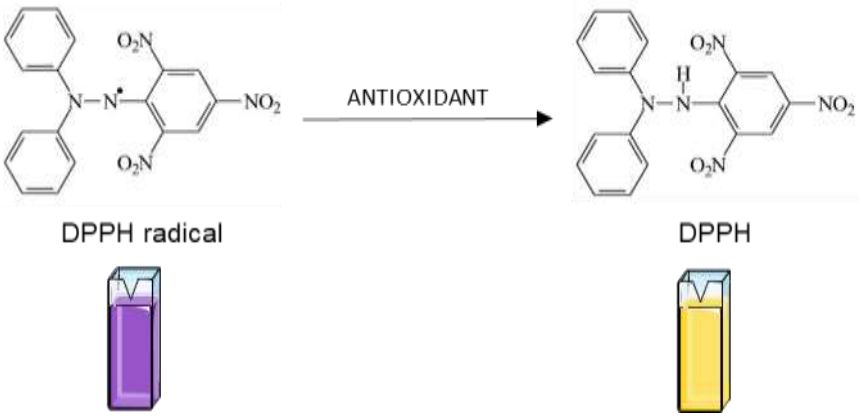


Figure 29. Reaction of scavenging activity of DPPH free radical. Above is the chemical structure of the free radical, with the modification suffered in the presence of the antioxidant compound. Bottom, macroscopic colour of the reagent (violet, DPPH•) and product (DPPH, yellow).

This activity was firstly confirmed by Thin Layer Chromatography (TLC) as a qualitative assay by disposing 10 µL of lyophilised extract (20 mg/mL) in a Silicagel 60 F_{254nm} with plastic base (#105554, Merck KGaA, Darmstadt, Germany). The mobile phase was ethyl acetate:methanol:water (65:15:5 v/v/v). After separation, the plate was sprayed with a 0.4 mg/mL DPPH free radical solution to identify antioxidant activity as yellow spots.

Then, those extracts that were antioxidants were tested quantitatively, in order to select those with the highest activity.

- Reagent preparation

  Qualitative assay: DPPH• 0.4 mg/mL in methanol.

Quantitative assay: DPPH• 0.02 mg/mL in methanol.

- Sample preparation

The lyophilised extracts OV1 – OV6 were diluted in its correspondent solvent at ten serial concentrations (1,000 – 1.95 µg/mL). Rosmarinic acid (#R4033, Sigma–Aldrich Co., St. Louis, MO) and Butylated hydroxytoluene, BHT (#47168, Sigma–Aldrich Co., St. Louis, MO) were used as positive control at the same concentrations.

Finally, digestion fractions from the three previously performed *in vitro* gastrointestinal digestion processes (OV1, OV2 and OV6; mouth, stomach and intestine fractions) were also analysed, as well as intestinal fractions from the correspondent positive controls: RA–Int and BHT–Int, at the same concentration as the crude extracts.

- Analyse conditions

Three 96–well plate (*n* = 18) were prepared for each extract (**Figure 30**).

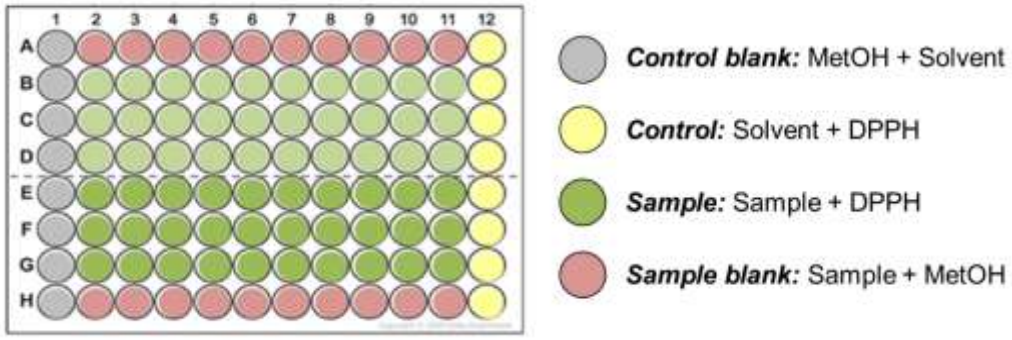


Figure 30. 96–well plate design for the antioxidant activity assay: distribution and colour legend to explain the content of each well with an image of the result.

Every plate needs a *control blank* (150 µL methanol and 150 µL solvent), a *control* (150 µL solvent and 150 µL DPPH•) and a *blank* for the sample (*sample blank*, 150 µL sample and µL methanol). Methanol was used for the *blanks* since it is the solvent of DPPH•.

- Data process

The reaction was monitored every 15 min for 90 min where absorbance was registered at 517 nm in order to calculate scavenging activity (percentage of inhibition, %)) with the following formula:

$$Scavenging\ activity\ (\%) = \left(1 - \frac{Abs_{sample} - Abs_{sample\ blank}}{Abs_{control} - Abs_{control\ blank}}\right) x\ 100$$

In which Abs_{sample} is the absorbance at 517 nm of the reaction in presence of sample (sample dilution + DPPH• solution), $Abs_{sample\ blank}$ is the absorbance of the blank for each sample dilution (sample dilution + DPPH• solvent), $Abs_{control}$ is the absorbance of control reaction (sample solvent + DPPH• solution) and $Abs_{control\ blank}$ is the absorbance of the blank (DPPH• solvent + sample solvent).

Values in each point were expressed as EC_{50}, the concentration in which the 50% of the free radical DPPH• is reduced. Furthermore, by using EC_{50} values it was calculated the index of antioxidant activity (AAI) for each extract [253]:

$$AAI = \frac{final\ DPPH\ concentration\ \left(\frac{\mu g}{mL}\right)}{EC_{50}\left(\frac{\mu g}{mL}\right)}$$

Results were catalogued in terms of antioxidant strength of the compound according to an independent of DPPH• concentration universal scale for this method [253].

- Statistical analysis

EC_{50} values were generated with GraphPad Prism, v6.01 (GraphPad Software, La Jolla, CA). Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups. Besides, a stabilisation point in time was considered when there was a $p > 0.50$ between consecutive values.

1.1.2 RESULTS

This assay is a good selection for a first approach of bioactivity and it is already perfectly set up by the Pharmacognosy research team from University of Navarra [222]. First, a qualitative assay was carried out to determine if the extracts present antioxidant activity against DPPH free radical. **Figure 31** shows TLC with the six extracts revealed with DPPH• solution. Yellow colour indicates antioxidant activity.

Figure 31. TLC (mobile phase: ethyl acetate–methanol–water (65:15:5 v/v/v)) of the six extracts post–sprayed with DPPH• solution to detect qualitative antioxidant *in vitro* activity. The yellow spots correspond to the positive result of the antioxidant activity against the DPPH free radical.

All extracts showed yellow spots on TLC. At first glance, OV2 to OV6 showed high antioxidant activity, with OV1 being the least antioxidant. Considering that the DPPH• reaction on TLC is only a qualitative approach, quantitative assay were performed (**Tables 22** to **27**).

Table 22. OV1 (H_2O, cold extraction). Antioxidant activity (%) and EC_{50} (µg/mL). Data expressed as means ± SD of triplicate analysis. Values with different letter show significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Value in bold means EC_{50} max (stabilisation point).

	Time (min)					
[Extract] (µg/mL)	15	30	45	60	75	90
125	100.95±5.96	101.45±6.20	101.61±6.85	101.51±7.24	101.37±7.15	101.66±8.43
62.5	98.41±4.11	98.67±4.02	99.07±4.17	99.31±4.40	99.45±4.70	99.64±5.34
31.25	96.31±4.15	97.86±3.35	98.48±3.25	99.01±3.47	98.85±4.14	98.88±4.96
15.62	86.33±6.65	93.37±0.88	90.55±7.10	92.33±6.35	94.40±3.44	95.05±5.79
7.81	68.35±1.99	74.60±2.13	79.89±0.65	82.65±2.70	83.43±1.34	86.66±3.99
3.91	37.57±1.33	42.10±0.95	42.48±0.74	44.09±0.60	44.97±0.60	51.86±0.70
1.95	26.19±0.90	25.95±0.69	27.22±0.48	26.93±0.45	30.10±0.69	31.15±0.82
0.98	22.70±1.99	18.84±0.70	19.07±0.54	18.13±0.51	11.20±0.41	10.82±0.48
$\overline{EC}_{50}$ (µg/mL)	5.03±0.37^{b}	4.29±0.12^{b}	4.11±0.25^{b}	**3.91±0.21^{a}**	3.77±0.08^{a}	3.39±0.14^{a}

Table 23. OV2 (H_2O, hot extraction). Antioxidant activity (%) and EC_{50} (µg/mL). Data expressed as means±SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$).Value in bold means EC_{50} max (stabilisation point).

	Time (min)					
[Extract] (µg/mL)	15	30	45	60	75	90
125	99.25±1.85	99.47±2.00	99.96±2.03	99.63±2.26	99.97±2.74	99.93±3.02
62.5	98.33±1.58	99.02±1.39	99.83±1.42	99.67±1.63	100.41±2.38	100.52±2.91
31.25	95.85±3.49	96.80±2.86	98.17±2.02	97.80±2.31	98.56±3.72	98.37±4.77
15.62	87.98±0.87	89.80±8.97	91.50±6.33	89.78±5.20	94.79±2.77	95.84±2.65
7.81	81.06±1.80	83.55±9.24	81.49±4.78	77.19±0.62	87.72±5.67	89.92±3.85
3.91	56.53±0.72	60.13±1.03	63.24±1.38	64.73±2.57	64.97±0.84	65.30±0.87
1.95	37.99±0.91	42.81±2.60	46.99±0.70	45.43±2.21	41.94±1.10	41.99±0.90
0.98	26.78±0.58	28.71±0.84	33.50±0.74	33.27±0.75	34.81±0.78	35.38± 0.66
$\overline{EC}_{50}$ (µg/mL)	3.11±0.07^{c}	2.75±0.32^{b}	**2.52±0.15^{a}**	2.41±0.07^{a}	2.34±0.13^{a}	2.26±0.06^{a}

Table 24. OV3 (EtOH 50%. cold extraction). Antioxidant activity (%) and EC_{50} (µg/mL). Data expressed as means±SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Value in bold means EC_{50} max (stabilisation point).

	Time (min)					
[Extract] (µg/mL)	15	30	45	60	75	90
125	100.36±2.90	100.08±3.31	99.94±3.53	99.42±3.94	99.19±3.88	99.15±3.94
62.5	101.67±1.24	101.83±1.71	101.83±1.80	101.67±2.02	101.36±2.21	101. 34±2.00
31.25	97.69±3.12	99.44±2.45	100.01±3.12	99.88±3.43	98.94±5.04	99.50±4.12
15.62	79.46±0.67	94.99±8.33	97.25±6.04	98.88±4.21	97.16±2.68	98.98±2.34
7.81	59.61±1.06	63.74±1.50	67.87±1.32	72.35±0.94	75.86±1.58	77.88±3.98
3.91	46.07±1.13	50.18±1.20	50.89±0.99	50.86±1.08	51.27±2.05	58.23±1.61
1.95	35.01±0.84	36.61±0.64	38.70±0.95	39.95±0.82	42.02±1.65	42.20±1.77
0.98	21.50±0.96	25.31±0.61	26.33±1.06	24.77±0.82	27.57±1.23	28.49±2.03
$\overline{EC}_{50}$ (µg/mL)	4.73±0.17^{b}	4.00±0.32^{b}	**3.65±0.17^{a}**	3.45±0.11^{a}	3.21±0.15^{a}	2.85±0.15^{a}

Table 25. OV4 (EtOH 50%. hot extraction). Antioxidant activity (%) and IC50 (µg/mL). Data expressed as means±SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Value in bold means EC_{50} max (stabilisation point).

	Time (min)					
[Extract] (µg/mL)	15	30	45	60	75	90
125	100.9±4.19	100.10±4.95	100.09±5.34	99.52±5.70	99.27±5.99	98.93±5.99
62.5	98.71±1.95	99.24±3.30	99.06±3.90	98.80±4.17	98.34±4.27	97.87±4.37
31.25	92.24±6.87	95.34±3.65	97.26±3.10	98.00±3.63	98.42±4.18	98.12±4.26
15.62	86.86±1.13	82.94±1.36	79.50±0.83	93.36±3.23	90.69±1.08	92.83±3.02
7.81	59.06±1.41	63.69±2.30	69.06±1.39	82.02±1.11	81.84±1.11	85.51±3.72
3.91	46.20±2.31	56.04±0.99	59.77±1.76	64.46±1.39	65.64±1.41	69.66±0.93
1.95	33.82±1.98	45.52±1.73	45.43±0.99	41.64±1.12	44.23±1.67	46.37±1.04
0.98	13.05±3.83	14.09±1.25	18.76±0.91	16.95±1.65	20.63±1.00	20.65±1.05
$\overline{EC}_{50}$ (µg/mL)	5.11±0.16^{b}	4.14±0.11^{b}	3.65±0.23^{b}	2.80±0.06^{b}	**2.66±0.04^{a}**	2.41±0.13^{a}

Table 26. OV5 (EtOH. cold extraction). Antioxidant activity and EC_{50} (µg/mL). Data expressed as means±SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Value in bold means EC_{50} max (stabilisation point).

	Time (min)					
[Extract] (µg/mL)	15	30	45	60	75	90
125	105.29±0.65	105.21±0.64	105.35±0.59	105.51±0.71	105.49±0.64	105.71±0.67
62.5	104.89±0.77	105.14±0.59	105.16±0.45	105.41±0.71	105.28±0.65	105.43±0.67
31.25	102.98±1.83	104.36±0.85	104.71±0.73	105.21±0.89	105.28±0.68	105.50±0.64
15.62	90.40±8.90	100.97±6.86	103.29±7.71	105.23±6.09	106.00±6.18	106.37±5.29
7.81	71.11±1.38	67.81±6.37	72.48±7.36	77.24± 6.10	78.11±8.20	80.10±8.31
3.91	50.46±1.81	51.00±2.27	51.02±1.31	50.90±1.26	49.53±1.18	50.19±1.41
1.95	36.66±0.94	36.27±0.76	38.89± 1.23	38.91±1.45	41.78±2.17	35.37±1.68
0.98	19.45±0.87	22.52±2.46	21.71± 1.18	29.01±0.80	29.26± 0.88	28.00±1.32
$\overline{EC}_{50}$ (µg/mL)	4.05±0.22^{b}	3.82±0.27^{b}	3.58±0.38^{b}	**3.22±0.19^{a}**	3.15±0.34^{a}	3.28±0.29^{a}

Table 27. OV6 (EtOH, hot extraction). Antioxidant activity and EC_{50} (µg/mL). Data expressed as means±SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Value in bold means EC_{50} max (stabilisation point).

	Time (min)					
[Extract] (µg/mL)	15	30	45	60	75	90
125	102.28±2.57	103.23±2.05	103.53±2.16	103.83±2.20	103.95±2.29	104.22±2.38
62.5	93.13±8.65	100.46±2.76	101.46±2.70	101.66±3.04	101.85±3.46	101.94±3.86
31.25	77.40±0.40	98.97±3.65	100.36±2.74	98.24±4.70	100.33±2.93	101.11±2.70
15.62	75.38±0.95	98.25±4.76	99.26±4.35	96.71±6.71	97.56±5.88	99.95±4.83
7.81	72.78±0.80	92.45±2.55	94.80±1.52	92.23±4.55	94.07±3.65	96.09±1.85
3.91	57.07±1.14	59.93±0.96	66.24±1.01	76.89±0.93	78.56±0.91	79.43±1.05
1.95	50.26±1.71	51.76±1.54	53.16±1.26	53.10±1.99	49.69±1.41	46.62±1.07
0.98	36.56±1.88	36.98±0.73	33.85±1.55	31.82±2.13	29.40±2.09	25.08±1.57
$\overline{EC}_{50}$ (µg/mL)	2.40±0.27[b]	**2.05±0.04[a]**	2.16±0.09[a]	2.02±0.08[a]	1.96± 0.07[a]	1.83±0.06[a]

According to the results, the classification of the most antioxidant to the least antioxidant extract is OV6 > OV2 ≥ OV4 > OV5 > OV3 > OV1.

The results showed the importance of method of extraction, hot or cold. The differences between OV1 and OV2 (aqueous extracts) were statistically highly significant (EC_{50} = 3.91 ± 0.21 *vs.* 2.52 ± 0.15 µg/mL, $p < 0.001$). The same as for OV3 and OV4, hydroalcoholic extracts (3.65 ± 0.17 *vs.* 2.66 ± 0.04 µg/mL, $p < 0.001$) and for OV5 and OV6, ethanolic extracts (3.22 ± 0.19 *vs.* 2.05 ± 0.04 µg/mL).

When the comparison is made *per* groups of extracts that share the same method of extraction but different solvent: within the cold maceration, the three extracts were statistically different: OV1, OV3 and OV5 (EC_{50} = 3.91 ± 0.21. 3.65 ± 0.17, 3.22 ± 0.19 µg/mL, respectively with $p < 0.001$). However, the hot maceration profile was different, OV2 and OV4 were not statistically different (EC_{50} = 2.52 ± 0.15, 2.66 ± 0.04 µg/mL, p = 0.177); whereas OV2–OV4 vs. OV6 (2.05 ± 0.04 µg/mL, $p < 0.001$) were statistically different.

Table 28. BHT and Rosmarinic acid (RA). Antioxidant activity and EC_{50} (µg/mL) calculated for positive controls. Data expressed as means±SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Values in bold means EC_{50} max (stabilisation point).

	Time (min)					
$\overline{EC}_{50}$ (µg/mL)	15	30	45	60	75	90
BHT	324.66±15.19[b]	210.16±2.63[b]	149.86±4.07[b]	131.72±4.11[b]	98.46±2.74[b]	**78.07±4.31[a]**
RA	**1.71±0.04 [a]**	1.69±0.05 [a]	1.67±0.05 [a]	1.66±0.05 [a]	1.64±0.04 [a]	1.63±0.04 [a]

The results showed that the six extracts were more antioxidant than BHT: EC_{50} of extracts < 4.00 µg/mL against 78.07 ± 4.31 µg/mL of BHT. In a previous study with oregano, the methanolic extract containing 23.53 mg/g of rosmarinic acid [13], also showed to be very significantly ($p < 0.001$) more antioxidant than BHT: DPPH• EC_{50} = 4.65 ± 0.12 µg/mL against 465.24 ± 0.03 µg/mL, respectively. The other positive control (rosmarinic acid)

presented a significant higher activity, the EC_{50} being more comparable to the results of the extracts: 1.71 ± 0.04 μg/mL.

In fact, as Brand–Williams recommends [252], the reaction was monitored over time to establish a kinetic scale depending on the stabilisation time–point of the reaction [252]. A sample is considered *fast* antioxidant if the stabilisation point of the reaction is reached before 30 min, *intermediate* antioxidant if it stabilizes between 30 and 60 min and *slow* antioxidant if it needs more than 60 min to stabilize.

Five of the six extracts (OV1, OV2, OV3, OV5 and OV6) were intermediate antioxidants (stabilisation points between 30 and 60 min). OV4 was the extract that required the longest time to stabilize the reaction: stabilisation point at 75 min, presenting a kinetic behaviour similar to BHT (slow kinetic). Rosmarinic acid showed a fast kinetic reaction (**Table 29**).

Table 29. Classifications of extracts of *O. vulgare* and BHT in terms of antioxidant activity.

Sample	Stabilisation (min)	$\overline{EC}_{50}$ (μg/mL)	Kinetic	AAI	Strength
OV1	60	3.91±0.21	Intermediate	5.11	Very strong
OV2	45	2.52±0.15	Intermediate	7.93	Very strong
OV3	45	3.65±0.17	Intermediate	5.47	Very strong
OV4	75	2.66±0.04	Slow	7.52	Very strong
OV5	60	3.22±0.19	Intermediate	6.19	Very strong
OV6	30	2.05±0.04	Intermediate	9.75	Very strong
BHT	90	78.07±4.31	Slow	1.02	Strong
RA	15	1.71±0.04	Fast	11.69	Very strong

EC_{50} values can be internationally comparable thanks to a proposed antioxidant activity index (AAI), which is obtained as a result of dividing the concentration of DPPH• in the final solution (20 μg/mL in this study) by the value of EC_{50} [253]. This index might determine the strength of the antioxidant activity, regardless of the concentration of DPPH•. According to the current classification, plant extracts are considered *poor* antioxidants when AAI < 0.5, *moderate* when AAI is between 0.5 and 1.0, *strong* if AAI is between 1.0 and 2.0 and *very strong* antioxidants when AAI > 2.0.

Antioxidant activity index (AAI) was also calculated to determine the strength of antioxidant activity of the extracts, regardless of the concentration of DPPH• (**Table 29**). The results showed that the six extracts were *very strong antioxidant*, with their AAI > 2, with OV1 being the lowest with 5.11 and OV6 being the highest with 9.75. However, BHT could be considered as a *slow moderate–strong antioxidant* compound, whereas rosmarinic acid was a *very strong antioxidant,* like the extracts.

The *in vitro* DPPH• assay was also performed with the gastrointestinal digestion samples from OV1, OV2 and OV6 extracts. BHT and Rosmarinic acid (RA) were also subjected to the gastrointestinal digestion process to obtain positive controls (**Table 30**). From

rosmarinic acid, only the intestinal fraction was analysed, oral and stomach data are not available.

Table 30. Digestion process for BHT and Rosmarinic acid (RA). Data related to method and bioaccesibility (%), calculated from final mass (mg) and initial mass (mg).

	Step	Tampon Sol. used	Vol. added (mL)	Mass adjustment (mg)	Enzyme mass (mg)	Total extra mass added (mg)	Initial mass (mg)	Final mass digested (mg)	Bioaccesibility (%)
BHT	Mouth	$NaHCO_3$ 1 M	0.20	16.80	0.18	16.98	501.1	515.4	100.00
	Stomach	HCl 3 M	1.2	131.26	27.20	158.46	497.6	655.6	100.00
	Intestine *Abs.*	$NaHCO_3$ 1 M	0.6	50.40	73.93	124.33	498.5	437.80	**70.29**
	Intestine *Non–abs*.							184.50	
RA	Mouth	$NaHCO_3$ 1 M	0.40	33.60	0.18	33.78	–	509.30	–
	Stomach	HCl 3 M	0.20	21.82	27.20	49.02	–	595.60	–
	Intestine *Abs.*	$NaHCO_3$ 1 M	0.40	33.60	73.93	107.53	499.3	480.17	**79.12**
	Intestine *Non–abs.*							123.04	

BHT showed lower bioaccesibility comparing to the extracts, whose value was approximately 90 % for OV1 and OV2, and 80 % for OV6. Throughout the digestion process, the rosmarinic acid solution changed colour depending on the pH, being pink in the stomach. In the end, the intestinal absorbable fraction presented intense brown colour and little sediment (intestinal non–absorbable fraction) was found after centrifugation (123.04 mg), which means a high bioaccesibility of the compound (79.12 %). Previous studies that performed *in vitro* gastrointestinal digestion obtained a similar bioaccesibility of rosmarinic acid (76 %) by centrifugation of intestinal solution under the same conditions [192].

Before antioxidant quantification, TLC revealed with DPPH• solution was first performed with the three samples from the digestion step, OV1, OV2, OV6, BHT and rosmarinic acid (**Figure 32**).

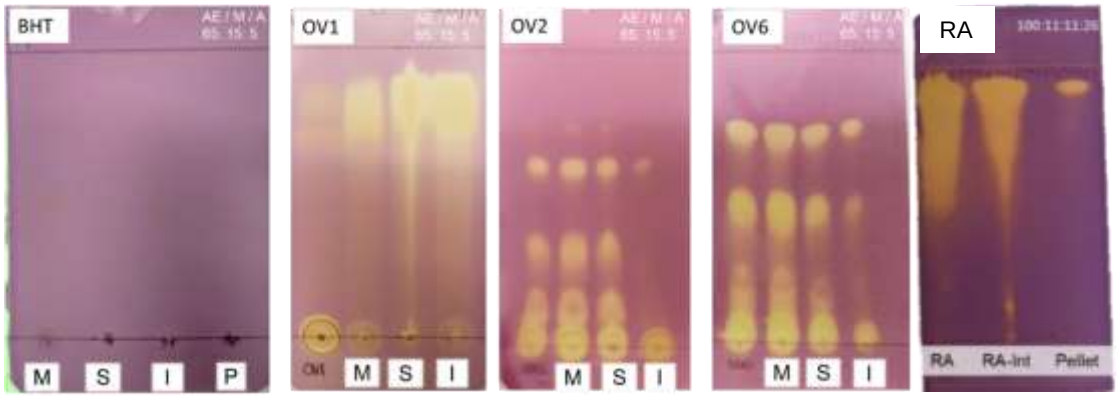


Figure 32. TLC sprayed with DPPH• solution. Mobile phase used: Ethyl acetate:Methanol:Water (65:15:5 v/v/v) for BHT and extracts, and Ethyl acetate:Glacial acetic acid:Formic acid:water (100:11:11:26 v/v/v/v/) for Rosmaric acid (RA). M: mouth/bucal digestion fraction); S: stomach digestion fraction; I: intestinal absorbable fraction; :P pellet or non–absorbable intestinal fraction.

In TLC (**Figure 32**), BHT did not show any yellow spot after applying DPPH• revealing solution after *in vitro* gastrointestinal digestion, while the rest of samples (OV1, OV2, OV6 and Rosmarinic acid) tend to retain this activity. An *in vitro* DPPH• quantitative assay was performed and the results obtained are showed in **Tables 31** to **33.**

Table 31. OV1 before and after each *in vitro* digestion step (Mouth, Stomach, Intestine). Antioxidant activity expressed as EC_{50} (µg/mL). Data expressed as means ± SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Values in bold means EC_{50} max (stabilisation point).

	Time (min)					
Sample	15	30	45	60	75	90
OV1	5.03 ± 0.37 [a]	4.29 ± 0.12 [a]	4.11 ± 0.25 [a]	**3.91 ± 0.21 [b]**	3.77 ± 0.21 [b]	3.39 ± 0.02 [b]
Mouth	6.94 ± 0.05 [a]	6.25 ± 0.02 [a]	5.19 ± 0.01 [a]	**5.08 ± 0.02 [b]**	4.95 ± 0.02 [b]	4.61 ± 0.02 [b]
Stomach	4.47 ±0.32 [a]	3.97 ± 0.13 [a]	3.34 ± 0.05 [a]	**3.18 ± 0.04 [b]**	3.16 ± 0.02 [b]	3.06 ± 0.02 [b]
Intestine	6.53 ± 0.03 [a]	5.58 ± 0.02 [a]	4.90 ± 0.03 [a]	4.02 ± 0.03 [a]	3.18 ± 0.02 [a]	**2.91 ± 0.04 [b]**

In terms of kinetics (**Table 31**), OV1 showed a slow stabilisation point (60 – 90 minutes). However, in the last steps of the gastrointestinal digestion process, its EC_{50} value improved (lowered) with statistical differences to the crude extract: 2.91 ± 0.04 and 3.91 ± 0.21 µg/mL, respectively with $p < 0.001$. Like OV1, all steps of digestion showed a strong index of antioxidant activity (AAI > 2.0: 3.93 (mouth), 6.29 (stomach) and 6.87 (intestine).

OV2 presented an intermediate kinetic behaviour throughout the *in vitro* gastrointestinal digestion process, with the exception of the stomach, which reached the stabilisation point 15 min later, at 60 min of reaction (**Table 32**). The antioxidant activity in the mouth and stomach –with no statistical differences between them, $p = 0.515$– showed significant differences with crude extract: EC_{50} 2.95 ± 0.02 (mouth), 2.92 ± 0.01 (stomach) and 2.52 ± 0.07 (OV2) µg/mL, with $p = 0.010$. As already happened in OV1, intestinal absorbable fraction significantly improved the *in vitro* antioxidant activity of the crude extract: EC50 1.23 ± 0.02 µg/mL (intestine), with $p < 0.001$ with OV2, OV2–Mouth and OV2–Stomach.

Table 32. OV2 before and after each *in vitro* digestion step (Mouth, Stomach, Intestine). Antioxidant activity expressed as EC_{50} (µg/mL). Data expressed as means ± SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Values in bold means EC_{50} max (stabilisation point).

	Time (min)					
Sample	15	30	45	60	75	90
OV2	3.11 ± 0.07 [a]	2.75 ± 0.32 [a]	**2.52 ± 0.07 [b]**	2.41 ± 0.15 [b]	2.34 ± 0.13 [b]	2.26 ± 0.06 [b]
Mouth	4.04 ± 0.03 [a]	3.32 ± 0.03 [a]	**2.95 ± 0.02 [b]**	2.83 ± 0.02 [b]	2.74 ± 0.03 [b]	2.63 ± 0.02 [b]
Stomach	5.04 ± 0.04 [a]	3.45 ± 0.03 [a]	3.15 ± 0.02 [a]	**2.92 ± 0.01 [b]**	2.73 ± 0.03 [b]	2.63 ± 0.01 [b]
Intestine	1.63 ± 0.32 [a]	1.38 ± 0.03 [a]	**1.23 ± 0.02 [b]**	1.14 ± 0.02 [b]	1.09 ± 0.02 [b]	1.06 ± 0.03 [b]

The AAI values for OV2 after digestion showed that the extract was a strong antioxidant agent. In all cases, the AAI was higher than 2.0, 6.78 (mouth), 6.85 (stomach) and 16.26 (intestine).

Being OV6 the most antioxidant crude extract, after the *in vitro* digestion process it did present a significant loss of activity (**Table 33**): EC_{50} 2.05 ± 0.04 (OV6) and 7.84 ± 0.68 (intestine) µg/mL, with $p < 0.001$. Regarding kinetics, the oral and stomach digestion fractions needed more time to reach the stabilisation point (90 and 75 min, respectively), being classified as slow antioxidants; while the intestinal fraction maintained the kinetic behaviour of the crude extract by stabilizing the reaction at 30 min.

Table 33. OV6 before and after each *in vitro* digestion step (Mouth, Stomach, Intestine). Antioxidant activity expressed as EC_{50} (µg/mL). Data expressed as means ± SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Values in bold means EC_{50} max (stabilisation point).

	Time (min)					
Sample	15	30	45	60	75	90
OV6	2.40 ± 0.46 [a]	**2.05 ± 0.04 [b]**	2.16 ± 0.06 [b]	2.02 ± 0.07 [b]	1.96 ± 0.09 [b]	1.83 ± 0.08 [b]
Mouth	18.17 ± 0.30 [a]	12.07 ± 0.22 [a]	9.70 ± 0.17 [a]	8.35 ± 0.13 [a]	7.56 ± 0.15 [a]	**7.09 ± 0.14 [b]**
Stomach	9.43 ± 0.43 [a]	6.63 ± 0.26 [a]	5.63 ± 0.22 [a]	5.08 ± 0.19 [a]	**4.70 ± 0.14 [b]**	4.49 ± 0.12 [b]
Intestine	8.68 ± 0.66 [a]	**7.84 ± 0.68 [b]**	7.38 ± 0.69 [b]	7.07 ± 0.71 [b]	6.87 ± 0.70 [b]	6.73 ± 0.70 [b]

Before digestion, OV6 had an AAI index of 9.76, being a very strong antioxidant extract. After digestion, these values were significantly lower than the crude extract, 7.09 (mouth), 4.70 (stomach) and 7.84 (intestine) µg/mL, with $p < 0.001$ to OV6. Despite the loss of antioxidant activity after digestion, OV6 could still be considered as very strong antioxidant in the intestine (AAI > 2).

BHT after digestion did not show any colour transformation in TLC (**Figure 32**) and the inhibition percentages did not reach even 20 % (which was considered as baseline in previous DPPH• blank tests [254]). Therefore, the use of this compound as positive control of antioxidant activity *in vitro* after the digestion process was not recommended.

Rosmarinic acid reached stabilisation point relatively quickly, at 15 min with EC_{50} = 1.71 ± 0.04 µg/mL and its intestinal absorbable fraction 15 min later with no statistically differences (p = 0.999) (**Table 34**).

Table 34. Rosmarinic acid before (RA) and after (RA–Int) *in vitro* gastrointestinal digestion (Intestine). Antioxidant activity and EC_{50} (µg/mL). Data expressed as means ± SD of triplicate analysis. Values with different letter present significant differences (p < 0.05) and same letter indicates no significant differences (p > 0.05). Values in bold means EC_{50} max (stabilisation point).

Sample	Time (min)					
	15	30	45	60	75	90
RA	**1.71 ± 0.04** [a]	1.69 ± 0.05 [a]	1.67 ± 0.05 [a]	1.66 ± 0.05 [a]	1.64 ± 0.04 [a]	1.63 ± 0.04 [a]
RA–Int	2.84 ± 0.11 [a]	**1.88 ± 0.15** [b]	1.84 ± 0.11 [b]	1.76 ± 0.12 [b]	1.72 ± 0.14 [b]	1.69 ± 0.15 [b]

According to these results, rosmarinic acid retains its antioxidant activity *in vitro* after simulating a gastrointestinal digestion process and the biochemical changes that it could entail, being a very strong antioxidant (AAI > 2.0; 11.69 –before digestion– and 10.64 –after intestinal digestion–).

1.2 ABTS• *in vitro* assay

As a complement to DPPH• antioxidant determination, the Trolox equivalent antioxidant capacity (TEAC) method, also known as the ABTS radical cation decolourization assay, was performed *in vitro*. This assay determines, through a simple and inexpensive protocol, the ability of an antioxidant compound to counteract the free radical ABTS. Unlike other common *in vitro* antioxidant tests to determine this activity, this method does not require enzymes or special conditions [243]. In addition, the method could be applicable to the study of water–soluble and lipid–soluble antioxidants, offering a potentially versatile antioxidant action inside the human body.

1.2.1 MATERIAL AND METHODS

- Experimental basis

The ABTS• *in vitro* assay was carried out according to García–Herreros *et al.* [219]. It is based on the formation of ABTS cation radical that exhibits a colour change measurable by spectrophotometry at 741 nm (**Figure 33**).

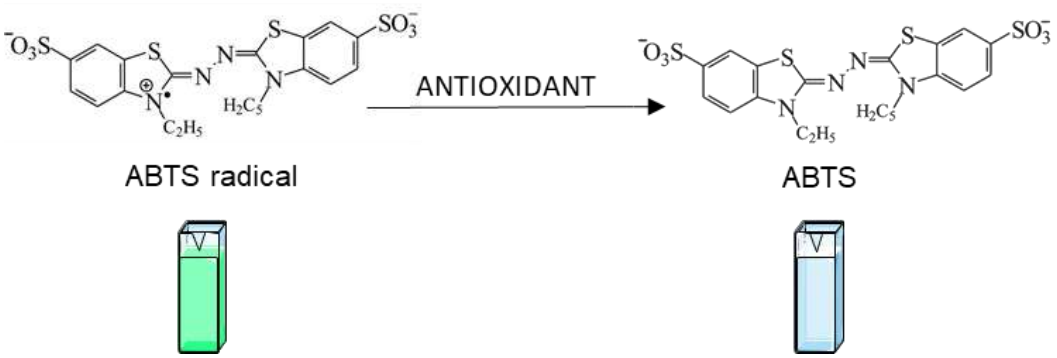


Figure 33. Chemical basis of the ABTS• assay: colorimetric reaction in presence of antioxidant compound.

- Substrate preparation

First, a solution of $K_2S_2O_8$ 140 mM in water was prepared with a tablet of ABTS• (#10102946001, Sigma–Aldrich Co., St. Louis, MO) and kept in the dark for 12 h. The ABTS free radical is generated through the following reactions:

$$S_2O_8^{2-} + ABTS \rightarrow SO_4^{2-} + SO_4^{-*} + ABTS^{+*}$$
$$SO_4^{-*} + ABTS \rightarrow SO_4^{2-} + ABTS^{+*}$$
$$S_2O_8^{2-} + 2ABTS \rightarrow 2SO_4^{2-} + 2ABTS^{+*}$$

- Sample preparation

Once the free radical was generated, the six extracts (OV1 – OV6) were resuspended in distilled water at 1 mg/mL. Distilled water was used as negative control.

Digestion fractions of the three previously performed *in vitro* gastrointestinal digestion processes (OV1, OV2 and OV6; mouth, stomach and intestine fractions) were also analysed.

- Analyse conditions

In a 96–well plate, 182 µL of the reagent solution (substrate) were mixed with 18 µL of each sample or control and allowed to react for 6 min. Then, the absorbance at 741 nm was measured with a FLUOStar Omega spectrofluorometric analyser (BMG Labtechnologies, Offenburg, Germany).

- Data process

Assay was performed in triplicate and the inhibition percentage was calculated using the following formula:

$$\%\, I = \frac{Abs_{control} - Abs_{t=1}}{Abs_{control}}\ x\ 100$$

Where *% I* is the inhibition percentage, $Abs_{control}$ the absorbance of control and $Abs_{t=1}$ the absorbance at time 1.

Finally, results were expressed in terms of Trolox (6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid, TE), a water–soluble analogue of vitamin E that can be used as an antioxidant standard (mg TE per 100 mg of lyophilised extract) [243]. Data transformation was obtained by extrapolation from the Trolox calibration curve whose equation was $y = 0.2802x + 0.8694,\ R^2 = 0.9952$, where *y* is the inhibition percentage (*% I*) and *x* corresponds to Trolox concentration (mM).

Intestinal absorbable fractions were also subjected to this complementary *in vitro* antioxidant test.

- Statistical analysis

Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

1.2.2 RESULTS

The complementary *in vitro* ABTS• antioxidant assay was performed with the six crude extracts at 1 mg/mL concentration and the results were expressed as the amount of Trolox (TE) per mg of lyophilised extract, after substituting the data in the Trolox calibration curve (**Figure 34**).

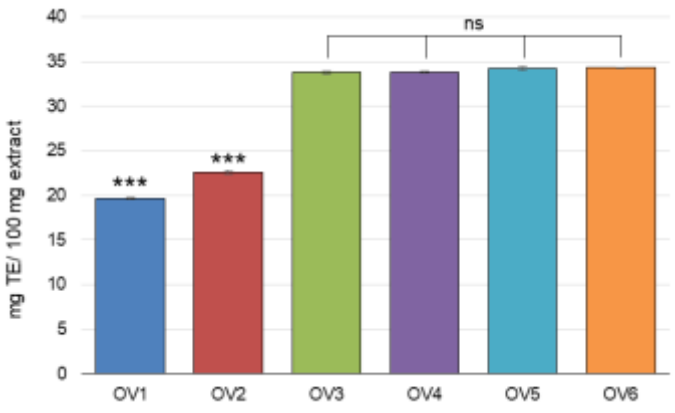


Figure 34. ABTS• results for the extracts expressed as mg TE/100 mg extract ± SD. *ns* indicates non–statistical differences and ***, $p < 0.001$. All extracts were statistically compared with each other.

OV1 (19.64 ± 0.10 mg TE in 100 of mg extract) and OV2 (22.59 ± 0.18 mg TE) were the least antioxidant extracts with statistical differences ($p < 0.001$). The aqueous extracts were also significantly different from the rest of the extract, which did not show statistical

differences between them: 33.75 ± 0.13 (OV3), 33.81 ± 0.08 (OV4), 34.24 ± 0.20 (OV5) and 34.28 ± 0.09 (OV6) mg TE per 100 mg of extract, with $p > 0.05$.

This assay was repeated with the digested extracts (OV1, OV2 and OV6) in order to verify the antioxidant activity of the intestinal absorbable fraction. The samples obtained from the three digestion steps were analysed through this *in vitro* assay (**Figure 35**).

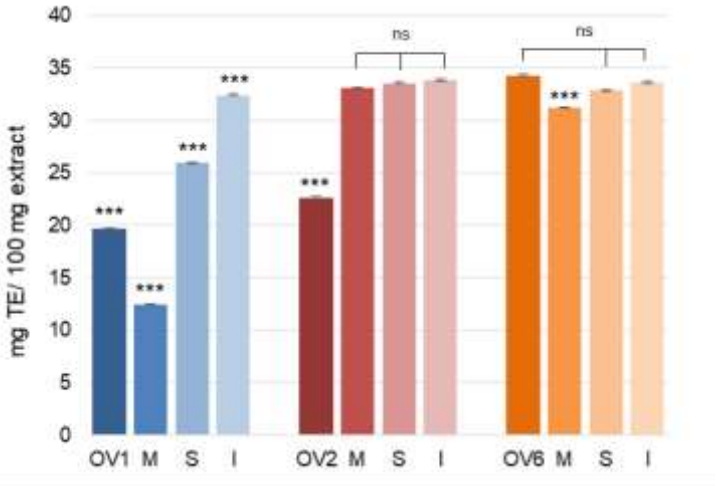


Figure 35. ABTS• results for OV1, OV2 and OV6 at different stages of digestion (mean ± SD, mg TE per 100 mg extract). M: Mouth/oral digestion); S: stomach digestion); I: intestinal absorbable fraction of digestion). Within same extract, *ns*: no significant statistical differences observed ($p > 0.05$) and ***: statistical differences ($p < 0.001$).

As shown (**Figure 35**), OV1 increased antioxidant activity from 19.64 ± 0.10 to 32.41 ± 0.10 mg TE per 100 mg extract, as well as OV2: from 22.59 ± 0.18 to 33.87 ± 0.14 mg TE per 100 mg extract, with $p < 0.001$. Unlike the aqueous extracts, OV6 was able to maintain the antioxidant activity in a 98.10 %: crude extract 34.28 ± 0.09 and intestinal fraction, 33.63 ± 0.17 mg TE per 100 mg extracts, with no statistical differences observed ($p = 0.998$). In a previous study [177], an extract of *O. vulgare* obtained by hot maceration in methanol showed, after the *in vitro* digestion process, a conservation of 98.47 % of the *in vitro* antioxidant activity measured through the ABTS• assay (from 42.06 to 41.5 mg TE per 100 mg extract).

2 HYPOGLICAEMIC ACTIVITY

One of the main diseases of worldwide concern is non–insulin dependent *Diabetes miellitus* (NIDDM), caused by an insulin resistance or a decreased secretion by Langerhans β cells of this hypoglycemic hormone.

In Spain, 13.8 % of population suffers from Diabetes and 47 % of real diabetics remain undiagnosed. The World Health Organisation (WHO) [255] predicts that by 2035, 22 % of

diabetic worldwide population will double. Moreover, a study carried out by *Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas del Instituto de Salud Carlos III in collaboration with the Sociedad Española de Diabetes (SED) and the Federación Española de Diabetes (FED*) [256] concluded that 3.6 % of glycaemic data obtained from pharmacies was altered and 7.9 % people tested were insulin intolerant.

After ingestion, sugars and carbohydrates are digested and reduced to glucose, which is absorbed in the intestine. This increase of glycaemia (glucose levels in blood) promotes the release of hormone insulin in the pancreas that lowers glucose levels. The β–cells in pancreas secrete insulin so that glucose can enter into cells as a cell nutrient. In addition, insulin stimulates the formation of glycogen in the liver and thus blood glucose to normal values. The antagonistic hormone produced by the endocrine side of the pancreas, glucagon, stimulates glycogen degradation when there is a lack of glucose intake and glucose is needed. This is a simplification of endocrine feedback to control glycaemia and body energy balance. However, as occurs with the antioxidant reactions in the body, these can also be altered giving rise a worldwide–extended disease: Diabetes. There are three main types of diabetes, depending on the cause of the problem [257]:

- **Type I diabetes.** 5–10 % of all diabetic patients have this type of the disease [255]. Its cause is autoimmune since the organism itself destroys the β–cells of the pancreas. Type I diabetes is a non–curable disease that appears suddenly and whose patients are generally young and of normal weight. As a treatment, patient needs to inject insulin because this function of the pancreas is partially or totally disabled. At first glance, it may seem an easy treatment, but the challenge relies on the patient who must be able to calculate the dose of insulin required for each glucose intake. Although engineering and pharmaceuticals have played such a helpful role with diabetics (insulin pumps, different types of insulin depending on the speed of action, mobile applications with glycaemia predictions…), science continues to search for a cure in theories such as the microencapsulation of β–cells, transplantation or genetic theories.

- **Type II diabetes.** Almost all diabetic patients in the world suffer from this type of metabolic diabetes (90 – 95 %) [255]. Generally, these patients are adults whose risk factors (overweight, sedentary lifestyle, non–healthy diet or family history) lead their bodies to insulin resistance. This disease can be suffered silently and asymptomatically without medical diagnosis. Therefore, glycaemic monitoring is important to keep resistance under control with hygienic–dietary changes in lifestyle. As prevention, glycaemic can be periodically monitored in pharmacies or MD's and takes the Findrisk test (questioning the

risk of suffering type II diabetes in 10 years). A low score in this test does not mean a lack of pre–diabetes or diabetes. Treatment begins with daily oral medicines that affect different pathways (DPP–4, GLUT–1, α–glycosidase…). If glycaemia and HbA1c (glycosylated haemoglobin that reflects mean glycaemia within last 2 – 3 months) values remain under control, monotherapy is sufficient. However, when the patient presents glycaemic alterations –reflected by a high HbA1c value (> 7 %) – more than one type of hypoglycaemic treatment is needed and sometimes insulin is part of this treatment.

- **Gestational diabetes.** Last but not least, there is a third type of diabetes that can appear during pregnancy. Both mother and child may develop diabetes if the glycaemia is not controlled during pregnancy.

For all the three types of diabetes, patient education and selection of the optimal treatment for them are important. When the disease is not treated well, blood glucose is so high that it can cause tissue damage due to overproduction of superoxide in cells, leading to potentially fatal complications such as retinopathies, limb amputations, neuropathies, renal and cardiovascular (CVD) pathologies or even premature death.

Among antidiabetic treatments (**Table 35**), inhibition of the *α–glucosidase* pathway has been shown to be effective in delaying intestinal absorption of polysaccharides and disaccharides. This enzyme, located in the membrane–bound epithelium of the small intestine, is responsible for the cleavage of glucose from ingested disaccharides.

Table 35. Summary of current oral treatments for type II diabetes with their potential side effects and precautions/contraindications [258]. HbA1c = Glycosilated haemoglobin (goal values < 5.5 % for non–diabetic patients and < 6.5 % for diabetic patients).

Medication	HbA1c reduction	Potential adverse effects	Precautions/contraindications
Alpha–glucosidase inihibtors: ***Acarbose*** *Miglitol*	0.5 – 0.8 %	Flatulence, diarrhoea, abdominal bloating	Avoid when creatinine clearance < 25 mL / min Most effective when given with starchy, high–fiber diet Reverse hypoglycaemia with glucose, not sucrose
Biguanides: *Metformin*	1.0 – 1.3 %	Nausea, diarrhoea, abdominal floating	Especial warning for GFR 30 – 44 mL /min and < 30 mL /min
Dipeptidyl–peptidase–4–inhibitors (DPPIV inhibitors) *Suffix: –gliptin*	0.5 – 0.9 %	Headache, pancreatitis	*Linagliptin* does not require dosage adjustment in renal insufficiency *Saxagliptin* dosage adjustment when administered with concomitant CYP3A4 inhibitors
Glucagon–like peptide–1 receptor agonists *Suffix: –glutide*	0.8 – 2.0 %	Nausea, vomiting, sense of fullness Weight loss 1 – 4 kg	Exenatide not recommended if GFR < 30 mL
Meglitinides *Suffix: –glinide*	0.5 – 0.9 %	Hypoglycaemia	Metabolized primarily by the liver (CYP3A4 and CYP2C9)
Sodium–glucose transporter 2 inhibitors *Suffix: –agliflozin*	0.5 – 0.9 %	Increased urinary tract and genital infections, increased LDL Weight loss of 0.7 – 3.5 kg	Dosage adjustment required in renal insufficiency
Sulfonylureas *Glimepiride* *Glipizide* *Glyburide*	0.4 – 1.2 %	Hypoglycaemia, weight gain	Dosage adjustment required in renal insufficiency
Thiazolidinedione *Suffix: –glitazone*	0.5 – 1.4 %	Weight gain, oedema	Contraindicates in patients with congestive heart failure Decrease concomitant insulin dose at initiation

Despite the antidiabetic efficacy of acarbose (lowering HbA1c, **Table 35**), digestive problems such as flatulence (probably affecting more than 1 in 10 people), stomach pain and diarrhoea (probably affecting fewer than 1 in 10 people) are the main side–effects that limit it success in prescribing [259]. In addition, acarbose causes rare side effects (likely to affect less than 1 in 100 people): nausea, vomiting, increasing liver enzymes and indigestion [260]. Many plants with *α–glucosidase* inhibitory activity have been shown to generate less side effects [13,189,261–263].

Considering type II diabetes as one of the main pathologies of the metabolic syndrome, the six extracts were tested to inhibit this enzyme through an *in vitro* assay. The same *in vitro* test was repeated with the intestinal fractions of the previously performed digestions (OV1, OV2 and OV6), since this enzyme is human and is found in the intestine.

2.1 MATERIAL AND METHOD

- Experimental basis

According to Matsui [264] and Shuyuan *et al.* [263], the inhibitory activity of *α–glucosidase* can be measured thanks to an analogous disaccharide (4–Nitrophenyl–α–*D*–glucopyranoside –*p*–NPG–, used as substrate). The enzyme breaks down the α chemical bond of the substrate, so that carbohydrate D–Glucopyranoside and the phenolic compound 4–Nitrophenyl are released. 4–Nitrophenyl turns yellow at pH 6.8, making it quantifiable at 405 nm (**Figure 36**).

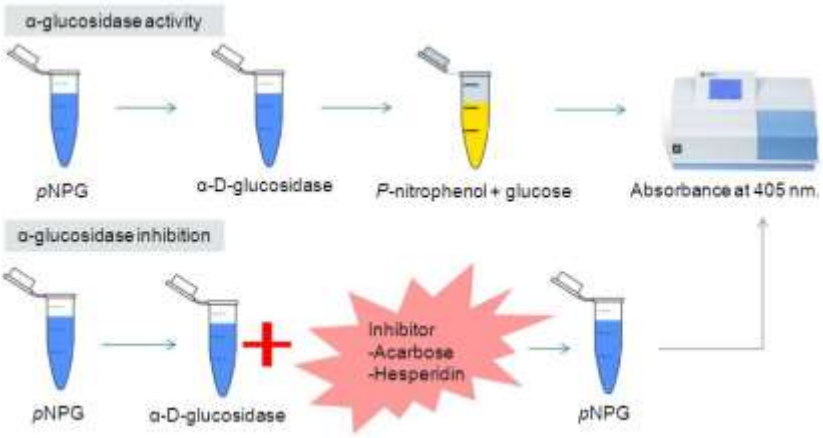


Figure 36. Scheme of the inhibition and activity of *α–glucosidase*. Reactive p–NPG (4–Nitrophenyl–α–D–glucopyranoside) is an analogous disaccharide that is broken down by the enzyme into P–nitrophenol (a compound with a yellow colour measurable at 405 nm) and glucose. In the presence of any *α–glucosidase* inhibitor (e.g. acarbose), a yellow coloration would not result as the enzyme is removed.

The more inhibitory activity the extract has, the less yellow the solution will have (measurable, lower absorbance value at 405 nm). To enhance colour, a basic solution must be added (optimal efficient medium pH 6.8) [265] so that there are enough electrons for the *p*–nitrophenolate anion to make resonance and stabilize [266]. This protocol was established in the laboratory with the help of Hugo Lana.

- Enzyme preparation

The enzyme *α–glucosidase* needs to be stored at –20 ºC, and kept cold during handling. The enzyme was obtained from *Saccharomyces cerevisae* (#G0660, Sigma–Aldrich Co., St. Louis, MO) and the optimal pH is 6.8, the same as the intestinal one [267]. The enzyme was resuspended in 0.1 M PBS buffer, pH 6.8, at a concentration of 0.5 U/L –within the range suggested 0.2 U/L–1U/L [268].

- Substrate preparation

As the enzyme, substrate (4–Nitrophenyl–α–*D*–glucopyranoside – #*N*1377, Sigma–Aldrich Co., St. Louis, MO) must be stored at –20 ºC and kept on ice while handling. After several titration tests performed, the optimal substrate concentration was 1 mM in 0.1 M PBS buffer and pH 6.8.

- Stop solution preparation

It is necessary a solution to stop the reaction and measure the inhibitory activity avoiding biodegradation. 80 µL of 0.15 M Na_2CO_3 was added to each well after the reaction occurred.

- Sample preparation

The different lyophilised extracts were diluted in 0.1 M PBS buffer, pH 6.8 at six serial concentrations (1,000 – 0.03125 µg/mL). Acarbose (#A8980, Sigma–Aldrich Co., St. Louis, MO) was used as the positive control at the same concentrations and the sample solvent (PBS) was used for the negative control. Finally, the intestinal digestion fractions of OV1 were also analysed, as well as the intestinal fractions of the corresponding positive control (Acarbose–Int at the same concentrations).

- Analysis conditions (**Figure 37**)

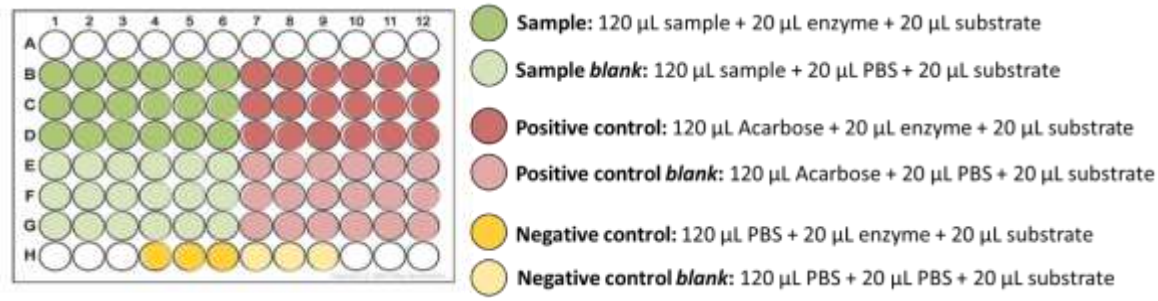


Figure 37. 96–well plate design for the antidiabetic assay: distribution and a coloured legend to explain the content of each well with a photo of the result.

Every plate needs a *positive control* (120 µL Acarbose) and a *negative control* (120 µL PBS) with the corresponding blanks. 20 µL PBS was used for *blanks* instead of enzyme (20 µL 0.5 U/L in PBS) and the volume of substrate was constant for each condition (20 µL).

Sample, acarbose, PBS and enzyme were added to the 96–well plate and incubated for 15 min at 37 ºC with shaking. Then, substrate was added according to the design. It was incubated under the same circumstances for an additional 15 min. After the incubation time, 80 µL of the stop solution were added to each sample.

- Data process

The absorbance measurement was made with the Power WaVe XS de BioTek® spectrophotometer at 405 nm.

1. Calculation of the oxidation inhibitory percentage for each kinetic measurement of each plate with the following formula:

$$Inhibitory\ (\%) = \left(1 - \frac{\left((Abs_S - Abs_{SB}) - (Abs_C - Abs_{CB})\right)}{Abs_C - Abs_{CB}}\right) x100$$

Where, *Inhibitory* (%, percentage of inhibition) is obtained from the absorbance of the sample (Abs_S) without its blank (Abs_{SB}: Sample Blank Absorbance) against the absorbance of the negative control (Abs_C) without its blank (Abs_{CB}: Negative Control Blank Absorbance).

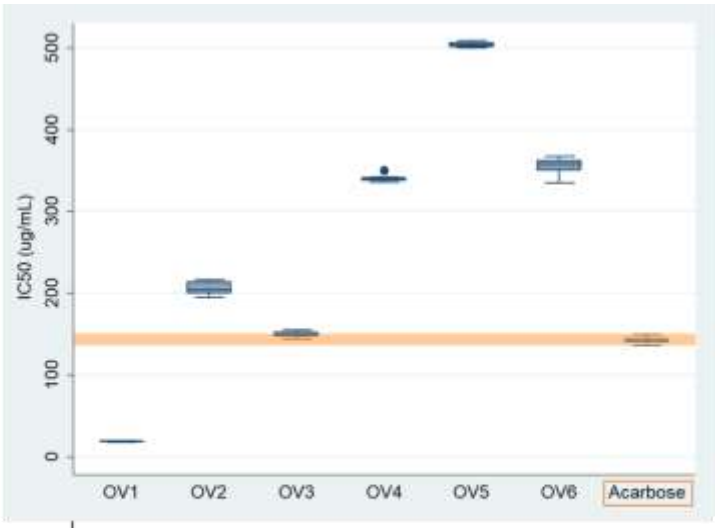
2. Calculation of IC_{50}, corresponding to the concentration where the 50% of the substrate is degraded by the enzyme, as an indirect quantitative measure of the inhibitory activity. IC_{50} values were generated with GraphPad Prism, v6.01 (GraphPad Software, La Jolla, CA).

- Statistical analysis

Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

2.2 RESULTS

α–glucosidase inhibition percentages were calculated for each measured concentration with the formula showed above. Then, the inhibitory percentages were transformed into a concentration at which 50 % of the enzyme was inhibited (IC_{50} in µg/mL). Acarbose was used as positive control and reference compound (**Figure 38**).



	Sample						
	OV1	OV2	OV3	OV4	OV5	OV6	Acarbose
IC50 (µg/mL)	18.31 [a]	205.17 [b]	149.40 [c]	339.56 [d]	503.80 [e]	355.00 [d]	141.47 [c]
SD	0.30	7.36	3.36	3.60	2.69	10.05	3.57

Figure 38. *In vitro* inhibitory *α–glucosidase* effect results expressed as IC_{50} for all extracts and positive control. Table below shows the mean and SD of values. Values with different letters show significant differences ($p < 0.05$) and the same letter indicates that there are no significant differences ($p > 0.05$).

The results showed statistical differences ($p < 0.001$) between most of the extracts. Acarbose was shown to inhibit 50 % of the enzyme at 141.47 ± 3.57 µg/mL. From that reference concentration (orange band in the boxplot), only OV1 showed a significantly lower IC_{50} (18.31 ± 0.30 µg/mL, $p < 0.001$), which means that a concentration 7.72 times lower is needed to produce an effective effect as acarbose. From its part, OV5 presented the highest IC_{50} value (503.80 ± 2.69 µg/mL), followed by OV6 and OV4 (355.00 ±10.05 and 339.56 ± 3.60 µg/mL, $p = 0.095$ among them), OV2 (205.17 ± 7.36 µg/mL) and OV3 (149.40 ± 3.36 µg/mL), with OV3 not significantly different from acarbose (149.40 ± 3.36 µg/mL, $p = 0.501$).

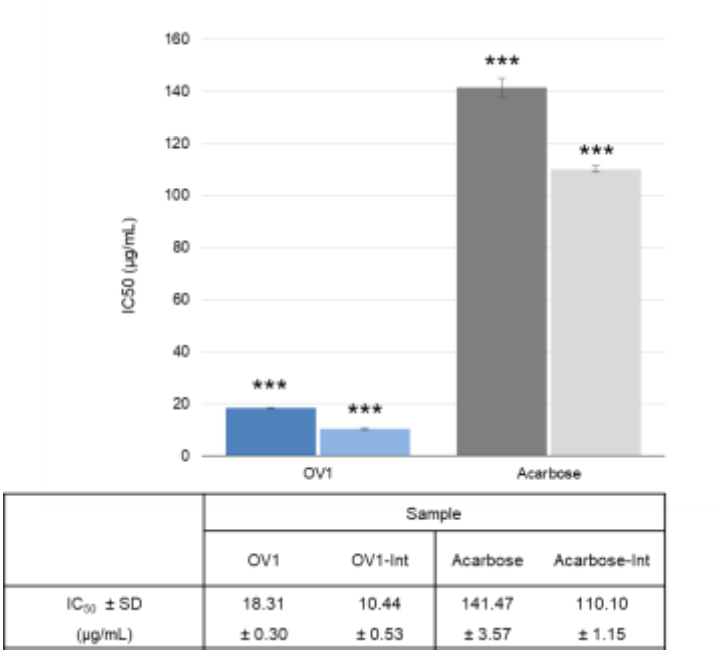
Previous studies with the commercial oregano species [269], whose extract was prepared with 80 % ethanol as solvent, showed an *in vitro* inhibition of this enzyme of 63.33 ± 2.04 % at 400 µg/mL [269], being inhibition of OV6 at that concentration 59.85 ± 0.18 % in the current study.

As explained, *α–glucosidase* is an enzyme physiologically located in the lumen. Therefore, intestinal fractions after the *in vitro* digestion process might show more accurate inhibitory results than crude extracts. Because it is the inhibitoriest extract, the hypoglycaemic activity of OV1 was also determined after the *in vitro* gastrointestinal digestion process. In order to compare with a positive control, acarbose was also subjected to the *in vitro* gastrointestinal digestion process (**Table 36**). Acarbose showed a high bioaccesibility (71.61%) after the gastrointestinal digestion process.

Table 36. Digestion process for acarbose with the three steps: mouth, stomach and intestine (with absorbable and non–absorbable (non–abs.) fractions).

Acarbose	Buffer Solution used	Vol. added (mL)	Mass adjustment (mg)	Enzyme mass (mg)	Total extra mass added (mg)	Initial mass (mg)	Final mass digested (mg)	Bioaccesibility (%)
ORAL	$NaHCO_3$ 1 M	0.12	10.08	0.18	10.26	499.00	509.30	100.00
STOMACH	HCl 3 M	1.00	109.38	27.20	136.58	500.30	595.60	100.00
INTESTINAL Abs.	$NaHCO_3$ 1 M	0.50	42.00	73.93	115.93	500.20	441.13	**71.61**
INTESTINAL Non–abs.							175.00	

Contrary to the results of published studies [269], the intestinal fractions showed lower IC_{50} value after digestion compared to the results of the extracts before digestion (**Figure 39**).



	Sample			
	OV1	OV1-Int	Acarbose	Acarbose-Int
IC_{50} ± SD (µg/mL)	18.31 ± 0.30	10.44 ± 0.53	141.47 ± 3.57	110.10 ± 1.15

Figure 39. *α–glucosidase* inhibitory activity expressed as IC_{50} (mean ± SD µg/mL) before and after digestion: Statistical differences are indicated in graph with *** ($p < 0.001$).

Therefore, the most hypoglycaemic extract (OV1) showed an improvement in hypoglycaemic activity after digestion, being even more hypoglicemiant than the positive control: 18.31 ± 0.30 µg/mL before digestion and 10.44 ± 0.53 µg/mL after digestion, with $p < 0.001$ – with all values. The significant improvement of this activity in the intestinal absorbable fraction leads to consider OV1 as a potentially future oral alternative to acarbose in treatment of type II diabetes. No similar published were found studies against this enzyme using intestinal absorbable fractions of plant extracts.

3 LIPID–LOWERING ACTIVITY

Cholesterol is waxy, fat–like substance present in the cell membrane that also circulates in the blood in diverse particles containing lipids and proteins (lipoproteins) [233]. This substance fulfills several functions in the human body: structural (fluidity of the cell membrane and lipid rafts), metabolic (steroid formation) and signalling (cell growth, proliferation, migration and cancer risk) [233]. Cholesterol can be endogenous synthethised in the liver by the mevalonate metabolic pathway or come from exogenous intake, meat and dairy products, mainly [233]. Ingested dietary fats are absorbed in the intestine in the form of quilomicrons, which are lipoproteins with a high content of triglycerids [270]. These are transported to liver, where they are metabolised. Serum contains thres types of lipoproteins: low density lipoprotein (LDL), high density lipoprotein (HDL) and very low density lipoproteins (VLDL) [271].

Due to its hydrophobic structure, cholesterol is transported in blood by these lipoproteins and remains in the arteries in the form of LDL–c, whose high values can cause atherosclerosis by entrapment in the sub–endothelial space of the arteries [228,271,272]. Cholesterol transported as LDL–c from the liver to extra hepatic tissues should not be elevated, while HDL–c –that mobilizes cholesterol back to the liver– should higher, the better because it presents cardioprotective, antioxidant and anti–inflammatory properties [228,272]. As medical advice, these serum parameters should be periodically monitored in the prevention and treatment of hypercholesterolemia, which often leads to the development of atherosclerosis and, consequently, to cardiovascular pathologies and the development of a chronic inflammatory state. Through changes in diet and lifestyle, the patient can sometimes modify the values preserving health status. However, other times these recommendations are not enough or non–modifiable risk factors lead the patient to a high blood lipid content.

Endogenous cholesterol is produced by the mevalonate metabolic pathway, where the enzyme HMG–Coa reductase catalyses one of the first reactions that produces mevalonic acid [231]. HMGR (3–hydroxy–3–methylglutaryl–CoA reductase) is a transmembrane glycoprotein located in the endoplasmic reticulum that catalyzes the reduction of HMG–CoA into coenzyme A (CoA) and mevalonate. Generally, the LDL receptor and oxidized cholesterol species control enzyme activity through synthesis, degradation and phosphorylation to maintain mevalonate concentrations; and for this reason, it has been the target of hypolipemiant drugs [230]. Competitive inhibitors of this enzyme induce expression of LDL receptors in the liver, LDL catabolism is increased and so, cholesterol is reduced [233].

Statins (rosuvastatin, lovastatin, atorvastatin, pravastatin, simvastatin…) are the main group of compounds capable of inhibiting HMG–CoA reductase. Although these molecules are obtained by synthetic chemistry, their origin is found in the natural bioactive compound found on red rice yeast: monacolin K [228,230,233].

Although oregano extracts did not present this bioactive compound, this inhibitory activity was also tested *in vitro* as part of its possiblea pharmacological activity in the prevention of metabolic syndrome and cardiovascular diseases (CVD).

3.1 MATERIAL AND METHODS

- Experimental basis

The hypolipemiant activity was tested by inhibiting the enzymatic activity of HMG–CoA reductase, as a result of the decrease in the absorbance of $NADP^+$ (reduced by nicotinamide adenine dinucleotide phosphate) in the presence of the HMG–CoA substrate (**Figure 40**).

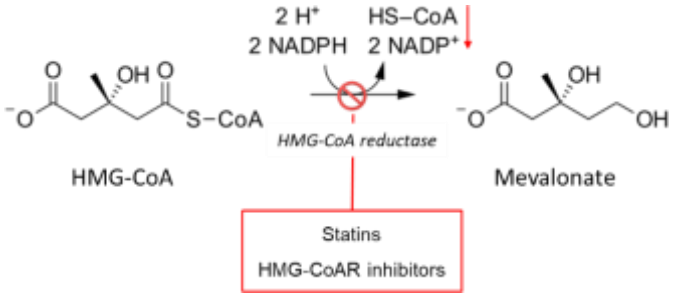


Figure 40. HMG–CoA reductase pathway. The enzyme (HMG–CoA reductase) reduces HMG–CoA (substrate) to mevalonate, which requires two NADPH. By inhibiting this reaction, NADP+ production is reduced and the amount of NADPH not consumed can be monitored in experimental research.

The reaction was reproduced *in vitro* using a commercial kit (#CS1090–1KT, Sigma–Aldrich Co., St. Louis, MO), following indications from the manufacturer and the previously published protocol [229,273,274]. This kit contains β–Nicotinamide adenine dinucleotide 2′–phosphate reduced tetrasodium salt hydrate (NADPH, #N6505, Sigma–Aldrich Co., St. Louis, MO), 5x assay buffer (#A5981, Sigma–Aldrich Co., St. Louis, MO), substrate solution (HMG–CoA, #S7447, Sigma–Aldrich Co., St. Louis, MO) and HMG–CoA reductase (catalytic domain, #H8789, Sigma–Aldrich Co., St. Louis, MO).

- Preparation of the substrate solution

10 µL NAPH, 407.5 µL of 5x buffer and 30 µL of HMGCo–A (substrate solution) from the commercial kit were placed in a 2 mL tube. These quantities refers to a single measure and condition.

- Preparation of the sample

The OV1 intestinal sample (OV1–Int) was dissolved in distilled water at 1 mg/mL and 50 µL were added to each tube containing substrate solution, per condition. As a negative control, distilled water was used, corresponding to 100 % of the enzyme activity.

- Preparation of the enzyme

2.5 µL of the enzyme (HMG–CoA reductase) were added per tube.

- Analysis conditions

The reaction was measured at different times: 0, 1, 2, 4 and 6 min from the start of the reaction (enzyme addition to tube). 80 µL of methanol were added to stop the reaction at the correspondent measurement times. The assay was performed in triplicate.

The amount of NADPH was measured by HPLC–DAD (LiChroCART® 250–4 LiChrospher® 100 RP–18 (5 µm) [274]). Twenty five microliters of the sample were injected and the analytical method consisted of a gradient composed of solution A (100 mM KH_2PO_4), and solution B (methanol) as follows: 0 min, 95% A, 5% B; 15 min 70% A, 30% B; 20 min, 20% A, 80% B, 23 min, 20% A, 80% B, with a flow of 0.8 mL/min. Detection was performed at 340 nm.

- Data process

The AUCs from the peaks in HPLC–DAD at 340 nm were used to calculate thepercentage of inhibition through following formula:

$$I\,\% = 100 - \frac{\Delta\, AUC_{sample}}{\Delta\, AUC_{control}}\; x\; 100$$

Where $\Delta\, AUC_{sample}$ is the slope (m) of the linear function obtained from sample results of AUC over time (AUC = m t + n) and $\Delta\, AUC_{control}$, the slope from the linear function obtained from results of AUC over time of control, considering 100 % of activity of the enzyme.

- Statistical analysis

Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

3.2 RESULTS

As a consequence of the high price of the commercial kit and to avoid of sample and kit reagent waste, only intestinal fractions of OV1 were used to test the inhibitory activity of HMG–CoA reductase, as it is the most hypoglycemic extract. There is a high evidence reported on the relationship between type II diabetes and the increase in LDL–c in the serum of patients, which suggests that compounds capable of controlling glycaemia and insulin resistance might have a potentially inhibitory activity of HMG–CoA reductase. The samples were only analysed at one concentration (1 mg/mL), so the IC_{50} value could not be calculated as in other previous activities. The processing data of the final values of the slope and the percentage of the final values of inhibition are presented in **Table 37**.

Table 37. Results of HMG–Coa inhibitory activity of the intestinal fraction of OV1 and control in terms of AUC at each time point of monitoring. The slope of the graph is also shown, as intermediate data for the calculation of the percentage of inhibition, which is indicated in last column with respect to the control (100% of reaction, 0 % inhibition).

	AUC					*slope*	**Percentage Inhibition (%)**
Time (min)	**0**	**1**	**2**	**4**	**6**		
Control	62,242.75 ± 1,052.23	60,320.38 ± 995,20	57,809.72 ± 997,52	53,356.73 ± 999,54	47,810.03 ± 1,063.12	–2,426.6 ± 0.95	0
OV1–Int	28,816.95 ± 1,247.51	26,898.19 ± 1,315.49	25,098.18 ± 1,362.50	24,300.33 ± 1,397.41	21,639.81 ± 1,236.19	**–**1,095.9 ± 2.75	**54.84 ± 0.11**

Compared to the control, which corresponds to the entire expression of the enzyme, the steeper the slope, the lower the inhibitory activity. As shown, OV1 presented a lower slope control (**Table 37**). After calculations, OV1 inhibited HMG–CoA reductase *in vitro* by 54.84 ± 0.11 %. To conclude with solid results, it would be interesting to repeat this test with OV1 comparing the activities with a positive control such as a statin.

A previous study using same conditions [274], obtained an IC_{50} of 0.20 ± 0.02 μg/mL for simvastatin and 10.89 ± 0.12 for rutin – flavonoid used as standard in that study. The IC_{50} value for OV1–Int could not be determined as only one concentration was tested. However, OV1–Int would potentially inhibit 54.84 ± 0.11 % of the enzyme at 86.20 μg/mL, the concentration of *Annona cherimola* leaves in decoction required being higher (137.3 ± 37.5 μg/mL) to produce 50 % of inhibition (IC_{50} value) [274].

4 ACETYLCHOLINESTERASE INHIBITOR ACTIVITY

Among age–related diseases, Alzheimer's disease (AD) is the most common cause of dementia. This chronic neurodegenerative disease develops slowly and progressively, causing the deterioration of intellectual capacity in various Wernicke areas: learning and memory, language abilities, reading and writing, praxis, interaction with environment and

personality changes. The earliest stages begin with short–term memory loss. Detection is such an important step in this progressive disease, because medicine can only delay the neurodegenerative progression. Risk factors for developing AD include both genetic (*APOE4* gene variants) and environmental factors (age, depression, metabolic syndrome: hypertension, diabetes and hyperlipidaemia).

From a physiopathology point of view, synapse loss is thought to be caused by Tau neurofibrillary tangles (intracellular accumulation of abnormal neuronal cytoskeletal components) and extracellular senile plaques of β–amyloid peptide, derived from β–amyloid precursor protein (APP) (**Figure 41**).

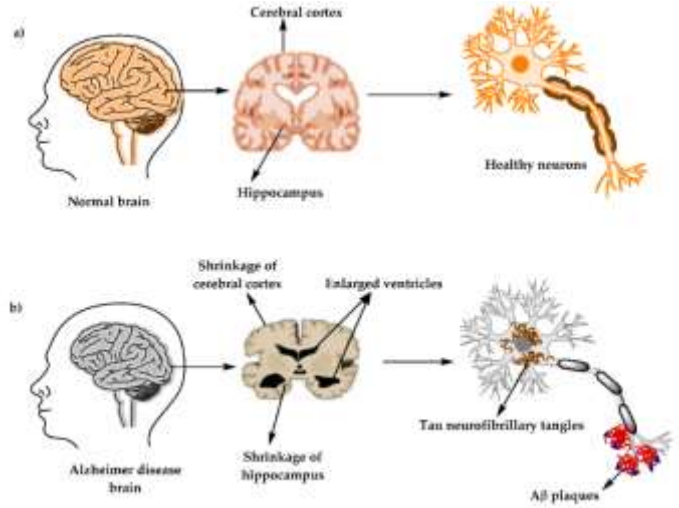


Figure 41. a) Normal brain and healthy neuron. b) Alzheimer's disease (AD) brain and neuron with Tau tangles and Aβ plaques. [275].

Since 1970–1980s, science has focused on "The Cholinergic hypothesis of AD" [276] because of highly consistent findings of some altered selective neurotransmitter systems in AD patients . A presynaptic reduction of acetylcholine (ATCh) was found in patients with AD., among treatments for Alzheimer's, acetycholinesterase inhibitors, that increase this neurotransmitter in the neocortical synaptic space, are the most common. The most important are donepezil, galantamine and rivastigmine, especially used in early stages of the disease [277].

Moreover, memantine (NMDA antagonist) is generally used in the advanced stage of AD, as dysfunction in the glutamate system was found to be linked with Aβ peptide related oxidative stress. In this sense, since has been shown that aging and oxidative stress (production of Reactive Oxygen Species – ROS) are involved in AD, antioxidants might be potentially adjuvants in the treatment of Alzheimer's [278].

Most treatments are still in clinical trials, such as those that target the β–Amyloid cascade by increasing Aβ42/Aβ40 ratio. This pathway might potentially be the near future as

biomarker in both diagnosis and treatment [279]. Previous studies link antioxidant and anti–aging properties with a delay in the development of dementia [280]. Among the hypothetical pathways involved in Alzheimer's neurodegenerative disease, previous studies tested medicinal plants against acetylcholinesterase enzyme *in vitro*, one of them being oregano [13,183,281,282].

4.1 MATERIAL AND METHODS

- Experimental basis

The antiacetylcholinesterase activity of a plant extract is based on the original method proposed by Ellman *et al*. [283], with several adaptations [284]. In this work, the assay was performed in individual spectrophotometry cuvettes.

Cholinesterase activity is measured indirectly by quantifying the concentration of 5–thio–2–nitrobenzoic acid (TNB) ions formed in the reaction between the reagent thiol 5,5′–dithiobis–2–nitrobenzoic acid (DTNB) and thiocholine, a hydrolysis product of the substrate (i.e., acetylthiocholine [ATCh]) by the cholinesterase. The formation of the yellow ion of 5–thio–2–nitrobenzoic acid (TNB) can be measured at 405 nm (**Figure 42**).

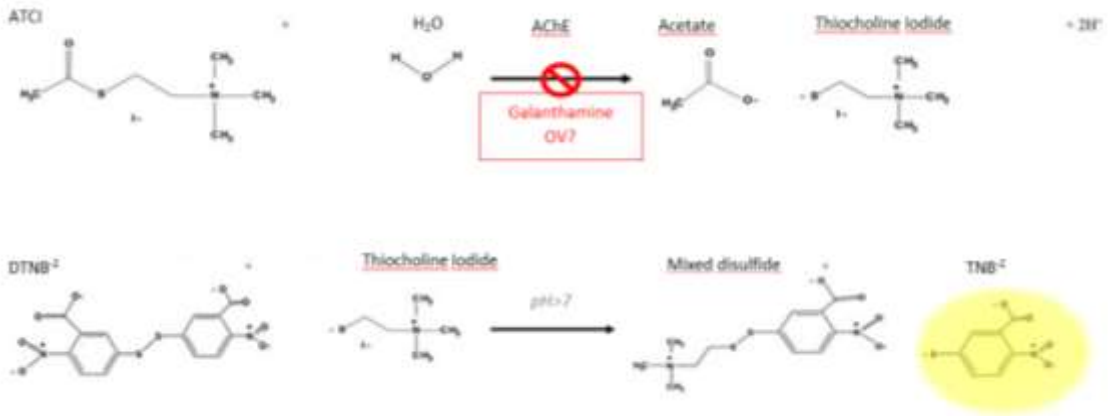


Figure 42. Biochemical reaction showing inhibition of the enzyme acetylcholinesterase (AChE). As a result of the first reaction, enzyme breaks down the substrate acetylthiocholine (ATCI) into acetate and thiocholine iodide. To measure enzyme activity, Ellman's reagent (DTNB) is added in medium to react with thiocoline iodide from the first reaction producing a yellow compound measurable at 405 nm (TNB).

- Buffer preparation

Two buffer solutions at pH 8 were needed:

1. Buffer A with salts (Ellman´s reagent): Total volume 200 mL: 584.5 mg NaCl + 866.90 mg $MgCl_2$ 6 H_2O in 200 mL distilled water.
2. Buffer B without salts (for enzyme preparation): 6.075 g Tris (T3) in 1,000 mL of distilled water at final pH 8.

- Preparation of the enzyme

Storage temperature must be –20 ºC, keeping the enzyme on ice while handling. The concentration for the enzyme (AChE, #C1682, Sigma–Aldrich Co., St. Louis, MO) was 0,22 U/L in buffer A.

- Preparation of the substrate preparation

Acetylthiocholine iodide (AChI, #A5751, Sigma–Aldrich Co., St. Louis, MO) was stored in the fridge at 2–8 ºC and dissolved in water at 15 mM.

- Preparation of Ellman's reagent

DTNB solution at 1.2 mg/mL (#D8130, Sigma–Aldrich Co., St. Louis, MO) was prepared in buffer A.

- Preparation of the samples

The six extracts (OV1 – OV6) were dissolved in distilled water at different concentrations from 1 mg/mL to 0.1 mg/mL. The digested extracts were also tested at the same concentrations for all three digestion steps: oral, stomach, and intestinal. Galantamine – one of the treatments for Alzheimer's in the market – was used as positive control at the same concentrations. Distilled water was used as a negative control.

- Reaction and analysis conditions

Each reaction, corresponding to one measurement, was performed in spectrophotometry cuvettes by mixing 100 μL of inhibitor (sample, galantamine or distilled water), 325 μL of buffer B, 475 μL of DTNB dissolved in buffer A and 25 μL of enzyme (in buffer B). After 5 min of incubation at room temperature, 75 μL of colourimetric substrate (ATCI in distilled water) were added. Absorbance at 405 nm was monitored over time (25 measures every 10 seconds). Each condition was tested in triplicate.

- Data process

The calculation of the Inhibitory percentage of enzyme inhibition for each kinetic measurement of each plate was carried out using the following formula [283]:

$$Inhibitory\ (\%) = \left(1 - \frac{Abs\ Abs_S}{Abs\ Abs_C}\right) x100$$

Where Abs_S corresponds to slope of absorbance of sample and slope Abs_C, to slope obtained from absorbance of negative control.

The absorbances were recorded and a graph absorbance (*y*–axis) – time (*x*–axis) was made. The slope obtained from equation of the line described by data (with $R^2 > 0.9500$) was used to calculate percentage of inhibition for that concentration. The slope of the line described by data was used to calculate percentage of inhibition through the formula previously shown and IC_{50} (mg/mL) were calculated with GraphPad. The IC_{50}, corresponding to the concentration (*x*) where the 50 % of substrate is degraded by the enzyme (*y* = 50), was used as an indirect quantitative measure of the inhibitory activity.

- Statiscal analysis

Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

4.2 RESULTS

Within traditional medicine, oregano has been used to prevent and treat neurodegenerative diseases related to memory, such as Alzheimer's. Science has been able to demonstrate this activity in research field, finding different pathways where oregano [238,262,285] and its components [262,286] proved to be effective for Alzheimer's.

The extracts showed a different behaviour in the inhibition of the enzyme acetylcholinesterase *in vitro* (**Figure 43**). Galantamine, as a positive control, inhibited 50 % of the enzyme at a concentration of 0.434 ± 0.004 mg/mL. From that reference, OV2, OV3 and OV4 showed a statistically lower activity, showing IC_{50} values higher, 0.594 ± 0.009 mg/mL (OV2), 0.563 ± 0.005 mg/mL (OV3) and 0.515 ± 0.008 mg/mL (OV4), with $p < 0.001$ between them. On the other hand, OV1, OV5 and OV6 were more active than the control, with IC_{50} values of 0.311 ± 0.012 mg/mL (OV1), 0.361 ± 0.008 mg/mL (OV5) and 0.175 ± 0.015 mg/mL (OV6), with $p < 0.001$ between them.

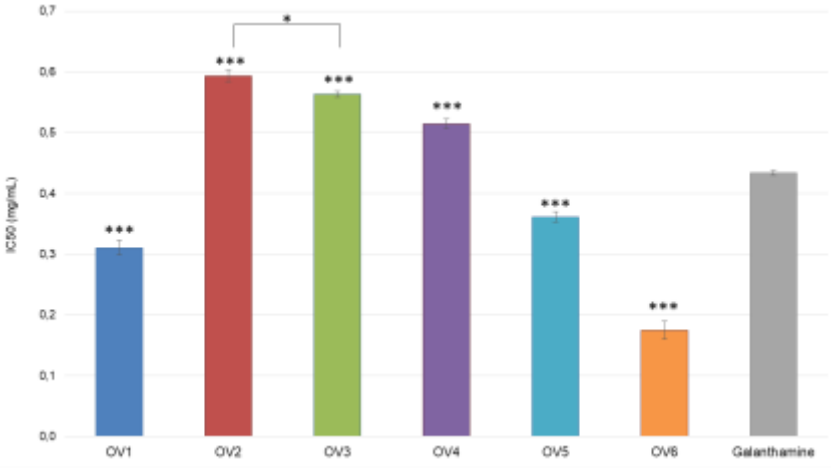


Figure 43. Acetylcholinesterase inhibitor activity of six extracts of *O. vulgare* and galanthamine expressed as IC_{50} (mean ± SD mg/mL) of the six extracts of *O. vulgare* and positive control. * corresponds to statistical differences ($p < 0.05$) and *** to statistical differences ($p < 0.001$).

Dinis *et al.* [287] concluded in a previous study that rosmarinic acid was capable of inhibiting at least 50 % of the enzyme at 0.439 ± 0.025 mg/mL, this value being in line with those obtained for oregano extracts (**Figure 43**). However, previous studies carried out with *O. vulgare* concluded that when extracting with 80 % of methanol there was an inhibition of 20.11 ± 3.56 % at 10 mg/mL [13]. Parallel to this result, another study with extract prepared with ethanol, did not reach 50 % of inhibition even with a concentration of 1 mg/mL [288]. As always, methodology could vary – although it should not – and vegetal material of origin might have a different nature and secondary metabolites. For instance, galantamine showed on a previous study to inhibit the enzyme 72.96 ± 0.26 % at 25 μg/mL, whereas in this study 50 % of inhibition was obtained at 434 μg/mL of galantamine. Nevertheless, some of the extracts (OV1, OV5 and OV6) showed significant higher activity than the positive control, so the intestinal fractions of the most active extracts were also tested against the enzyme. **Figure 44** shows the results of intestinal fractions and crude extracts.

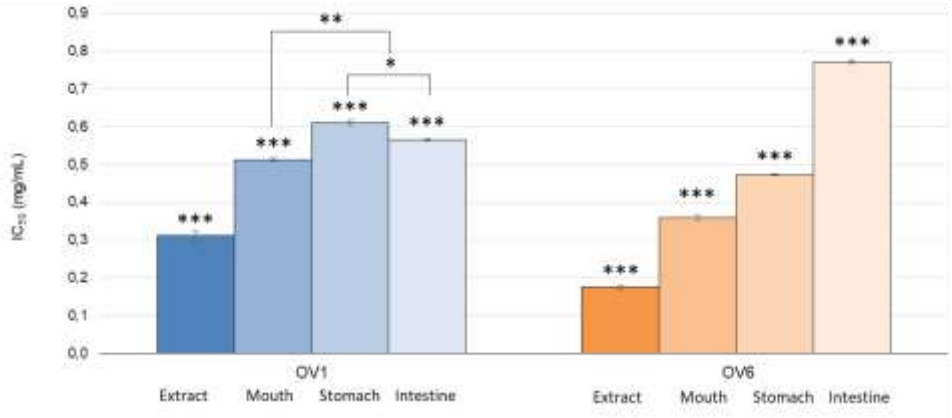


Figure 44. IC_{50} values (mean ± SD mg/mL) along digestion: extract (before), mouth (oral digestion sample), Stomach (stomach digestion sample) and intestine (intestinal absorbable fraction). * corresponds to statistical differences with $p < 0.05$, ** corresponds to $p < 0.01$ and *** corresponds to $p < 0.001$.

OV1 showed a tendency to lose bioactivity in the first two steps of digestion, recovering some bioactivity in the last step, because the IC_{50} in the intestinal fraction was significantly lower than the stomach sample: 0.311 ± 0.012 (OV1), 0.512 ± 0.005 (mouth), 0.610 ± 0.008 (stomach) and 0.586 ± 0.004 mg/mL (intestine), with significant differences in each digestion step compared to the control ($p < 0.001$).

OV6 – the most active inhibiting this enzyme among the rest of extracts – showed this tendency to lose bioactivity throughout digestion, but with a significantly greater loss: 0.175 ± 0.015 (OV6), 0.408 ± 0.006 (mouth), 0.573 ± 0.002 (stomach) and 0.773 ± 0.005 mg/mL (intestine), with $p < 0.001$.

In this sense, OV6 would be potentially be the most active extract before digestion but this activity was very significantly lost after the digestion process (four times less active in the intestine than *ex–corpore*), while OV1 only suffered a loss of 88.42 %. A previous study with *Mentha spicata* extract [287], showed a reduction on this activity from an IC_{50} of 0.72 mg/mL before digestion to 1.9 mg/mL after digestion. Oral administration might cause a loss of compounds and bioactivity. Nevertheless, more pre–clinical studies are out with *O. vulgare* needed, as there is little well–established evidence for its traditional use for memory.

5 ANTI–INFLAMMATORY *IN VITRO* ACTIVITIES

The human body has a physiological defense reaction against any aggression called inflammatory response, whose symptoms may include pain, redness, warmness and oedema in the patient. Within acute inflammation, three phases develop from the appearance of the lesion: initial, vascular and cellular phase (**Figure 45**).

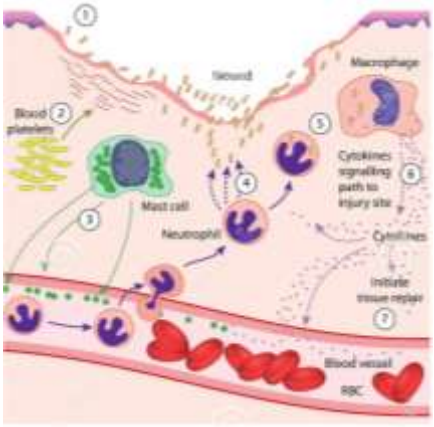


Figure 45. Mechanism of acute inflammation from wound injury (1) to tissue repair (7). The initial phase comprises the release of blood platelets (2); then, the vascular phase begins with the secretion of factors into the blood vessels from mast cells (3), the arrival of neutrophils (4) and macrophages that secrete cytokines (6) to end in the cellular phase with the tissue repair by the immune system (7) [289].

The inflammation cascade generally begins with IL–1 –activated membrane phospholipids producing arachidonic acid, rapidly degraded into prostaglandins through the cyclooxygenase (COX) pathway and leukotrienes through the lipo–oxigenase pathway. Products of the COX pathway cause vasodilation and platelet aggregation (**Figure 45–2**). Then, in the vascular phase of the mast cell–modulated inflammation (**Figure 45–3**), the body develops a modification of the local microcirculation where the injury has occurred: vasoconstriction of the arterioles, vasodilation –giving redness and warmness to the area injured– and contraction of endothelial cells. Through other complex mechanisms, the body recruits different physiological mediators form neutrophils, which secrete factors to counteract damage Finally, in the cellular phase, macrophages secrete cytokines (IL–1 for example, **Figure 45–5** and **6**) responsible for the immune system response to repair the tissue and return to normal (**Figure 45–7**). Depending on the damage and bleeding, the coagulation system must be activated to produce thrombin and restore the stat of homeostasis. Therefore, substances capable of stopping inflammation at any time could be assembled as anti–inflammatories. Non–steroidal anti–inflammatory drugs (NSAIDs) act by effectively inhibiting COX in the inflammatory cascade. But, as been seen, there are more systems involved in inflammation [290].

During inflammation, the first system to suffer damage is the cell membrane, whose vulnerability could be related to the regulation of the volume and water content in the cell. Therefore, according to previous studies, the red blood cell membrane is similar to the lysosome membrane and, therefore, its preservation from haemolysis might provide insights into the inflammatory process by delaying tissue damage [290]. In fact, red blood cells (RBC) have an with important role in the inflammation caused by atherothrombosis, a complication of the metabolic syndrome (uncontrolled dyslipidaemia and type II diabetes, among other modifiable factors) (**Figure 46**).

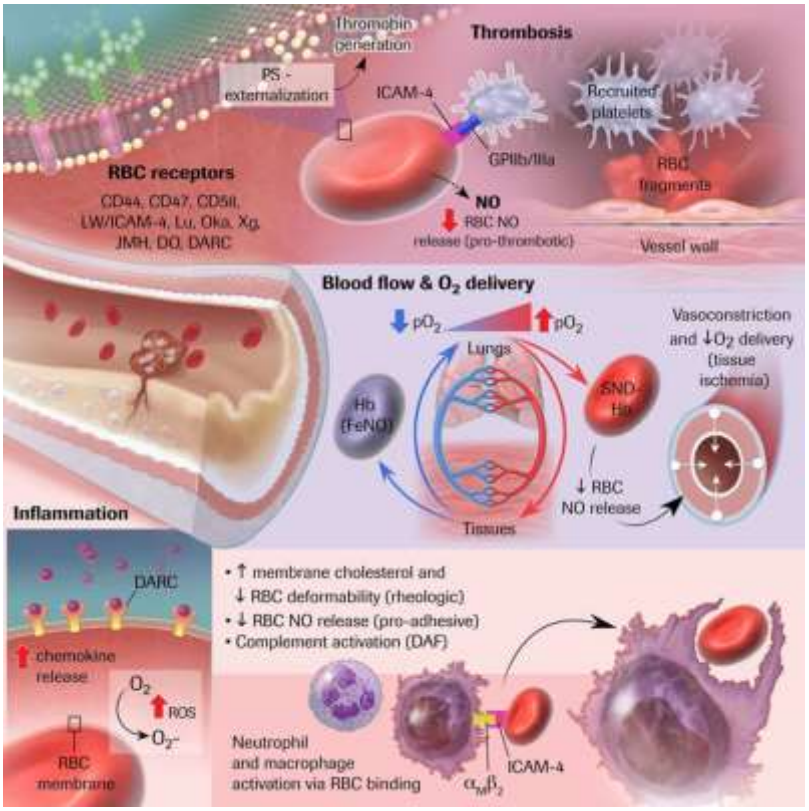


Figure 46. Thombosis, blood flow and oxygen supply, and inflammation in the vessels in atherothrombosis; importance of the role of Red Blood Cells (RBC) [291].

Generally, erythrocytes (red blood cells, RBC) are physiologically linked to the transport and exchange of iron and oxygen from the lungs to the tissues. However, the recruitment of platelets, as part of the initial phase of inflammation, occurs thanks to RBC receptors such as ICAM–4, which bind to GBIIb/IIa from platelets. This receptor in the RBC membrane also activates neutrophils and macrophages by binding to the $\alpha_M\beta_2$ receptor (**Figure 46**).

Reactive oxygen species, such as those produced in the release of cytokines in inflammation, have been shown to damage this membrane, thereby disabling the activating role of RBCs in the anti–inflammatory pathway. Therefore, any compound capable of preserving the RBC membrane might improve the body reaction against inflammation. This potential anti–inflammatory effect might occur if that compound is well transported into blood cells by albumin, the primary transporter in blood. *In vitro*, these two effects can be measured through the protein denaturation test and the RBC membrane stabilisation test.

5.1 PROTEIN DENATURATION

In biochemistry, the denaturation of a protein is a process in which the higher structures degraded to their native state as a consequence of the application of some external stress,

extreme pH conditions, addition of compound (organic solvent), agitation and radiation or heat. Among the main proteins present in human body, albumin comprises more than 50 % of the proteins in plasma. This protein synthetized by living organisms is essential for maintaining osmotic pressure (electrolytes and pH) and transports most of the compounds throughout the blood.

In experimental research, the protective effect of a compound against albumin denaturation can be measured *in vitro* with the clinical purpose of studing the anti–inflammatory activity of that compound.

5.1.1 MATERIAL AND METHODS

- Experimental basis

The protective effect of crude extracts against protein denaturation was evaluated following Reshma *et al.* method with some modifications carried out in our laboratory [292]. Bovine serum albumin (BSA) presents a quaternary structure at room temperature and can be denatured by heating (increasing the temperature to 60 ºC), taking the protein to a simpler inactive structure (**Figure 47**). The presence of certain compounds might protect albumin from denaturation, preserving the 3D structure.

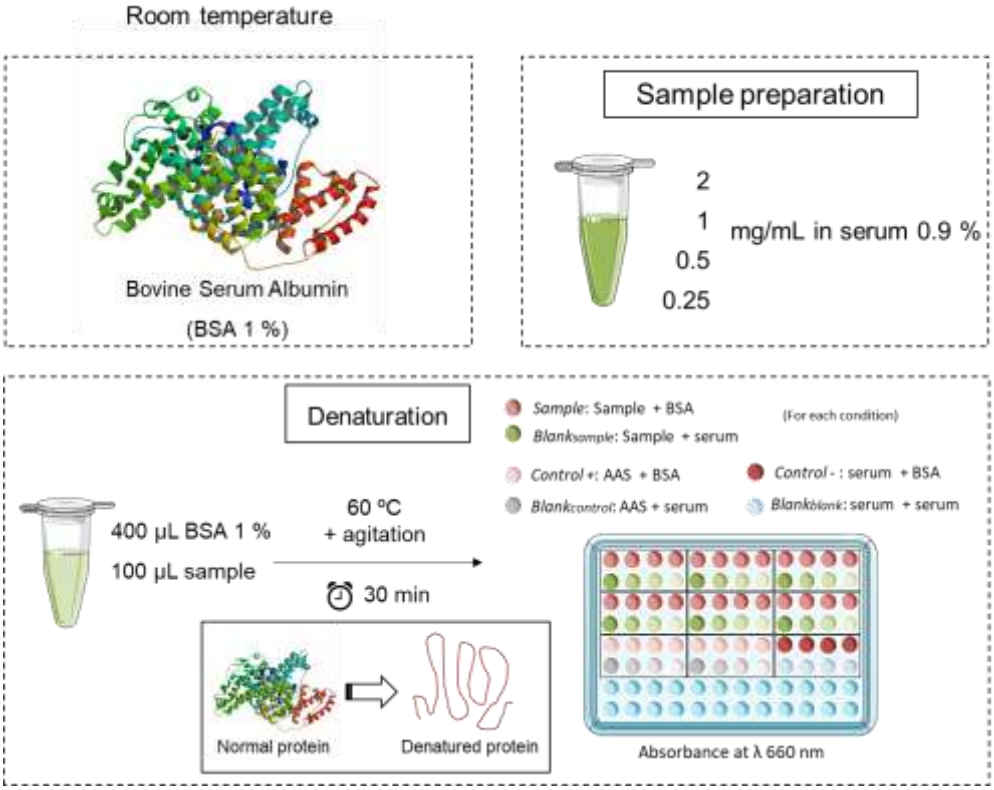


Figure 47. Scheme of the protein denaturation protocol. Bovine Serum Albumin (BSA) presents 3D structure at room temperature, whereas after denaturation by heat shock, the denatured protein presents a simpler linear structure. The samples were prepared at different serum concentrations. A mixture of sample and BSA was incubated at 60 º C for 30 min with shaking and absorbances were monitored at 660 nm.

- Substrate preparation

Serum Bovine Albumin (BSA, #A9418, Sigma–Aldrich Co., St. Louis, MO) was used as the substrate, as it is the main transport protein in human blood that undergoes heat denaturation. 1 % solution in commercial 0.9 % saline solution for perfusion (serum 0.9 %; CN: 999789, ERN laboratory) was used.

- Sample preparation

The six extracts (OV1 – OV6) were dissolved in 0.9 % saline solution at different concentrations (2, 1, 0.5, 0.25 mg/mL).

Acetylsalicylic acid in two pharmaceutical forms was used as a positive control at the same concentrations. Hereby are the components of each of them:

- Aspirin ® 500 mg tablets BAYER (CN: 712786)
    - Active Pharmaceutical Ingredient: 500 mg ASA.
    - Excipients: cellulose in powder, cornstarch.
- A.A.S 500 mg tablets SANOFI (CN: 672905)
    - Active Pharmaceutical Ingredient: 500 mg ASA.
    - Excipients: sacarine, manitol (E421), cornstarch, arabic gum and orange flavour (maltodextrin, sacharose, modified starch (EI450), butylhydroxianisol (E320)).

The tablets were crushed and the powder was used to prepare the solutions in 0.9 % saline solution, neglecting the excipients. 0.9 % saline solution was used as a negative control. Experiments was performed in triplicate.

- Analysis conditions

100 µL of each sample (50, 100, 200 and 400 µg/mL) were mixed with 400 µL of 1 % BSA and incubated at 60 ºC for 30 min in a 96–well plate, prepared as shown in **Figure 47**:

- Sample: 100 µL sample + 400 µL BSA 1 %
- Sample *blank*: 100 µL sample + 400 µL serum 0.9 %
- Positive control: 100 µL A.A.S or Aspirin® + 400 µL BSA 1 %
- Positive control *blank*: 100 µL A.A.S or Aspirin® + 400 µL serum 0.9 %
- Negative control: 100 µL serum 0.9 % + BSA 1 %
- Negative control *blank*: 100 µL serum 0.9 % + 400 µL serum 0.9 %

- Data process

The reaction was performed in triplicate and quantified at 660 nm with a spectrophotometer. Percentage of denaturation was calculated using the following formula:

$$\%\ Denaturation = \frac{Abs_{sample} - Abs_{sample\ blank}}{Abs_{control} - Abs_{control\ blank}}\ x\ 100$$

Where Abs_{sample} corresponds to the absorbance of the sample with albumin; $Abs_{sample\ blank}$, to the absorbance of the sample without albumin; $Abs_{control}$, to the absorbance of positive control with albumin and $Abs_{control\ blank}$, to the absorbance of the positive control without albumin.

- Statistical analysis

Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

5.1.2 RESULTS

Albumin is the most abundant serum protein produced by the liver, whose functions comprise more than transport of compounds along blood vessels. Previous studies have demonstrated its essential role as marker for diseases such as pneumonia, rheumatoid arthritis and infection, among others. As it is a protein, the denaturation produced by extreme conditions such as stress or the presence of toxic compounds would physiologically disable this protein. Previous studies have studied the decrease of albumin in inflammation, showing a lower synthesis in the liver in chronic patients and a possible denaturation in acute inflammation. In addition, commonly used anti–inflammatories such as acetylsalicylic acid (A. A. S., Aspirin ®) have shown a dose–dependent capacity to thermally induce protein denaturation.

In the experimental design, high temperature was selected as the trigger for albumin denaturation, following the performance of previous studies that included this test within research of *in vitro* anti–inflammatory effect of plant extracts compared to Aspirin®. **Table 38** shows the percentages of denaturation of the six extracts at different doses and the two positive controls.

Table 38. Results of albumin denaturation expressed in percentages (mean ± SD %) for each condition. AAS: positive control, generic aspirin (CN: 672905); ASP: Aspirin®, from Bayer laboratory (CN: 712786). Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$).

	Percentage of denaturation (%) per sample							
Concent. (µg/mL)	**OV1**	**OV2**	**OV3**	**OV4**	**OV5**	**OV6**	**AAS**	**ASP**
50	47.8 ± 12.3^{a}	62.0 ± 1.8^{b}	64.1 ± 4.8^{b}	43.9 ± 1.3^{a}	56.7 ± 2.8^{c}	49.0 ± 1.3^{a}	159.1 ± 20.2^{j}	366.9 ± 17.2^{g}
100	43.6 ± 11.8^{a}	56.5 ± 1.5^{c}	40.0 ± 4.0^{a}	52.9 ± 9.0^{c}	66.9 ± 2.9^{b}	68.6 ± 2.1^{b}	336.1 ± 9.2^{f}	374.8 ± 24.7^{g}
200	34.1 ± 2.5^{a}	47.0 ± 2.0^{a}	39.9 ± 2.8^{a}	61.9 ± 1.5^{b}	76.1 ± 2.3^{d}	74.2 ± 3.4^{d}	323.7 ± 17.6^{f}	362.7 ± 13.1^{g}
400	17.4 ± 2.0^{i}	42.4 ± 8.4^{a}	50.7 ± 3.1^{c}	71.9 ± 1.1^{d}	95.2 ± 9.2^{e}	91.6 ± 24.7^{e}	415.4 ± 12.5^{h}	415.8 ± 14.4^{h}

Both positive controls showed a significantly high percentage of albumin denaturation, independent of concentration. Although there were differences between the positive controls, with A.A.S at 50 µg/mL being less harmful than the rest of conditions (159.1 ± 20.2 %, with $p < 0.001$ respect rest of results), all of them were not protective of the albumin to heat shock (**Table 38**).

On the contrary, the extracts showed to be better inhibitors of albumin denaturation. OV1 showed an expected behaviour being statistically lower the percentage of denaturation at high doses: 47.8 ± 12.3 % at 50 µg/mL, 43.6 ± 11.8 at 100 µg/mL, 34.1 ± 2.5 % at 200 µg/mL (with $p > 0.05$ between them) and statistically lower at maximum dose of 400 µg/mL (17.4 ± 2.0 %, with $p < 0.001$ with rest of results). These results were the lowest among the extracts, considering OV1 as potentially preserver of albumin. OV2 showed a similar behaviour but with higher percentages of denaturation: from 62.0 ± 1.8 % at 50 µg/mL to 42.4 ± 8.4 % at 400 µg/mL, which was no statistically different from the results of OV1 at 50, 100 and 200 mg/mL ($p > 0.05$).

Then, OV3 presented better albumin protective capacity at medium concentrations (40.0 ± 4.0 % and 39.9 ± 2.8 % at 100 and 200 µg/mL, without statistical differences with OV1 at medium–low doses, $p > 0.05$) than at extreme concentrations (64.1 ± 4.8 % and 50.7 ± 3.1 % at 50 and 400 µg/mL). The rest of the extracts showed a behaviour and result opposite to that of OV1: greater denaturation at higher concentration, from 40–50 % to 90 % for OV5 and OV6 and 71.9 % for OV4. In a previous published study with methanol extract of *O. corniculata* L. [293], the highest dose tested produced greater albumin denaturation, being 65.43 ± 1.13 % at 400 µg/mL. In addition, aspirin also showed greater albumin denaturation than the extract, even at the lowest doses tested. Although the ethanolic extracts of *O. vulgare* (OV4, OV5 and OV6) showed direct proportionality

between dose and albumin protein denaturation, previous studies with aqueous extracts of some other medicinal plants also found this correlation.

Albumin is a protein produced by the liver responsible for the transport of important substances along the arteries that regulates the water content of tissues and cells. Inflammation occurs through many pathways, but this protection of albumin from denaturation might be better related clinically to transport of the extracts in blood than to remediation of inflammation. However, if administrated topically, albumin role water in regulating water content might be clinically relevant in preserving epithelial inflammation damage. According to the results, OV1, OV2 and OV3 would be the only ones recommended for either oral or topical administration due to the good preservation of albumin from denaturation.

5.2 RED BLOOD CELL MEMBRANE STABILISATION

Red blood cells, also known as haematites or erythrocytes, are the most common type of blood cells whose main function is the transport of oxygen. These non-nucled cells are also involved in other physiological functions as seen above (**Figure 46**). Red blood cells are deformable, flexible with a three–layered membrane rich in carbohydrates, phospholipids and cholesterol. Membrane proteins confers flexibility and durability to RBC, in addition to fulfilling their physiological functions of transport and cell adhesion, relevant in inflammation. However, these proteins can be denatured by many factors, leading to inactivation of these important blood cells.

5.2.1 MATERIAL AND METHOD

- Experimental basis

The stabilisation of the RBC membrane stabilisationwas tested to verify the protective effect of the extract against haemolysis, following Reshma *et al*. method [292] with some modifications (**Figure 48**).

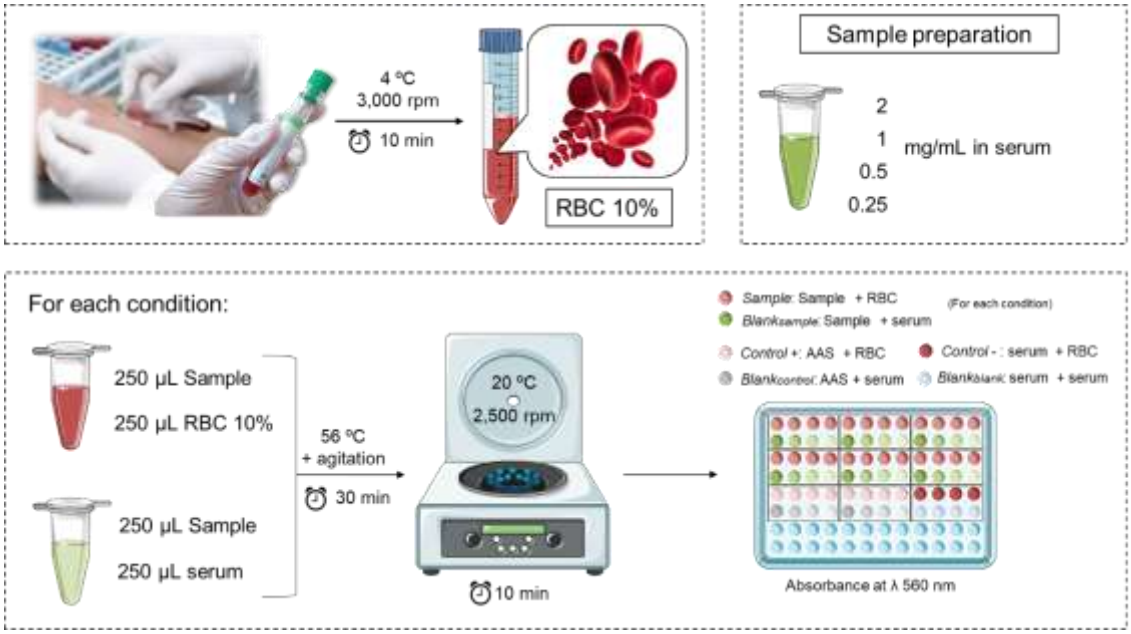


Figure 48. Scheme of the of RBC membrane stabilization methodology, from sample collection to measurement at 560 nm.

- Collection and preparation of RBC

Different subjects (young Caucasian volunteers who had not taken NSAIDs within the previous 10 days, sex was not taken into account) had blood drawn into Vacutainer® BD heparin tube. First of all, three volunteers were considered enough for a first attempt. However, one of the subjects had to be excluded from the study. With the results from the other two subjects, the ideal sample size for the study was calculated though a bilateral study using the following formula:

$$n \geq \frac{2\left(Z_\alpha + Z_\beta\right)^2 x\, S^2}{d^2}$$

Where Z_α and Z_β correspond to the desirable risk (for a two–sided test, $Z_\alpha = 1.960$ and $Z_\beta = 0.842$), S^2 corresponds to the variance of the control group (two subjects from the first samples collected) and d indicates desired precision in the test.

Blood samples were processed independently for each subject. Vacutainer tubes were centrifuged (3,000 rpm at 4 ºC for 10 min). The supernatant was discharged, and the pellet was washed and centrifuged two more times (3,000 rpm at 4 ºC for 10 min) to obtain RBC from the blood sample (**Figure 48**). The final pellet was diluted at 10 % in commercial 0.9 % saline solution for perfusion (CN: 999789, ERN laboratory).

- Sample preparation

The extracts (OV1 – OV6) were diluted to four different concentrations (800, 400, 200 and 100 µg/mL) in 0.9 % saline solution. Aspirin® was used as positive control (tested at the

same concentrations) and saline solution as negative control. In addition, blank (without RBC) of each concentration were also taken into account.

- Analysis conditions

The reaction was performed by mixing RBC and sample (1:1 v/v; final concentrations would be same as **albumin denaturation**) and incubated at 56 ºC for 30 min. After heat shock, the samples were centrifuged (10 s at 2,500 rpm). A 96–well plate was prepared as shown in **Figure 48**:

- Sample: 250 µL sample + 250 µL RBC 10 %
- Sample *blank*: 250 µL sample + 250 µL serum 0.9 %
- Positive control: 250 µL A.A.S or Aspirin® + 250 µL RBC 10 %
- Positive control *blank*: 250 µL A.A.S or Aspirin® + 250 µL serum 0.9 %
- Negative control: 250 µL serum 0.9 % + RBC 10 %
- Negative control *blank*: 250 µL serum 0.9 % + 250 µL serum 0.9 %

- Data process

The absorbances were monitored at 560 nm by spectrophotometry in order to calculate the percentage of membrane stabilisation (%) using the following formula:

$$RBC\ membrane\ stabilization\ (\%) = \left(1 - \frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}}\right) x\ 100$$

In which Abs_{sample} is the absorbance at 560 nm of the reaction in the presence of sample (sample dilution + RBC 10 %), Abs_{blank} is the absorbance of the blank for each sample dilution (sample dilution + serum) and $Abs_{control}$ is the absorbance of the control reaction (serum + RBC 10 %).

- Statiscal analysis

Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

5.2.2 RESULTS

The second *in vitro* assay was the preservation of the RBC membrane. As explained in **Figure 48**, these cells are involved in the transport of oxygen by haemoglobin, whose

conservation in available form could be guaranteed thanks to the antioxidant activity of extracts. Besides, ROS produced by cytokine signalling in inflammation can also damage the RBC membrane. **Table 39** shows mean and SD of results expressed as percentage of haemolyses.

Table 39. Results of albumin denaturation expressed in percentages (mean ± SD %) for each condition. AAS: positive control, generic aspirin (CN: 672905); ASP: Aspirin®, from Bayer laboratory (CN: 712786). Letters indicate no statistical differences ($p > 0.05$) between results with same letter (from [a] to [n]) and statistical differences ($p < 0.001$) between results with different letter.

Percentage of hemolyse (%)

Concentration (mg/mL)	OV1	OV2	OV3	OV4	OV5	OV6	AAS	ASP
50	34.71 ± 1.37[a]	42.43 ± 6.68[e]	32.71 ± 2.96[a]	27.77 ± 1.86[b]	24.66 ± 2.45[b]	22.24 ± 1.26[c]	127.55 ± 4.10[i]	141.54 ± 9.49[l]
100	23.74 ± 2.61[b]	35.41 ± 2.90[a]	27.06 ± 1.82[b]	24.24 ± 2.08[b]	29.16 ± 2.95[b]	35.14 ± 2.95[a]	100.37 ± 2.58[j]	111.58 ± 3.14[m]
200	15.93 ± 1 .53[c]	19.60 ± 3.30[c]	24.55 ± 0.78[b]	20.25 ± 1.14[c]	40.58 ± 5.41[e]	84.12 ± 1.98[h]	89.47 ± 2.65[h]	95.02 ± 2.15[j]
400	8.74 ± 0.77[d]	12.36 ± 0.76[f]	41.61 ± 1.94[e]	38.69 ± 0.69[e]	52.92 ± 3.31[g]	45.35 ± 2.04[e]	66.27 ± 1.66[k]	76.01 ± 2.33[n]

The positive control showed better preservatuion of the RBC membrane than albumin denaturation in the previous asssay, but only at higher doses: 66.27 ± 1.66 % of haemolysis in AAS at 400 µg/mL and 76.01 ± 2.33 % in Aspirin® at same dose.

OV1 and OV2 presented the expected behaviour [292], already observed in the positive control, but with a percentage of haemolysis lower than 45 %. Again, OV1 would be the best recommended extract for this activity and at a higher dose, less haemolysis was observed 8.74 ± 0.77 %, against 12.36 ± 0.76 % of OV2 at the same concentration (400 µg/mL).

Within the hydroalcoholic extracts, OV4 showed an opposite behaviour for low doses: 27.77 ± 1.86 % of haemolysis at 50 µg/mL, 24.24 ± 2.08% at 100 µg/mL and 20.25 ± 1.14 µg/mL, but there was greater haemolysis at 400 µg/mL = 38.69 ± 0.69 %. This change in behaviour at high dose was also observed for OV3, with the lowest percentage of haemolysis at 200 µg/mL (24.55 ± 0.78 %) and by doubling the dose, the sample suffered greater haemolysis with 41.61 ± 1.94 %. In this sense, the use of these two extracts might be recommended better preservation of the RBC membrane, up to 200 µg/mL.

In contrast, OV6 produced the highest percentage of haemolysis at 200 µg/mL = 84.12 ± 1.98 % (with no statistical differences with A.A.S. at the same concentration = 89.47 ± 2.65 %, with $p = 0.056$), while for the other concentrations this percentage was 22.24 ± 1.26

(50), 35.14 ± 2.95 % (100) and 45.35 ± 2.04 (400). The next least effective extract was OV5 with percentages of haemolysis ranging from 24.66 ± 2.45 % at 50 µg/mL to 52.92 ± 3.31 % at 400 µg/mL.

In addition to the albumin denaturation results, OV5 and OV6 would not be recommended for further studies regarding this activity, since albumin and RBC membrane would not be preserved. In this line, OV4 could also be discarded, while OV3 would be better at medium concentrations. However, OV1 and OV2 produced the lowest haemolysis. Indeed, this preservation of the RBC membrane might play a physiological role in addition to the very antioxidant activity of the aqueous extracts themselves, when they are transported along the vessels. Adding this activity to the rest of bioactivities tested, the aqueous extracts would be the most versatile ones, whose doses must be adjusted to establish a safe therapeutic window for the applicable cells.

6 CHEMICO–PHARMACOLOGICAL CORRELATION

Sometimes it is noteworthy to observe and measure the relationship between the tested variables to understand the effect produced by the tested sample. Statistical analysis with a correlation might respond to the hypothesis or extend the application of the observed effect to the results obtained.

This type of statistical analysis is one of the recurrent techniques in data analysis, usually stimated before the incorporation of more complex models in the study that is being carried out. Two variables are associated when one variable gives information about the other. For example, the higher the intake of grass–based food and the higher the LDL–c levels, the greater the risk of suffering from cardiovascular disease (dyslipidaemia, diabetes type II, atherosclerosis, stroke…) [271]. Besides, as result of the conection between the different systems that encompass the human body, it has been shown that these pathologies are also connected [142,228,294]. As explained above, type II diabetes appears due to insulin resistance, which can be produced by different modifiable and non–modifiable factors, whose correlation to disease was determined [295].

After testing *in vitro* the different activities under study and theoretically correlated with metabolic syndrome and ageing, it is necessary to compare and associate the results obtained separately. Therefore, a correlation was mead between all of them and the chemical characterisation through Pearson's correlation.

6.1 MATERIAL AND METHODS

- Experimental basis

Correlation is a type of association between two countable variables that evaluates the trend in the data (positive or negative). This calculation provides to measure the direction and magnitude between two variables. In a correlation, a positive value indicates a positive direct relationship, while a negative value indicated negative indirect relation between the variables. The magnitude indicated the strength of the link, being values between -1 and 1. The closer to the unit, the stronger the relationship, which on a graph is generally observed as a smaller dispersion of the values, as shown in **Figure 49**.

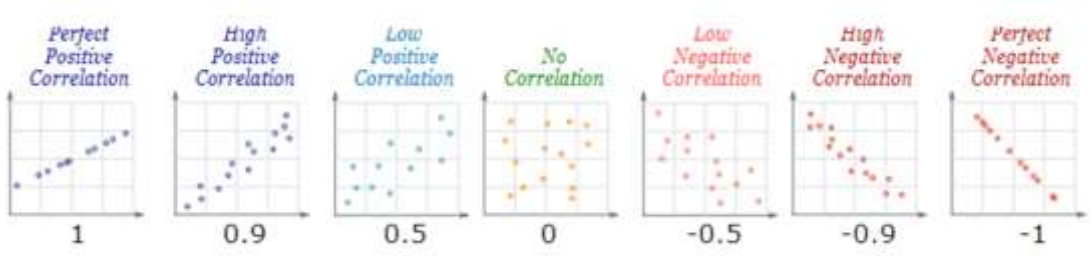


Figure 49. Different types of results in a lineal correlation expressed visually in a graph.

One of the most widely used coefficients for calculating lineal correlation is Pearson's, which assumes that the trend must be linear, there are no outliers, and the variables must be numeric with a reasonable number of values.

- Data process and statistical analysis

The data from the *in vitro* pharmacological activities resulting from the assays performed all the above in this Chapter were compared in pairs to determine if there is a correlation in activity. All data described normality fulfilling thr requirements for a Pearson correlation test.

Results were compiled in Excel and processed in Stata v.12 (StataCorp LLC, College Station, TX) using Pearson correlation with Boferoni's adjustment.

6.2 RESULTS

As described in **Chapter I**, the solvent and temperature selected for the preparation of the extracts affected the quantity of compounds extracted in the six different extracts (OV1 – OV6). Accordingo to the quantification by HPLC–DAD (*see Chapter I, chemical quantification)*, rosmarinic acid was the main compound, thus there is the possibility of considering it as the reference compound to standardize the chemical composition of *O. vulgare* extracts for future studies. However, cold maceration in water did not result effective in extracting this compound and so, it was decided rest to take into consideration the rest of the chemical group of compounds for correlation with pharmacological activities, as the first conclusion of the *in vitro* part of this study before beginningt with *in vivo* studies. **Table 40** compiles the Pearson correlations found, indicating the test involved in the comparison.

Table 40. Correlation matrix (Pearson correlation coeficients). **RA**: Rosmarinic acid (mg RA/100 mg extract); **TPC**: Total Phenolic Compounds (mg GA/100 mg extract); **DPPH•** (IC_{50} ug/mL); **ABTS•** (mg TE/100 mg extract); **a–gl**c: *α–glucosidase* inhibition (IC50 ug/mL); **AChE**: Acetylcholinesterase inhibition (IC50 ug/mL); **Albumin***X*: denaturation albumin at *X* ug/mL extract (50, 100, 200 and 400; percentage denaturation); **RBC***X*: Red Blood Cell haemolyse at *X* ug/mL extract (50, 100, 200 and 400; percentage haemolyse). Bold values indicate that the correlation is significant at the 0.05 level.

	Flavo.	Cinna.	RA	Salvian.	DHBA	Siríng.	ΣComp.	TPC	DPPH•	ABTS•	a–glc	AChE	Alb50	Alb100	Alb200	Alb400	RBC50	RBC100	RBC200
Cinna.	–0.2335	1																	
RA	**0.8484**	0.1544	1																
Salvian –	0.5469	0.0589	**0.7462**	1															
DHBA	**0.9546**	–0.3865	0.6579	0.3189	1														
Siríng.	**0.7603**	–0.4477	0.428	0.0368	**0.8569**	1													
ΣComp.	0.297	0.3415	0.5117	0.5074	0.1439	–0.3618	1												
TPC	**0.9025**	0.1355	**0.9852**	0.6842	**0.7482**	0.4956	**0.527**	1											
DPPH•	–0.5105	–0.1115	–0.7081	–0.5815	–0.34	0.1097	**–0.912**	–0.6996	1										
ABTS•	0.407	0.4198	0.6211	0.0305	0.2939	0.3541	0.141	0.6081	–0.3271	1									
a–glc	0.0114	**0.7578**	0.4912	0.2466	–0.2175	–0.3357	0.5241	0.4227	–0.5354	**0.7314**	1								
AChE	0.4378	0.198	0.342	0.5061	0.4037	0.371	–0.0583	0.3835	0.1665	–0.1014	–0.2056	1							
Alb50	0.2494	–0.1322	0.3403	0.6061	0.1164	0.2982	–0.3655	0.2405	0.1993	–0.0207	–0.0954	0.5348	1						
Alb100	–0.0986	0.4152	0.3574	0.3236	–0.3244	–0.5676	0.6832	0.2641	**–0.7269**	0.3824	**0.8275**	–0.5057	–1732	1					
Alb200	–0.0006	0.5866	0.4369	0.1503	–0.2006	–0.3617	0.5908	0.3745	–0.6436	0.6967	**0.9554**	–0.4481	–0.2664	**0.9101**	1				
Alb400	0.1556	0.557	0.5632	0.1734	–0.0435	–0.129	0.4597	0.5022	–0.5807	**0.8536**	**0.9517**	–0.3392	–0.1229	**0.903**	**0.9637**	1			
RBC50	0.0781	–0.3249	–0.1367	0.407	0.1175	0.0514	–0.1444	–0.1211	0.2659	**–0.7773**	–0.6809	0.6639	0.4753	–0.5501	**–0.7851**	**–0.8133**	1		
RBC100	0.3422	–0.1847	0.5856	**0.778**	0.1251	–0.1377	0.5223	0.4827	**–0.7233**	0.0505	0.3101	–0.1309	0.3874	0.6428	0.3831	0.3478	0.0586	1	
RBC200	0.1562	–0.1482	0.3617	0.0573	0.0484	–0.0422	0.3405	0.2942	–0.6172	0.5137	0.5131	**–0.7435**	–0.1802	**0.7328**	0.7096	0.7034	**–0.7115**	0.5909	1
RBC400	0.1972	0.4753	0.4922	–0.0317	0.0648	0.1862	0.0663	0.4466	–0.2432	**0.9688**	**0.7954**	–0.2247	0.0093	0.4758	**0.7601**	**0.8947**	**–0.8327**	0.0644	0.5591

Despite rosmarinic acid being the main compound quantified, only flavonoids –another prevalence group– and salvianilic acids showed a significant positive correlation with the main compound (0.8484 and 0.7462, respectively with $p > 0.05$). Moreover, dyhydroxybenzoic acids also showed a strong positive correlation with rosmarinic acid (0.9546) and with the predecessor group in the shikimic acid route, the syringic acids (0.8569). However, none of the compounds stood out significantly from the rest in the sum of total compounds (non–significant values < 0.600), but their values were strongly related with the total phenolic content in the extracts determined by the quantitative method of Folin–Ciocalteau (0.527).

In terms of bioactivity, the global amount of the compounds generally influenced the antioxidant activity to counteract the DPPH free radical (the higher the amount of compounds, the lower the IC_{50}, strong negative correlation with –0.912). Regarding the method of extraction, many previous studies have opted for organic solvents to obtain a greater amount of compounds and so, a greater antioxidant activity. Despite the published results that relate each of the groups to the strong antioxidant activity of an extract, it could be stated that instead of attributing the antioxidant activity to a single compound, the very strong antioxidant effect of the extracts would be due to a synergy between the compounds. A recent published study [296] determined a high positive correlation between total phenolic compounds and antioxidant effects resulted from different assays,which agrees with the results shown in **Table 40** (–0.696 for DPPH• and 0.6081 for ABTS•). However, the antioxidant capacity is the only directly related to the amount of compounds extracted, since no other activity of those analyzed showed significant Pearson's correlation values.

Emphazising the individual influence of each of the chemical groups in the bioactivities, caffeic acid derivatives could be related to hypoglycaemic activity (0.7578). This type of compounds are generally associated with antioxidant activity [128,178,208], and there are also studies that states that a diet rich in cinnamic acids can prevent the appearance of hassles in diabetic patients [297]. OV1 was shown to be potentially more active in inhibiting *α–glucosidase* than the marketed product used as positive control (acarbose), even after gastrointestinal digestion in the site of action. Likewise, in the *in vitro* anti–inflammatory activity tests, OV1 is shown to be the most respectful extract with the RBC membrane of and albumin denaturation. As expected, these two assays demonstrated to be highly correlated in **Table 40**.

Along with the rest of the results, extracts could cooperate in the inflammatory process because the preservation of RBC and albumin was also correlated with hypoglycaemic activity. This link could be considered such a positive result for clinical practice, that the

extracts might provide a better oxygenation of the extremities and the ability to repair damaged inflamed tissues in exacerbations of type II diabetes. In addition, this type of diabetic patients usually suffers from dyslipidaemia and heart problems, whether vascular or directly related to the heart. Due to the lack of results for lipid–lowering activity, this variable could not be added to the correlation study in **Table 40**.

Ageing is another important risk factor in type II diabetes, but, according to the results of the extracts, the rest of activities related to age were not directly correlated. Nevertheless, antioxidant acticivites were roundaboutly related to anti–inflammatory properties. In this sense, and coinciding with the hypothesis presented in the chapter (*see Chapter II, anti–inflammatory activity)*, the antioxidant activity of the extracts could positively benefit the preservation of the RBC membrane and the active form of oxygen, thus avoiding the formation of free radicals. Previous studies determining this activity tend to select extracts based on antioxidant activity. Moreover, assays of denaturation of albumin and haemolyse against heat shock are usually complemented with antioxidant activity assays based on nitric oxide free radicals scavenging. Reshma *et al*. [292] concluded that concentration of a methanolic extract of *Oxalis cormuculata* was directly proportional to the antioxidant activity (determined by complementary assays) and anti–inflammatory parameters. Inflammation is not only an acute process caused by a trauma, where researcher ought to engage the study of COX [298]. Metabolic complications can cause chronic inflammation in the patient, generally vascular. The resulting relationship between antioxidant, anti–inflammatory and indirectly, hypoglycaemic activity might prevent aggravations in the patient.

7 CITOTOXICITY

Sometimes, the fine line between life and death is based on the substance and/or the dose administrated. The papyri of Eberes (1,500 BC), Hippocrates (V Century BC) and Dioscorides (I Century AC) were some of the names related to the study of toxic products (most of them plants). *Dosis sola facit venenum* – The dose makes the poison – Paracelso (1493 – 1541). With this affirmation, the characteristic of being something toxic changed from qualitative to quantitative (amount administrated). Paracelso was the first to relate the activity of a substance to the dose and considered experimentation essential to discover the response of the living organism to the toxicon (the action of the poison).

From a conceptual point of view, it is important to distinguish between toxic action (sum of processes associated to a chemical compounds that leads to a toxic effect) and toxic effect (harmful effect as a result of some physiological imbalance caused by a substance that

can be reversible or irreversible) [152]. A toxic substance has different entrances into the organism: mouth for ingestion (orally), skin (or blood) for intraperitoneal, endovenous, subcutaneous and dermal (local–topic) and lungs for inhalation. Different organs would be exposed to that toxic substance and the elimination would be different (faeces, urine, secretory glans or exhaled air) depending on the route of administration (**Figure 50**).

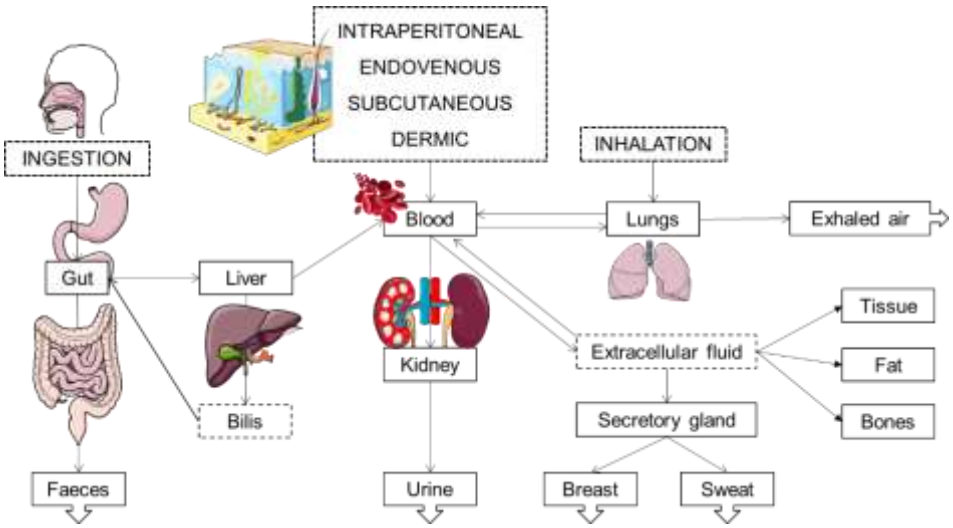


Figure 50. Possible routes to be followed by a substance in a living organism.

Ingestion is one of the principal routes where gut is the first affected system, followed by the liver. Any oral administration follows the same route: stomach (liberation of drug) – intestine (absorption and excretion) – blood (transport) – liver (metabolism) – kidney (excretion).

In this sense, it was necessary to test the toxicity in these target organs (intestine and liver) so that dose of extracts does not lead to toxicity. Despite the fact that oregano is not toxic when used as condiment, it would be worthwhile to test the cytotoxicity of the extracts because *dosis sola facit venenum*.

Within the preclinical phase, cell lines are appropriate to start testing for toxicity to humans. When selecting the appropriate cell line, these are the criteria to consider before starting the experiments [299]:

- Species: Non–human and non–primate cell lines have fewer biosafety restrictions.
- Functional characteristics: The purpose of the experiment might help decide the functional characteristics. For example, liver cell lines may be more suitable for toxicity testing.
- Finite or continuous: Continuous cell lines are often easier to clone and maintain, whereas finite may give more options for expression of correct functions.

- Normal or transformed: Transformed cell lines usually have an higher growth rate and plating efficiency, are continuous, and require less serum in the medium, but they have undergone a permanent change in their phenotype through genetic transformation.
- Growth conditions: such as growth rate, saturation density, cloning efficiency and, ability to grow in suspension.

Caco–2 cell line: the Caco–2 cell line are adenocarcinoma epithelial cells from a human Caucasian male (72 years of age) generally used in permeability assays, because these cells are able to express a variety of carrier proteins present in various endothelial and epithelial barriers of the body. *In vitro* cytotoxicity assay on this cell line may determine the therapeutic window (toxicity limits) for further oral administration of the extracts.

HepG2 cell line: the HepG–2 cell line are human hepatoma cells commonly used *in vitro* as an alternative to human hepatocytes for studies concerning drug metabolism and hepatotoxicity.

A375 cell line: the A375 cell line are epithelial cells from a 4–year–old human female with malignant melanoma, generally used for tumourigenicity assays. In this case, the cytotoxicity of the extracts in this human skin cell line was analysed as for their topical administration.

7.1 MATERIAL AND METHODS

Cell culture and MTT assay were carried out according to Serralheiro *et al.* method [273] and American Type Culture Collection (ATCC) recommendations [300].

- Experimental basis

The MTT (3–[4,5–dimethylthiazol–2–yl]–2,5 diphenyl tetrazolium bromide) assay consists of the measurement of cytotoxicity and the determination of mitochondrial activity through the conversion of MTT into formazan crystals by the living cells (**Figure 51**).

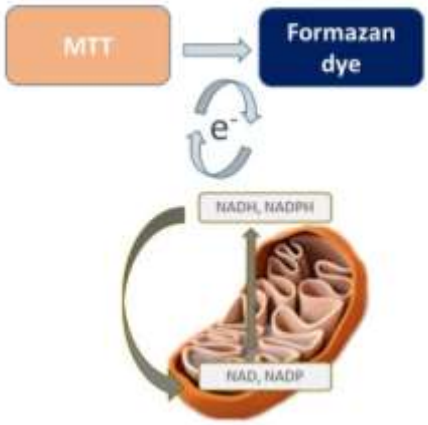


Figure 51. Reaction that takes place in MTT cytotoxicity assay.

- Cell culture

Caco2 cells (ATCC#HTB37) were grown in Roswell Park Memorial Institute medium (RPMI; #R7388 Sigma–Aldrich Co., St. Louis, MO) supplemented with 10 % Fetal Bovine Serum (FBS, #F7524, Sigma–Aldrich Co., St. Louis, MO), 100 U/mL penicillin and 100 U/mL streptomycin mix suitable for cell culture (#P4333, Sigma–Aldrich Co., St. Louis, MO and L–glutamine 2 mM (#G8540, Sigma–Aldrich Co., St. Louis, MO). This medium was denominated complete medium.

100 µL of cell solution at 5 x 10^4 cells/mL were added per well, incubated 72 h at 37 ºC with an atmosphere of 5% CO_2 and renewed every 48–72 h (Figure 53).

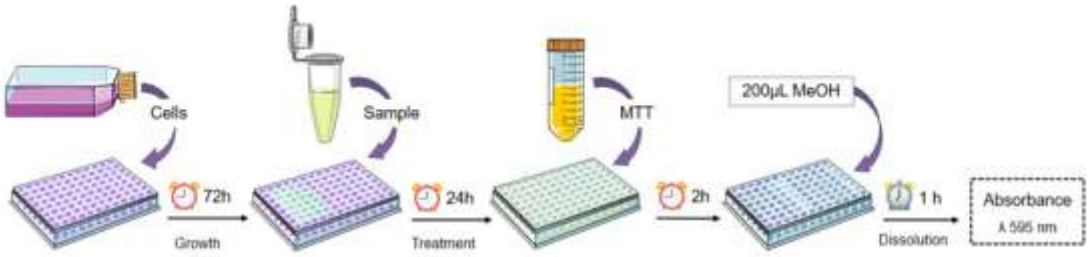
According to ATCC growing recommendations and previous studies, MTT assay was performed for HepG–2 cell line and A375 cell line using Dulbecco′s Modified Eagle′s medium (DNEM medium – # D5030, Sigma–Aldrich Co., St. Louis, MO) instead of RPMI.

- Sample preparation

Samples were dissolved in RPMI medium (without supplementation, denominated as *free medium*) at different concentrations (0.01, 0.05, 0.10, 0.50 and 1 mg/mL). The lack of the sample was used as negative control and rosmarinic acid and doxorubicin as positive control at the same concentrations as the samples.
Intestinal fractions were only tested on the Caco–2 cell line. Crude extracts and intestinal fractions (OV1–Int, OV2–Int, OV6–Int and RA–Int) were tested on the HepG–2 cell line. Crude extracts were tested on the A375 cell line.

- Reaction and analyse conditions

According to **Figure 52**, three different types of assays were perfomed: one of them, the standard MTT method described in all published studies, and two new variants of this protocol with modifications in sample dispensing time (*life* MTT and *growing* MTT).



Type of assay	72h	24h	2h	1h
Normal	/	sample	MTT	MeOH
Growing	sample	/		
Life	sample	sample		

Figure 52. Experimental design of the MTT assay. Table below schematically shows the growth characteristics for each of the MTT variants (MTT normal, growing and for life). Then, the addition of MTT was the same for all types of methods.

Normal MTT counts with 72 h of cell growth without treatment, followed by RPMI medium replacement and addition of 100 µL of sample solution (in RPMI medium) incubated for 24 h. In *growing* MTT, the cells grew with the treatment, but it was not administered during the 24 h *normal* MTT does. In the other variant of *normal* MTT method, *life* MTT, the assay was carried out by adding sample to cells at each time of the experiment: when growing for 72 h and as in the *normal* MTT assay for 24 h (**Figure 52**).

Then, cells were washed twice with PBS and 100 µL of MTT solution (at 1 mg/mL in *free medium,* #M2003, Sigma–Aldrich Co., St. Louis, MO) were added per well. After 2 h of incubation, the content of the 96–well plate was replaced by 200 µL of methanol per well, so that the formazan crystals dissolve and the toxicity can be measured by the absorbance of the solution generated at 595 nm after 60 min.

- Data process

The absorbances were measured and the percentage of cytotoxicity was calculated with the following formula:

$$\%\, Toxicity = \frac{Absorbance_{sample}}{Absorbance_{control}} x\ 100$$

Normal, *growing* and *life* MTT were performed in triplicated for each condition and the results expressed as percentage of toxicity found in HepG–2 and A375 cells, separately. Due to time constraints and some obstacles with this cell line, the Caco–2 MTT assay was replicated once with *n* = 6 per condition.

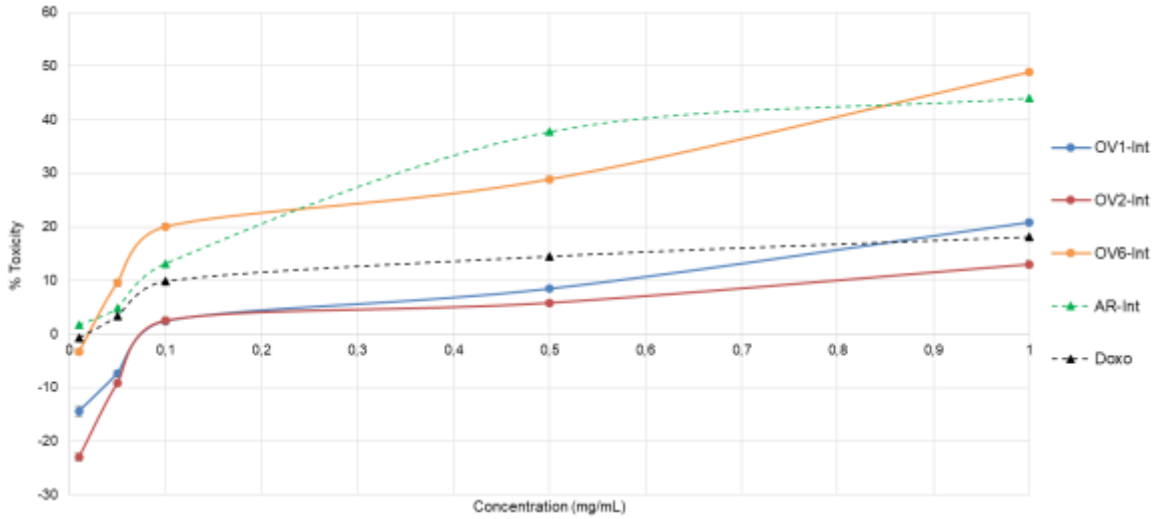
- Statistical analysis

Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

7.2 RESULTS

7.2.1 Caco–2 cell line

The MTT assay will determine the potentially toxic concentrations of the extracts in the Caco–2 cell line. Since Caco–2 physiologically corresponds to lumen, the intestinal fractions of the extracts after *in vitro* gastrointestinal digestion were tested (OV1–Int, OV2–Int and OV6–Int), as well as the intestinal fraction of Rosmarinic acid (RA–Int) for positive control to compare with doxorrubicin. The results were expressed as a percentage of toxicity (**Figure 53**).



% Toxicity *per* concentration (mg/mL)					
Sample	0.01	0.05	0.1	0.5	1
OV1-Int	-14.38 ± 1.02^{a}	-7.35 ± 0.40^{b}	2.42 ± 0.60^{c}	8.46 ± 0.27^{d}	20.83 ± 0.67^{e}
OV2-Int	-22.98 ± 0.75^{f}	-9.20 ± 0.32^{g}	2.50 ± 0.59^{c}	5.82 ± 0.49^{h}	12.99 ± 0.67^{i}
OV6-Int	-3.30 ± 0.49^{j}	9.55 ± 0.66^{d}	20.02 ± 0.54^{e}	28.86 ± 0.54^{k}	48.87 ± 0.46^{l}
AR-Int	1.67 ± 0.40^{m}	4.86 ± 0.64^{h}	13.14 ± 0.35^{i}	37.74 ± 0.94^{n}	44.05 ± 0.33^{o}
Doxo	-0.71 ± 0.65^{p}	3.38 ± 0.72^{h}	9.87 ± 0.35^{d}	14.50 ± 0.65^{i}	18.14 ± 0.33^{q}

Figure 53. Results for cytotoxicity in Caco–2. Graph shows the percentage of toxicity (mean ± SD %) at the concentrations tested. As shown in the legend, blue corresponds to OV1 intestinal fraction (OV1–Int); red, to OV2 intestinal fraction (OV2–Int); orange, to OV6 intestinal fraction (OV6–Int); dashed green to positive control rosmarinic acid (RA–Int) and dashed black line to doxorrubicin (Doxo). The table below shows the percentages of toxicity (mean ± SD %) per concentration tested of the samples (0.01, 0.05, 0.1, 0.5 and 1 mg/mL). Values

with different letter show significant differences ($p < 0.05$) and the same letter indicates no significant differences ($p > 0.05$).

The results show the percentage of toxicity for each extract at the concentrations tested (0.01, 0.05, 0.1, 0.5 and 1 mg/mL). RA–Int was used as positive control and toxicity was shown to be less than 50 % for all concentrations tested: from 1.67 ± 0.40 % at 0.01 mg/L to 44.05 ± 0.33 % at 1 mg/mL. On the other hand, doxorrubicin – the other positive control – presented less toxicity than RA–Int (18.14 ± 0.33 % at the highest concentration tested (1 mg/mL)). In general, the toxicity was directly proportional to the concentration: the higher concentration, the higher the percentage of toxicity, with different trends. Doxorubicin was not only less toxic for Caco–2 but also more moderate because line described in the graph presented a lower slope or at least, horizontal asymptote had a lower toxicity value ($y < 20$ % for doxorrubicin and $y < 50$ % for rosmarinic acid). This mathematical fact could be explained as different safety limits with a wider therapeutic window for doxorrubicin (gradient maintenance and flat slope) than RA–Int, where the toxicity changed quickly from 0.1 to 0.5 mg/mL (from 13.14 ± 0.35 % to 37.74 ± 0.94 %, respectively).

At low doses, the extracts presented similar percentages of toxicity per group of extraction solvent. The aqueous extracts (OV1–Int and OV2–Int) were practically not significantly different with no toxic effect up to 0.5 mg/mL with 8.46 ± 0.27 % and 5.82 ± 0.49 %, respectively with $p = 0.789$. On the other hand, OV6–Int presented a non–significant higher toxicity than aqueous extracts, maintaining up to the maximum of 48.87 ± 0.46 % at the maximum concentration tested (1 mg/mL), which almost doubles toxicity observed at the half concentration of 0.5 mg/mL (28.86 ± 0.54 %).

Therefore, OV1–Int and OV2–Int were the least toxic extracts regardless of concentration (up to 1 mg/mL which is the upper limit tested). Conversely, OV6—Int would be recommended to be used at low dose – if selected for oral formulation – because toxicity in Caco–2 cells was close to 50 % at 1 mg/mL. Until now, no previous studies with these characteristics were carried out with *O. vulgare*, but its oral safety and therapeutic use already suggested low cytotoxicity in the lumen. Within the study of hypoglycaemic activity of diverse natural compounds [301], flavonoids such as quercetin and rutin at different concentrations showed high cell viability (around 100 %) in the MTT assay performed in Caco–2. Then, the enzymatic activity tested on these cells improved with time of exposure. In that sense, the lack of toxicity in Caco–2 of OV1–Int supports its safety as a hypoglycaemic oral treatment. Further studies in this line should contemplate Caco–2 permeability and enzymatic assays, which in this work could not be performed due to various inconveniences.

7.2.2 HepG–2 cell line

The results of the MTT assay were expressed as percentage of toxicity found in the cells at the different concentrations tested.

First, the standard MTT protocol was performed for all six extracts, rosmarinic acid and doxorubicin as positive controls. **Figure 54** below shows a compilation of the results of the three MTT assays in HepG–2.

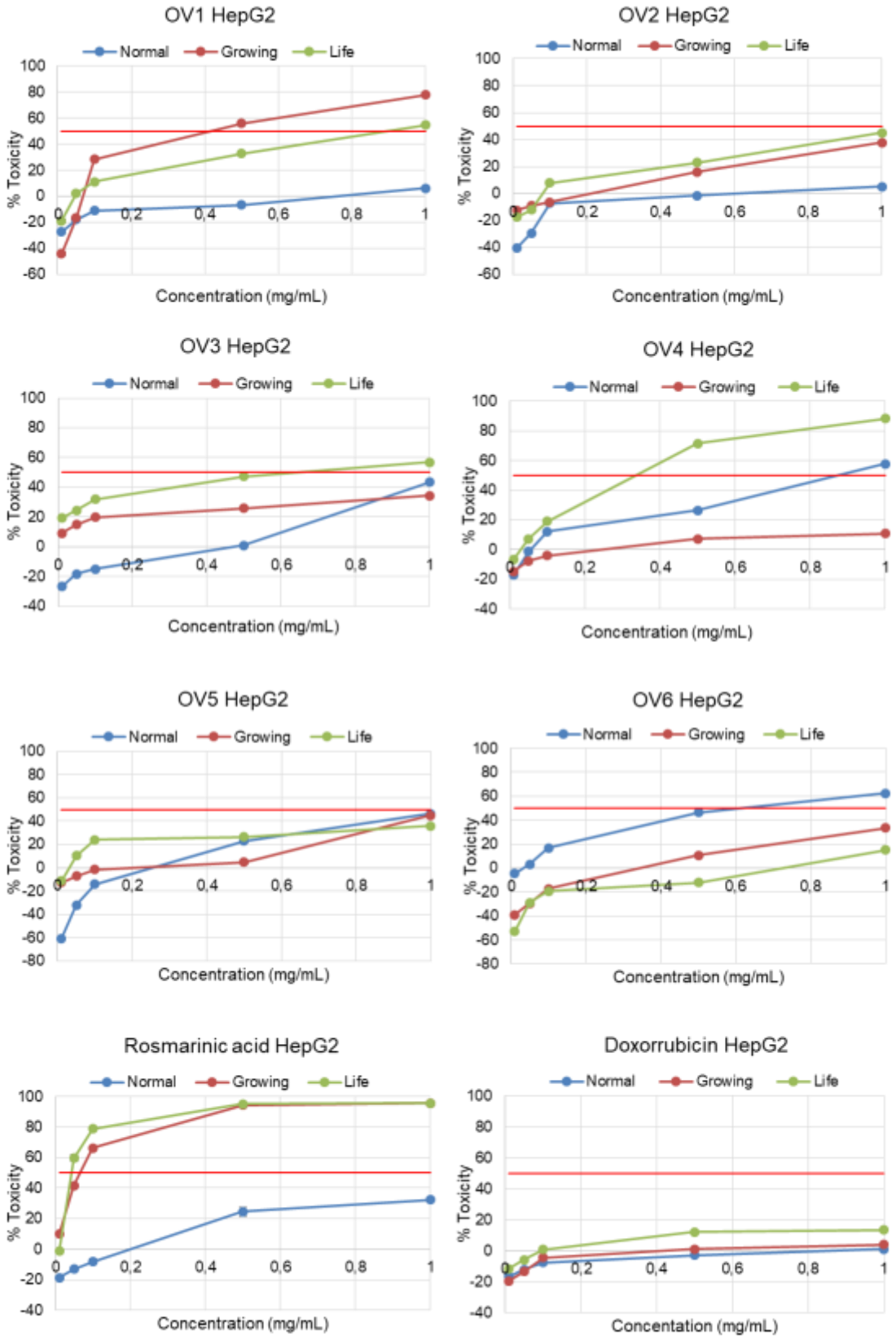


Figure 54. Results for cytotoxicity in HepG–2 (mean ± SD %) at concentrations tested. Percentage of toxicity (mean ± SD %, y–axis) per concentration (mg/mL, x–axis). Blue line corresponds to *normal* MTT assay; red, for *growing* MTT method and green for *life* MTT method.

Samples that exceeded 50 % toxicity (red line in **Figure 54**) might be considered toxic at that concentration. At first glance, all the extracts respected the red line at doses lower than 0.4 mg/mL, with OV2, OV3 and OV5 as the most respectful ones at higher doses. OV4 and OV6 were the only extracts that exceeded 50 % toxicity at high concentrations, in red at 1 mg/mL: > 0.6 mg/mL for OV6 (62.53 ± 0.62 % at 1 mg/mL) and > 0.8 mg/mL for OV4 (57.72 ± 1.00 % at 1 mg/mL), with $p = 0.03$. The rest of the samples did not present remarkable levels of toxicity, although OV5 and OV3 were close to that value at 1 mg/mL: 43.49 ± 1.0 % and 46.27 ± 0.86 %, respectively with $p = 0.232$.

In relation to the positive controls, again rosmarinic acid showed to produce higher levels of cytotoxicity than doxorrubicin at high concentration (from 0.1 mg/mL administered). As shown in the graph, doxorrubicin was the least toxic for HepG–2 cell line, even at highest concentration tested, because line described by the data was the lowest among the samples tested. Then, OV1 and OV2 showed low toxicity (< 10 %) at any concentration tested, being 6.15 ± 0.80 % and 5.12 ± 0.92 % at 1 mg/mL being the maximum registered, respectively, with no statistical differences ($p = 0.908$). In this sense, OV1 and OV2 could be grouped with doxorubicin, OV3 and OV5 as slightly more toxic than rosmarinic acid, and OV4 and OV6 as toxic at high concentrations. From a therapeutic point of view, these results are also important for lipid–lowering activity as cholesterol is synthetized in the liver through HMG–Coa reductase pathway. OV1 and OV2 – non–toxic extracts – showed potentially inhibitory activity of this enzyme.

Moreover, the other two variants of the *normal* MTT showed different toxic effects on cells (**Figure 54**). The sample was added during the growing of cells for 72 h and no sample was administered during the standard 24 h of treatment. In this sense, *growing* MTT (first variant of this cytotoxicity assay carried out) showed different results for the extracts. Contrary to what was expected, the positive controls showed opposite behaviours. Rosmarinic acid showed high levels of toxicity even at low concentrations, being greater than 50 % from 0.1 mg/mL (66.20 ± 0.52 %) and greater than 90 % from 0.5 mg/mL (94.21 ± 0.62 % and 95.69 ± 1.09 % at 0.5 and 1 mg/mL), whereas doxorrubicin did not even exceed 5 % toxicity at any concentration, being the highest registered 3.93 ± 1.05 % at 1 mg/mL.

OV1 was the only extract whose concentration tested exceeded 50 % toxicity: 55.87 ± 0.76 % at 0.5 mg/mL and 77.96 ± 053 % at 1 mg/mL. In this sense, it would be recommended to use OV1 in low concentrations or to avoid prolonged exposure of the cells to it. Another extract with similar toxicity limitation by concentration would be OV5, because the level of toxicity increased from 4.68 ± 1.04 % at 0.5 mg/mL to 45.14 ± 0.39 %

at 1 mg/mL. In this sense, OV5 should not be used at concentration higher than 0.5 mg/mL, regardless exposure, since this behaviour was also previously observed in *normal* MTT.

The rest of the extracts presented low levels of toxicity at high concentration, being 37.71 ± 0.81 % (OV2), 34.33 ± 0.11 % (OV3) and 33.32 ± 0.75 % (OV6). Despite this non–significant difference in toxicity results at 1 mg/mL between OV3 and OV6 (p = 0.721), OV2 and OV6 could be considered more similar with increasing concentration, since the line described by the data in the graph showed a similar trend. On the contrary, OV3 presented a higher percentage of toxicity at low concentration (9.15 ± 0.70 % at 0.01 mg/mL, 14.84 ± 0.75 % at 0.05 mg/mL and 19.85 ± 0.59 % at 0.1 mg/mL), while OV2 and OV6 did not present any toxicity (mathematically the values were below 0 %, but in practice this is lack of toxicity). Besides, OV4 – which did not present significant differences with doxorubicin and OV5 at 0.1 mg/mL: < 0 % with p = 0.105 – showed to maintain the level of toxicity below 15 %. On the graph, the OV4 data described a saturated line that exhibited a mathematical horizontal asymptote in the same way as doxorubicin. In other words, OV4 and doxorubicin could be considered safe for HepG–2 cells up to 1 mg/mL.

Once the toxicity of extract administered at different time points in HepG–2 has been analysed, the last group of results in **Figure 54** shows the effect produced in this cell line when dispensing is continued (*Life* MTT method). Rosmarinic acid presented high toxicity even at very low concentrations, being greater that 50 % from 0.05 mg/mL: 59.65 ± 0.22 % at 0.05 mg/mL, 78.89 ± 1.04 % at 0.1 mg/mL, 95.18 ± 0.76 % at 0.5 mg/mL and 95.74 ± 0.72 % at 1 mg/mL. On the other hand, doxorubicin showed no toxicity to cells even at any concentration, the highest registered being 13.28 ± 0.42 % at 1 mg/mL.

The extracts exhibited different behaviours compared to the previous cytotoxicity MTT modalities. OV6, which could be considered toxic for cells in *normal* MTT and increased in toxicity when growing from 0.5 mg/mL, did not show any toxicity effect at any concentration when the exposure to cells is long–lasting: highest toxicity level at *life* MTT = 15.13 ± 1.19 %, with non–significant differences to positive control (13.28 ± 0.42 %) with p = 0.374. The other ethanolic extract also presented low levels of toxicity levels, maintaining values below 50 % as in the previous variants of MTT. When exposing cells to OV5 at each time point of growth, the maximum toxicity observed was 35.74 ± 0.60 % at 1 mg/mL. In this sense, it could be said that OV5 and OV6 lower their toxicity when the exposure to cells is longer than 24 h and during growth.

The aqueous extracts, which presented different toxic effect in the previous MTT modalities, recovered the behaviour of the partners in *life* MTT by describing a similar trend of the data in the graph. However, OV1 was shown to be more toxic than OV2: graphically,

the blue line is above the red line and mathematically the OV1 values were above OV2, even exceeding 50 % toxicity at 1 mg/mL: 54.70 ± 0.28 %.

Hydroalcoholic extracts presented different toxic effects to the cells. At low concentrations, OV4 presented less toxicity than OV3: 7.07 ± 0.34 % and 24.33 ± 0.61 % at 0.05 mg/mL, with $p < 0.001$. However, OV4 exceeded 70 % toxicity from 0.1 mg/mL (71.52 ± 0.43 % at 0.5 mg/mL and 88.43 ± 0.49 % at 1 mg/mL), whereas OV3 slightly exceeded 50 % at 1 mg/mL, being non–significantly different from OV1 (56.90 ± 0.34 % and 54.70 ± 0.23 % at 1 mg/mL, respectively with $p = 0.87$).

In general, the toxicity of all of them increased with concentration and rosmarinic acid showed to be more toxic than expected, but it was chosen as a positive control because it is one of the main compounds present in the extracts. Nevertheless, in MTT performed as standard protocol, toxicity remained below 50 %. However, when administered during cell growth (or prolonged exposure), toxicity levels exceeded 50 % at low concentration. The results showed no statistical difference for toxicity at 0.5 mg/mL ($p = 0.828$) and 1 mg/mL ($p = 0.998$) in *growing* MTT and *life* MTT. Already in Caco–2, this compound showed to be more toxic than OV1, OV2, OV3 and doxorubicin, by limiting its use to low doses for cell safety.

On the contrary, doxorubicin again behaved as an optimal positive control and, as shown, no toxicity value exceeded 20 % toxicity in any variant of the cytotoxicity assay. In fact, prolonged cell exposure (*life* MTT) showed slightly higher toxicity levels at high concentrations than the other two MTT variants performed, which were nont significantly different at 0.5 mg/mL ($p = 0.075$) and 1 mg/mL ($p = 0.105$).

Despite the low toxicity effect of OV1 and OV2 in Caco–2, in liver, the aqueous extracts presented lower toxicity in n*ormal* MTT than in the new variants performed (g*rowing* MTT and *life* MTT). The administration of OV1 should not be recommended for long–term treatments to avoid hepatotoxicity, unless the concentration is less than 0.5 mg/mL, because the toxicity overpass 50 % for g*rowing* MTT and in *life* MTT at 1 mg/mL, while after only 24 h a non–toxic effect was produced even at 1 mg/mL. Similarly, OV2 would be recommended in same way, although values did not exceed 50 % toxicity but they were close at 1 mg/mL for *growing* MTT and *life* MTT, with $p = 0.030$.

OV3 presented less toxicity in *normal* MTT in the other two variants of this method, although in *growing* MTT the toxicity levels were maintained as the concentration increased. In fact, the values did not exceed 50 % toxicity even at 1 mg/mL, where *growing* MTT assay showed levels greater than 50 % toxicity. In this sense, the use of this extract should be limited in exposure and concentration to avoid toxicity to cells in the liver, despite

the fact that no toxicity was found in Caco–2 cells, where the highest toxicity was 25.65 ± 0.76 % at 1 mg/mL and and the trend was to show a borderline toxic effect at that point.

OV4 presented toxicity in *normal* and *life* MTT at concentrations greater than 0.5 mg/mL and 1 mg/mL, respectively. However, when it was administer only during the cell growth, this toxicity was reduced and maintained. In this sense, it would be good not to use high concentrations of this extract or to avoid its use for cells. Indeed, in Caco–2 this extract was one of the most toxic, with the highest toxicity level being 47.68 ± 0.90 % at 1 mg/mL.

OV5, the most toxic compound for the Caco–2 cell line, showed less toxic effect on the HepG–2 cell line, although the three MTT variants presented toxic levels close to 50 % at 1 mg/mL. Therefore, this compound may not be recommended for oral use, unless the dose is less than 0.1 mg/mL and the treatment is time limited (no prolonged exposure because toxicity was higher in *life* MTT assay).

OV6, which produced a similar toxicity in Caco–2 than OV4, presented greater toxicity when MTT was performed in a standard way, being toxic from 0.5 mg/mL. However, its presence during the first part of cell growth provided less toxicity, being even less if the cells were exposed all the time to this compound. As a result, low chronic oral treatment of OV6 could potentially be considered as safe in order to avoid intestine and liver toxicity (**Figure 54**).

As a complement to the cytotoxicity study in cell lines typical of an oral administration, intestinal absorbable fractions were also tested in liver cells (HepG–2). **Figure 55** shows different graphs of the crude extract and intestinal fraction of it (graph below) in the three variants of MTT method: *normal* (in blue), *growing* (in red) and *life* (in green).

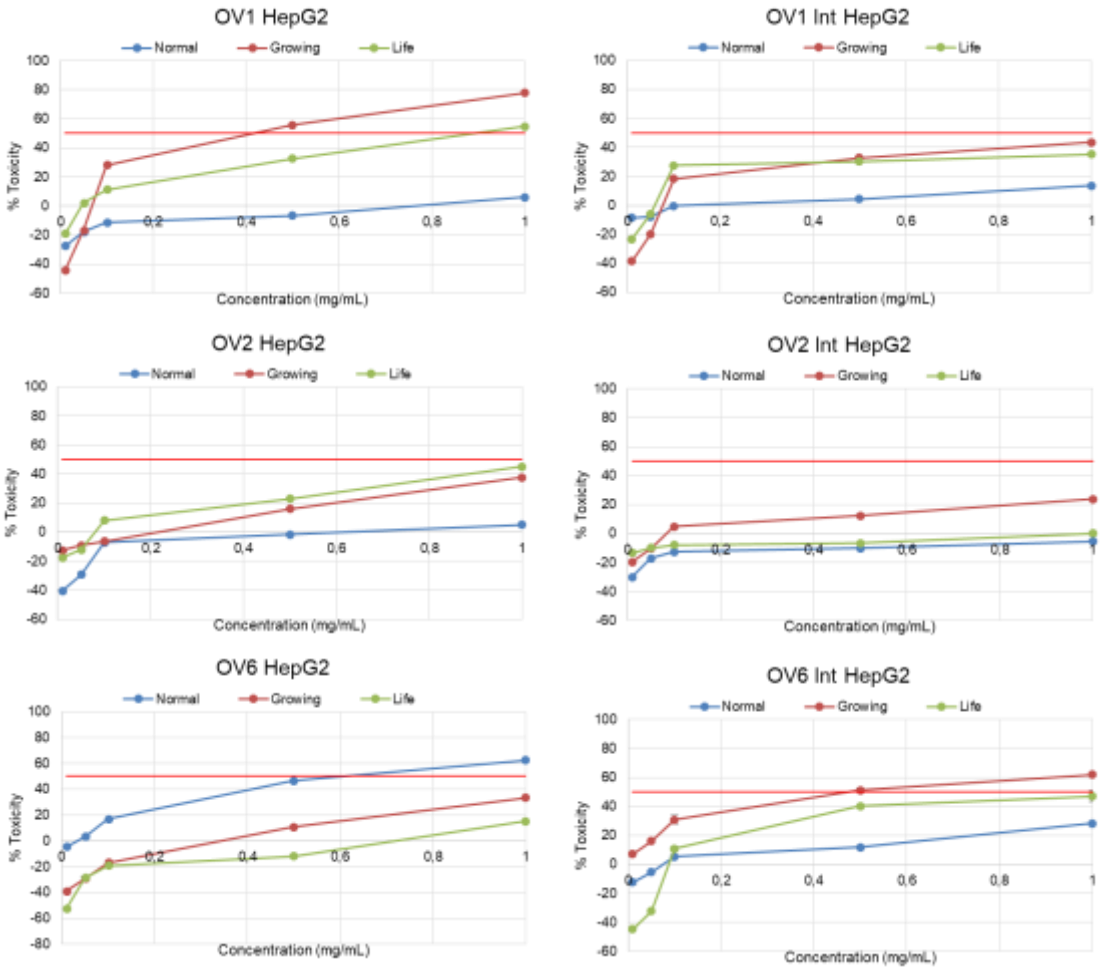


Figure 55. Results of cytotoxicity in HepG–2. Percentage of toxicity (mean ± SD %, y–axis) per concentration (mg/mL, x–axis). Blue line corresponds to *normal* MTT assay; red, for *growing* MTT method and green for life (*life* MTT method).

The graphs provided an overview of cytotoxicity levels of the extracts in HepG–2 before and after digestion. Toxicity observed in OV1 in crude extract in growing and *life* MTT assays, especially at high concentrations (0.5 and 1 mg/mL), pretended to be reduced since date did not exceed the 50 % toxicity red line on the graph. Still, results in the *normal* MTT method were significantly less toxic than in the other two variants, which could provide a benefit for the cold macerated aqueous extract of *O. vulgare* L., under the tested conditions. Then, that cytotoxicity observed for the crude OV6 extract changed after digestion, since *normal* MTT results did not overpass the red line at any concentration. Unfortunately, this fact could not change previous recommendations for OV6: low concentrations of this extract (< 0.5 mg/mL) might assure no toxic effect in oral administration of the extract.

Contrary to these effects and improving toxicity levels, the intestinal fraction of OV2 presented almost no toxic effects after normal and prolonged exposure in the HepG–2 cell line. The toxic effect showed to be increased up to 25 % at 1 mg/mL (maximum observed)

if the cells were exposed only at growing time, but reduced to 0 % at 1 mg/mL when presence of OV2 is mantained in time. Translated to a hypothetical oral administration, OV2 could be provided for chronic treatment with potentially low hepatotoxicity, since neither the crude extract nor intestinal fraction showed cytotoxicity in this assay. The lack of documented research using this method in literature made difficult the comparison of results with other extracts from this medicinal plant. Nonetheless, published studies demonstrated an *in vivo* hepatoprotective effect of extracts of *O. vulgare* that are in consonance with the results obtained here. Anyhow, the extent use of oregano as medicinal plant for centuries already suggested not obtaining toxic levels such as those found for rosmarinic acid, for example. The cytotoxicity assays not only demonstrated the absence of toxic effect of *O. vulgare* administrated orally, but also confirmed this lack of effect for the current extracts prepared. The decision on orally administrated selection might contemplate all the bioactivities tested *in vitro* before testing *in vivo*. OV5 did not show high toxic levels (over 50 %) in HepG–2, but it had a low protective effect for albumin and red blood cells *in vitro*. Contrary to the potential benefits of extraction method of OV4 (high temperature and mix in polarity of solvents), the results on bioactivity were mediocre with the disadvantage of slightly toxicity on liver cells. That effect might possibly require limitations on the concentration of OV6. Despite the antioxidant properties of this extract, results on rest of *in vitro* tests were also irrelevant. Thus, taking into account bioactivities, the best for oral administration would be aqueous (OV1 with some limitations and OV2) and cold–hydroalcoholic (OV3).

7.2.3 A375 cell line

Not many more bioactivities been tested for the six extracts prepared in this study, given the interest in oral administration. As a preliminary attempt at the potential use of the extracts as a skin remedy, a few different activities were tested. First, the cytotoxic effect on epithelial cells (A375 cell line) to determine safety of the extract, and then, the *in vitro* activities related to blood flow (albumin denaturation and RBC membrane preservation).

The cytotoxicity of the extracts in A375 cell line was determined using the MTT assay. The results were expressed as percentage of toxicity and the values of the three variants of the method were compiled in one graph per extract (**Figure 56**).

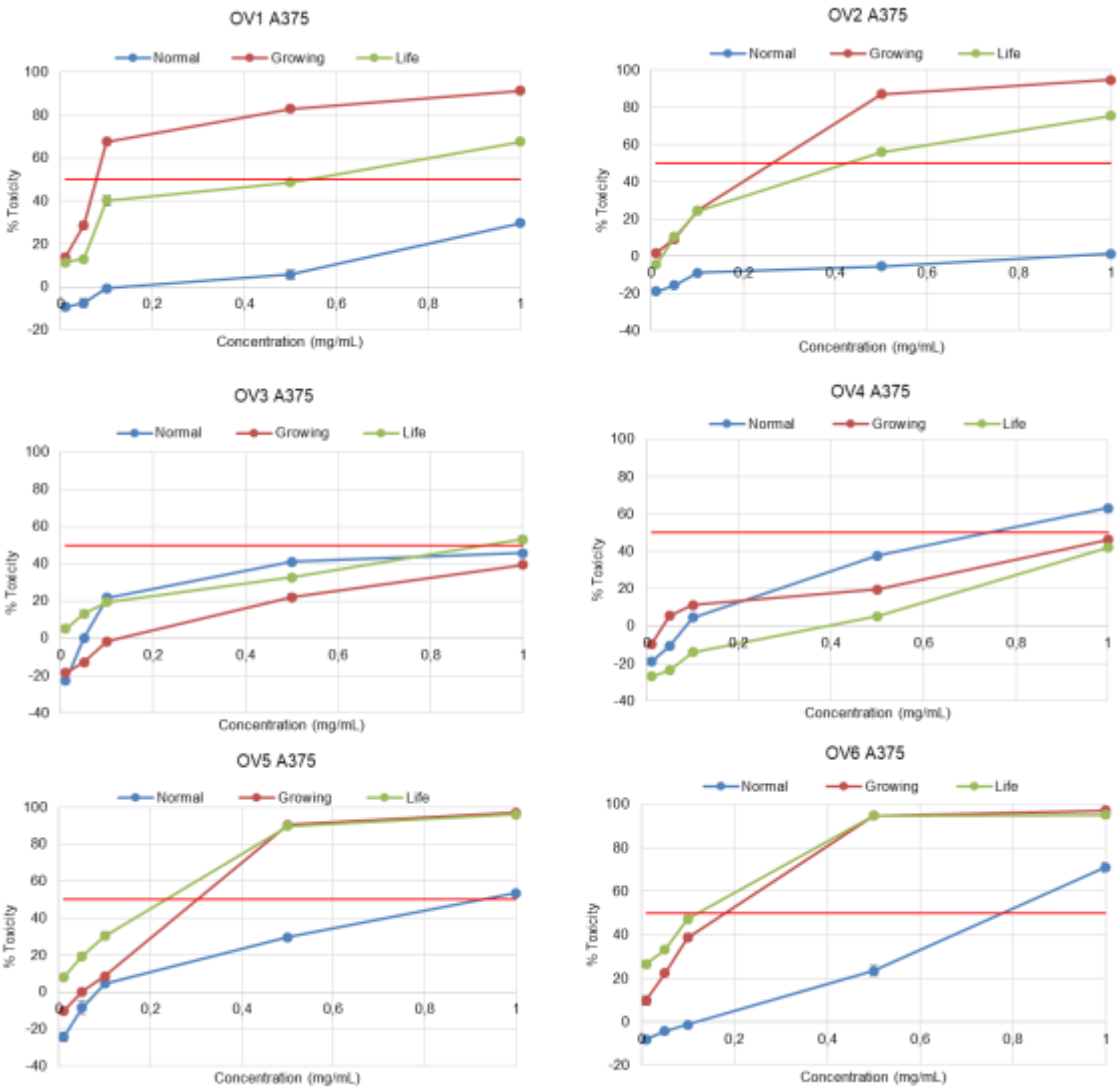


Figure 56. Results of MTT assay in A375 for six extracts in the three modalities of MTT assay. Percentage of toxicity (mean ± SD %, y–axis) per concentration (mg/mL, x–axis). Blue line corresponds to *normal* MTT assay; red, for *growing* MTT method and green for *life* MTT method.

The red line indicates 50 % of toxicity (**Figure 56**), a value above which extract could be considered potentially toxic for that cell line [302]. OV3 and OV4 were the most respectful extracts, regardless of exposure, since the values did not exceed the line as for the other extracts. Only the high concentration of both extracts showed slightly toxic values, being close to 50 %: OV3 *normal* = 45.76 ± 0.26 %, *growing* = 39.50 ± 0.42 % and *life* = 53.22 ± 0.82 %; OV4 *normal* = 63.18 ± 0.79 %, growing = 46.20 ± 0.47 % and *life* = 42.13 ± 0.76 %, with $p < 0.001$ among all of them, except OV4 *growing* and OV3 *normal* with $p = 0.212$. Besides, the data from OV3 administered for 24 h showed a tendency for the toxic effect to stabilize as the slope decreases. Prolonged exposure to OV3 at high doses should be avoided, because, despite the slightly toxic value obtained, it exceeded 50 %. On the contrary, OV4 presented better results for prolonged exposures of the extract (*life* andg

growing) than the *normal* MTT assay, where the tendency at high concentrations is potentially toxic. Nevertheless, these hydroalcoholic extracts of *O. vulgare* also presented low toxicity in cells with a significant antiproliferative effect.

Contrary to this effect of hydroalcoholic extracts, OV2 did not produce toxic effect in the short exposure method (*normal* MTT), even with higher toxicity percentages. In this case, by adding the sample only for 24 h once cells have grown, the toxicity levels were practically null, being below 0 % up to 0.5 mg/mL and 1.13 ± 0.36 % at highest concentration tested (1 mg/mL). However, the presence of OV2 during cells growth (or in long–term administration) produced high levels of skin cell toxicity: 87.07 ± 0.32 % (0.5 mg/mL) and 94.64 ± 0.29 % (1 mg/mL) in *growing* MTT and 55.81 ± 0.32 % (0.5 mg/mL) and 75.32 ± 0.81 % (1 mg/mL) in *life* MTT. OV2 could be safely suggested for cutaneous use for short–term applications.

In parallel, in the *normal* MTT method, OV1 did not produce any toxicity. On the contrary, *growing* and *life* presented percentages of toxicity greater than 50 % at the highest concentration *growing* 1 mg/mL = 91.39 ± 0.31 % and *life* 1 mg/mL = 67.69 ± 0.74 %, with $p < 0.001$. Besides, when OV1 was administered at lower concentrations only during growing, toxicity increased, with the percentages of toxicity being 67.73 ± 1.55 % at 0.1 mg/mL and 82.89 ± 0.95 % at 0.5 mg/mL. If this administration is maintained as in *life* MTT method, those percentages remain below 50 %: 40.14 ± 0.48 % at 0.1 mg/mL and 48.75 ± 1.15 % at 0.5 mg/mL. Lower concentrations (0.01 and 0.05 mg/mL) did not present much toxicity for A375 in these two variants of the method (below 30 %). In fact, the effect produced by the latter did not present statistically differences to the toxicity produced in *normal* MTT at 1 mg/mL = 29.81 ± 1.03 %, with $p = 0.712$.

Ethanolic extracts (OV5 and OV6)ould be potentially toxic. Administered during growth, the percentages of toxicity were greater than 50 %, even greater than 90 % for 1 mg/mL administered long–term and during growth. Besides, at those concentration, no significant differences were found between *growing* and l*ife* for the same extract. OV5 caused 90.45 ± 0.29 % and 89.80 ± 0.25 % of toxicity at 0.5 mg/mL with $p = 0.122$ and 97.12 ± 0.21 % and 96.15 ± 0.27 % at 1 mg/mL with $p = 0.989$. In the same way, toxicity of OV6 at 0.5 mg/mL was 94.79 ± 0.28 %, 94.79 ± 0.30 % ($p = 0.120$), and at 1 mg/mL, 97.26 ± 0.25 % and 95.13 ± 0.52 % ($p = 0.989$). At lower concentrations, *life* registered higher toxic percentages than g*rowing* and *normal* for same concentration, and OV6 higher values than OV5. Nevertheless, according to the results, these two extracts should not be recommended for topical use. Previous studies, suggested an ethanolic extract of *O. vulgare* as anti–acne topical remedy [303]. In that study, the plant material was somewhat

similar (leaves and OV was prepared from aerial flowered parts). So the selected extraction method was more like OV5; despite the fact that the proportions were different, there was the possibility of low extraction in their method or rapid saturation of the extraction solvent (10 g in 100 mL). Besides, the lack of chemical characterisation hinders the comparison. Nevertheless, cytotoxicity in this cell line was not carried out and their study was focused on anti–inflammatory and antibacterial activity.

There is a previous published study with hydroalcoholic extract (1:1 v/v) of *O. vulgare* cultivated in Greece, which performed cytotoxic assay on A375 cells [304]. Although the MTT assay was performed differently and higher concentrations of the extract were tested (2, 4, 6 and 10 mg/mL), results showed a significant antiproliferative activity in A375 [304]. The preparation of that extract could be comparable to OV3 (cold maceration hydroalcoholic extract), but the chemical composition did not show to be the same, especially due to the absence of rosmarinic acid, one of the main bioactive compounds of OV3. Nevertheless, the results found in that cytotoxicity assay could resemble the ones obtained for OV3 and OV4.

CHAPTER III:

STUDY OF *in vivo* PHARMACOLOGICAL ACTIVITIES of *Origanum vulgare* L. ssp. *vulgare* flowered aerial parts extracts

A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales.

Marie Curie

Among the *in vivo* models available for pre–clinical research experiments, *Caenorhabditis elegans* is one of the most widely used. *C. elegans* are small, free–living nematodes with potential benefits for answering questions in the fields of genetics, biology, and neurobiology. Going back to the last Century, in 1963 [305], Sydney Brenner proposed this *in vivo* model as a solution to those "classical problems of molecular biology", especially in development and nervous system [305,306].

Nowadays, *C. elegans* is widely used in a wide range of research fields and as simple and inexpensive tool to further study *in vivo* physiological responses to compounds, especially related to anti–aging properties. Among the experimental advantages of using this model, it is important to highlight that maintenance of worms only requires humidity, standard temperature (between 10 – 25 ºC), atmospheric oxygen and bacteria as a food source.

C. elegans is a multicellular eukaryotic organism that belongs to the *Rhabditidae* family. It is a non–segmented nematode approximately 1 mm long and 70 μm diameter, vermiform, bilaterally symmetrical in appearance and with a cutaneous integument [305]. Anatomical structure of adults have epidermis, muscles, nervous system and digestive system. All these systems are similar to other nematodes with a cylindrical, non–segmented body shape, with an outer tube (body wall: cuticle, hypodermis, excretory system, neurons and muscles) and an inner tube (pharynx, intestine and gonad). The particularity of this *in vivo* model is that the worms are transparent and externally covered by a cuticle, secreted by cells of the epithelial layer (epidermal, multinucleate and syncytial cells). This is a protective specialized extracellular matrix layer made of collagen, lipids and glycoproteins [307]. This layer also determines the shape of the body and provides anchor points for muscle contraction for its crawling movement.

A thin muscular *lamina* separates the epithelial layer from the nervous system. The muscles receive input from the neurons, mostly located in the head, around the pharynx with smaller muscles without striations to provide the pharyngeal pump. *C.elegans* feeds through the mouth but the pharynx is the main responsible for ingestion thanks to the fact that it is a practically autonomous organ (**Figure 58**). The pharynx is connected to the lumen of the intestine. The digestive system of these worms is practically a tube whose content is excreted to the outside through a rectal valve that connects the gut to the rectum and anus.

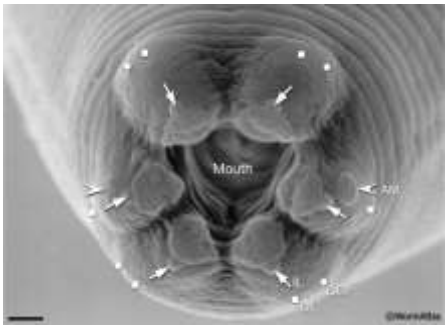


Figure 58. Entrance of food source of *C. elegans*. Scanning electron micrograph (SEM) of adult *C. elegans* showing the six symmetrical lips surrounding the opening of the mouth and the sensilla of the lip region. (OL) Outer labial; (CEP) cephalic; (AM) amphid; (IL) inner labial. 9000x. Scale bar: 1 µm. [307].

Genetically, *C. elegans* presents five autosome pairs and two sex chromosomes, being possible two types of sexuality: hermaphrodite (XX) and male (X0, with a 0.05 % of prevalence) [308]. They have different anatomy: males present single lobed gonads with specialized tails, whereas hermaphrodites have two ovaries, oviducts, spermatheca and uterus. The males can be distinguished by their tail [309]. Most of the cultivated ones (99.95 %) are usually hermaphrodites. When a worm is inseminated by itself, around 300 eggs can be produced, being the totality of new generation hermaphrodites. However, if the insemination is sexual (by a male, with a natural probability of 0.1 – 0.2 %) the amount of eggs could increase up to 1,000 eggs, being half–male, half hermaphrodites [309]. The advantage of self–reproduction is that it provides an easy way to study genetically identical offspring (isogenic population) as well as, count with statistically enough amounts of individuals for study.

The strains are cheap to breed and can be frozen, allowing for long–term storage. Moreover, thanks to be the first multicellular organism with a complete genome sequence [310], mutations and molecular identification of many key genes can be studied to identify behavioural defects. Indeed, at least 38 % of the protein–coding genes in *C. elegans* have predicted orthologs in the human genome [311], 60 – 80 % of human genes have predicted orthologs in the *C. elegans* genome [312], and 40 % of genes known to be associated with human diseases have clear orthologs in the *C. elegans* genome [313].

Undoubtedly, one of the most important characteristics of *C. elegans* as an *in vivo* model is their short life cycle (**Figure 59**). Life cycle of *C. elegans* is very rapid: 2 – 3 days at 20 ºC from the egg to the next generation. Starting from embryogenesis – that lasts for 16 h –, the eggs are laid once they reach 24–cell stage (gastrula), with an impermeable eggshell from fertilization to protect them. After 9 h of ex–utero development, they hatch into first stage (L1) larva. Then, after 12 h, they grow up to 380 µm becoming L2, and so on until adult (after 34 h). Adult hermaphrodites spend all their self–produced sperm in the

reproductive period of 2–3 day and then can live for several weeks until they die of senescence (under normal conditions, no longer than a month).

L2 larvae can activate an alternative life cycle becoming L3 larval stage called *dauer* (took from German verb meaning *lasting*). The characteristic of this alternative stage is the resistance to chemicals of their cuticle. Somehow, the uncomfortable conditions that led L2 to enter into *dauer*, made them become better survivors in undesirable environments with stress or chemical agents. However, when *dauer* larvae are transferred to new plates with normal conditions (space and food source), the shield disappears and the larvae continue their development as slightly different L4 larvae.

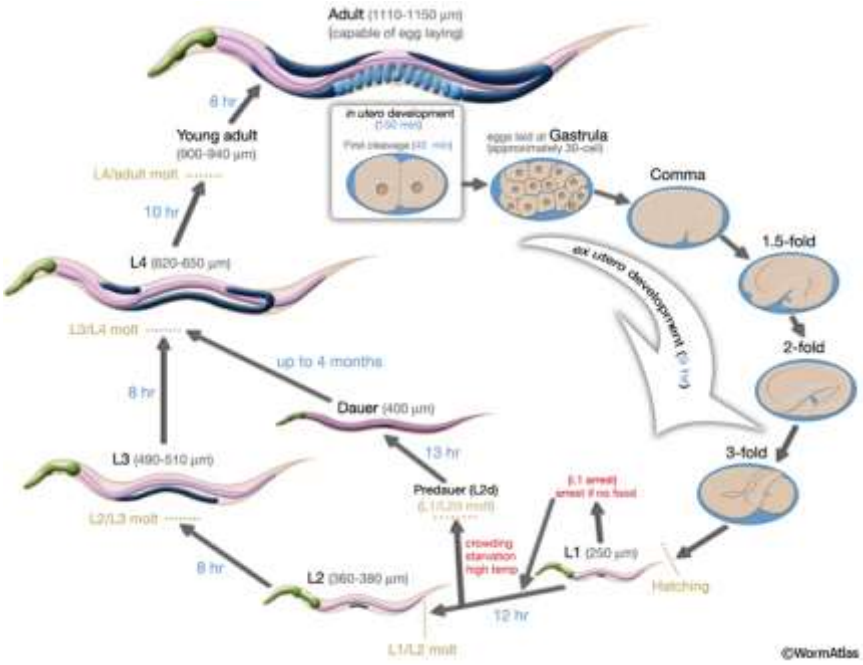


Figure 59. Cycle of life of *C. elegans*. Times, length and anatomical drawing given for each stage of development (times given correspond to 20 ºC; changes in temperature vary the times in growing) [314].

Under normal feeding conditions, the worms grow to 1 – 1.5 mm in the adult stage. If temperature, medium and strain are controlled, growing times can be perfectly controlled and calculated.

The length of the worms can also be used as a parameter of growth, indicating the stage (**Figure 59**). Generally, worms are grown in Petri plates and observed with a dissection lens. The eggs are easy to differentiate from the larval stages and the adults with eggs inside. Mean lengths for each stage are: Egg (100 µm), L1 (250 µm), L2 (360 – 3800 µm), L3 (490 – 510 µm), L4 (620 – 650 µm) and Adult (1,110 – 1,150 µm). The rest of stages might seem easy to distinguish in **Figure 60**, but in practise, human perception can lead to error.

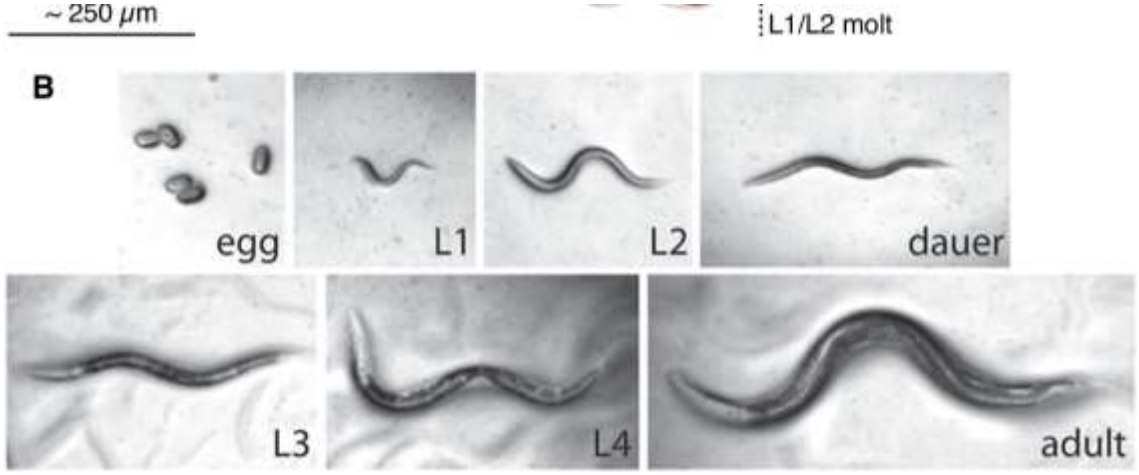


Figure 60. *C. elegans* cycle and picture of real worms regarding growing stage: egg, L1, L2, dauer, L3, L4 and adults [314].

1 PRELIMINARY ASSAYS

1.1 EXPERIMENTAL DESIGN

Before working with *C. elegans*, there were several factors to take into consideration:

- ***C. elegans* strain:** *C. elegans* was cultured according to previously reported guidelines [314]. N2 *Bristol* strain was used as wild–type reference provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota, MN).
- **Temperature of growing:** The speed of the growth depends directly on the temperature at which the worms are cultivated. For this work, 20 ºC (FOC 215i Cooled Incubator, VELP Scientifica, Usmate, Italy) was set up as temperature to grow *C. elegans*.
- **Medium of growing:** Solid Nematode Growth Medium (NGM) was used. *C. elegans* were grown in individual 9 cm diameter plates.
- **Food source:** Food source of *C. elegans* is *E. coli* OP50, a uracil auxotroph bacterium with limited growing in NGM. This bacteria grows in Lysogeny Broth (LB, Lennox), which is a non–selective medium for *E. coli* and coliforms.
- **Sample concentration range:** Aqueous extracts (OV1 and OV2) will be used for *in vivo* pharmacological activities, as a comparison between the two aqueous extracts with potential *in vitro* activities. Range of concentration to test of samples was established from 50 – 0.1 mg/mL so that EC_{50} values obtained *in vitro* would be represented. Real concentration would be diluted 1:110 (40 µL of sample in 4,000 µL of NGM).

- **Introducing sample in worm world:** Generally, assays are performed in 6–well cell culture plates with 4 mL of Nematode Growth Medium (NGM) per well at 20 ºC (FOC 215i Cooled Incubator, VELP Scientifica, Usmate, Italy). Two options were taken into consideration for the supplementation of plates with extracts of oregano whose advantages and disadvantages are compiled in **Figure 61.**

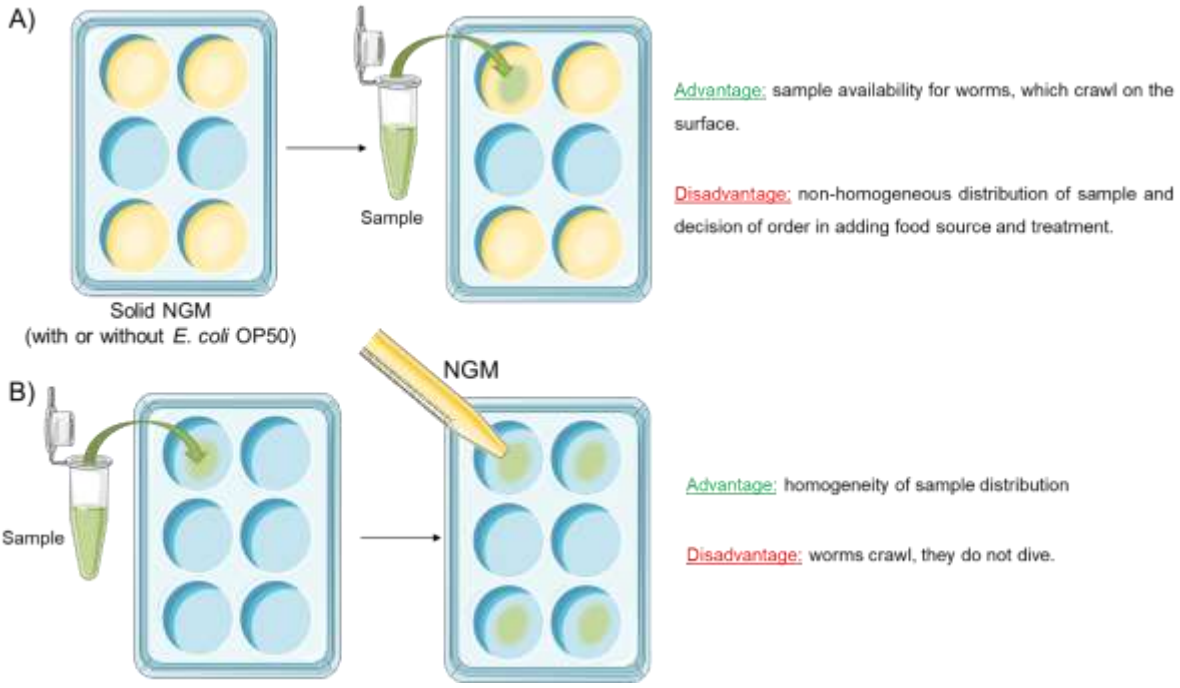


Figure 61. A) Introduction of the sample after NGM solidification – could be before or after adding food source. B) Introduction of the sample as part of NGM solution (before solidification).

On the one hand, adding the sample after solidification of NGM (**Figure 61–A**) allows better access to the sample as the worms crawl across the surface. However, as a drawback of this option, the homogeneity of the medium cannot be guaranteed and the worms may have difficulties in eating and crawling if sample compounds are too large. On the other hand, option B (**Figure 61–B**) offers homogeneity of the sample in NGM medium, but the worms will not have as much access to the sample since their movement is crawling and they do not dive. Both options were prepared, but the first one did not result homogeneous and the worms barely moved on the plate. Besides, there was a risk of decreased the intake – either food source or extract (**Figure 61–A**).

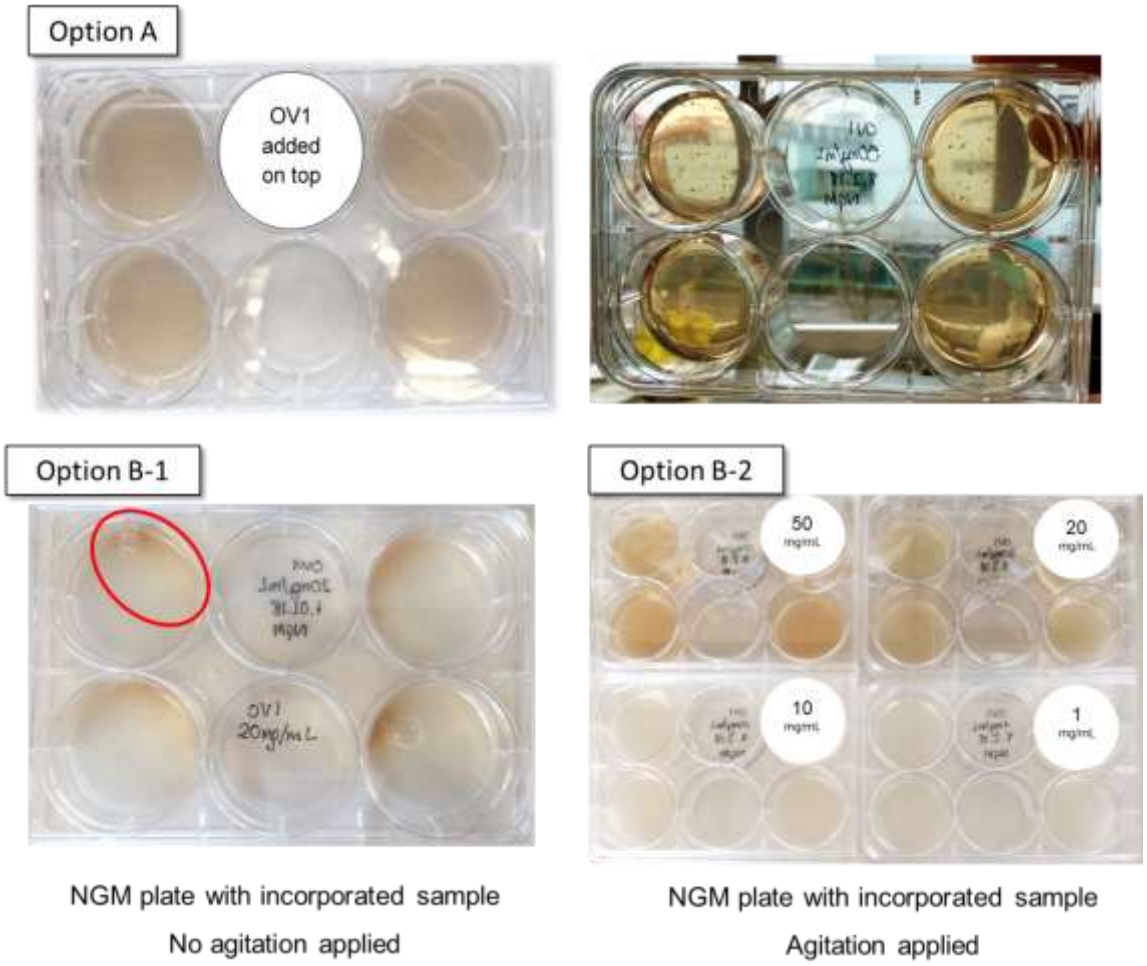


Figure 62. Six–well plate of NGM with sample added on top of NGM (Option A) and incorporated into NGM (Option B). In Option B–1, agitation was not applied – lack of homogeneity observed is marked with red circle – and in Option B–2, agitation was applied – plates from different concentrations are shown (from darker to lighter, 50, 20, 10 and 1 mg/mL).

The decision of the volume to incorporate to plate was made based on previous studies carried out by Dr. David Navarro–Herrera from the Department of Biochemistry & Genetics, University of Navarra [315].

40 µL of sample were added to the bottom of well before adding 4 mL of NGM (liquid). Then, the plates were agitated until the homogeneous distribution of the sample. Agitation was a crucial step to reach homogeneity; otherwise, the sample was not fully distributed (**Figure 62–B–1**).

1.2 SOLUTION PREPARATIONS

The total volumes of preparation are indicative and could be changed regarding needs while maintaining the proportions of the components. When working with *C. elegans* it is recommended to have an enough stock of OP50 seeded NGM 9 cm Ø plates and to

prepare a proper experimental design, taking into consideration the quantities necessary for optimal sterile conditions of the solutions.

1.2.1 Reagent solutions for stock

Before preparing NGM, some solutions need to be prepared and would be kept stored.

1. 5 mg/mL Cholesterol: 1 g of cholesterol (#C3045, Sigma–Aldrich Co., St. Louis, MO) in 200 mL of pure ethanol. Stored in 50 mL tubes at – 20 ºC.
2. 1 M $MgSO_4$: Under agitation mix 246.5 g of $MgSO_4 \cdot 7\ H_2O$ (#1374361, Sigma–Aldrich Co., St. Louis, MO) and 800 mL of H_2O type I. Flush to 1 L and prepare aliquots of 500 mL by filtering. Autoclave and keep stored at room temperature.
3. 1 M $CaCl_2$: 55.5 g of $CaCl_2$ (#C1016, Sigma–Aldrich Co., St. Louis, MO) dissolved in 800 mL of H_2O type I, under agitation. Flush to 500 mL, autoclave and keep stored at room temperature.

- ✓ 1 M KPO_4 pH 6 Buffer: 128.19 g of KH_2PO_4 (#P0662, Sigma–Aldrich Co., St. Louis, MO) + 13.24 g $K_2HPO_4 \cdot 3H_2O$ (#P5504, Sigma–Aldrich Co., St. Louis, MO) + 800 mL H_2O type I. Flush to 1 L, adjust pH to 6 with KH_2PO_4 / KOH and prepare aliquots of 500 mL. Autoclave ant keep stored at room temperature.

4. LB ampicillin: 10 g of LB Broth (#L3522, Sigma–Aldrich Co., St. Louis, MO; containing 10 g /L tryptone, 5 g/L Yeast extract and 5 g/L NaCl) in 500 mL of H_2O type II. Autoclave for 20 min. Under sterile conditions (flame), add 0.5 mL ampicillin and keep stored at 4 ºC.

1.2.2 Nematode Growth Medium (NGM) for plates

Total volume = 1 L

- ✓ 3 g NaCl
- ✓ 17 g agar
- ✓ 2.5 g peptone

  type II H_2O up to 1 L

Autoclave for 50 min.

Under sterile conditions (flame) add:

- ✓ 1 mL 1 M $CaCl_2$
- ✓ 1 mL 5 mg/mL Cholesterol in ethanol

- ✓ 1 mL 1M $MgSO_4$
- ✓ 25 mL 1 M pH 6 KPO_4 Buffer
- ✓ 8 – 10 mL Nystatin (#N3503, Sigma–Aldrich Co., St. Louis, MO)
- ✓ 1 mL 5 mg/mL Ampicillin

With sterile pipet, spare NGM onto plates. Regarding type of plate, these are the recommendations for volumes to add:

- ✓ 9 cm Ø plates: 25 mL per plate (~ 40 plates with 1 L NGM prepared)
- ✓ 6 well plates: 4 mL per well

Let the plates at room temperature overnight for 6 well plates and for 3 days for 9 cm Ø plates, until solidification. Plates can be stored at 4 ºC.

Before using for worms, OP50 (food source) needs to be added:

- ✓ Let grow 20 µL of OP50 in 5 mL LB ampicillin, overnight at 37 ºC under agitation.
- ✓ Under sterile conditions (flame), seed OP50 onto plates in the centre of the plate:
  - 400 µL for 9 cm Ø plates
  - 100 µL per well for 6–well plates
- ✓ Let dry next to flame and they are ready to be stored at 4 ºC or used for *C. elegans* experiments.

This seeding OP50 protocol can be carried out from already prepared frozen OP50 stock:

- ✓ In LB ampicillin plates, inoculate single colony of OP50 by streaking technique, under sterile conditions.
- ✓ Let grow overnight at 37 ºC.
- ✓ Pick 10 colonies and add each one to 5 mL of LB ampicillin solution.
- ✓ Let grow overnight at 37 ºC under agitation.
- ✓ Keep stock stored at – 80 ºC.

Note: OP50 in LB ampicillin solution could be stored at 4 ºC if very frequent work with *C. elegans*, but needs to be regrown in new LB ampicillin solution at 37 ºC overnight every use (20 µL of this solution per 5 mL of new LB ampicillin solution).

1.2.3 M9

Total volume = 1 L

- ✓ 3 g KH_2PO_4
- ✓ 7.52 g $Na_2HPO_4 \cdot 2\ H_2O$
- ✓ 5 g NaCl

- ✓ 800 mL type I H_2O

Flush to 1 L, prepare aliquots of 500 mL (or 50 mL to better maintain sterile conditions) and autoclave.

Add 1 mL 1M $MgSO_4$ under sterile conditions and keep store at room temperature. It is recommended to be used within 2 weeks of preparation.

1.2.4 Freezing buffer

Under sterile conditions, mix 70 mL of M9 (previously autoclaved) and 30 mL of pure glycerol (previously autoclaved). Keep stored at room temperature.

1.3 ORDINARY PROTOCOLS TO WORK WITH *C.elegans*

When purchasing *C. elegans*, they come grown on a small NGM plate. The first recommendation is to grow them in 9 cm Ø plate, reproduce them – check phenotype if necessary – and prepare stock. Therefore, this section compiles the three main ordinary protocols for the maintenance of *C. elegans*.

Besides, conditions such as growing time and growth temperature would be for N2 wild type at 20 ºC, other experimental conditions might need changes in these variables but not in the technical performance of the protocols.

1.3.1 SINCHRONIZATION

Cultured *C. elegans* plates are needed for synchronization. To work for the first time – from new purchased litter – turn the plate where *C. elegans* came on and plate it on the new NGM OP50 seeded plate. If working from an old stored *C. elegans* plate, cut a piece of NGM and place it on the new NGM plate backwards. Incubate for 3 – 4 days at 20 ºC so that new NGM plate has *C. elegans* for experiments (**Figure 63**).

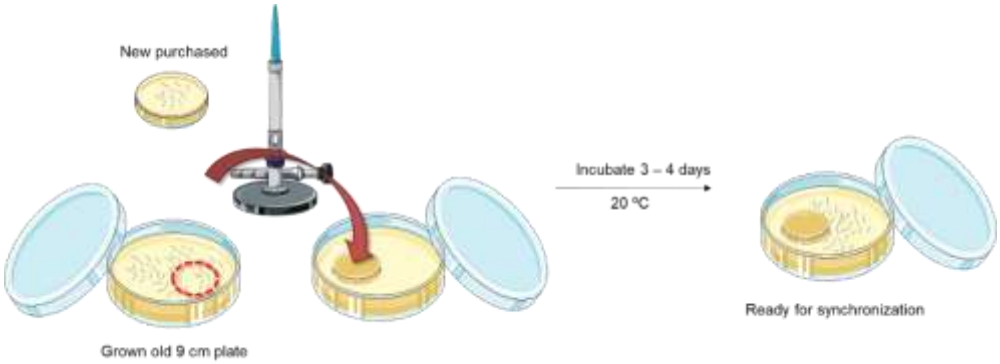


Figure 63. Start of synchronization of *C. elegans* in 9 cm diameter plates. At 20 ºC, they need 3 – 4 days to be ready for synchronization.

For synchronization, at least three plates with *C. elegans* with high amount of eggs and adults with eggs would be needed, because eggs are the only form of *C. elegans* that is resistant to bleach and the perfect stage to reset growth.

1. Under sterile conditions, add 5 mL of PBST to each plate and try to release eggs from surface with a planting handle. Collect the liquid with sterile pipet and pour it into a 15 mL tube. Repeat twice. From each plate, one tube.
2. Centrifuge at 1,250 rpm for 4 min.
3. Retire supernatant (aspiration up to 2 mL mark).
4. Add 10 mL of PBST and repeat steps 2, 3 and 4.
5. Centrifuge at 1,250 rpm for 4 min.
6. Add 10 mL sodium hypochlorite (bleach) 12 %.
7. Agitate manually for 2 min and let aside on ice for 2 min; repeat twice this step.
8. Centrifuge at 1,250 rpm for 4 min.
9. Retire supernatant (aspiration up to 0.5 mL mark).
10. Add 10 mL of sterile H_2O.
11. Centrifuge at 1,250 rpm for 4 min.
12. Retire supernatant (aspiration up to 2 mL).
13. Repeat three times steps 10, 11 and 12 (retiring up to 0.5 mL mark in the last step).
14. Add 10 mL of M9 and split in two.
15. Add 5 mL of M9 to each new M9 tube.
16. Incubate overnight at 20 ºC under agitation.

Before plating, the eggs might have hatched – check under the lens – and so, all worms would be L1–synchronised.

1.3.2 PREPARING STOCKS

Conditions needed:

- ✓ Low quantity of bacteria
- ✓ No contamination
- ✓ High presence of L1 and L2 (only freezing survival stage)
- ✓ Egss on plate
- ✓ Proper phenotype

Three 9 cm diameter plates are needed for one stock batch (4 cryotubes).

1. Add 5 mL of M9 to each plate and softly agitate to release worms from medium.
2. Collect the liquid and pour it into a 15 mL falcon tube.

3. Centrifuge at 1,200 rpm for 5 min.
4. Retire the supernatant, add 15 mL of M9 and centrifuge at 1,200 rpm for 5 min. Repeat three times.
5. Add 2 mL of freezing buffer and prepare aliquots in cryotubes that would be kept stored at – 80 º C.

Note: A control of freezing is needed to be done as verifying test after 48 h:

Let the cryotube thaw at room temperature and discharge it onto NGM OP50 seeded 9 cm Ø plate. At least 10 worms should be alive to accept that batch stock.

1.4 ANTIBIOGRAM

The strain of *E. coli* OP50 used is resistant to ampicillin. For this reason, it is recomended to test the samples by antibiogram assay before the experiment with *C. elegans*, even more so when there is evidence of antibiotic activity of the samples [5,105,316].

1.4.1 MATERIAL AND METHODS

For each extract were used two LB agar 9 cm plates: one seeded with *E. coli* OP50 resistant to ampicillin and the other with *E. coli* OP50 sensible to ampicillin (**Figure 64**).

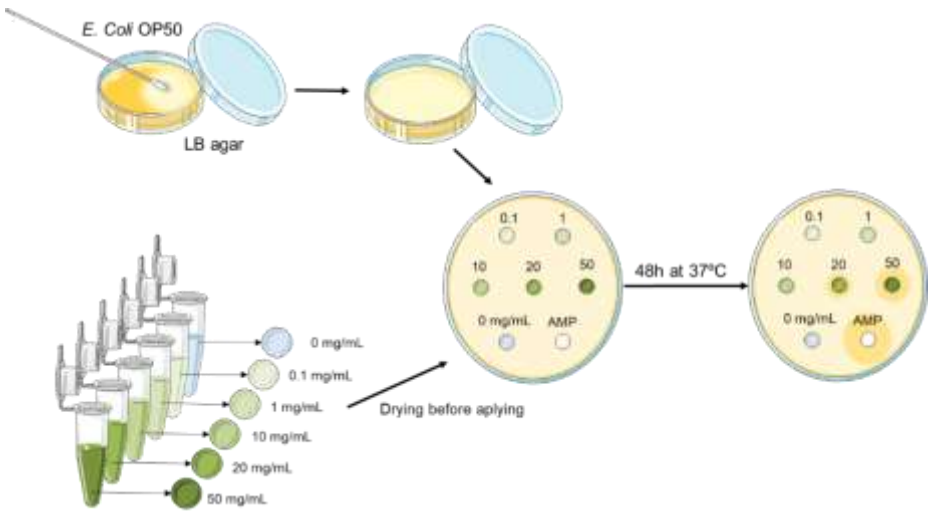


Figure 64. Antibiogram with plant extracts at different concentrations. AMP: ampicilin.

20 µL of extracts dissolved in distilled water at different concentrations (0.1, 1, 10, 20 and 50 mg/mL) were tested. Distilled water was used as a negative control and ampicillin (#A9393, Sigma–Aldrich Co., St. Louis, MO) as a positive control. The solutions were poured onto the plate, and after drying, 6 mm diameter antibiogram disks (#WHA2017006, Sigma–Aldrich Co., St. Louis, MO) were disposed and incubated at 37 ºC for 48 h. (**Figure**

64). The disks that presented a halo of inhibition were considered positive and the diameter of the halo was measured to quantify this activity. Antibiogram was performed in duplicate for each extract.

1.4.2 RESULTS

O. vulgare has traditionally been indicated for cough and digestive disorders thanks to its digestive, antimicrobial, expectorant, antiseptic and antispasmodic properties [317]. Previous studies confirmed its antimicrobial activity, including strains of *E. coli* [29,216,285,316]. Most of this antibacterial activity focuses on the essential oils of *O. vulgare*. However, the aqueous extract from leaves of O. *vulgare* formulated as nanoparticles showed more than 10 mm zone of inhibition against *E. coli* and *E. coli* EP, among other microorganisms tested [60]. Moreover, Martins *et al.* [127] demonstrated antibacterial activity against *E. coli* of the infusion, decoction and hydroalcoholic extract of commercial *O. vulgare* L. at 20 mg/mL. Although the *E. coli* used as food source was the OP50 strain, different from the used on previous studies [60,127,193], antibiogram was decided to be performed to rule out antimicrobial activity of OV1 and OV2.

The results showed that the extracts did not present any inhibitory activity at these concentrations, since halos did not appear in any of the plates (**Figure 64**). However, the positive control (ampicillin in *E. coli* OP50 sensitive plate) showed inhibitory halos in all replicates, with an average diameter of 1.35 ± 0.07 cm (**Figure 65**).

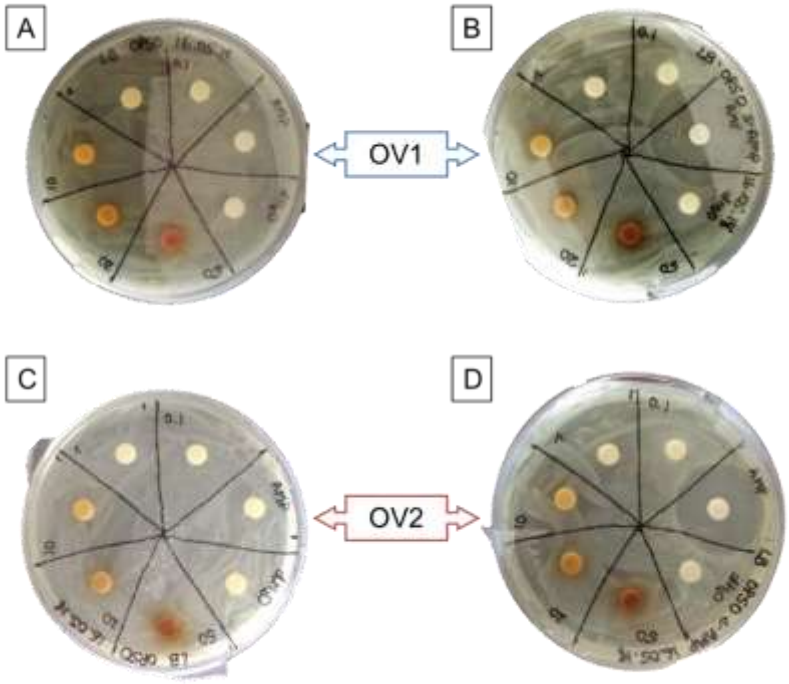


Figure 65. Antibiogram plates. OV1: cold aqueous extract (upper plates) and OV2: heated aqueous extract (lower plates). A: OV–C concentrations at *E. coli* OP50 ampicillin resistant plate (AMP). B: OV–C concentrations in *E. coli* OP50 ampicillin sensitive plate (S–AMP). C: OV1 concentrations in *E. coli* OP50 ampicillin resistant plate (AMP). D: OV2 concentrations in *E. coli* OP50 ampicillin sensitive plate (S–AMP).

As the results indicated, OV1 and OV2 did not inhibit *E. coli* OP50 at the concentrations tested, allowing the use of this extracts and this food source for *in vivo* testing without interferences.

1.5 PHARYNGEAL PUMPING RATE AND GROWING

C. elegans uses *E. coli* as a food source while crawling in medium and movement of the pharynx can be an indication of ingestion [318]. This simple test consists of recording and then counting the swallowing movement of the worms to see if additional dietary supplementation with extracts modifies the maintenance and normal growth of the worms. [319,320].

1.5.1 MATERIAL AND METHODS

- Sample preparation

OV1 and OV2 extracts were dissolved in water at 1 and 10 mg/mL concentration (middle concentrations tested in antibiogram assay). Distilled water was used as negative control.

4 µL of each solution were added to 4 mL NGM per well before solidification. Then, L1 synchronised worms were placed onto wells.

- Analyse conditions and data process

After 48 h, they were recorded with a video–camera connected to the microscope and pharyngeal movements per minute were counted. Photographs were also taken to determine the length of the worms.

- Statistical analysis

Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

1.5.2 RESULTS

OV1 and OV2 extracts presented non–significantly pharyngeal pumping rate a pharyngeal pumping rate without significant differences compared to the control (**Table 41**).

Table 41. Results of pharingeal pumping rate and growing length of *C. elegans* treated with different concentrations of OV1 and OV2. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$).

	Control	OV1		OV2	
Concentration (mg/mL)	0	1	10	1	10
Movements/min	201 ± 2[a]	199 ± 2[a]	198 ± 2[a]	203 ± 2[a]	200 ± 1[a]
Length					
L3: 490– 510 µm	498.40 ± 1.35[b,c]	496.70 ± 0.95[b]	496.10 ± 0.99[b]	500.50 ± 1.96[c]	501.50 ± 1.08[c]
L4: 620–650 µm	631.90 ± 1.66[d]	628.21 ± 1.99[e]	626.93 ± 1.73[e]	634.25 ± 2.62[d]	636.72 ± 1.77[d]
Adult: 1,110–1,150 µm	1,145.41±2.55[g]	1,138.86±1.75	1,137.72±2.21	1,145.93±1.20[g]	1,146.10±2.13[g]

At the L3 stage, all worms grew the same and no significant differences were found between the extracts and the control. Small negligible differences begin to appear at the L4 stage, where worms fed with OV1 showed lower length: 628.21 ± 1.99 µm with 1 mg/mL and 626.93 ± 1.73 µm with 10 mg/mL, compared to 631.90 ± 1.66 µm for the control, with $p < 0.05$. The L4 worms fed with OV2 grew a little more in net value, but without significant differences ($p > 0.05$): 634.25 ± 2.62 µm with 1 mg/mL and 636.72 ± 1.77 µm with 10 mg/mL. The differences were statistically different to OV1 results ($p < 0.05$).

These small significant differences were maintained at the stage of adultness: control = 1,145.41 ± 2.55 µm, OV1 (1 mg/mL) = 1,138.86 ± 1.75 µm, OV1 (10 mg/mL) = 1,137.72 ± 2.21 µm, OV2 (1 mg/mL) 1,145.93 ± 1.20 µm and OV2 (10 mg/mL) = 1,146.10 ± 2.13 µm.

Results of OV1 showed significant differences compared to control and OV2 ($p < 0.05$), but within range of growth for adult stage (1,110 – 1,150 µm). The results of OV2 did not showed differences compared to control.

In conclusion, the worms eat, grow and move in a non–dose–dependent manner, since the results at different concentrations were within the normal length values of each test. Now the worms are eating and growing, are they eating the extract or are they just eating *E. coli* avoiding OV1 and OV2?

2 ANTIOXIDANT ACTIVITY *IN VIVO*

When working with a single chemically–synthetized pure compound and *C. elegans*, traceability whithin the worms can be done with the help of compounds with fluorescence. The fact that the worms are transparent provides a better quantification of the intake of the molecule of study. However, the extracts are composed by more than a single compound, making it difficult to label all of them.

If the worms are fed with *E. coli* that is on the plate in the same amount as in control plate, how could intake of *E. coli* be differentiated from the intake of the extract? The pharyngeal pumping rate does not discriminate compounds. The worms are eating but there is no way to detect if they are eating the extract, let alone quantify their intake.

What is generalised to do is to start directly with functional assays such as ROS or lifespan and attribute the positive result obtained to the fact that worm is eating the extract. However, is this observed effect produced by the extract inside the worm (oral administration) or by the presence of the extract in the growing medium (environmental effect or hypothetical topical administration)?

To answer that question and be able to firmly attribute the future functional effects tested in treated worms to the fact that *O. vulgare* extracts were eaten, a new method to quantify antioxidant activity inside the worm was developed [254]. The direct activity observed would be chemically confirmed in a new complementary variant of the original method (not published yet).

2.1 MATERIAL AND METHODS

2.1.1 DPPH• *in vivo* ASSAY

Antioxidant activity *in vivo* was quantified as a new method developed in the laboratory and described by de Torre *et al* [254]. This idea arose from the fact that *C. elegans* is a

transparent organism and the DPPH• method is a colorimetric assay that could give information of both extract intake and *in vivo* antioxidant activity of that extract.

- *C. elegans* growth

C. elegans was cultured as described previously and the strain used was N2 *Bristol.* All assays were performed in 6–well cell culture plates with 4 mL of Nematode Growth Medium (NGM) per well at 20 °C.

- Sample preparation

The same concentrations tested on antibiogram assay were prepared for OV1 and OV2 (0, 0.1, 1, 10, 20 and 50 mg/mL in distilled water) and 40 µL of each condition were added per well. Rosmarinic acid was used as a positive control and glucose as a negative control, at the same concentrations. The OV2 intestinal fraction obtained from digestion performed *in vitro* (OV2–Int) was also tested and quantified *in vivo*. The sample was dissolved at the same concentrations as the crude extract under the same conditions. The intestinal fraction of rosmarinic acid was used as a positive control (for the antioxidant activity) *after* digestion at the same concentrations as the sample (0, 0.1, 1, 10, 20 and 50 mg/mL in distilled water).

- Analyse conditions

100 µL of *E. coli* OP50 were seeded on each well as the worm food source and 2,000 L1 synchronized worms were placed onto each well. After 48 h, they were collected with 2 mL of sterile water per plate, washed three times and pelleted by centrifugation (314 g / 4 min / 20 ºC). Then, worms werewas resuspended in 1.75 mL of sterile water and crushed for 20 s at maximum power using Ultraturrax T25. Final solution was filtered through a 0.45 µm filter (**Figure 66**).

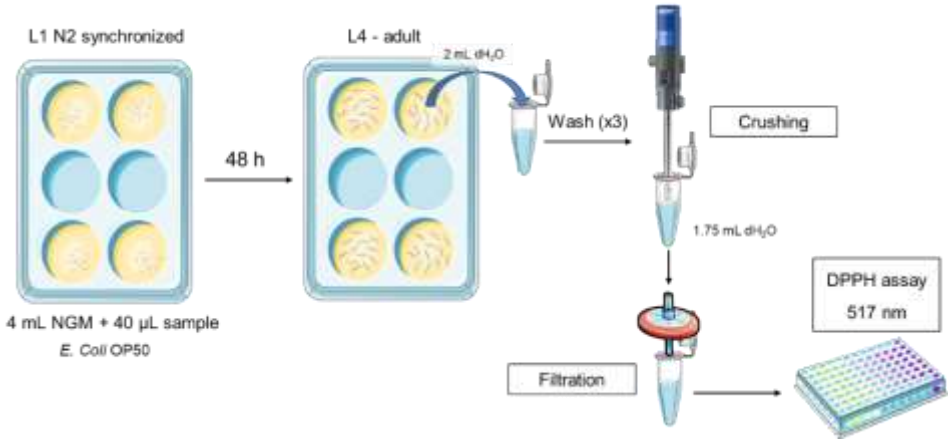


Figure 66. Method to quantify antioxidant activity *in vivo*. L1 N2 synchronised worms grew up to L4–adult stage on NGM plates containing sample and food source (*E. coli* OP50). After collecting, they were crushed and solution was filtrated.

In a 96–well plate, 150 µL of each solution was mixed with 150 µL of DPPH• solution at 4 mg/mL, in the same manner as the DPPH• *in vitro* assay.

- Data process and Statistical analysis (*see Chapter II, DPPH• in vitro assay*).

2.1.2 CHEMICAL *in vivo* DETERMINATION

C. elegans treatment and conditions were the same as for assay described above: L1–N2 synchronized worms grown for 48 h at 20 ºC on NGM plates with treatment (extract at 0.1, 1, 10, 20 and 50 mg/mL concentration). Then, L4–adults were collected in 2 mL distilled water, washed three times and crushed with Ultraturrax. Finally, crushed worms were re–suspended in 0.5 mL of distilled water (**Figure 67**).

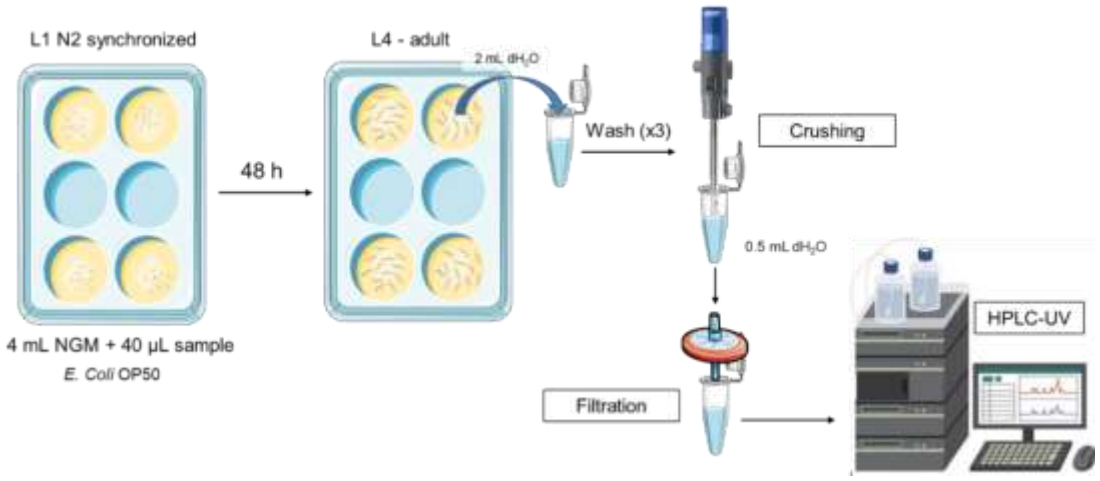


Figure 67. Adaptation of the simple and reliable method to quantify antioxidant activity *in vivo* to chemical quantification inside the worms.

HPLC–DAD conditions were the same as for chemical characterisation and quantification was carried out in the same way (*see Chapter I, chemical quantification*).

2.2 RESULTS

The *in vitro–in vivo* correlation (IVIVC) is difficult to predict, even more in plants where there is more than one active compound. As a solution, *C. elegans* is a good, simple and inexpensive *in vivo* model to evaluate several activities *in vivo* [305]. Most of them are functional and require time and expensive equipment. Nonetheless, this is a simple and reliable method useful to quantify antioxidant activity of the compounds inside the worms [254]. Based on previous *in vitro* bioactivity results, the aqueous extracts (OV1 and OV2) were versatile active extracts that fit with the traditional use of oregano [127,317] and recommendations of EMA for other species of oregano (*O. majorana* L. [321] and *O. dictamnus* L. [322]): preparation in infusion or decoction.

Once the food source of the worms did not show alterations when applying OV1 or OV2 at those concentrations, the antioxidant activity inside the worms was determined after 48 h of treatment. The results expressed in EC_{50} (µg/mL) along time are shown in **Table 42**.

Table 42. Antioxidant activity *in vivo* by the DPPH•· method expressed in EC_{50} (mean ± SD µg/mL). Data expressed as means ± SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Values in bold means EC_{50} max (stabilisation point).

	EC_{50} (µg/mL)					
Sample	**15**	**30**	**45**	**60**	**75**	**90**
OV1	283.40 ± 22.24^{a}	159.62 ± 15.00^{a}	**117.53 ± 11.64^{b}**	93.58 ± 7.40^{b}	80.24 ± 2.72^{b}	72.54 ± 2.28^{b}
OV2	46.64 ± 1.03^{a}	32.86 ± 2.38^{a}	**27.13 ± 1.60^{b}**	24.59 ± 0.61^{b}	20.56 ± 0.34^{b}	16.28 ± 0.42^{b}
RA	11.03 ± 0.49^{a}	9.62 ± 0.65^{a}	**8.98 ± 0.30^{b}**	8.50 ± 0.33^{b}	8.09 ± 0.31^{b}	8.04 ± 0.25^{b}
Glucose	–	–	**–**	–	–	–

Both extracts reached stabilisation after 45 min of reaction, with the same intermediate kinetic behaviour as *in vitro* assay. In the same way, the heat aqueous extract (OV2) presented significantly higher *in vivo* antioxidant activity (27.13 ± 1.60 µg/mL) than the cold aqueous extract (OV1) with 117.53 ± 11.64 µg/mL. Previously on antioxidant *in vitro* determination, OV2 also showed higher significant scavenging activity against DPPH free radical than OV1, 2.53 ± 0.18 and EC_{50} = 4.11 ± 0.25 µg/mL, respectively.

The simulation process of a gastrointestinal digestion was *in vitro* performed with the most antioxidant activity aqueous extract of *O. vulgare*: OV2 showing a 94.85 % of bioaccesibility. Not only was quantitative value ameliorated (2.53 ± 0.18 µg/mL *vs*. 1.63 ± 0.32 µg/mL, with $p < 0.001$), even compared to the positive control (RA Int. (30') = 1.88 ±

0.15 µg/mL and OV2 Int. (15') = 1.63 µg/mL, with $p < 0.001$) but also kinetics changed from stabilisation at 45 min to only 15 min to stabilise the reaction. Taking this assay into an *in vivo* organism such as *C. elegans* (**Figure 68**), the antioxidant activity in terms of DPPH• is also significantly ameliorated after *in vitro* gastrointestinal process.

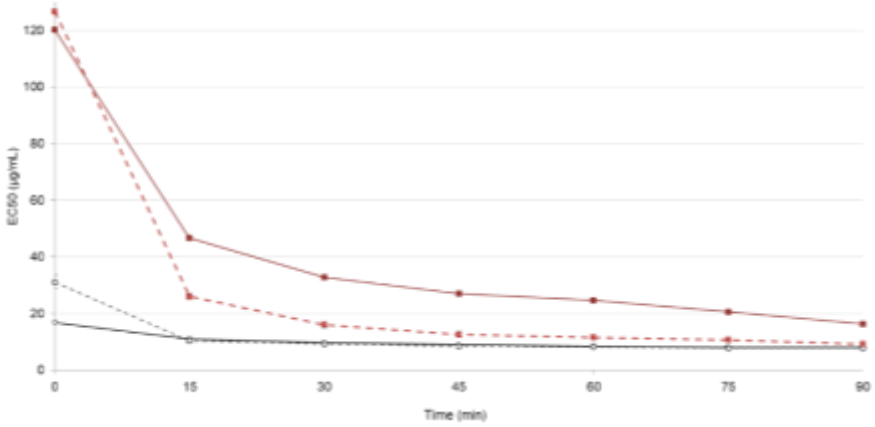


Figure 68. Antioxidant activity *in vivo* by the DPPH• method expressed in EC_{50} (mean ± SD µg/mL). Red line to hot aqueous extract (OV2) and grey line to positive control (RA, Rosmarinic acid). Dashed line of each colour correspond to intestinal fraction (OV2–Int for OV2 and RA–Int for positive control). Antioxidant activities of the extracts showed differences statistical in all time–points.

Kinetics was intermediate for crude and digested extract, being statistically different between them: OV2 EC_{50} (45') = 27.13 ± 1.60 µg/mL and OV2 Int. EC_{50} = 16.08 ± 1.01 µg/mL, with $p < 0.001$. No significant differences were found on the positive control before and after digestion: RA (45') = 8.98 ± 0.30 µg/mL, RA Int (45') = 8.49 ± 0.59 µg/mL, $p > 0.05$.

Antioxidant activity *in vivo* was a technique published in 2019 as part of the current work. In this sense, there are still no results from other studies using this technique to compare yet. Besides, studies of *O. vulgare* in *C. elegans* use different nature of extract or essential oils and different working conditions for the worms (liquid medium [177]). These facts make comparison to other studies using this *in vivo* technique unfeasible. Nevertheless, the results might be in line to previous published ones where rosmarinic acid [318,323] and other *Lamiaceae* plants containing this compound [177,324], which showed to be antioxidant *in vitro* activity and enhance the physiological response of *C. elegans* to oxidative stress [318,325,326]. Unlike other studies, here the physiological response of the worms treated with the extracts would be warranted, since the antioxidant activity inside the worm has been monitored.

The bioactivities of medicinal plants can be attributed to certain chemical compounds with demonstrated activity *perse*, like rosmarinic acid [323,327], one of the main compounds in the extracts prepared for this study. Once the chemical profile is known – already analysed in *Chapter I* – the designation can be easier to establish. Positive *in vivo* activity results of

extract already have a distinct identity, meaning that such bioactivity exists within the worm. However, is there any objective evidence that the internal activity is directly thanks to the compounds in the extract or simply their presence in the environment of the worms leads them to activate certain antioxidant metabolic mechanisms?

The simple and reliable method to quantify antioxidant activity *in vivo* was upgraded to be able to analyse the chemical identification of what the worms ate. The modification on volume – making sample more concentrated in worms – was carried out to adjust the sample to the low limit of detection of the HPLC–DAD. After several attempts, 0.5 mL was the adequate volume to resuspend crushed worms for chemical characterisation after filtration. For medicinal plants research and complex chemical compounds that cannot be fluorescently tagged, this application of the method may help with the demonstration of the intake of the treatment and so, later, firmly attribute the observed physiological responses to the compound.

First of all, the negative control did not show any peak, which means that no interferences from additional compounds (parts of worms or food source) remain in the samples whose *in vivo* activity was tested. The positive control showed a sharp peak (λ_{abs} = 329.4 nm) at 37.21 min corresponding to the pure compound given to worms: rosmarinic acid.

The extracts presented several peaks that were identified and quantified as previously with crude extracts *in vitro (see Chapter I, chemical quantification*). **Table 43** shows the most remarkable ones, including rosmarinic acid in OV2, and its comparison with the amount *in vitro* (*Chapter I*).

Table 43. Semi–quantification of the most remarkable chemical compounds of OV1 and OV2 inside the worms identified by HPLC–DAD. RA: Rosmarinic acid. Values expressed as mg of standard per 100 mg. Peaks correspond to peak numbers of the raw extract from **Chapter I**.

Extract	Amount of compound (mean ± SD mg/100mg)				
	Concentration given *in vivo* (mg/mL)	DHBA derivates		Flavonoids	RA
		Peak **9**	Peak **21**	Peak **15**	Peak **32**
OV1	**Crude *in vitro***	6.81 ± 0.03	–	1.42 ± 0.09	–
	10	1.23 ± 0.03	–	0.91 ± 0.02	–
	20	1.88 ± 0.02	–	1.18 ± 0.02	–
	50	3.76 ± 0.02	–	1.36 ± 0.01	–
OV2	**Crude *in vitro***	–	9.55 ± 0.01	1.93 ± 0.05	31.83 ± 1.12
	10	–	6.51 ± 0.01	0.45 ± 0.01	15.46 ± 0.01
	20	–	7.71 ± 0.01	0.59 ± 0.01	17.24 ± 0.02
	50	–	9.52 ± 0.01	0.67 ± 0.01	23.60 ± 0.02

Integrated AUC values were transformed into mg of standard per 100 mg of extract to be comparable to the raw extract. Three main type of compounds were selected, because of its bioactive relevance and greater presence: DHBA derivates (peak **9** and **21**), flavonoid (peak **15**) and rosmarinic acid (peak **32**). Broadly, the values increased with given

concentration: the greater the amount of sample on plate (higher concentration given), the more probable it was that the worm had eaten it.

2,5–DHBA (peak **9**), the dyhidroxybenzoic acid *per excellence* of OV1 (with 6.18 ± 0.03 mg/100 mg), was identified *in vivo* at 3.76 ± 0.02 mg/100mg, in worms treated with 50 mg/mL of OV1. The other compound found within the same worms was a flavonoid (peak **15**) in an amount of 1.36 ± 0.01 mg/100 mg. Compounds detected inside the worms might also verify the intake of OV1 by this model, although the presence was not directly reflected in antioxidant activity.

On the contrary, the worms treated with OV2 did reflect the presence of the compounds on antioxidant activity since EC_{50} value was lower than OV1 (**Table 43**). Even though more compounds were detected for OV2, only the main peaks were semi–quantified in order to confirm their presence inside the worms (**Table 43**). 3,4–DHBA (peak **21**) presented significant preservation inside the worms, better than 2,5–DHBA in OV1. However, a higher quantity of flavonoids (peak **15**) was found inside worms treated with OV1 than those that grew on OV2 plates. Assuming that the worms have the capability to pick their food, those with OV2 might have had more compounds for selection since OV2 is chemically more complete than OV1. One of those compounds missing in OV1 is rosmarinic acid, with such an antioxidant activity *in vivo*. Rosmarinic acid inside worms treated with OV2 might be responsible for the *in vivo* antioxidant activity observed (**Figure 68**). When pure rosmarinic acid was administered to *C. elegans* (positive control), these were the quantities detected on HPLC–UV: 37.33 ± 0.02 % for 50 mg/mL dose, 15.74 ± 0.05 % for 20 mg/mL and 9.50 ± 0.09 % for 10 mg/mL. Crude OV2 contains 31.83 ± 1.12 % of rosmarinic acid, but the presence of this compound inside the worms could be nearly comparable to that found in worms treated with 100% rosmarinic acid. Does this fact mean rosmarinic acid from OV2 is more attractive for worms or that the worms fed with only *E. coli* and rosmarinic acid on plate were tired of the diet? Nevertheless, there was rosmarinic acid and DHBA (2,5–DHBA for OV1–treated and 3,4–DHBA for OV2–treated) inside the worms, meaning they ate the treatment.

3 24 h SURVIVAL

C. elegans is a nematode covered by a cuticle that protects the living organism from hostile conditions, even existing the possibility to redirect its growth into a dauer form. These hostile conditions can be used for assays that quantify resistance to different types of stress conditions (**Figure 69**), where the capacity of survival of the worms against known

stress (such as oxidative stress, heat, cold, osmotic stress, hypoxia, hyperoxia, ultraviolet radiation, endoplasmic reticulum stress, and heavy metals exposure) is tested [328].

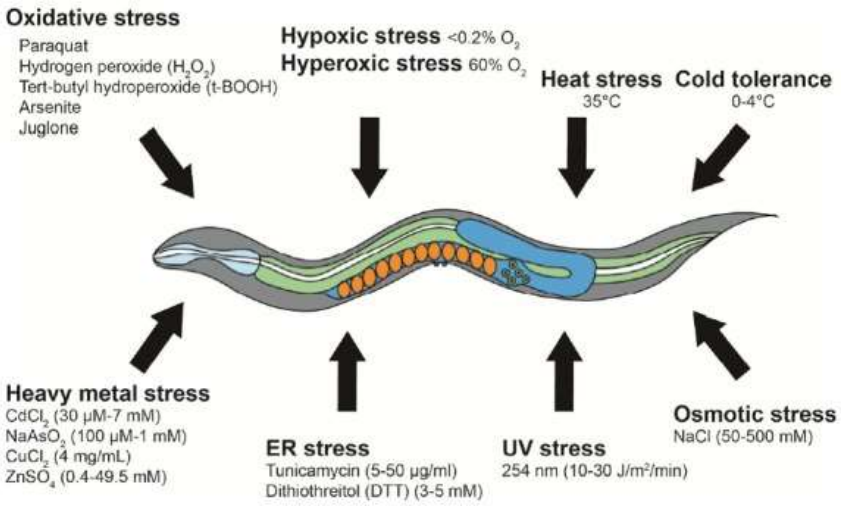


Figure 69. Abiotic stress resistance assays [329].

The main activity investigated in this work is antioxidant, as part of prevention of age–related diseases. Therefore, among the stress conditions available to change in the *C. elegans* normal environment, stress induction with a pro–oxidant compound was used to determine the protective effect produced by different concentrations of the extract.

This work focuses on the beneficial effect of the prepared extracts. Would they protect the worms from a 24 h exposure of a lethal dose of a pro–oxidant?

3.1 MATERIAL AND METHODS

- Experimental design

After testing internal antioxidant activity in the worms, protective activity of the extracts against an oxidant compound was determined after 24 h exposure. Experimental design and protocol is shown in **Figure 70**.

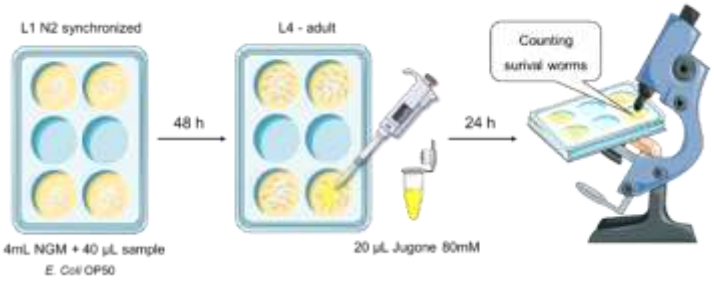


Figure 70. 24h survival assay in *C.elegans* against juglone lethal effect.

- Sample preparation and Analyse conditions

N2 wild strain of *C. elegans* was grown and treated under the same conditions as the antioxidant *in vivo* assay previously described. 50 L1 synchronized worms were put onto NGM 6–well plates supplemented with 0.01 % (v/v) extract of known concentration (0, 0.1, 1, 10, 20 and 50 mg/mL in water) and *E. coli* OP50 as food source. After 48 h of treatment, 20 µL of a lethal dose of juglone (80 mM) in ethanol were added to the plates. Because juglone is dissolved in ethanol, effect of this solvent on the worms was firstly tested.

- Data process

The surviving worms were counted after 24 h of exposure and the results were expressed as a percentage of surviving worms, calculated using the following formula:

$$Survival\ percentage\ (\%) = \frac{number\ of\ worms\ alive}{total\ worms}\ x\ 100$$

Non–treated worms (0 mg/mL) condition was used as a negative control.

- Statistical analysis

The experiment was performed in triplicate. Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

3.2 RESULTS

Juglone is dissolved in ethanol, a solvent that might be toxic for worms. For that reason, a preliminary assay under the same conditions (medium, worms, time and extract concentration) was necessary to discard lethal effect of solvent instead of juglone (**Figure 71**).

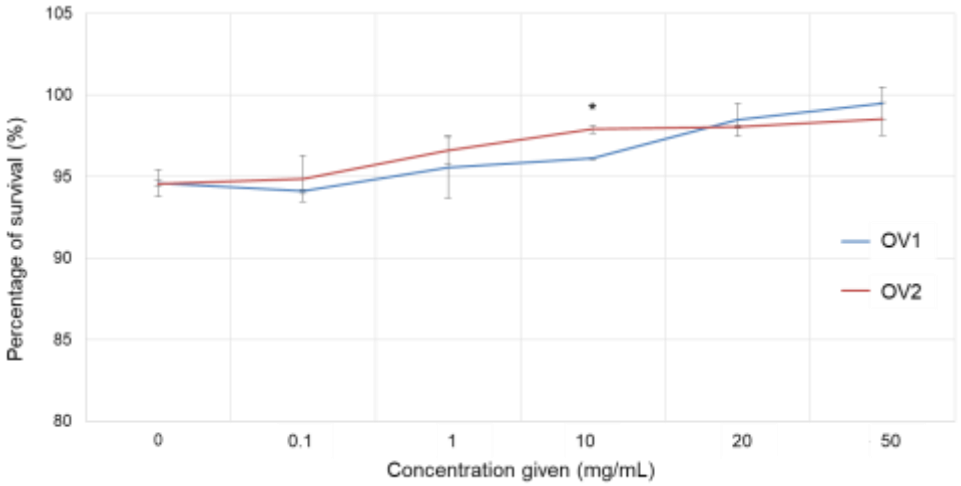


Figure 71. Percentage of survival (mean ± SD %) of worms treated with different concentrations of the extracts after 24 h of ethanol exposure. OV1: cold aqueous extract; OV2: heated aqueous extract. Y–scale was zoomed in (from 80 % to 105 %, to include SD bars) in order to appreciate differences between extracts. X–values correspond to concentration given as treatment (mg/mL). * corresponds to $p < 0.05$.

Survival rates after 24h of ethanol exposure were greater than 90 % at any given concentration. Only OV1 and OV2 presented statistical differences at 99 µg/mL (10 mg/mL given concentration), with survival percentages of 97.89 ± 0.22 % for OV2 and 96.09 ± 0.07 % for OV1, with $p = 0.041$. Higher concentrations did not present statistical differences ($p > 0.05$) between the extracts, although values for OV1 are higher.

In this sense, solvent for juglone maintained survival over 90 %, so ethanol could be discarded as toxic for *C. elegans* under these conditions. The results for the survival rate after 24 h of treatment with a juglone lethal dose are shown in **Figure 72**.

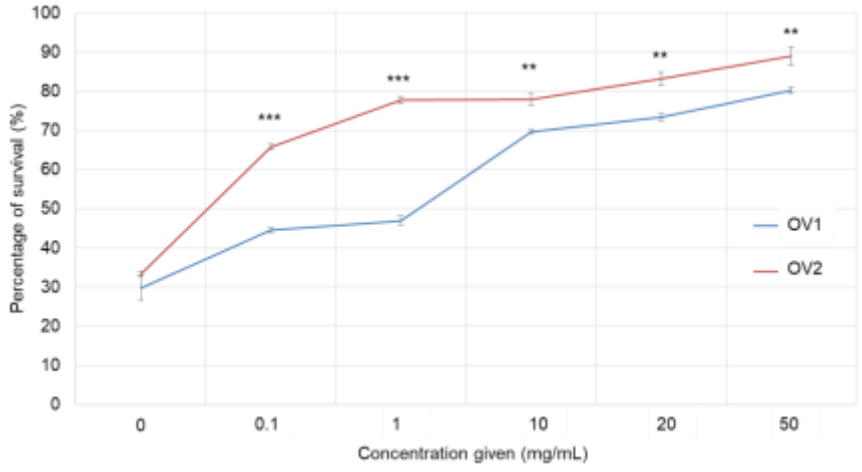


Figure 72. Percentage of survival (mean ± SD %) of worms treated with different concentrations of the extracts after 24h of juglone exposure. OV1: cold aqueous extract; OV2: heated aqueous extract. X–values correspond to real concentrations of the extracts (µg/mL). ** corresponds to $p < 0.01$ between the extracts and *** corresponds to $p < 0.01$. Within each extract, consecutive concentration values showed statistical differences ($p < 0.001$).

At first glance, both extracts produced a positive effect on *C. elegans*, since the percentage of surviving worms increased significantly when applying the sample. Worms treated with OV2 presented significantly higher survival rate at any concentration ($p < 0.01$), with the exception of non–treated worms (control): 29.79 ± 3.15 % (OV1) and OV2 (0 mg/mL) = 33.34 ± 0.61 %, $p = 0.203$. Previous studies performed with same concentration of juglone (80 mM for 24 h) also presented a survival rate for control of around 30 % [177,330]. In one of those studies [177], the methanolic extract of *O. vulgare* did not present statistical differences with respect to the control in the increase in survival rate, whereas OV1 and OV2 showed a significant dose–dependant increment.

Within the same extract, differences were non–significant ($p > 0.05$) between consecutive concentrations of OV2 from 1 mg/mL to 20 mg/mL: 77.73 ± 0.88 %, 77.95 ± 1.57 % and 83.25 ± 1.73 %. The survival rate of different concentrations of OV1 was not significantly different ($p > 0.05$) at 10 mg/mL (69.63 ± 0.39 %) and 20 mg/mL (73.35 ± 0.93 %). Besides, the value of 50 % survival of the worms was statistically different for the two extracts: 19.44 ± 0.55 µg/mL for OV1 and 0.39 ± 0.03 µg/mL for OV2, with $p < 0.001$.

4 REACTIVE OXYGEN SPECIES (ROS) ACCUMULATION

Since the Ageing Theory postulated by Harman in 1956 [247], interest has increased in the study of reactive oxygen species (ROS) generation and cellular oxidative damage that leads to ageing–related diseases. The aim is to redirect aged–human body to a youthful balance in ROS production that has increased with age [244].

Generation of ROS takes place in mitochondria, where 0.4 % and 4 % of oxygen consumption is converted into ROS [331]. When intra and extra–mitochondrial scavenging mechanisms are altered to minimize damage, ROS may accumulate and disease appears. As in many other fields of clinical research, *C. elegans* is a superb *in vivo* model to analyse the effects of mitochondrial function and ROS accumulation.

4.1 MATERIAL AND METHODS

- Experimental basis

After testing the antioxidant effect on *C. elegans*, the accumulation of ROS inside the worms was tested under the same conditions by applying a fluorescent reagent. CM–H_2DCFDA is a cell–permeant chloromethyl derivative of a reduced form of fluorescein cleaved by an intracellular esterase that produces a non–fluorescent compound. ROS can

oxidise this compound into a highly fluorescent compound: 2',7'–dichlorofluorescein (DCF) [332] (**Figure 73**).

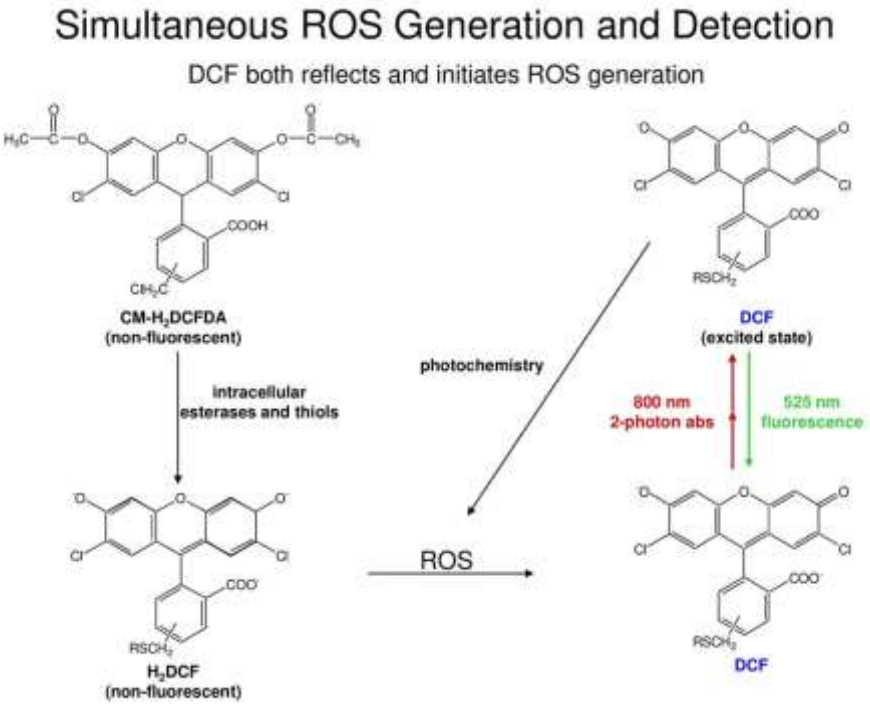


Figure 73. Experimental basis of the simultaneous ROS generation and detection, [332].

- <u>*C. elegans* growth</u>

Worms were treated in the same way as for the *in vivo* antioxidant activity assay.

- <u>Sample preparation</u>

OV1 and OV2 extracts at two concentrations (1 and 5 mg/mL) were tested. Again, rosmarinic acid was used as a positive control at the same concentrations and lack of treatment as a negative control. Besides, as a complementation of antioxidant *in vivo* activity of intestinal fractions, the ROS accumulation in worms was also determined after treating them with 1 mg/mL of OV2 intestinal fraction.

- <u>Analyse conditions</u>

After 45 h of treatment – so that worms are in L3 stage with no eggs inside – worms were picked, washed and CM–H_2DCFDA was added and incubated for 30 min at 20 ºC. Then, tubes containing worms were centrifuged at 1,400 rpm for 4 min and pellet was re–suspended in 1 mL of PBS. 25 µL of the mixture were placed on each 2 % (0.1 % azide) agarose slide, covered and observed under the lens with green fluorescence light (**Figure 74**).

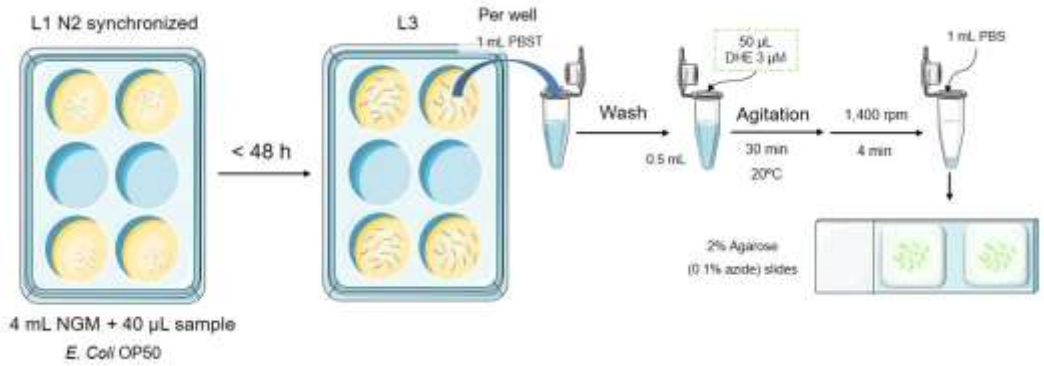


Figure 74. Schema for ROS accumulation in the *C. elegans* assay.

- Data processing

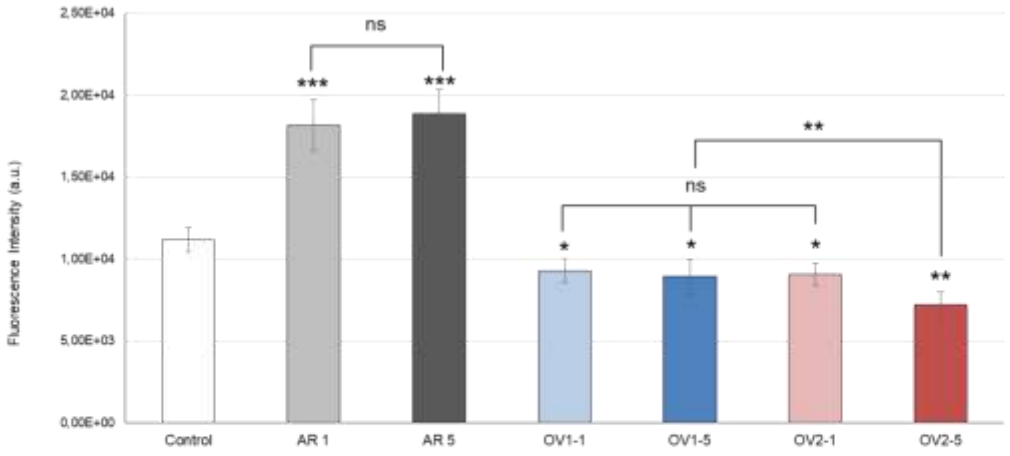
Photographs of a minimum of 25 worms were taken for each condition in order to be able to quantify fluorescence. The experiment was performed in triplicate and the results were expressed as a percentage with respect to the negative control (non–treated worms), since the images and the measurement of the fluorescence intensity of H_2DCFDA in live *C. elegans* can be confused with the presence of autofluorescence from the animal's intestine.

- Statistical analysis

Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

4.2 RESULTS

The worms showed different fluorescence intensity after adding H_2DCFDA depending on the extract given as treatment and the dose (1 or 5 mg/mL). **Figure 75** shows a representative image of each condition.



	Control	AR 1 mg/mL	AR 5 mg/mL	OV1 1 mg/mL	OV1 5 mg/mL	OV2 1 mg/mL	OV2 5 mg/mL
Fluorescence Intensity (a.u. mean ± SD)	11,152.12 ± 742.50 [a]	18,145.03 ± 1,571.74 [b]	18,845.64 ± 1,477.78 [b]	9,287.88 ± 736.74 [c]	8,948.06 ± 1,022.77 [c]	9,044.67 ± 677.57 [c]	7,224.73 ± 772.18 [d]
Percentage regarding control (%)		+ 62.70%	+ 68.99%	-16.72%	- 19.76%	- 18.90%	- 35.22%

Figure 75. Intensities measured from ROS accumulation assay given as intensity of fluorescence (mean ± SD a.u.). Percentage of increment (+) or decrement (–) was calculated with respect to the control (100%). AR corresponds to positive control, rosmarinic acid. *ns* indicates no statistical differences ($p > 0.05$), * indicates statistical differences with $p < 0.05$, ** indicates statistical differences with $p < 0.01$ and *** indicates statistical differences with $p < 0.001$. A. u. means arbitrary units.

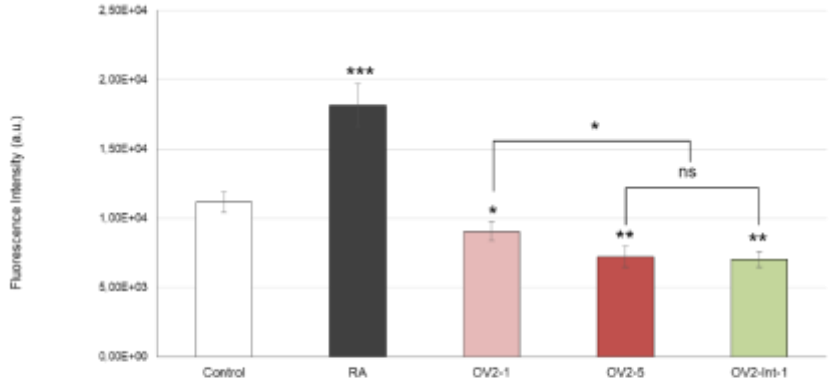
With the rest of the images, the intensity of fluorescence was quantified (as arbitrary units, a. u.) and statistically compared between the conditions. The worms treated with rosmarinic acid (**Figure 74**) emitted significantly more fluorescence than the control (11,152.12 ± 742.5 a.u, with $p < 0.001$) ones, regardless of the concentration (18,145.03 ± 1,571.74 at 1 mg/mL and 18,845.64 ± 1,477.78 a.u. at 5 mg/mL , with $p = 0.195$). On the other side, OV1 at both concentrations and OV2 at 1 mg/mL showed significantly lower fluorescence than the control ($p < 0.05$): 9,287.88 ± 736.74 a.u. (OV1–1), 8,948.06 ± 1,022.77 a.u. (OV1–5), 9,044.67 ± 677.57 a.u. (OV2–1), with no significant differences between them ($p > 0.05$). However, statistical differences with $p < 0.01$ were observed for OV2 at 5 mg/mL with control and rest of extracts (7,224.73 ± 772.18 a.u.).

AR (at 1 or 5 mg/mL) increased 62.70 % the amount of ROS, whereas OV1 (at 1 mg/mL or 5 mg/mL) and OV2 at low dose (1 mg/mL) reduced in 18.90 % the amount of ROS in the worms. Moreover, OV2 at higher dose (5 mg/mL) was able to duplicate this percentage compared to the other extracts tested, being the decrement 35.22 %.

This effect could be defined as a ROS–protection or even a ROS reduction on the accumulation, in consonance to the results of scavenging activity *in vivo*. ROS accumulation assay was performed by fluorescence, showing a significantly lower intensity in the worms treated with OV2 at 5 mg/mL (– 35.22 %) than those that received an OV1 treatment or OV2 at 1 mg/mL. Nevertheless, both extracts at any of the doses tested

produced a reduction in the fluorescence intensity, which can be attributed to a reduction in the ROS accumulation, while the positive control increased this amount. This result showed to be opposite to the rest of the antioxidant activity observed in Rosmarinic acid. Being a very antioxidant pure compound, the results could be related to the fact that high doses of rosmarinic acid might activate other mitochondrial metabolism pathways, like scavenging enzymes responsible for ROS detoxification. Indeed, pure rosmarinic acid produced high cytotoxicity at the higher concentrations tested on cells (*see Chapter II, cytotoxicity*), supported by results from other researchers [229]. This toxicity might also be explained as an over–physiological stressful condition caused by a high concentration of rosmarinic acid. Previous studies found a similar conversely behaviour on roots [333], where the fluorochrome was not able to penetrate and aggregate inside the mitochondria due to depolarized membranes (low ΔΨm) leading to a strong ROS accumulation in cells treated with rosmarinic acid [333].

The ROS accumulation assay was also performed for intestinal absorbable fraction of OV2 at 1 mg/mL, because 5 mg/mL was consider a high dose and to check if at that dose there was any significant improvement in the reduction of this accumulation (**Figure 76**).



	Control	AR 1 mg/mL	OV2 1 mg/mL	OV2 5 mg/mL	OV2 Int. 1 mg/mL
Fluorescence Intensity (a.u. mean ± SD)	11,152.12 ± 742.50 [a]	18,145.03 ± 1,571.74 [b]	9,044.67 ± 677.57 [c]	7,224.73 ± 772.18 [d]	7,019.17 ± 559.52 [d]
Percentage regarding control (%)		+ 62.70	- 18.90	- 35.22	- 37.06

Figure 76. Intensities measured from ROS accumulation assay given as intensity of fluorescence (mean ± SD). Percentage of increment (+) or decrement (–) was calculated with respect to control (100 %). AR corresponds to positive control, rosmarinic acid OV2 Int. corresponds to intestinal fraction of OV2. *ns* indicates no statistical differences ($p > 0.05$), * indicates statistical differences with $p < 0.05$, ** indicates statistical differences with $p < 0.01$ and *** indicates statistical differences with $p < 0.001$. Table below shows exact data, where same letters indicates no statistical differences between results ($p > 0.05$).

The results for the crude extract showed to be significantly different at 1 mg/mL (OV2 1 mg/mL = 9,044.67 ± 677.57 a.u. and OV2 Int. at 1 mg/mL = 7,019.17 ± 559.52 a.u., with $p < 0.05$) but did not show any significant difference comparing to the crude extract at 5 mg/mL ($p = 0.998$). Expressed as a percentage of reduction on the ROS accumulation,

OV2 at 1 mg/mL produces a reduction of 18.90 %, while after digestion this reduction is similar to the observed when giving five times more raw extract to the worms (OV2 at 5 mg/mL = 35.22 % and OV2 Int. at 1 mg/mL = 37.06 %, with p = 0.998).

Although previous studies did not obtain these results with a positive control, some researchers support the idea of plant material synergy, meaning that the whole extract appears to be more effective than individual compounds – like as rosemary and rosmarinic acid in ROS accumulation [188,324]. Results so far have shown that OV2 – containing a 33 % of rosmarinic acid and other compounds – retains its antioxidant properties inside the worms in terms of scavenging activity and ROS accumulation. Would they be strong enough to increase the life expectancy of worms as part of anti–aging treatment?

5 HYPOGLYCAEMIC ACTIVITY *IN VIVO*

The simple and reliable method designed for the direct measurement of the antioxidant activity of a compound in *C. elegans* was adapted to other bioactivities of interest already tested *in vitro* in this study. Hereby, protocol of antioxidant activity *in vivo* was adapted so that hypoglycaemic activity could also be measured, since OV1 showed to inhibit alphaglucosidase better *in vitro* than commercial anti–diabetic treatment.

5.1 MATERIAL AND METHODS

- Experimental design

Experiment could be explained as the combination of two of the already performed tests (**Figure 77**): Antioxidant activity *in vivo*, with some modifications and Hypoglycaemic activity *in vitro*: *α–glucosidase* inhibition (see *Chapter II*, *hypoglycaemic activity in vitro*).

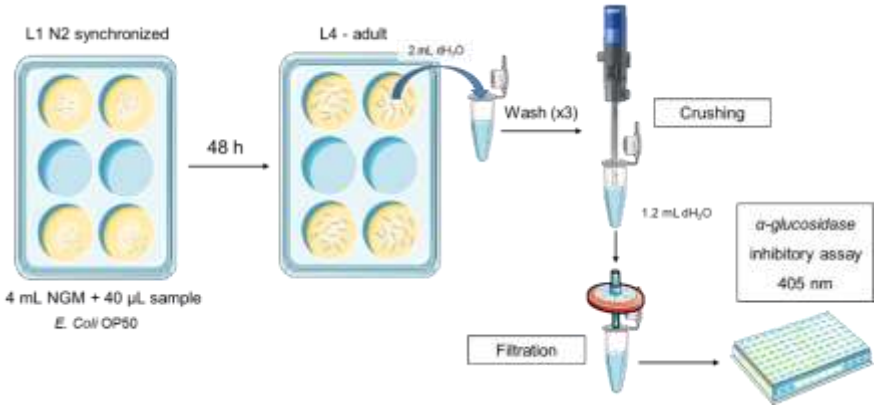


Figure 77. Scheme of the adapted method for hypoglycaemic activity *in vivo*.

- Sample preparations

The same concentrations tested on antibiogram assay and antioxidant *in vivo* assay were prepared for OV1 and OV2 (0, 0.1, 1, 10, 20 and 50 mg/mL in distilled water). Acarbose was soluble in NGM, so it could be selected as a positive control at the same concentrations as the samples.

- Analyse conditions

1,000 L1–N2 synchronised worms grew up to L4–adult stage on NGM plates containing the sample (40 µL per well of OV1 and OV2 tested at the same concentrations: 0.1, 1, 10, 20 and 50 mg/mL) and the food source (*E. coli* OP50). Acarbose was given to the worms at the same concentrations as a positive control. After 48 h, worms were picked with 2 mL of dH2O, washed three times, resuspended in 1.2 mL, crushed with Ultraturrax for 20 s and filtered. Finally, hypoglycaemic *in vitro* activity assay was performed in the same manner as previously described (see *Chapter II, hypoglycaemic activity in vitro*).

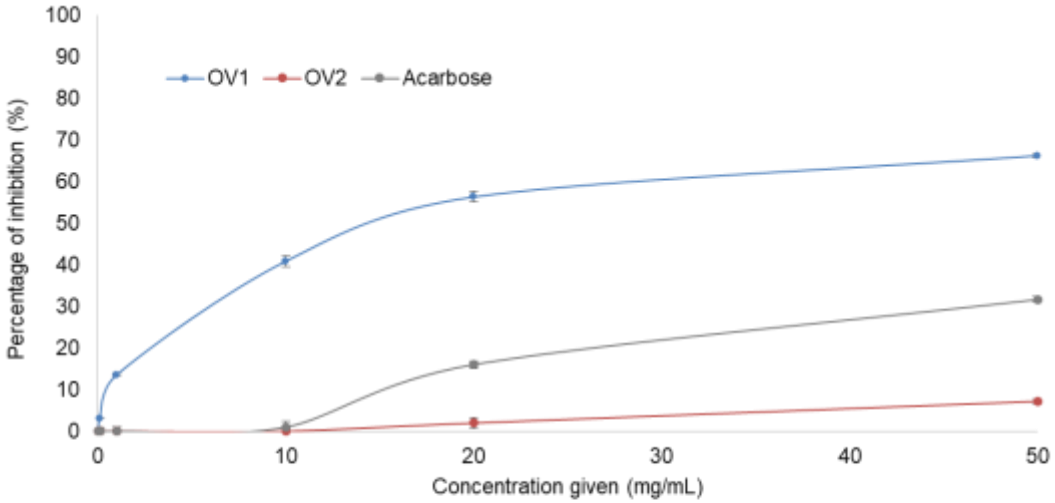
- Statistical analysis

The experiments were performed in triplicate. Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro–Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (95 % CL) or post–estimation margins to check interaction among groups.

5.2 RESULTS

Given the current prevalence of diabetes in society [257], research of natural compounds able to regulate glycaemia and insulin–resistance is nowadays covering importance in industry, as alternative or co–adjuvant treatment to existing hypoglycaemic medicines. In pre–clinical steps of research, *C. elegans* is be considered a promising *in vivo* model for the study of the molecular mechanism of glucose as part of anti–diabetic bioactivity [334]. However, as for antioxidant activity, the effects of the compounds are attributed *a posteriori* [334–337], after a physiological response of the worms rather than directly measuring preservation of activity inside the worm.

Only the results from OV1 reached 50 % of inhibition of the enzyme. Due to the low activity of acarbose and OV2, the results are shown as a percentage of inhibition (mean ± SD %). IC_{50} could not be calculated for samples under the conditions of the assay (**Figure 78**).



Percentage of inhibition (%) per concentration given (mean ± SD mg/mL)

Sample	0	0.1	1	10	20	50
OV1	0.00 ± 1.01[a]	3.06 ± 0.08 [b]	13.58 ± 0.33 [c]	40.85 ± 1.32 [d]	56.29 ± 1.35 [e]	66.22 ± 0.41 [f]
OV2	0.00 ± 0.95 [a]	0.00 ± 0.89 [a]	0.00 ± 0.98 [a]	0.00 ± 1.18 [a]	1.97 ± 1.42 [a]	7.18 ± 0.54 [g]
Acarbose	0.00 ± 0.97 [a]	0.00 ± 0.25 [a]	0.01 ± 0.40 [a]	0.98 ± 1.33 [a]	16.03 ± 0.89 [c]	31.66 ± 0.63 [h]

Figure 78 Results of hypoglycaemic activity *in vivo* for OV1, OV2 and positive control (acarbose) given as percentage of inhibition (mean ± SD %). Table below shows values for percentage of inhibition (mean ± SD %) per concentration. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$).

OV1 showed to be the most active extract tested, even twice more than the positive control at the highest concentration: 66.22 ± 0.41 % and 31.66 ± 0.63 % at 50 mg/mL, respectively ($p < 0.001$). Acarbose – the positive control – did not reach 50 % of inhibition, meaning that a higher concentration of this compound would be needed to produce any hypoglycaemic effect. Indeed, enzyme inhibition started at 20 mg/mL or more given to the worms: 16.03 ± 0.89 % at 20 mg/mL and doubled (31.66 ± 0.63 %) at 50 mg/mL. OV2 did not even pass 10 % inhibition at any of the concentrations tested, being 7.18 ± 0.54 % at 50 mg/mL and practically null for the rest of the concentrations tested.

As it is an adaptation of our method developed in 2019 [254], foreign results have not yet been published for comparison. However, a previous study with *O. vulgare* collected in Morocco concluded that the hot aqueous extract at 20 mg/kg given for 2 weeks was sufficient to normalise glycaemia in serevely diabetic rats [32]. Compared with the results obtained, the hot aqueous extract of this plant might reduce glycaemia through other pathway. Nevertheless, OV1 was significantly more active than acarbose both *in vitro* and *in vivo*.

6 LIFESPAN

Lifespan is the icing on the cake of antioxidant and anti–aging research. The Free–radical Theory [247] defends that the formation of free radicals shortens life expectancy, in somehow because of the fast develop of age–related diseases such as Alzheimer's or metabolic syndrome and the high risk of suffering a cardiovascular episode. The development of medicine in early diagnosis, surgery and treatment – also thanks to pharmaceutical products as well – has increased the life expectancy of humans in the last centuries. This generational progress in quality and longevity of life is difficult to study for human species. If you want to test a compound to determine if it improves life–expectancy, *C. elegans* is also an optimal *in vivo* model for lifespan assays in pre–clinical research, thanks to the short life of worm generation and the possibility of extrapolation to humans because of genetic similarities.

6.1 MATERIAL AND METHODS

The lifespan assay was performed by following recommendations by Navarro *et al*. (2017) [315]. The principal objective of the experiment is to determine the life expectancy of the worms depending on the administered dose of the extracts (OV1 and OV2). The basis of the experiment lies in counting worms and recording when they die.

- Strain of *C. elegans*

N2 *C. elegans* wild strain was used. Once L1 synchronized worms grew (48 h, 20 ºC), L4–adults would be transfer on plates with 40 µL of 5–fluro–2'–deoxyuridine (FuDR) 40 mM. FuDR is a compound to prevent hatching of eggs.

- Growing control

In this study, worms would stay in same plate for days – until death – and natural food source (*E. coli* OP50) would be limited to an initial quantity 200 µL instead of 100 µL, which was sufficient according to previous studies [315].

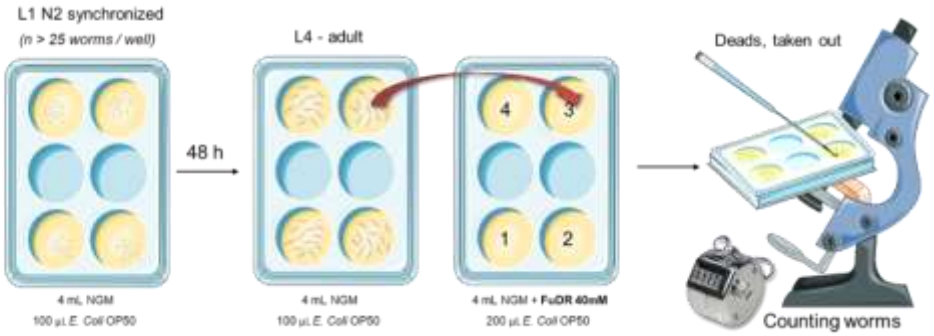
- Samples preparation

OV1 and OV2 would be administered in the same way as so far: 40 µL per well, incorporated on NGM. Concentrations to test would be 20 and 2 mg/mL for OV1 and 5 and 0.5 mg/mL for OV2.

- Analyse conditions

Three type of lifespan were performed (**Figure 79**):

1. **Normal assay:** the sample to test is administered to worms once they have grown: L4–adults are generally transfered to the plate containing the substance of study.
2. **Life assay:** the sample was chronically administrated to worms: from L1 to death worms were in presence of the extract.
3. **Growing assay:** the sample was administered to worms only during growth and removed in adulthood: they grew on plates with the extracts (from L1 to L4–adult) and then, were transferred (L4–adult) to plates without extracts until death.



	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
	Synchronization	*L1 synchronized*		*Transfer*			
Normal		NGM		NGM + sample			
Life		NGM + sample		NGM + sample			
Growing		NGM + sample		NGM			

Figure 79. Scheme for lifespan assays. L1 N2 synchronised worms (25 per well) were grown until L4–adult and then transferred to NGM plates containing FuDR 40mM – egg–display inhibitory substance. Then, dead and alive worms were counted every two days.

The lifespan assays started with the synchronization of the worms (Day 0). Then, 25 L1 synchronized N2 worms were placed per well onto NGM supplemented with *E. coli* OP50 (100 µL per well). 6–well plates were used for the experiment; only the 4–corner wells were used to avoid problems with the inner wells. For those plates containing the sample (*life* and *growing* assays), OV1 (2 or 20 mg/mL in distilled water) or OV2 (0.5 or 5 mg/mL in distilled water) was added to the medium (40 µL per well). The plates were incubated at 20 ºC for 48 h for growing.

On day 3, the L4–adult worms were transferred to the plates containing NGM 0.001 % of FuDR 40 mM supplemented with 200 µL of food source and, those that required (*normal* and *life* assays), 40 µL of the correspondent extract. The *growing* plates were prepared without extracts, as part of the experimental design (**Figure 78**).

- Data process

From the time point of the transfer of the worms (day 3), the dead worms were registered every 2 days: those that did not respond to wire platinum touch were considered dead and removed from the plate. The experiment ended when no live worms were left. In addition, length measurement and pharyngeal pumping rate were also monitored along the first days of the experiment as a control of growth.

The surviving worms were expressed as a fraction of surviving worms, calculated using the following formula:

$$Survival\ fraction = \frac{number\ of\ worms\ alive}{total\ worms}$$

- Statistical analysis

This study was performed in triplicate (replica 1: starting on 26th February 2019; replica 2: starting on 10th February 2020 and replica 3: starting on 17th February 2020). Statistical analysis was carried out by introducing data in Oasis 2 webpage, which compares statistically the survival curves by a Log–Rank test – a contrast of data based on Chi–squared distribution (χ^2). The graphs were confectioned with Microsoft Excel 2013.

6.2 RESULTS

As for the rest of *in vivo* bioactivities tested, OV1 and OV2 were the extracts selected for life experiments because of their *in vitro* properties. According to the results obtained on previous assays performed, OV2 presented significant lower EC_{50} in the antioxidant *in vivo* assay (**Table 42**), a significant lower ROS accumulation at 5 mg/mL (**Figure 78**) and a higher survival rate against juglone (**Figure 71**). In other words, the lower dose of OV2 would potentially produce the same effect as OV1. For those reasons, OV2 was tested at 0.5 and 5 mg/mL, whereas OV1 was tested at higher concentrations 2 and 20 mg/mL.

The experiment had to be stopped due to Covid–19 restrictions and so, data was registered until 25th day for the third replicate and 31st day for the second replicate. First replicate was the only one that could be completed: until the last worm that died on day 49th. However, according to **Figure 80**, it can be observed that results from the three replicates overlap within the same conditions, especially for the control (black line in **Figure 80**). This fact points that results obtained in the incompleted replicates (2nd and 3rd) would be of the same order than the results from the first replicate.

According to the data registered, the worms with lack of treatment (control) died earlier than the ones that received treatment (last worm died on 33rd day), meaning that the

extracts expand the life expectancy of worms. The last surviving worm died on 49th day, receiving OV2 5 mg/mL for *Life* assay.

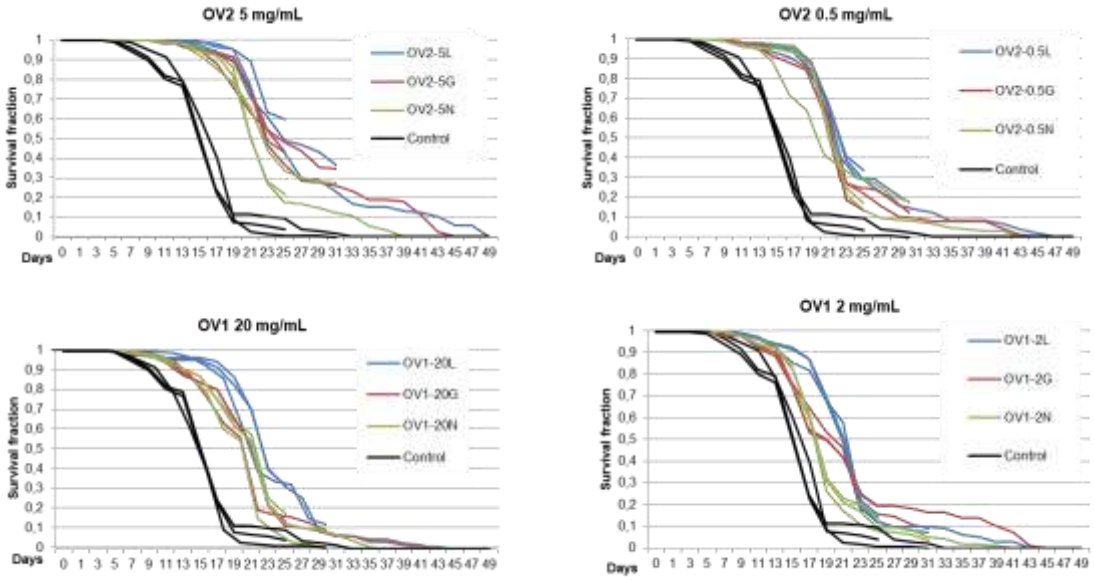


Figure 80.1st, 2nd and 3rd replicate of lifespan assays. The results are given as surviving fraction per condition over time (days).

The worms without oregano treatment (control), showed similar life expectancy in the three replicates, since 50 % of them died between day 15th and 17th. Considering this result as a normal life expectancy of *C. elegans*, any treatment with oregano showed to increase lifespan. All the treatments were able to maintain with life 50 % of worms at least during 21 – 23 days; with the exception of OV1 at 2 mg/mL at adulthood. This was the least effective treatment, since the slope in the graph was closer to the control in time (50 % of survival rate on 19th day) (**Figure 80**). At first glance, OV2 at 5 mg/mL showed increase better the life expectancy of worms, followed by OV2 0.5 mg/mL and OV1 20 mg/mL.

The third replicate was started later in time than the second replicate, so experiment had to end even earlier without finishing: but still four worms were alive in control (day 25). The maximum death rate registered was for the control (96.26 %), followed by the OV1 treatments except OV1 at 20 mg/mL for *Life* assay (death = 68.75 %) and including OV2 at 0.5 mg/mL only during growth (death = 86.36 %).

As calculated, OV2 given at 5 mg/mL presented a lower rate of dead worms than the rest of conditions, especially when given during growth of worms (*life* and *growing* assays). Then, the lower concentration of OV2 (0.5 mg/mL) presented a less number of deaths in the time of life expectancy than the control. It would mean an increment of around 20 % in the life expectancy of worms given OV2 at 0.5 mg/mL, preferably at adulthood because

growing assay presented the higher death rate in this group: 87.39 % against 82.14 % when given while *growing* and 81.81 % for *life*. Then, OV1 presented higher rate of deaths within 31 days, being 20 mg/mL given for *life* assay the lowest value: 88.18 % against the others that overpassed 90 %. Indeed, only one worm from the group that received OV1 at 20 mg/mL only in adulthood (*normal* assay) was still alive on the 31st day.

Not many complete conclusions can be drawn from the results of the three replicates, but they can be compared on 25th day of counting (**Table 44**):

Table 44. Survival rate (mean ± SD) on 25th day of the three replicates of lifespan assays for each condition. Values with different letter present significant differences (p < 0.05) and same letter indicates no significant differences (p > 0.05).

			Survival rate day 25th			
Sample	**Concentration (mg/mL)**	**Modality**	**1st**	**2nd**	**3rd**	**Mean ± SD**
OV2	5	Life	0.43	0.49	0.60	0.50 ± 0.08^{a}
		Growing	0.37	0.48	0.43	0.43 ± 0.05^{a}
		Normal	0.18	0.34	0.22	0.24 ± 0.08^{b}
	0.5	Life	0.29	0.30	0.34	0.31 ± 0.03^{b}
		Growing	0.22	0.25	0.14	0.20 ± 0.06^{c}
		Normal	0.13	0.30	0.17	0.20 ± 0.09^{c}
OV1	20	Life	0.32	0.34	0.31	0.32 ± 0.01^{b}
		Growing	0.12	0.16	0.13	0.14 ± 0.02^{d}
		Normal	0.13	0.08	0.17	0.13 ± 0.04^{d}
	2	Life	0.12	0.13	0.15	0.13 ± 0.02^{d}
		Growing	0.19	0.16	0.19	0.18 ± 0.02^{c}
		Normal	0.09	0.08	0.14	0.10 ± 0.03^{e}
Control			0.09	0.09	0.08	0.09 ± 0.01^{e}

On 25th day, the control had a very similar survival rate, being 0.09 (1st), 0.09 (2nd) and 0.08 (3rd) and mean = 0.09 ± 0.01. For rest of the conditions, the data was less reproducible than for the control, being the highest standard deviation 0.09: values of survival rate of 0.13, 0.17 and 0.30 for the same condition (OV2 0.5 mg/mL, *Normal* Lifespan) but different replicates (1st, 3rd and 2nd, respectively). In this sense, at that moment (day 25th), the results of OV1 presented better reproducibility between replicates than OV2, because the moment of maximum deaths had already passed. Then, since at 25th day only 9 % of the non–treated worms were alive, the treated worms seemed to survive longer, except those treated with 2 mg/mL of OV1 (survival rate 10 ± 3 %, with non–significant differences with control, p > 0.05). Moreover, worms treated with OV1 (10 – 18 %) died faster than with OV2 (20 – 50 %), with the exception of chronic treatment of OV1 at 20 mg/mL: 32 ± 1 % worms were alive. According to the free–radical theory, *in vivo* scavenging activity and decreased ROS accumulation inside worms of OV2 might explain the protection against natural death. However, the hypoglycaemic activity and 2,5–DHBA might play a secondary role in the survival of the worms. For final conclusions on the life expectancy of the tested

treatments, **Table 45** summarizes the best results of complete lifespan – data obtained from the 1st replicate.

Table 45. Result of lifespan (first replicate) for all conditions expressed as mean lifespan in days, age in days in percentage of mortality and linear interpolation of mortality curve. Values from same column with different letter present significant differences ($p < 0.05$) and same letter within same column indicates no significant differences ($p > 0.05$).

Sample	(mg/mL)	Modality	nº worms	Restricted mean			Age in days at % mortality				
				Days	S.E.	95% C.I.	25%	50%	75%	90%	100%
OV2	5	Life	107	27.75[a]	0.81	26.15 ~ 29.34	21.18[a]	22.90[a]	30.42[a]	43.12[a]	49.00[a]
		Growing	116	27.22[a]	0.79	25.69 ~ 28.76	21.00[a]	22.76[a]	31.67[a]	41.28[a]	45.00[a]
		Normal	114	23.70[b]	0.52	22.69 ~ 24.71	19.64[b]	21.14[b]	23.45[b]	33.20[b]	39.00[b]
	0.5	Life	108	25.26[a']	0.68	23.93 ~ 26.59	20.22[a]	22.20[a]	26.14[b]	34.10[b]	47.00[a]
		Growing	118	24.22[b]	0.57	23.10 ~ 25.34	20.11[a]	21.77[b]	23.83[b]	29.40[b]	45.00[a]
		Normal	105	23.19[b]	0.51	22.18 ~ 24.20	19.71[b]	21.03[b]	22.73[b]	26.75[b]	43.00[a]
Control			106	16.53[c]	0.50	15.54 ~ 17.51	13.31[c]	15.14[c]	16.97[c]	23.80[c]	33.00[c]
OV1	20	Life	113	24.40[b]	0.53	23.35 ~ 25.44	20.47[a]	22.38[a]	27.08[a]	28.86[b]	45.00[a]
		Growing	112	21.91[d]	0.60	20.74 ~ 23.08	17.75[b]	21.22[b]	22.78[b]	27.80[b]	43.00[a]
		Normal	101	21.61[d]	0.56	20.52 ~ 22.71	17.04[b]	21.27[b]	22.80[b]	28.80[b]	37.00[b]
	2	Life	106	23.32[b]	0.74	21.86 ~ 24.78	18.35[b]	21.06[b]	22.77[b]	34.60[b]	47.00[a]
		Growing	110	22.20[d]	0.88	20.47 ~ 23.93	14.94[d]	19.00[b]	22.95[b]	39.00[a]	45.00[a]
		Normal	119	19.84[e]	0.51	18.84 ~ 20.84	15.78[d]	17.78[d]	20.21[d]	24.80[c]	41.00[b]

On average, any of the treatments significantly expanded the life expectancy of worms, compared to the non–treated ones (16.53 days for control with $p < 0.001$). Half of the non–treated worms died by 17th day, whereas for any treatment with OV2 that day was 23rd. Indeed, the non–treated worms (control) showed significant differences from the treated worms in any mortality rate, with the exception of theworms treated with 2 mg/mL OV1 in the normal assay, where 10 % of the worms had non–significantly different age to control (24.80 and 23.80 days, with $p > 0.05$). *Grosso modo*, extracts should be recommended to be administered from very early stages, since the whole population of those treated before adulthood (*Life* and *Growing* conditions) died significantly later in time: 43 – 49 days, with $p < 0.001$. The differences between extracts were noteworthy as 25 % of worms died, where worms treated with OV1 at 2 mg/mL administrated only in adulthood began to die earlier (on 16th day comparing to 20th day of other conditions). However, 10 % of these worms were still alive from day 25th to 41st day. Inversely, exposure of adult worms to OV1 at higher doses (20 mg/mL) provided a comparable average survival rate to those receiving treatment at growing stage, but the last worm died earlier in time (37th day), when the rest of treatments passed 40th day. In this sense, the last day of experiment cannot be decisive for the life expectancy of a population, since that a single subject could have reached this value whereas the rest of population lived significantly less.

The highest doses of OV2 provided significantly the longest average longevity found in the worms, being 27.75 days when continuously dispensed and 27.22 days during growth of worms. As well, a chronic dose of 0.5 mg/mL of this extract did also significantly extended the lifespan of the worms with an average of survival rate of 25.26 days ($p < 0.01$ with high

doses of OV2). However, when OV2 0.5 mg/mL was discontinued in adulthood (*Normal*), the average number of worms died earlier (50 % = 21.14 days with $p < 0.001$ and restricted mean = 23.70 days, with $p < 0.001$).

At first glance, OV1 presented shorter lifespan than these treatments of OV2, but no statistical differences were found in the restrictive mean of chronically administration of the two extracts: 23.70 (OV2–5), 23.19 (OV2–0.5), 24.40 (OV1–20) and 23.32 (OV1–2) days, with $p < 0.001$. Giving OV1 for life significantly increased life expectancy of the average population and individually, they lived longer (45 and 47 days, respectively). Meanwhile, treating worms with OV1 for a specific period of life could potentially shortern the life expectancy of the average population, since growing and adult results of survival rate were significantly lower than the life results. In general, there was a similar behaviour for the longest–lived worm treated with OV2 or OV1: receiving treatment during growth could potentially provide a longer adulthood.

The effect of a treatment only during the early stages of *C. elegans* or since larvae birth to death has not been previously tested, so there are no published studies to compare with. Nuri *et al.* [338] found no significance on lifespan extension effect of Turkish plant extracts –with similarities in chemical composition with *O. vulgare* –, whereas another study demonstrated a 16 % lifespan extension with green tea extract. In any case, in consonance with results of a mixture of cranberry and oregano [339], OV1 and OV2 lived significantly longer than non–treated ones.

In summary, both OV1 and OV2 showed to improve the survival rate of *C. elegans* by protecting the worms from both the lethal dose of a pro–oxidant compound and time. For best results, treatment at early stages where organism is metabolically growing and developing could be relevant to the point of improving lifespan in 70 % for OV2 and 40 % for OV1. Thus, the reduction in risk of death in an *in vivo* model such *C. elegans*, may be translated in humans into a potentially lower risk of triggering age–related diseases.

CHAPTER IV:

PHARMACEUTICAL TECHNOLOGY

pre–formulation assays.

Simplicity is the ultimate form of sophistication.

- Leonardo da Vinci.

The word Phytotherapy etymologically comes from **Greek (phyton = plant, and therapeia = meaning treatment**) and comprises the treatment and prevention of diseases with plants, plant constituents and medicines containing plants. Regarding the use of the medicinal plants, some authors rather differentiate between the prophylactic and therapeutic effect of herbal medicine. No matter the purpose of the preparation, this medical field has been changing in recent decades. This new phytotherapy includes the advances in pharmaceutical technologies in preparations (creams, capsules, microencapsulation, tablets…), ensuring the quality, safety and efficacy of the drug according to Pharmacopeia and EMA.

Around 25 % of the current drugs prescribed in United States contain at least one active ingredient extracted from plant material and the World Health Organization (WHO) reported 80 % relief with natural remedies [340]. According to the WHO definition, *herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that can be wither whole plants or part of medicinal plants* [341]. Final herbal products can be composed by a single herbal medicine (such as Tanakene® with *Ginkgo biloba* L.) or a combination of different medicinal plants with a synergic purpose (such as Fisiocrem®).

Both patients and industry require solid oral dosage forms for their simplicity of administration and production: low costs, higher stabilities and ease of large–scale production [342]. Hereby are some of the conventional dosage forms for herbal medicines [340]:

- Granules: these oral pharmaceutical forms are prepared through a process in which the particles are assembled into larger, more stable structures that improve flowability, compression and uniformity of content [340]. Despite the advantages, the process can be costly in time, money and equipment [343]. Improvements with medicinal plants are being studied in order to optimize industrial production [344].

- Tablets: generally, the herbal material to be formulated is given as a fine hygroscopic powder that is difficult to compress [340]. A tablet is a compresed solid dosage form whose color, form and size can vary. Moreover, pharmaceutical technology has been able to design gastrorresistant or modified–release tablets, also suitable for plant extracts [345]. Most of the natural sleeping pills on the market with *Passiflora incarnata* L. and *Valeriana officinalis* L., among other sedative medicinal plants, benefit from this type of layered–tablets.

- Capsules: encapsulation could be considered as one of the simplest single oral dosage form without the problem of compressibility [340]. Besides, in practise, most patients

do not differenciate between capsule and tablet. In the capsules, drug substance (plant extract) is enclosed in an easy–to–handle, odourless and tasteless container, an effective way to mask the bad taste of herbal extracts.

Novel drug delivery systems for herbal medicines are increasingly being developed and appearing on the market: mouth–dissolving tablets, sustained and extended released formulations, mucoadhesive systems, transdermal dosage forms, microparticles, microcapsules, nanoparticles and implants [340,346–349]. Controlled release formulations with alkaloids of aconitum species or opioid analgesic are already patented [350], but these transdermal delivery systems might increase both production and purchase price. Thus, conventional topical formulations are still the choice of industry and patients, as for oral administration.

Among pharmaceutical forms for cutaneous use, creams and oinments are the simplest assortments to prepare where the drug substance is nude in the mixture [340]. Creams can be defined as an emulsion with a good skin penetration, whereas ointments tend to be less extensible and function as an effective skin barrier against moisture loss. The topical use of medicinal plants can date back to Greek times with the use of poultices.

1 ORAL ADMINISTRATION

From the point of view of the community pharmacy, the demand of these phytomedicines has increased as well as new products on the market. Part of this return to natural products might be associated either with concerns about side effects or lifelong dependency on chemical drugs that tries to find a solution in medicinal plants as prevention.

This work is related with different extracts from *O*. *vulgare*, a plant widely used in traditional medicine. However, EMA has not yet been published a monograph. For this reason, there is a small number of commercial pharmaceutical products that contain oregano.

In the Spanish (and European) market, most products contain essential oil of oregano, with a standard quantity of thymol and carvacrol. Pranarom, a Belgian natural pharmaceutical industry, produces microcapsules of essential oil of *O.vulgare* [351] (mainly containing carvacrol), indicated for respiratory pathologies and increase of the body defences. Even though this brand commercialises more products with oregano, these are the ones containing single aerial flowered parts of *O. vulgare*. Some others such as Oleocaps 2 [352] – a combination with more medicinal plants – are formulated for gastrointestinal problems thanks to the carminative properties of plants like *O. vulgare*.

Solgar, another pharmaceutical laboratory, offers oregano soft capsules containing 175 mg of oregano with a mixture with extra virgin olive oil and 17.5 mg of oil from oregano [353], where its chemical composition is purely thymol and carvacrol. Again, the main components that take advantage of oregano are its essential oils, ignoring other bioactive compounds such as rosmarinic acid.

Traditional phytotherapy contemplates the use of oregano as a tisane (infusion), which might not be the ideal way of administration for many costumers. The preparation of an infusion needs time and accessories (hot water, cup...), while some oral pharmaceutical forms are ready–to–take (capsules, powder sachets…). However, would the pharmaceutical form affect the bioactivity of oregano?

In this work, two simple oral pharmaceutical forms with hydroalcoholic extract of *O. vulgare* L. (OV3) were designed and the bioactivities were compared after the *in vitro* gastrointestinal digestion process.

1.1 MATERIAL AND METHODS

1.1.1 PHARMACEUTICAL FORM FORMULATION

- **Sample preparation**

The cold macerated hydroalcoholic extract (OV3, with at least 30 % of rosmarinic acid) was selected for the design of two oral pharmaceutical forms: divided powder and hard gelatin capsules.

- **Formulation design**

1. Dried divided powder

   Composition:

   - ✓ 500 mg of dried cold macerated hydroalcoholic extract (OV3) of *O. vulgare* L.
   - ✓ 1 mg Silicon dioxide E551 (#1.13126, Merck KGaA, Darmstadt, Germany)

2. Hard gelatin capsules

   Per capsule:

   - ✓ 250 mg of dried cold macerated hydroalcoholic extract (OV3) of *O. vulgare* L.
   - ✓ 1 mg Silicon dioxide E551 (#113126, Merck KGaA, Darmstadt, Germany)
   - ✓ Hard gelatin capsule nº 2 (#1117942, Acofarma, Madrid, Spain)

- **Quality control of formulations**

Quality control assays were performed in a small scale in three replicates as a control in the production for each oral preparation. According to Pharmacopeia (Real Farmacopea Española, 5th Edition [354]), the capsules should satisfy uniformity content (assay B, 2.9.6), uniformity mass (2.9.5) and disintegration assay (2.9.1; *performed in next headland*), whereas divided powder only needs to satisfy the uniformity content and the mass content for single–dose powder preparations [354,355].

Uniformity content and uniformity mass was carried out by measuring weight of each replicate. The standard deviation must not exceed 6.0 %.

1.1.2 *IN VITRO* GASTROINTESTINAL DIGESTION OF ORAL FORMULATIONS

The *in vitro* gastrointestinal digestion process was performed as previously explained with the crude extracts (*see Chapter I, in vitro gastrointestinal digestion*), with some modifications already published [355].

- **Sample**

Two *in vitro* digestions were simulated with:

1- Divided power containing 500 mg of dry powder previously dissolved in water.

2- Two capsules each containing 250 mg of extract.

Besides, a control with empty capsules was also performed. The disintegration assay (2.9.1), according to Pharmacopeia (Real Farmacopea Española, 5th Edition [354]) was performed by adding OV3 capsules and empty capsules separately to different solutions: water, water pH 2, water with stomach enzymes and water pH with enzymes (simulating physiological conditions).

1.1.3 CHEMICAL CHARACTERISATION

The chemical characterisation of the intestinal fractions (absorbable and non–absorbable) of both formulations was carried out using the different techniques previously described in this work): TLC, HPLC–DAD and determination of total phenolic compounds by *in vitro* Folin–Ciocalteau assay (*see Chapter I, qualitative and quantitative analysis*).

1.1.4 BIOACTIVITIES

- Properties of the intestinal fraction of the two formulations were also analysed in terms of the following bioactivities already performed in *Chapter II and III*:

- Antioxidant *in vitro and in vivo* activity
- Hypoglycaemic *in vitro* activity
- Hypolipidemiant *in vitro* activity
- Acetylcholinesterase *in vitro* inhibitory activity

- Cytotoxicity:
  - Caco–2 cells: MTT assay
  - HepG–2 cells: MTT assay

Assays were performed in triplicate.

1.2 RESULTS

EMA recommends the use of tisanes for oral use from other species of oregano (*O. majorana* L. [239] and *O. dictamnus* L. [322]). The aqueous extracts (OV1 and OV2) were already been tested *in vitro* and *in vivo* before and after a gastrointestinal digestion process, considering this oral administration as part of traditional phytotherapy.

As novelty in modern phytotherapy, the hydroalcoholic extract was selected for oral pharmaceutical formulation because of the heterogeneity in chemical profile of this extract (*see Chapter I*), *in vitro* bioactivity and low levels of cytotoxicity (*see Chapter II*). Besides, a previous study with commercial *O. vulgare* selected hydroalcoholic extract among other options to compare against infusion and decoction [127], with satisfactory results.

1.2.1 PREPARATION OF THE PHARMACEUTICAL FORMS: QUALITY CONTROL ASSAYS

The preparation of the OV3 extract showed a yield of 28.48 % (*see Chapter I, preparation of the extracts*). For other species of oregano, EMA recommends a maximum of 4 g of dried plant *per* dose (2 – 4 g *per* dose, once to twice a day before meals). Therefore, the daily dose of hydroalcoholic extract containing 33 % of rosmarinic acid was calculated to be 500 mg. For the capsules, the volume occupied by that mass was measured and the capsules n. 2 were selected, also because it is the standard size for human use.

Daily dose of dry powder (OV–P): 500 mg of this form dissolved in water once a day would provide a minimum of 170 mg of Rosmarinic acid.

Daily dose of capsules (OV–C): Two capsules once a day would provide a minimum of 170 mg of Rosmarinic acid.

Results of quality control assays of formulations were already published [355]:

The capsules must fulfil assay B of content uniformity (2.9.6) in the mean ± 15 %. This formulation met these requirements, being the total mass 311.15 ± 0.94 mg for individual fullfilled capsule with a content of 250.01 ± 0.64 mg. The uniformity mass assay (2.9.5) accepts a 10 % deviation for capsules less than 300 mg. This value in the formulation was 0.03 %. The divided powder preparations also met content uniformity and mass assays, being their values in the range of 502.04 ± 0.06 mg.

The disintegration of the capsules during the *in vitro* gastrointestinal digestion process also satisfied the established conditions for capsules and tablets (2.9.1) (i.e., capsules were disintegrated after 30 min at gastric conditions in a solution with HCl 0.1 M or gastric enzymes). The empty capsules, whose result for uniformity and mass assay was 61.14 ± 0.01 mg/mL, also met such requirements.

1.2.2 *IN VITRO* GASTROINTESTINAL DIGESTION PROCESS: BIOACCESSIBILITY

The simulation of a *in vitro* gastrointestinal digestion involves the recreation of physiological conditions by adding enzymes and pH, regarding the stage of the gastrointestinal gut: mouth, stomach and intestine. Adjustment of these conditions during *in vitro* gastrointestinal digestion for each formulation for oral administration was as follows (**Table 46**).

Table 46. Digestion of OV–P (dry powder formulation (500 mg of OV3)), OV–C (hard gelatin capsules (500 mg in 2 capsules of OV3) and capsules (empty capsules). Data related to method and bioaccesibility (%), calculated from final mass (mg) and initial mass (mg).

	Step	Tampon Solution used	Vol. added (mL)	Mass adjustment (mg)	Enzyme mass (mg)	Total extra mass added (mg)	Initial mass (mg)	Final mass digested (mg)	Biodis. (%)
OV–P	Mouth	$NaHCO_3$ 1 M	0.1	8.40	0.18	8.58	501.0	510.5	100.00
	Stomach	HCl 3 M	0.3	32.81	27.20	68.41	500.1	565.9	100.00
	Intestine *Absorbable*	$NaHCO_3$ 1 M	0.6	50.40	73.93	134.41	500.0	436.3	**87.26**
	Intestine *Non–abs.*							123.4	
OV–C	Mouth	$NaHCO_3$ 1 M	0	0.00	0.18	0.18	622.60	623.1	100.00
	Stomach	HCl 3 M	0.1	10.94	27.20	38.14	624.31	623.2	100.00
	Intestine *Absorbable*	$NaHCO_3$ 1 M	0.6	16.80	73.93	101.67	621.76	555.7	**89.37**
	Intestine *Non–abs.*							165.5	
Capsules	Mouth	$NaHCO_3$ 1 M	0	0.00	0.18	0.18	122.41	134.6	100.00
	Stomach	HCl 3 M	0.05	5.47	27.20	32.67	122.71	199.8	100.00
	Intestine *Absorbable*	$NaHCO_3$ 1 M	0.1	8.40	73.93	87.80	121.76	105.9	**86.97**
	Intestine *Non–abs.*							15.5	

The bioaccessibility of the two formulations (capsule and powder) – expressed as the percentage of the final concentration with respect to the initial concentration– was 87.26 % for the powder and 89.37 % for the encapsulated extract (**Table 46**). **Figure 81** shows the images of absorbable and non–absorbable intestinal fractions after lyophilisation for each formulation.

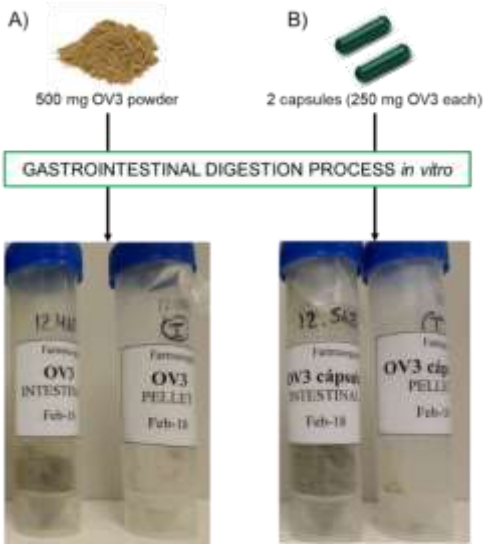


Figure 81. Oral formulations with OV3 subjected to the *in vitro* gastrointestinal digestion process and photographs of the lyophilised intestinal fractions. The left tube of each image corresponds to absorbable fraction and right, to non–absorbable fraction (pellet). A) OV–P: 500 mg OV3 powder. B) OV–C: 2 capsules of 250 mg OV3 each.

The empty hard gelatin capsules were completelly disaggregated, presenting a bioaccesibility of 86.97 % (**Table 46**). Pharmacological activity assays should be performed with the intestinal absorbable fraction of this sample to discard potential bioactivity.

1.2.3 CHEMICAL CHARACTERISATION

The chemical characterisation of the samples before and after *in vitro* gastrointestinal digestion was performed qualitatively (TLC) and quantitatively (HPLC–DAD), with the determination of total phenolic compounds (Folin–Ciocalteau).

1.2.3.1 QUALITATIVE CHARACTERISATION

According to previous TLC results, for qualitative characterisation, the mobile phase with ethyl acetate: glacial acetic acid: formic acid: water (100:11:11:26 v/v/v/v) was selected and NP reagent visualized at 36 nm in order to intensify these characteristic spots (**Figure 82**).

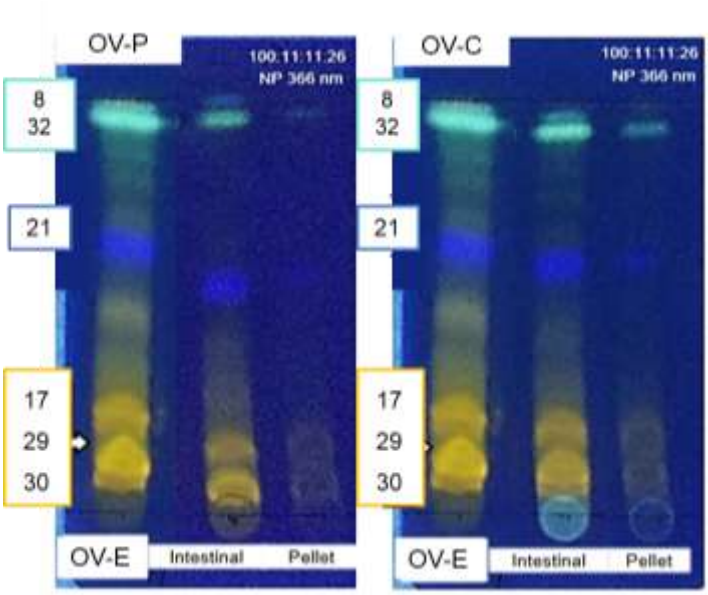


Figure 82. Thin layer chromatography (TLC) of the two formulations before and after the process of *in vitro* gastrointestinal digestion revealed with NP solution and observed at 366 nm. Mobile phase: ethyl acetate:glacial acetic acid:formic acid:water (100:11:11:26 v/v/v/v). OV–P: dryed powder; OV–C: hard gelatine capsules. OV–E: Crude extract, non–digested hydroalcoholic extract; Intestinal: intestinal fraction after digestion; Pellet: non–absorbable fraction after digestion. Colour of each compound corresponds to the colour observed on TLC with NP reagent: blue for phenolic acids, green for hydroxybenzoic acid and yellow–orange for flavonoids. The numbers corresponds to the main compounds found in HPLC–DAD (**Table 49**).

TLC did not show important differences between the two formulations (**Figure 82**). Exposure to the NP reagent allowed the identification by colour of the main compounds previously described in OV3 hydroalcoholic extract. Green is characteristic of

hydroxycinnamic acids: caffeic acid (peak **8** in HPLC–DAD) and rosmarinic acid (peak **32**); intense blue for dihydroxybenzoic acid (peak **21**) and yellow–orange for flavonoids (peaks **17**, **29**, **30**) (**Figure 82**) [174].

1.2.3.2 QUANTITATIVE CHARACTERISATION

Intestinal absorbable and non–absorbable fractions of OV3 solid oral formulations after gastrointestinal digestion were characterised by HPLC–DAD, allowing identification of the seven main compounds by retention time and UV spectrum using standards with the same conditions (*see Chapter I, chemical quantification, HPLC–DAD quantification*). Besides, the total phenolic compounds were also quantified by the Folin–Ciocalteau method under the same conditions as described in Chapter I *(Chemical quantification, total phenolic compounds in vitro test).*

The amount of the identified compounds was compared between the different samples and was expressed in mg of standard per 100 g of sample (**Table 47**).

Table 47. OV3 crude extract and digested oral formulations. Intestinal absorbable (intestinal) and non–absosbable (pellet) fractions of hard gelatine capsules and dried powder. Amount of each main compound (mg standard per 100 mg dry extract) before and after digestion grouped per type of compound. Intestinal bioaccesibility (%). Last row corresponds to results of Total Phenolic Compounds (TPC) from Folin–Ciocaleaut assay (mean ± SD mg TE per 100 mg extract). Same letter indicates no statistical differences ($p > 0.05$) in the amount of compound between formulations, whereas different letter indicates that there are statistical differences in the amount of compound between formulations ($p < 0.05$). Pellet was not taken into account for statistical analysis.

Type of compound	Compound (peak number)	(mg/100 mg dry extract) Bioaccessibility (%)				
		Crude Extract before digestion	Hard gelatine capsules		Dried Powder	
			Intestinal	Pellet	Intestinal	Pellet
Dihydroxy cinnamic acids	Caffeic acid (**8**)	1.87 ± 0.14[a]	1.63 ± 0.08[b] 87.16 %	0.20 ± 0.09 10.69 %	1.44 ± 0.05[c] 77.00 %	0.24 ± 0.10 12.83 %
	Rosmarinic acid (**32**)	34.10 ± 0.04[a]	18.8 ± 0.01[b] 55.13 %	0.5 ± 0.02 0.01 %	18.3 ± 0.01[b] 53.66 %	0.6 ± 0.01 0.02 %
Dihydroxy benzoic acids	3,4–DHBA (**21**)	18.04 ± 0.11[a]	10.76 ± 0.07[b] 59.64 %	0.41 ± 0.08 0.02 %	11.88 ± 0.09[b] 65.85 %	0.60 ± 0.05 0.03 %
Flavonoids	Luteolin glycoside (**17**)	7.21 ± 0.03[a]	5.95 ± 0.01[a] 82.52 %	0.91 ± 0.08 12.62 %	5.84 ± 0.02[a] 80.99 %	0.93 ± 0.06 12.90 %
	Luteolin glycoside (**29**)	7.85 ± 0.06[a]	6.54 ± 0.03[b] 83.31 %	0.98 ± 0.04 12.48 %	4.38 ± 0.02[c] 55.79 %	0.93 ±0.02 11.85 %
	Apigenin glycoside (**30**)	7.09 ± 0.09[a]	6.33 ± 0.03[b] 89.28 %	0.85 ± 0.02 11.99 %	3.84 ± 0.02[c] 54.16 %	0.88 ± 0.03 12.41 %
TPC (µg TE/ 100 mg extract)		285.25 ± 0.45[a]	233.33 ± 0.36[b]		209.55 ± 0.21[c]	

Despite the significant losses of phenolic compounds after the digestion process, the encapsulated extract showed a smaller decrease than the powder (18.20 and 26.53 %, respectively with $p < 0.05$).

The main compound detected in the hydroalcoholic extract (OV3) was rosmarinic acid (**32**)**:** 34.10 ± 0.04 mg in the crude extract. This compound had a non–significantly different bioaccessibility when encapsulated (55.13 %) and in powder (53.66 %) after the digestion process. In tendency of preservation, the bioaccessibility of Caffeic acid (**8**) was also significantly higher for the encapsulated extract (87.16 % and 77.00 %, for the encapsulated form and powder respectively ($p < 0.05$)).

3,4–dihydroxybenzoic acid (**21**), with a content 18.04 ± 0.11 mg in the crude extract was another phenolic acid detected. In this case, the bioaccessibility was higher in the powder (65.85 %) than in the encapsulated form (59.64 %), but with no statistical differences were found ($p > 0.05$).

The bioaccessibility of flavonoids (glycoside of luteolin (**29**) and the glycoside of apigenin (**30**)) also depended on the pharmaceutical form, being 83.31 % and 89.28 % for the encapsulated form and 55.79 % and 54.16 % for the powder respectively, with statistical differences between formulations ($p < 0.05$).

The first flavonoid that appeared in the chromatogram (luteolin glycoside, **2**) was potentially bioaccesible in both forms, with no statistical differences: 82.52 % for the encapsulated form and 80.99 % for the powder ($p > 0.05$).

The dyhydroxycinnamic acid derivatives showed a good preservation after the digestion process, being caffeic acid over 75 % for both formulations. In the previous chemical semi–quantification after the digestion process, the intestinal bioaccesibility of pure rosmarinic acid was 79.12 %. Moreover, despite not being formulated, intestinal absorbable fraction of the crude extracts presented 40 % of bioaccesibility in OV2 and 60 % in OV6, meaning that a loss of 50 % in OV3 oral formulations was in consonance with previous results. Then, both formulations seemed to release similarly low quantities of phenolic acids (56.79 % for capsules and 54.87 % for powder) and DHBA (43.55 % for capsule and 42.46 % for powder) in the intestine. However, in the case of flavonoids, the quantities depended heavily on the formulation (84.96 % for encapsulated form and 63.48 % for powder).

Concluding, the main phytocompounds potentially active in the pathways tested were preserved in both formulations after gastrointestinal digestion, with some differences. However, will these compounds retain the properties found in the crude extract?

1.2.4 BIOACTIVITIES

In the intestinal absorbable fractions of the OV1, OV2 and OV6 extract, previously described in *Chapter II* and *Chapter III*, some pharmacological activities even increased

after digestion. Following the same line, both oral formulations were assayed after a gastrointestinal digestion process.

1.2.4.1 ANTIOXIDANT ACTIVITY

OV3 was a very strong intermediate antioxidant *in vitro*, according to AAI and reaction kinetics (*see Chapter II, antioxidant activity DPPH•*).

Qualitative TLC assay with DPPH• showed that the two formulations retained antioxidant activity after the gastrointestinal digestion process (the purple DPPH• solution turns into yellow in presence of antioxidants, **Figure 83**).

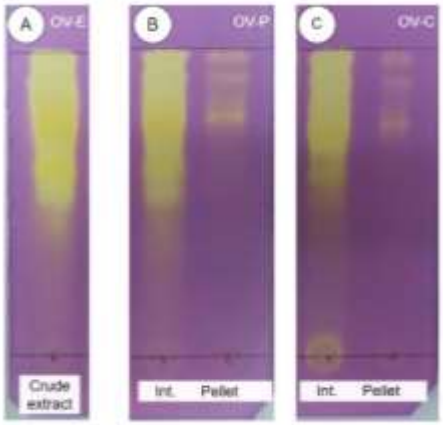


Figure 83. TLC (mobile phase: ethyl acetate – methanol – water (65:15:5 v/v/v)) of oral formulations after digestion compared to OV3, post–sprayed with DPPH• solution in order to detect qualitative antioxidant *in vitro* activity. Yellow spots correspond to positive result to antioxidant activity against DPPH free radical. (A) OV–E: non–digested hidroalcoholic extract (crude extract); (B) OV–P: extract in powder after gastrointestinal digestion; (C) OV–C: extract in capsules after gastrointestinal digestion; Int.: intestinal absorbable fraction; Pellet: intestinal non–absorbable fraction.

The antioxidant activity of the crude extract and the intestinal absorbable fractions from both formulations was also quantified by the DPPH• method, recording the absorbance values at 517 nm every 15 min for 90 min and calculating the EC_{50} values at each time point (**Table 48**). The activity of the non–absorbable fraction (pellet) was not quantified because this part will be excreted without being absorbed [355].

Table 48. OV1 before and after each *in vitro* digestion step (mouth, stomach, intestine). Antioxidant activity expressed as EC_{50} (µg/mL). Data expressed as means ± SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Values in bold means EC_{50} max (stabilisation point).

	Time (min)					
Sample	15	30	45	60	75	90
RA	1.71 ± 0.04 [a]	1.69 ± 0.05 [a]	1.67 ± 0.05 [a]	1.66 ± 0.05 [a]	1.64 ± 0.04 [a]	1.63 ± 0.04 [a]
RA–Int	2.84 ± 0.11 [a]	1.88 ± 0.15 [b]	1.84 ± 0.11 [b]	1.76 ± 0.12 [b]	1.72 ± 0.14 [b]	1.69 ± 0.15 [b]
OV–E	5.24 ± 0.22 [a]	4.06 ± 0.39 [b]	3.68 ± 0.19 [b]	3.47 ± 0.14 [b]	3.23 ± 0.15 [b]	2.88 ± 0.18 [b]
OV–C	4.21 ± 0.14 [a]	3.77 ± 0.30 [b]	3.52 ± 0.41 [b]	3.23 ± 0.31 [b]	3.03 ± 0.23 [b]	2.86 ± 0.18 [b]
OV–P	5.59 ± 0.63 [a]	5.23 ± 0.64 [a]	4.86 ± 0.58 [b]	4.57 ± 0.50 [b]	4.37 ± 0.45 [b]	4.23 ± 0.43 [b]

The reaction of the encapsulated extract after digestion stabilizes at the same time as the non–digested extract (p = 0.945 between EC_{50} at 30 min and EC_{50} at 45 min).The extract administered in powder has a similar behaviour but delayed in time (p = 0.999 between EC_{50} at 45 min and EC_{50} at 60 min).

The pharmaceutical form influenced the antioxidant activity of the hydroalcoholic extract (OV3). When encapsulated, the hydroalcoholic extract did not show statistical differences before and after digestion at the stability time point (4.06 ± 0.39 µg/mL and 3.77 ± 0.30 µg/mL, respectively (p = 0.981)). By contrast, the scavenging activity after digestion of the extract administered as dry powder was significantly increased, meaning a lower antioxidant activity (4.86 ± 0.58 µg/mL, p = 0.001). Both formulations show different antioxidant activities *in vitro* (p < 0.001), despite being a very strong antioxidant (AAI = 5.30 (OV—C) and 4.11 (OV—P)).

The antioxidant activities of the samples and the positive control were statistical different at all time–points (p < 0.001). As expected, the digested empty capsules (used as blank for encapsulated extract) and the blank sample (digestion without sample) did not show any antioxidant activity *in vitro*. For further information, scavenging activity (as % inhibition) was compiled and published in supplementary table S1 in de Torre *et al*. [355].

The other antioxidant activity test performed as a complementary *in vitro* test was with ABT• free radical [243]. **Figure 84** shows the results in terms of mg of TE per 100 mg of extract for the intestinal fractions of the oral formulations (powder and capsule) and the extract before digestion.

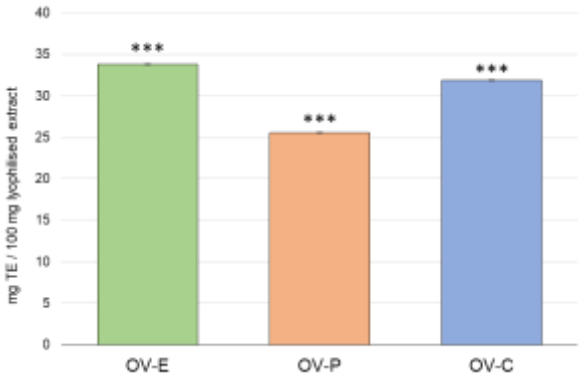


Figure 84. Results from ABTS• *in vitro* test expressed as amount of TE per 100 mg of lyophilised extract (mean ± SD mg/ 100 mg). OV–E: OV3 crude extract; OV–P: intestinal fraction of extract administrated as powder; OV–C: intestinal fraction of encapsulated extract. ***, statistical differences (p < 0.001).

The results after digestion were statistically different from the OV3 extract before digestion. As expected, the encapsulated extract presented lower losses after digestion than the powder (6.06 % and 32.35 %, respectively). Despite the losses, the capsule again showed better preservation of the antioxidant properties of the extract than the extract formulated

in powder. The general order of testing requires experimental *in vivo* models before testing in humans. Again, *C. elegans* would predict the *in vivo* preservation of bioactivities found *in vitro*. Taking advantage of the simple and reliable method to quantify antioxidant activity *in vivo* (*see Chapter III, antioxidant activity in vivo*, [254]), DPPH• scavenging activity inside the worms was monitored over time (**Table 49**). The results expressed in EC_{50} were represented as a function of time.

Table 49. OV1 before and after each *in vitro* digestion step (mouth, stomach, intestine). Antioxidant activity *in vivo* expressed as EC_{50} (µg/mL). Data expressed as means ± SD of triplicate analysis. Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). Values in bold means EC_{50} max (stabilisation point).

	Time (min)					
Sample	15	30	45	60	75	90
RA	11.03 ± 0.49[a]	9.62 ± 0.65[a]	8.98 ± 0.30[a]	8.50 ± 0.33[b]	8.09 ± 0.31[b]	8.04 ± 0.25[b]
RA–Int	10.34 ± 0.63[a]	9.22 ± 0.56[a]	8.49 ± 0.59[b]	8.17 ± 0.58[b]	7.91 ± 0.56[b]	7.88 ± 0.59[b]
OV–E	31.35 ± 1.41[a]	30.61 ± 1.50[a]	28.74 ± 0.58[b]	27.38 ± 0.62[b]	26.38 ± 0.70[b]	25.69 ± 0.42[b]
OV–C	26.33 ± 1.73[a]	24.34 ± 1.31[a]	21.14 ± 1.60[b]	18.92 ± 1.04[b]	18.23 ± 0.83[b]	17.59 ± 0.57[b]
OV–P	31.59 ± 2.18[a]	30.71 ± 2.50[b]	29.62 ± 1.84[b]	28.02 ± 1.61[b]	26.71 ± 1.66[b]	25.68 ± 1.23[b]

Before digestion, rosmarinic acid behaves as a slow antioxidant (stabilisation point between 60 and 75 min), while after digestion its kinetics were intermediate (between 45 and 60 min). The extract showed a different behaviour in stabilisation depending on the pharmaceutical form. The extract administered in powder showed similar results to rosmarinic acid, reaching a stabilisation earlier than the crude extract (before digestion: $p = 0.343$ between min 45 and 60; after digestion: $p = 0.825$ between min 30 and 45). By contrast, the encapsulated extract needed the same time as the non–digested extract to reach the stabilisation time point ($p = 0.912$ between min 45 and 60).

After stabilisation of the reaction, rosmarinic acid did not show statistical differences ($p = 0.054$) between the *in vitro* digested sample (RA–Int) and the pure compound (RA) (8.50±0.33 µg/mL and 8.49±0.59 µg/mL respectively).

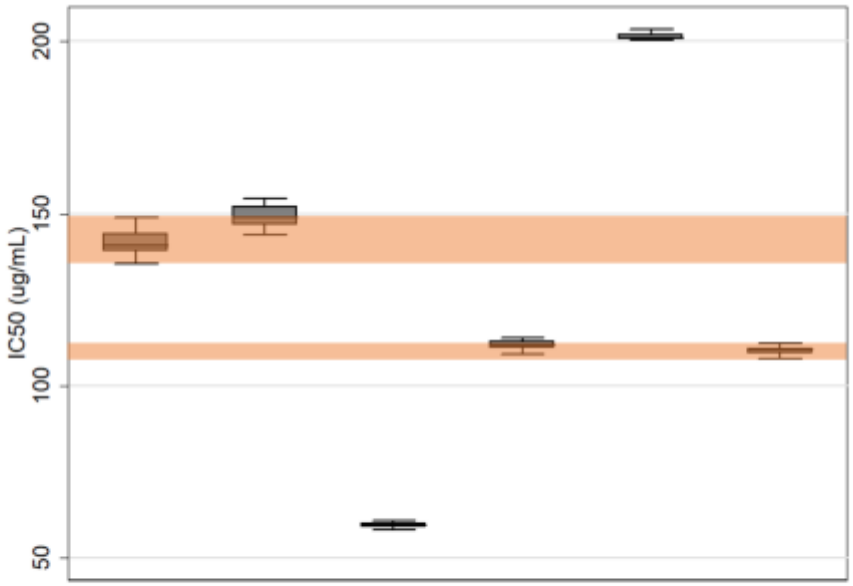
Again, the scavenging activity of hydroalcoholic extract seems to depend on the pharmaceutical form in which the gastrointestinal digestion was performed. Intestinal absorbable fraction of the extract as powder showed significantly lower activity ($p < 0.001$) *in vivo* than the encapsulate form at the stabilisation point (30.71±2.50 µg/mL and 21.14±1.60 µg/mL, respectively). When compared to the *in vivo* antioxidant activity of the crude extract, the powder form did not show differences ($p = 0.209$, 28.74±0.58 µg/mL before digestion and 30.71±2.50 µg/mL after digestion) but the encapsulated form showed significant differences with both of them ($p < 0.001$).

Furthermore, there were statistical differences between the samples and the positive control ($p < 0.001$). As expected, the digested empty capsules (used as blank for encapsulated extract) and the Blank sample (digestion without sample) did not show any

antioxidant activity *in vivo. For further information, scavenging activity (% inhibition) is compiled in supplementary table S2 in de Torre et al.* [355].

1.2.4.2 HYPOGLICAEMIC ACTIVITY

Although the hard gelatin capsules were completely disaggregated, the intestinal fraction of the empty capsules used as control in the digestion were also tested against *α–glucosidase* in order to discard any bioactivity of this formulation container. As shown in **Figure 85**, the empty capsules did not present any potentially inhibitory activity, being IC_{50} = 201.51 ± 1.12 µg/mL, with $p < 0.001$ for the rest of results.



	Acarbose	OV-E	OV-P	OV-C	Empty capsules	Acarbose Intestinal
IC_{50} (µg/mL) Mean ± SD	141.47 ± 3.57 [a]	149.40 ± 3.36 [a]	59.55 ± 0.85 [b]	111.86 ± 1.25 [c]	201.51 ± 1.12 [d]	110.10 ± 1.15 [c]

Figure 85. *α–glucosidase* inhibitory activity expressed as IC_{50} (mean ± SD µg/mL). Orange line represents positive control before (Acarbose) and after (Acarbose Int.) digestion. OV–E: non–digested hidroalcoholic extract (OV3 crude extract) OV–P: dry powder after gastrointestinal digestion; OV–C: capsules after gastrointestinal digestion; Empty caps: blank control of hard gelatine capsules after gastrointestinal digestion. Table below shows IC_{50} values (mean±SD µg/mL). Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$).

According to the results, two formulations after the digestion step presented higher bioactivity than the extract before digestion. The encapsulated extract (111.86 ± 1.25 µg/mL) did not present statistical differences with the intestinal fraction of the positive control: (110.10 ± 1.15 µg/mL) with $p = 0.282$. However, the extract in powder showed the highest hypoglycaemic because the IC_{50} value was the lowest (59.55 ± 0.85 µg/mL) with $p < 0.001$.

In a previous study with flavonoid fisetin from leaves of *Rhus succedanea* L [356], which was more active than acarbose, encapsularion in nanoparticles did not improved this inhibitory activity against *α–glucosidase*. Using the computational molecular docking approach, the authors explained their resuls in relation to the structural determinants involved on the interaction between flavonoids and the enzyme, considering that the 3',4'–dihydroxyl groups of B ring in flavonoids are crucial in enganging direct binding with the active–site residues. Since the flavonoid content was higher in the intestinal fraction of the capsule than in OV–P, our hypothesis relays on the fact that in OV–P, the binding site of flavonoids might be uncluttered and the chemical compounds are already completely released, comparing to the physical protection of the capsule as a formulation container. Thus, the extract given as powder clearly showed to enhance hypoglycaemic acitivty.

1.2.4.3 LIPID–LOWERING ACTIVITY

HMG–CoA *reductase* is the enzyme whose inhibition lead to a diminution of cholesterol levels. Among the existing drugs, statins are one of the most used for this pathology, and with a higher rotation in community pharmacy. In the previous test (*see Chapter II, hypolipidemiant activity*), the intestinal fractions of the aqueous extracts presented moderate inhibitory activity (OV1—Int = 54.84 ± 0.11 % and OV2—Int = 66.52 ± 0.06 %). However, under the conditions in which the assay was performed, none of the formulations of OV3 reached 50 % of inhibition but statistical differences were found. At a concentration of 50 µg/mL, the encapsulated extract inhibited 40.54 ± 1.32 % of the enzyme, whereas OV3 administrated as powder resulted in an inhibition percentage of of 6.57 ± 1.06, with $p < 0.001$.

Providing that this activity could be considered as a complement in bioactivity that could be of interest for further studies, the encapsulated extract could better help with the reduction of cholesterol, as part of the treatment for metabolic syndrome in elderly patients. Indeed, a potential treatment for age–related diseases should also contemplate the effect of delaying dementia. Henceforth, the intestinal encapsulated extract sample and the intestinal absorbable fraction of the extract administered in powder form were also tested against one of the most—well known pathways of dementia pathology: acetylcholinesterase inhibitory activity as a possible cause of Alzheimer's disease.

1.2.4.4 ACETYLCHOLINESTERASE INHIBITORY ACTIVITY

The inhibitory activity against acetylcholinestease enzyme was tested for the two solid oral formulations of OV3 after the *in vitro* digestion process. **Figure 86** below compiles final

IC_{50} values (mean ± SD mg/mL) and the increase in activity relative to the extract before digestion.

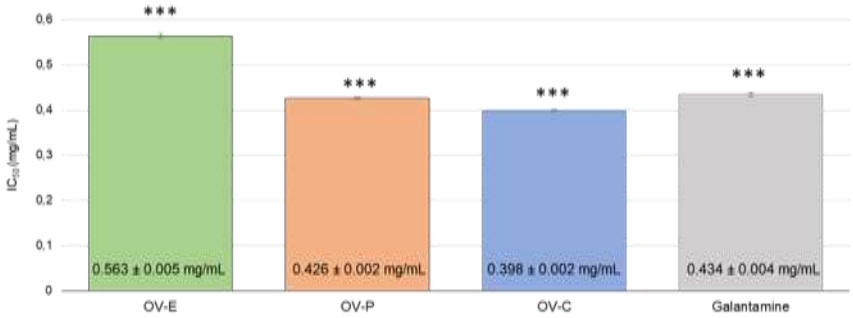


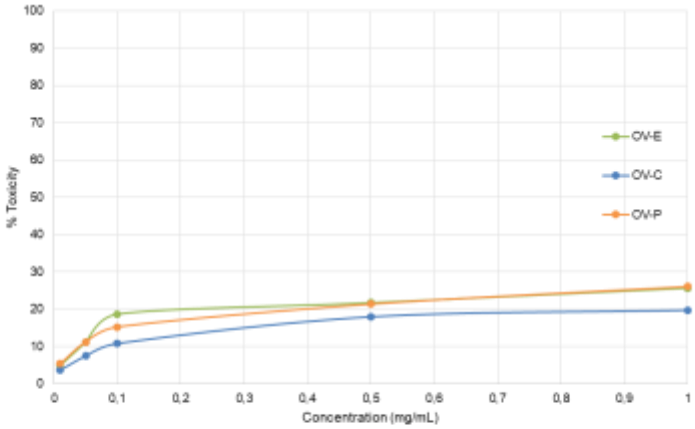
Figure 86. Acetylcholinesterase inhibitory activity of oral formulations expressed as IC_{50} values (mean±SD mg/mL). OV3–E: crude extract, OV3–P: dry powder extract ; OV3–C: encapsulated extract and Galantamine corresponds to positive control. ***: statistical differences among results ($p < 0.001$).

Despite being OV3 one of the least inhibitory extracts (*see Chapter II*), this activity presented a statistical significant increase after digestion, being 24 % for the powder and 29 % for the encapsulated extract (with $p < 0.001$). Besides, both formulations presented a lower IC_{50} value than the positive control: 0.426 ± 0.002 mg/mL (dry powder) and 0.398 ± 0.002 mg/mL (capsule) against galantamine (0.434 ± 0.004 mg/mL), with $p < 0.001$ among all of them.

1.2.4.5 CYTOTOXICITY

Caco–2 cell line

Following the methodology described in *Chapter II*, the cytotoxicity activity in the intestinal cell line Caco–2 was tested for both formulations previously subjected to a gastrointestinal digestion process. Intestinal absorbable remains of empty capsules was also analysed in order to discard possible activity of the container. To begin with, the intestinal fraction would be absorbed into the lumen after digestion so; the MTT *in vitro assay* was firstly carried out for Caco–2 cells, as a preliminary establishment of the safety dose for compounds liberated in the intestine (**Figure 87**).



% Toxicity per concentration (mg/mL)					
Sample	0.01	0.05	0.1	0.5	1
OV-E	4.91 ± 0.65^{a}	11.13 ± 0.53^{c}	18.66 ± 0.39^{e}	21.72 ± 0.42^{g}	25.65 ± 0.76^{h}
OV-C	3.65 ± 0.49^{b}	7.43 ± 0.35^{d}	10.80 ± 0.59^{c}	17.95 ± 0.61^{e}	19.74 ± 0.30^{g}
OV-P	5.37 ± 0.55^{a}	11.24 ± 0.39^{c}	15.25 ± 0.52^{f}	21.37 ± 0.46^{g}	26.05 ± 0.34^{h}

Figure 87. Cytotoxicity against Caco–2 of oral formulation expressed as percentage of toxicity (mean ± SD %, y–axis) per concentration (mg/mL, x–axis). OV3–E: crude extract; OV3–C: encapsulated extract after digestion; OV3–P: dry powder after digestion. Table below shows values of toxicity per concentration (mg/mL) (mean±SD %). Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$).

At first glance, none of the samples tested could be considered toxic to Caco–2 cells within the concentrations analyzed because the percentage of toxicity in the MTT assay did not even reach 30 % (a toxicity level greater than 50 % might be considered toxic [229]). Besides, the toxicity of the samples responded to a dose–dependance. The dry powder extract did not present any statistical difference with OV3 before digestion ($p > 0.05$), with the exception of 0.1 mg/mL where $p < 0.001$ (18.66 ± 0.39 % and 15.25 ± 0.52 %, respectively).

The encapsulated extract showed less toxicity, its percentages being significantly lower at the same concentration ($p < 0.001$). However, as shown in **Figure 87**, the toxicity determined at 0.05 mg/mL for OV–C ad OV–E was not significantly different than the toxicity of OV–P at 0.1 mg/mL (10.80 ± 0.59 %), with $p = 0.955$ with OV–E and $p = 0.317$ with OV–P. In this trend, the toxicity of OV–E at 0.1 mg/mL was not significantly different to toxicity of OV3–C at 0.5 mg/mL (17.95 ± 0.61 %) with $p = 0.945$. Lastly, 1 mg/mL of OV–C produced a similar toxic effect than OV–P and OV–E at half the concentration (0.5 mg/mL), with no significant differences: OV–C 1 mg/mL = 19.74 ± 0.30 % *vs.* OV–P 0.5 mg/mL = 21.37 ± 0.46 % and OV–E 0.5 mg/mL = 21.72 ± 0.42 %, with $p = 0.172$ and $p = 0.755$. A low toxic effect was found in both oral formulations, with the encapsulated extract being half as toxic as the powder.

HepG–2 cell line

Cytotoxicity was tested in HepG–2 cell line for the intestinal absorbable fractions of both solid oral formulations, under the same conditions as in *Chapter II*. Succeeding conceivable cytotoxicity to determine was for the liver: HepG–2 cell line. As in *Chapter II* with crude extracts, the standard MTT and the two novel variants of this cytotoxicity test were carried out with the two intestinal fractions of the oral formulations (**Figure 88**).

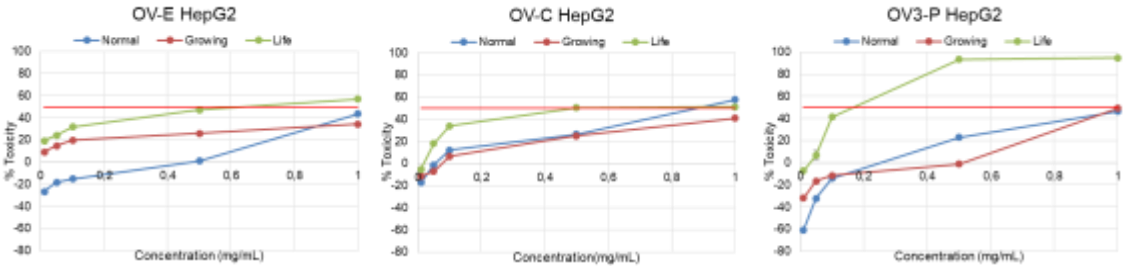


Figure 88. Results for cytotoxicity in HepG–2 (mean ± SD %) at concentrations tested. Percentage of toxicity (mean ± SD %, y–axis) per concentration (mg/mL, x–axis). Blue line corresponds to *normal* MTT assay; red, for *growing* MTT method and green for *life* MTT method. OV–E: Crude extract; OV–P: extract formulated as powder and OV–C: extract formulated as capsules.

The formulations presented greater toxicity at high concentrations (over 0.1 mg/mL) for prolonged exposure (*life* MTT method), being greater for powder: 93.19 ± 0.32 % at 0.5 mg/mL and 94.77 ± 0.28 % at 1 mg/mL, with p = 0.122. The rest of methods performed showed toxicity levels below 50 %, with the exception of the encapsulated extract at 1 mg/mL for the *normal* MTT assay: 57.72 ± 1.00 %. Nevertheless, at low concentrations (up to 0.1 mg/mL), results of toxicity were below 20 % of toxicity. Previous studies on HepG–2 determined the safety of rosmarinic acid – the main compound present in the formulations –, with a cytotoxicity of 34 ± 3% for 1 mg/mL in *normal* MTT assay [229]. This hepatoprotective effect could be observed correspondingly in OV3 administrated as powder (with at least 30 % of RA), being cytotoxicity at 1 mg/mL = 46.27 ± 1.86 %, whereas the cytotoxicity of the encapsulated extract was 57.72 ± 1.00 %, in the same conditions. Conversely, high levels of cytotoxicity were observed at high concentrations of powdered extract on prolonged exposure to the liver. In any case, these particular hepatotoxic findings should be deeply rooted in further pharmacokinetic studies with metabolite half–life determination.

2 TOPICAL ADMINISTRATION

As for oral administration, requests for natural products are increasing over the counter: antihemorroid creams with *Ruscus aculeatus* L. and *Aesculus hyppocastanum* L., painkiller and anti–inflammatory creams containing *Arnica montana* L., refreshing creams with vascular protective plants for heavy legs… Either because of their efficacy–safety or because of their symptoms, phytomedicines are gently featuring in society.

Furthermore, in the current market, there are plenty of products with medicinal plants for cosmetic purposes. The antioxidant properties of some medicinal plants can be used topically to preserve the skin from wrinkles. In this sense, many laboratories are looking for antioxidant compounds capable to penetrate the skin with higher preservation than vitamin C.

Oregano is rarely marketed for cutaneous use depite the fact that the EMA collects topical indications for the two species of oregano with monograph [321,322]. On the one hand, *O majorana* indicated for topical use as extract (1:5) with 96 % v/v ethanol and white petrolatum as extraction solvents for the relief of irritated skin around the nostrils. On the other hand, the EMA also contemplates cutaneous use of *O. dictamnus* as an herbal infusion or decoction for the relief of minor skin inflammations and bruises.

2.1 MOLECULAR CHANGES IN SKIN CELLS

The Fourier Transform Infrared (FTIR) spectrophotometer is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds, since it probes the molecular vibrations of functional groups [357]. The wavelength of light absorbed is characteristic of the chemical bond as can be seen from the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a sample can be determined. Taking this chemical tool into a biochemical environment, when the sample is a cell, chemical bonds (C–H, C=H, C=O…) of molecular components of cells can be analysed (lipids, proteins, phospholipids, charbohydrates…). Moreover, cells that receive a previous treatment might suffer molecular changes that this chemical technique could detect.

In *Chapter II*, the cytotoxicity of extracts in A375 was carried out in three different modalities. However, this complementary technique would indicate molecular changes produced in the cells by the extracts that lead cells to either apoptosis or life.

2.1.1 MATERIAL AND METHODS

- Experimental basis

As a deeper study of the effect produced in this cell line (A375), the molecular modifications in the cells were determined by FTIR, where each wavelength band can be attributed to a molecular group composing eukaryotic cells. According to previous studies [358], these are the main regions to take into consideration for the analysis of spectra and data obtained (**Table 50**).

Table 50. Correspondance of each wavelength range to cellular molecules.

Wavelength range (cm^{-1})	Chemical group	Cellular association	Reference
4,000 – 3,100	–OH N–H	*Overlapped by water absorption* *Not shown in graph*	357,359
3,050 – 2,800	CH2 CH3	Lipid acyl chains Proteins	359,360
2,800 – 1,800		*No absorption from cells*	
1,725	C = O ester	Phospholipids	360–362
1,650	C = O	Amide I	
1,540	N–H	Amide II	
1,480 – 1,300		Aminoacids side chains Fatty acids	
1,300 – 900		Carbohydrates RNA and DNA	359,363

Figure 89 shows an example of IR chromatogram and the areas to take into consideration for the interpretation of molecular changes, whose wavelength limits are shown above in **Table 50**.

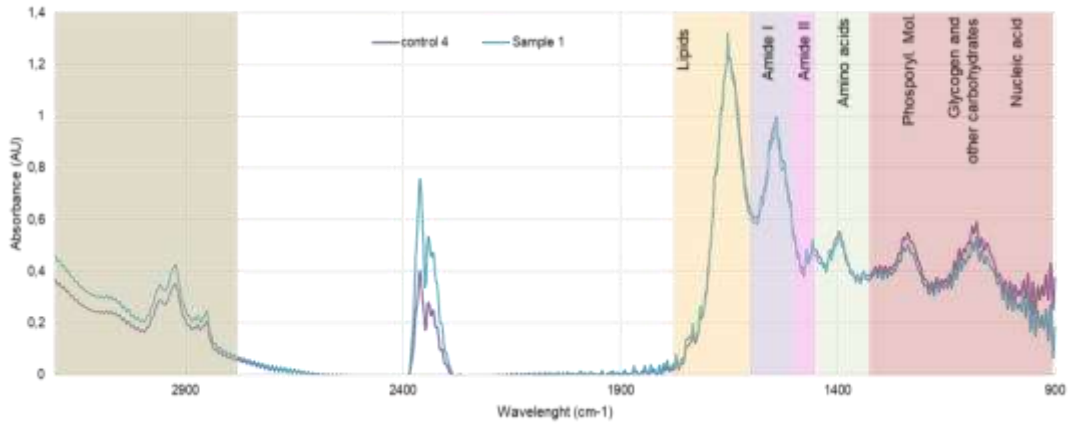


Figure 89. Example of a FTIR spectrum: control (purple) corresponds to control and Sample 1 (blue) to OV1 0.5 mg/mL as example of spectrum. Each fraction corresponds to the wavelength range attributed to a molecular group in cells due to the signal of bindings.

- A375 Cell culture

Cell culture was carried out following the protocol described previously (*see Chapter II, cytotoxicity in A375*).

- Sample preparation

The six crude extracts (OV1 – OV6) were dissolved in DNEM medium (without supplementation, denominated as *free medium*) at different concentrations (0.25 and 0.50 mg/mL). Lack of sample was used as a negative control.

- Analysis conditions

Molecular modifications were monitored by Fourier Transform Infrared (FTIR) after 24 h of treatment with the corresponding extract, according to previous studies carried out in the department of the University of Lisbon [229] (**Figure 90**).

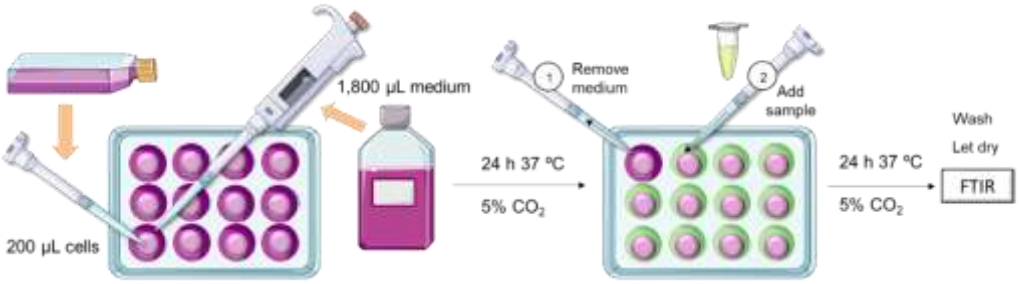


Figure 90. Scheme of FTIR method in cells: the cells were grown on a glass surface at 37 ºC and 5 % CO_2 for 24 h. Then, the medium was replaced by sample at different concentrations in fresh medium and incubated for 24 h. Molecular changes in the cells were determined by FTIR analysis after washing and cleaning the glass containing cells.

12–well plates were used, where the cells were grown in DMEM medium on a glass surface introduced into each well (**Figure 90**). Once they grew (48 h at 37 ºC with 5 % saturation of CO_2), extracts were added at two concentrations (0.25 and 0.50 mg/mL in the medium) to medium. Absence of treatment was used as a control.

After 24 h of treatment, the glasses were washed with water, cleaned with isopropanol, let dry and analysed on Thermo Electron Corp. ® Nicolet ™ FTIR spectrometer with DTGS detector. A resolution of 4 cm^{-1} in the range of 4,000 – 9,000 cm^{-1} were selected for spectra recording, and an empty calcium fluoride window was used as background.

Assay was performed in triplicate with at least three measures per condition and replica.

- Data process

Absorbances at different wavelength were transformed as proportions, regarding standard wavelength absorptions for the main chemical bonds attributed to the main molecular cell compounds:

- Lipids in the 1500–1300, 1800–1700 and 3000–2800 cm^{-1} spectral ranges.
- Carbohydrates and Nucleic acid in the 1300–950 cm^{-1} spectral range.
- Proteins in 1700–1600 cm^{-1} spectral range.

To simplify the discussion of results, they will be given as a ratio with respect to control (no treated cells).

2.1.2 RESULTS

The results of the FTIR spectrum from all the extracts (OV1–OV6) were transformed into intensity ratios of of each specific IR absorption band and the values of these ratios were expressed as a percentage of modification regarding the control for each condition (**Table 51**). In that sense, an increase or decrease of each ratio (corresponding to determining the molecular group in cell [363]) (**Table 51**). No previous studies were found that tested molecular changes in this cell line, but the results obtained were discussed in accordance with published studies in other cell lines [229,363].

Table 51. Results FTIR in A375 cell line for the six extracts expressed as ratios regarding control (absence of treatment). Each row corresponds to a molecular group of interest identified by wavelength range. Proportion to control (value = 1).

Sample	OV1		OV2		OV3		OV4		OV5		OV6	
Conc. [mg/mL]	**0.25**	**0.50**	**0.25**	**0.50**	**0.25**	**0.50**	**0.25**	**0.50**	**0.25**	**0.50**	**0.25**	**0.50**
CH_3/CH_2	0.71	0.96	0.96	0.96	0.96	0.89	0.99	1.00	1.01	1.01	0.93	0.96
CH_2/COOH	1.71	0.90	0.92	0.89	0.94	1.18	0.70	0.76	0.83	0.76	0.83	0.73
COOH/Amide II	1.33	1.00	1.08	1.19	1.15	1.13	1.65	1.41	1.39	1.43	1.59	1.70
Glyco/Amide II	0.53	1.41	0.80	1.34	1.56	1.48	1.23	1.21	1.15	1.07	1.15	1.22
PO_2–asym/ PO_2–sym	1.12	0.99	1.01	0.99	0.95	0.97	1.03	1.02	1.06	1.05	1.03	1.02
PO_2/Amide II	2.29	0.90	0.98	1.07	1.08	1.38	1.17	1.08	1.15	1.10	1.32	1.24
Amide I/Amide II	0.93	1.03	0.98	1.01	0.97	1.01	0.97	1.01	0.97	1.00	1.02	1.00
DNA/Amide II	0.64	0.83	0.88	0.92	1.03	0.87	1.08	1.04	1.04	1.03	1.02	1.05
Glyco/DNA	0.80	1.69	0.87	1.45	1.52	1.67	1.14	1.16	1.11	1.04	1.13	1.17
RNA/DNA	0.36	1.13	0.99	1.02	0.96	0.95	1.28	1.11	1.09	1.11	1.04	1.07
Protein/RNA	7.13	1.07	1.15	1.07	1.02	1.30	0.73	0.86	0.89	0.87	0.94	0.90

The ratio between $\upsilon_{as}(CH_2)$ and $\upsilon_{as}(CH_3)$ could be attributed to asymmetric stretching of those radical groups (bands at 2,922 cm^{-1} and 2,955 cm^{-1}), essentially present in lipids [364]. According to the results, no modification regarding control could be observed (ratio close to control = 1.00), except for OV1 at 0.25 mg/mL, where there was a 29 % reduction (**Table 51**). In previous studies with the HeLa cell line [302], the positive control cisplatin presented a minor increase in the length of the hydrocarbon chains, like OV1 at 0.25 mg/mL in A–375.

Cells are composed by different type of lipids and so the composition of lipids in A375 can be analysed by two ratios: COOH/Amide II and CH_2/COOH. This first one is the result of the stretching of the bands at 1733 cm^{-1}, mainly from membrane phospholipids, and at 1540 cm^{-1}, particularly from the amide II band. The second one (CH_2/COOH) could be associated with acyl chains, being the stretching band and the asymmetric stretching of CH_2 band. Both ratios were incremented in OV1 at 0.25 mg/mL (+33 % and +71 %

respectively) and practically equal to control at 0.5 mg/mL. OV2 presented a slightly diminution of acylchains (–8 % at 0.25 mg/mL and –11 % at 0.50 mg/mL) and an increment of membrane phospholipids higher at 0.5 mg/mL (+8 % and +19 %).

This behaviour was also observed in cells when being exposed to OV4, OV5 and OV6: increment of COOH/amide II (+65 % and +41 % (OV4), +39% and +43% (OV5) and +59% and +70% (OV6)) and diminution of acyl chains (–30 % and –24 % (OV4), –17 % and –24 % (OV5) and –17 % and –27 % (OV6)).

After OV3 treatment, acyl chains were reduced by 6 % at 0.25 mg/mL and increased by 18 % at 0.5 mg/mL, whereas membrane phospholipids were increased by 15 % and 13 %, respectively. These minor changes observed in OV2 and OV3 suggest less damage to cells, especially to the protective cell membrane. High variations in these two ratios regarding the control are related with a positive response to stress conditions. In this sense, OV2 and OV3 either did not harm the cells with stress, as much as the other extracts, or provided the cellular mechanisms to protect them against unfavourable conditions [364,365].

Another point indicative of the cell response to stress conditions is an increase in carbohydrates (ratio Glyco / Amide II), also observed in cisplatin [302]. The extracts that induced an increase in carbohydrates in the cells were OV1 (+41 %) and, OV2 (+34 %) at 0.5 mg/mL, OV3 (+56 % and +48 %,), OV4 (+23 % and +21 %), OV5 (+15 % and +7 % and OV6 (+15 % and + 22%) at both concentrations. The aqueous extracts (OV1 and OV2) at low doses (0.25 mg/mL) were the only ones that caused a decrease in carbohydrates in the cell (– 47 % and – 20 %, respectively). Despite the preservation of lipids with OV2 at both concentrations, this significant diminution in carbohydrates might indicate a bad survival to stress conditions when administering low doses of the extract. The OV3 extract presented the best response to stress conditions in terms of carbohydrates regardless of dose.

After analysing the structure and protection of the cell through membrane phospholipids and glycoproteins (carbohydrates), the next cell group of interest is the genetic material. The ratio υ_{as} (PO_2^-) / Amide II, with the band assigned at 1085 cm^{-1}, gives information about modifications in the phosphates of the nucleic acids [365]. The proportion of asymmetric and symmetric PO_2^- was practically the same as in the control, except for the OV1 results at 0.25 mg/mL where there was an increment of 12 %, which could be also related to the increment in 129 % in tthe phosphates of nucleic acids. Previous studies related the decrease in phosphates to a possible induction of apoptosis of the cell [366]. The rest of the extracts did not present relevant modifications regarding the control, with the

exception of OV3 at 0.5 mg/mL (+38 %) and OV6 at both concentrations (+ 32 % and + 24 %). Moreover, the proportion of amides (Amide I / Amide II) was practically the same as in the control for all conditions (ratio close to 1.00).

Continuing with the analysis of genetic material, OV1 at 0.25 mg/mL was the compound that more alterations provoke in the cells: – 36 % in amides II of DNA, –20 % in carbohydrates linked to DNA, – 74 % of RNA / DNA and a highly significant increment in proteins of RNA (+713 %). A presence of proteins seven times higher than in normal cells was observed, which could belong to structures such as enzymes. The rest of the extracts presented a moderate effect on proteins and genetic material (RNA and DNA), with the relevant increment of the ratio of glycoproteins and DNA of OV2 (+45 %) at 0.5 mg/mL and OV3 (+52 % and (+67 %) at two doses, 0.25 mg/mL and 0.5 mg/mL). In OV2 and OV3, the genetic material was not affected *per se*, preserving the essence of the cell. As seen in previous studies [229,363], the significant changes in the cell membrane might reflect the viability of the cells under that treatment, rather than the genetic material that can be intact. In other words, OV2 and OV3 seemed to provoke fewer changes compared to the control.

In summary, OV2 and OV3 were the extract whose impact provoked less stress to this skin cell line, compared to the rest of the extracts.

2.2 PHARMACEUTICAL FORM FORMULATION

2.2.1 MATERIAL AND METHODS

Four pharmaceutical formulations were designed for cutaneous use. Two of them containing 45 mg / 100 g of OV2 for acute use as anti–inflammatory, and the other two, containing OV3 at 15 mg / 100 g with indication of long–term use as venotonic.

OV2 Cream

Formulation:

- Aqueous extract of *O. vulgare* (OV2)	0.045 g
- Neo PCL ® O/W	22 g
- Sweet almond oil	3 g
- Propilenglicol	5 g
- Sodium Nipagin	0.1 g
- Distilled water	q.e.f. 100 g

Preparation:

1. Oil phase (OP): In a laboratory capsule, melt: 22 g of neo PCL (#Mg97451, Guinama, Valencia, Spain) and 3 g of sweet almond oil (#1200003, Acofarma, Madrid, Spain) in a water bath at 70 ºC.
2. Aqueous phase (WP): In another capsule, dissolve 0.1 g of sodium nipagin (#1162522, Acofarma, Madrid, Spain), 0.045 g of OV2 extract and 5 g of propilenglicol (#1167055, Acofarma, Madrid, Spain) in 69.85 g of distilled water.
3. When both phases present the same temperature measured with a termometer, add WP to OP in small portions with continuous mixing.
4. Mix with a mechanical homogeneizer until the complete homogenization and place in an aluminium tube.

Warning: this is an emulsion preparation, so temperatures need to be the same and continuous mixing could be considered the key to avoid damage.

OV2 Stick

Formulation:

- Aqueous extract of *O. vulgare* (OV2)	0.045 g
- Ricin oil	64 g
- Bee white wax	15 g
- Jojoba oil	15 g
- Stearic acid	3 g
- Propilenglicol	2 g

Method of preparation:

1. Melt in a capsule 15 g of bee white wax (#Mg97220, Guinama, Valencia, Spain) and stearic acid (#Mg97836, Guinama, Valencia, Spain) in a water bath at 70 ºC.
2. Melt the oils: 64 g of ricin and jojoba (#Mg94489, Guinama, Valencia, Spain) in a beaker.
3. In a mortar, mix 2 g of propilenglicol (# 1167055, Acofarma, Madrid, Spain) and 0.045 g of OV2 extract until a paste is formed.
4. Introduce the mortar in the water bath and add the rest of mixes (oils and wax with stearic acid) over the paste.
5. Mix until homogenization and add the final mixture into the stick template.

OV3 Gel

Formulation:

- 50 % ethanolic extract of *O. vulgare* (OV3)	0.015 g
- Carbopol	0.5 g
- Menthol	0.25 g
- Sodium Nipagin	0.1 g
- Genciana violet 10 %	2 drops
- Distilled water	q.e.f. 100 g
- Trietanolamine q.e. pH adjustment to 6 – 6.5	

Method of preparation:

1. In a laboratory capsule, dissolve 0.1 g of sodium nipagin (# 1162522, Acofarma, Madrid, Spain) in 99.13 g of distilled water.
2. Add 0.25 g of menthol (#1159615, Acofarma, Madrid, Spain) into previous mix.
3. Add 0.015 g of OV3 extract into the previous mixture.
4. Add 0.5 g of carbopol (#Mg90385, Guinama, Valencia, Spain) and let chill for 24 h.
5. With a pHmeter, adjust pH to 6 – 6.5 with trietanolamine (#1187025, Acofarma, Madrid, Spain).
6. Add 2 drops of genciana violet (#1193394, Acofarma, Madrid, Spain), as colour intensity desired.
7. Mix with a mechanical homogeneizator until being homogenized and place in a proper packaging material such as a dispenser bottle.

OV3 Fluid body milk

Formulation:

- 50 % ethanolic extract of *O. vulgare* (OV3)	0.015 g
- Cetiol V ®	5 g
- Propilenglicol	5 g
- Lanette wax SX ®	3 g
- Sodium Nipagin	0.1 g
- Distilled water	q.e.f. 100 g

Method of preparation:

1. Oil phase (OP): In a laboratory capsule, melt 3 g of wax lanette SW ® (#1118884, Acofarma, Madrid, Spain) and 5 g of cetiol V ® (# 1118933, Acofarma, Madrid, Spain) in a water bath at 70 ºC.
2. Aqueous phase (WP): In another capsule, dissolve 0.1 g of sodium nipagin (# 1162522, Acofarma, Madrid, Spain), 0.015 g of OV3 extract and 5 g of propilenglicol (# 1167055, Acofarma, Madrid, Spain) in water.

3. When both phases present same temperature (measure with a thermometer), add WP to OP.
4. Mix with mechanical homogeneizator until complete homogenization and place in a proper packaging material such as a dispenser bottle.

Warning: this is an emulsion preparation, so temperatures need to be the same and continuous mixing could be considered the key to avoid damage.

2.2.1.1 QUALITY CONTROL ASSAYS

As part of the quality control of the topical formulations designed, several assays were carried out with the four formulations:

- The data of the organoleptic characteristics were collected in a subjectively observable way by determining the colour, the texture in the skin and the smell.
- The pH was determined with a pH–meter.
- The viscosity was determined with a HAAKE viscotester 550 equipment in The Department of Chemistry (University of Navarra), using SV2 mould at controlled temperature of 33 ºC, since it is considered the standard human physiological temperature in the epidermis. The results of viscosity were registered by Rheo Win Job Manager software and expressed in terms strength needed from the equipment to mix the topical sample (MPa/s) along time (gel, cream and body milk).
- The extensibility assay was carried out as dictated by RD 175/2001 (23rd February), which regulates the correct preparation and control of magisterial formulations and pharmaceutical preparations in a Pharmacy [367].

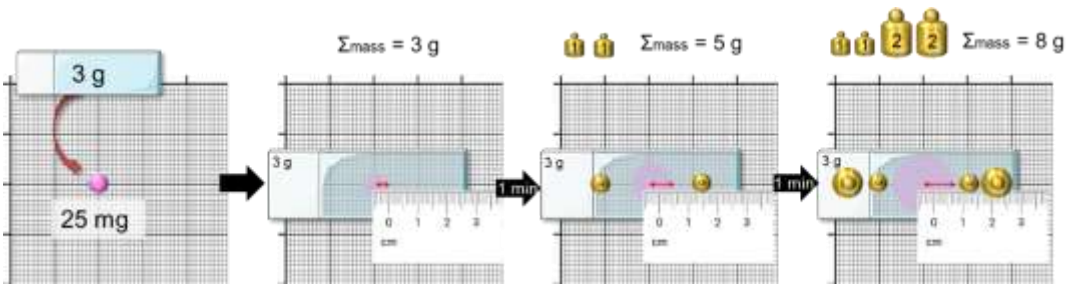


Figure 91. Realization of extensibility quality control for topical formulation.

As shown in **Figure 91**, 25 mg of sample were disposed on a chart paper, covered by glass slide of known mass (3 g) where different known weighs were added on the sides (2 and 3 g). The radius of the circle formed for the different added weights (3, 5, 9 g) was recorded after 1 min to calculate areas. The assay was performed at room

temperature with *n* = 15 for each topical formulation (gel, cream and body milk). Statistical analysis was carried out as for bioactivities in *Chapter II.*

- The uniformity of content was expressed as bioactivity: *in vitro* antioxidant activity after post–formulation recovery from formulation. According to the nature of the extracts, 25 g of each formulation were dissolved in 100 mL of solvent of extraction: distilled water for stick and cream (OV2 extract) and ethanol:water 1:1 v/v for gel and body milk formulation (OV3 extract). Sonication was needed until homogeneity of the mixture, which was vacuum–filtered colleting the liquid. Liquid was later lyophilised in order to be able to weigh for bioactivity assays. Two TLC were prepared and sprayed with NP solution for visualization of chemical composition and the other with DPPH• solution for qualitative antioxidant assay,as part of the qualitative control (*see Chapter I, qualitative chemical characterisation, TLC*) and *in vitro* antioxidant activity was also quantified over time (*see Chapter II, antioxidant activity, DPPH• assay*).

2.2.2 RESULTS

Even though the EMA does not have a monograph for *O. vulgare* L. yet , recent studies have demonstrated the potential use of this medicinal plant topically.Thanks to its antioxidant activity, essential oil of *O. vulgare* was recently suggested to be as a natural active ingredient potentially used for skin–ageing retardation [199]. Some other studies propose the use of *O. vulgare* as a potential anti–acne topical Nano emulsion [190], thanks to the antimicrobial activity of the essential oil. This antimicrobial activity, especially against *P. acnes* (responsible for acne vulgaris, a skin inflammation disorder), was also taken into consideration in the study of an ethanolic extract of *O. vulgare* with anti–inflammatory properties that might enhance the effect of the topical remedy [303].

According to the results obtained, OV2 and OV3 were selected for the design of a topical pharmaceutical form by the following reasoning:

First, the EMA recommends the topically use of other species of oregano prepared as either infusion/decoction or hydroalcoholic extract. For this reason and the toxicity found in MTT assay, OV5 and OV6 were discard for the design of a pharmaceutical form for cutaneous use. Then, OV1 and OV4 showed good results in the rest of the assays performed (low toxicity in A375 and similar anti–inflammatory behaviour: low hemolysis and low denaturalization of albumin). However, FTIR results made OV1 be discarded for topical use. Then, the modifications observed in OV2, and OV3, especially in the genetic material, lead us to select them for the design of a pharmaceutical form for topical administration.

Besides, the FTIR results showed different behaviour of cells when OV2 was administered in low doses of 0.25 mg/mL. Therefore, OV3 could be administered at either 0.25 mg/mL or 0.5 mg/mL, whereas a dose of 0.5 mg/mL would be recommended for OV2 administration.

The results of the MTT assay showed that OV2 reached 50 % of toxicity at 0.2 mg/mL in the *Growing* variant of method (72 h of exposure) and 0.4 mg/mL in the *Life* variant of the method (72 + 24 h of exposure). For that reason, OV2 should not be recommended for long–term use, or number of applications could be less.

Then, the anti–inflammatory assays showed that OV2 protected better to RBC membrane and albumin when administered at high doses, whereas OV3 produced higher haemolysis at highest dose. **Figure 92** shows the appearance of the four formulations prepared:

- OV2: high dose (0.4 – 0.5 mg/mL), short–term use
    - Cream (**Figure 92–A**)
    - Stick (**Figure 92–B**)
- OV3: preferably low dose, long–term use (chronic pathologies)
    - Hydrogel (**Figure 92–C**)
    - Fluid body milk (**Figure 92–D**)

Figure 92. Appearance of topical formulations. **A.** Cream with OV2; **B.** Stick with OV2; **C**. Hydrogel with OV3 and **D.** fluid body milk with OV3.

The cream and stick were selected as suitable topical pharmaceutical formulations for OV2 because they are best applied in small concentrated areas for acute use. In the design of the formulation, the selected concentration was 45 mg per 100 g of formulation. The formulation of the **cream** was an O/W emulsion, where oil phase was formed by Neo PCL ®, an autoemulsionable base providing consistence to the emulsion, and sweet almond oil, composed mainly by oleic acid that provides mousterising, nourishing, demulcent and lubricating properties to the formulation. The extract, distilled water as solvent, sodium

nipagin as a preservative and propilenglicol, a hygroscopic organic compound formed by hydration of propilen oxide, used as solvent mousteriser, formed the aqueous phase. Propilenglicol is a commonly used excipient in cosmetics and pharmaceutical industry thanks to its capacity to dissolve a wive variety of compounds, better than glycerin [368]. According to manufacturer's recommendations [369] and pharmaceutical technology theory, an emollient cream should contain 3 % sweet almond oil, 5 % propilenglicol and, for the desired consistence, 22 % Neo PCL ® O/W.

As seen in **Figure 92**, the **stick** formulation presented the desired and expected higher consistence than cream, obtained thanks to the selection of excipients. Bees wax was used as stiffening agent to provide solidity to formulation. Among the emollients, ricin oil – used since Egyptian times – presents many properties depending on quantity and administration (laxative and purgant when oral administrated, eye pain relieving…); but in topical formulations provides emollient and moisturizing qualities. Jojoba oil, used to smooth the skin and unclog hair folicules in cosmetics, was another emollient in the formulation, along with stearic acid, an effective surfactant, stabilizer and thickener agent [368]. Lastly, propilenglicol was again used, but in lower proportion (2 %), maintaining its role as a solvent.

The two other formulations were desired to be more fluid with a relief effect, containing 15 mg/100g of OV3. On one hand, Carbopol Ultrez ® was used to form the **gel**, since the particles of this polimer change their configuration by increasing their volume and viscosity of the liquid when dissolved in water. Trietanolamine was used to adjust the pH of the formulation so that this effect could chemically occur and nipagin sodium, as preservative. Lastly, genciana violet was the colorant added as tone desired and menthol was selected for the scence and cooling sensation when applying, that could work in a venotonic remedy.

On the other hand, **body milk** formulation was another emulsion, whose oil phase was formed by Lanette wax SX ® as emulsifying and Cetiol V, an oily liquid similar to skin–fats that confires fluidity and permeability to the formulation. In the aqueous phase, OV3 was dissolved in distilled water with propilenglicol as solubilizer moisturizer agent and nipagin as preservative.

Table 52 compiles the observed organoleptic characteristics and the pH determination of the four formulations.

Table 52. Organoleptic characteristics (texture, colour, odour) and pH of the four topical formulations.

Extract	Formulation	Texture	Colour	Odour	pH
OV2	**Cream**	Not greasy	White	Neutral	5.90 ± 0.05
	Stick	Easy thin lay disposed on skin; not greasy	Bone	Neutral	*n.d.*
OV3	**Gel**	Refreshing	Light translucent violet	Menthol	5.58 ± 0.02
	Body milk	Fluid	Light pink	Neutral	5.91 ± 0.03

The appearance of the four formulations was the desirable one with an optimal pH for cutaneous use. Nevertheless, the texture information provided was subjective and this characteristic would be objectively determined through the viscosity and extensibility assay.

For the viscosity test, Sensor SV2 system was selected since is primarily used to measure the viscosity of high viscosity liquids and pastes such as greases, creams, ointments, plastisols,… working in the low to medium shear rate range. Six cm^3 of sample were needed to fill the gap between the inner cylinder (rotor, 10.1 mm radius and 19.6 mm height) and the outer cylinder (cup of 11.55 mm of radius) [370]. The results were compiled in a graph (**Figure 93**) showing the strength needed by the rotor to mix the topical formulation (*y*: viscosity in MPa/s) along time (*x*: Time in s).

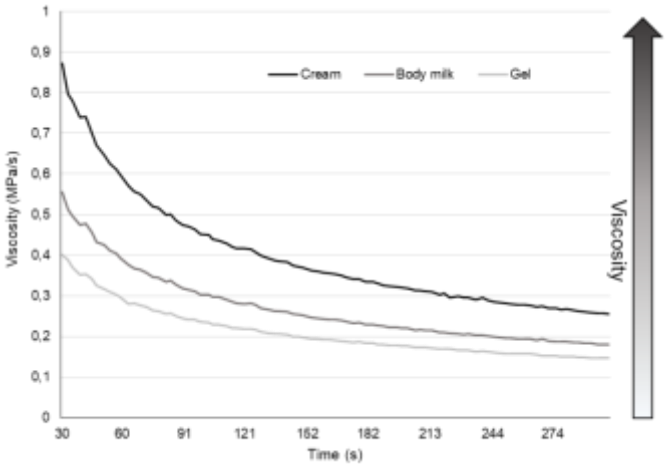


Figure 93. Viscosity expressed in MPas/s along time. From darker to lighter grey: cream, body milk and gel. Narrow on right reminds that higher position of curve in graph means higher viscosity.

The higher the curve is situated on the graph, the higher the viscosity of the formulation presents. The body milk, cream and gel are formulations applied on skin after taking a small amount, whereas the stick was meant to be rubbed on skin leaving a thin lay on the epidermis that would be absorbed over time. For this reason, viscosity measurement was

not considered for the stick formulation. In decreasing order, cream was the most viscous formulation, followed by body milk and gel, with values of 0.592 MPa/s, 0.122 MPa/s and 0.056 MPa/s, respectively, at 141.8 s.

Gels tend to be more spreadble than creams, so the viscosity should be lower, as resulted for published anti–inflammatory topical formulations where gel–creams had a viscosity of 671.9 ± 4.20 and gel, 95.30 ± 4.80 (Pa/s) [371]. As intended, the application of cream would be limited to certain inflammatory situations, whereas venotonic topical formulations (gel and body milk) could be daily applied with a little massage on heavy legs.

The extensibility was also determined and results are compiled in **Table 53**. The glass cover used was the same all the time with a known mass of 3 g.

Table 53. Extensibility expressed as area described by each formulation (mean ± SD, mm^2) after adding known weigh (3, 5 and 8 g). Values with different letter present significant differences ($p < 0.05$) and same letter indicates no significant differences ($p > 0.05$). n.d.: not detected.

	Area described (mm^2) per total weigh added (g)		
Formulation	**3**	**5**	**9**
Cream	7.98 ± 2.14^{d}	54.85 ± 9.06^{a}	58.18 ± 9.23^{a}
Stick	n.d	n.d	n.d
Gel	12.56 ± 0.01^{f}	232.21 ± 27.92^{b}	353.29 ± 49.30^{c}
Body–milk	19.04 ± 2.04^{e}	211.86 ± 13.35^{b}	336.02 ± 28.75^{c}

Again, the extensibility of the stick was not determined for similar practical reasons as viscosity. As weighs were added on the glass cover, the larger the area described was, the more extensible the formulation was. The most viscous formulation (cream) showed lower and limited extensibility, being areas 54.85 ± 9.06 mm^2 with 5 g and 58.18 ± 9.23 mm^2, with no statistical differences ($p = 0.208$). Then, body–milk and gel presented larger areas, meaning higher extensibility, with no statistical differences between the two less viscous formulations: 211.86 ± 13.35 mm^2 and 232.21 ± 27.92 mm^2 with 5 g, respectively ($p = 0.052$). Indeed, only with glass curse (3 g of pressure) cream showed half area than body–milk: 7.98 ± 2.14 mm^2 and 19.04 ± 2.04 mm^2, respectively ($p < 0.001$). Conversely, the gel remained in the middle of the formulations with low weigh on it (12.56 ± 0.01 mm^2). Both topical venotonic formulation presented desirable physical conditions such as low viscosity and high extensibility.

Finally, the uniformity of the mass content was determined as bioactivity of the extract. **Figure 94** shows two TLC; one revealed with NP (observed at 366 nm) and the other with DPPH•, as qualitative antioxidant preliminary assay.

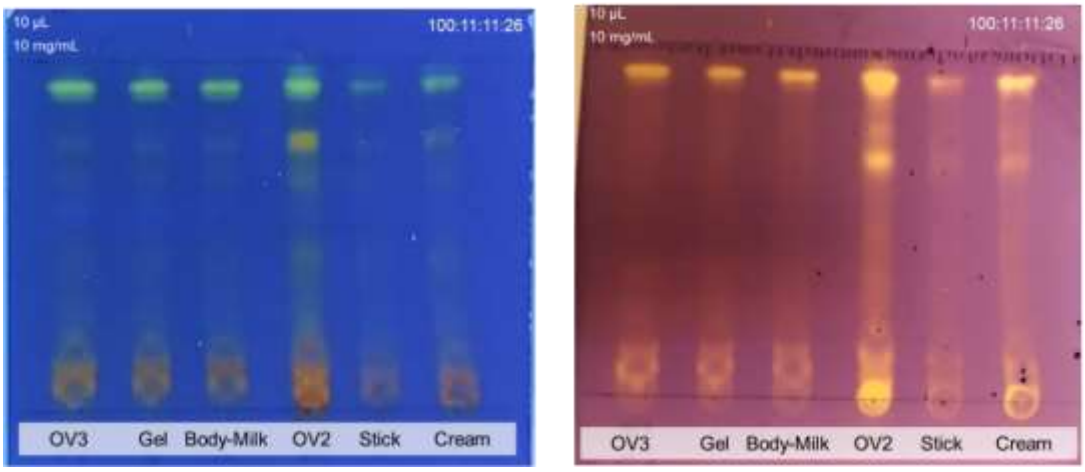


Figure 94. TLC of the four topical formulations and crude extracts they were made of (OV2 and OV3). Mobile phase: Ethyl acetate: Glacial acetic acid: Formic acid: Water (100:11:11:26 v/v/v/v/). On left, TLC sprayed with NP and observed at 366 nm. On right, TLC sprayed with DPPH• for qualitative antioxidant activity.

Chemically, the TLC revealed with NP showed a presence of main compounds in the four formulations in comparation with the crude extracts (OV2 and OV3). A green spot at Rf = 0.80, identified as rosmarinic acid, was observed in the four formulations, as well as yellow–orange spots with low Rf (next to baseline), related to flavonoids (*see Chapter I, qualitative chemical characterisation, TLC*). The qualitative DPPH• assay verified that antioxidant activity was preserved after formulation.

In vitro quantification of antioxidant activity was used as a control assay for uniformity of content in topical formulations. **Figure 95** compiles the EC_{50} values at stabilisation point of the rescued extract of each formulation compared to the crude extract.

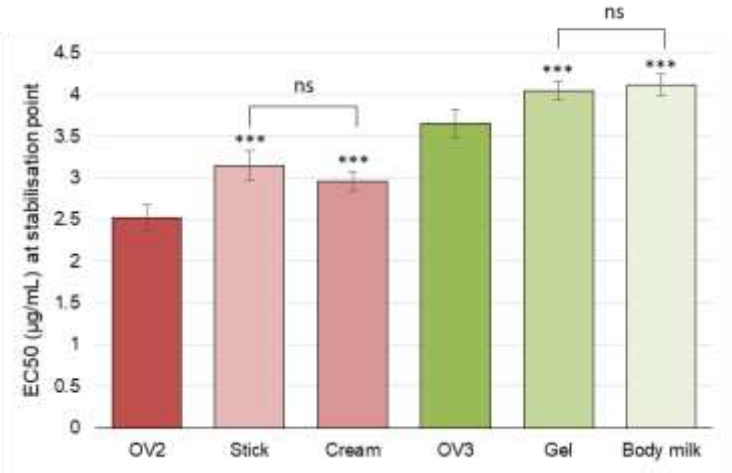


Figure 95. *In vitro* quantification of the antioxidant activity of the rescued extract from topical formulations given as EC_{50} at stabilisation point (mean ± SD, µg/mL). ***: statistical differences ($p < 0.001$) to control (crude extract); *ns*: no significant differences found between formulations.

No statistical differences were found between formulations of the same extract and all four could still be very strong antioxidants. However, the losses in the extraction were reflected

in the activity since the EC_{50} values were statistically different from those of the crude extract. In OV2, EC_{50} increased from 2.52 ± 0.15 µg/mL at stabilisation point to 3.18 ± 0.18 µg/mL and 2.96 ± 0.12 µg/mL, respectively with $p > 0.05$). The formulations with OV3 (EC_{50} = 3.65 ± 0.17 µg/mL) lowered their activity to 4.05 ± 0.11 µg/mL in gel and 4.11 ± 0.13 µg/mL in bodymilk, with $p > 0.05$ between them. Extrapolating the antioxidant activity *in* vitro, 75 – 90 % of content of the extract was recovered and retained the very strong antioxidant property of the crude extracts. Indeed, the antioxidant activity of OV2 formulations was even more antioxidant than the crude extract of OV3 (EC_{50} = 3.65 ± 0.17 µg/mL), OV5 (3.22 ± 0.19 µg/mL) or OV1 (3.91 ± 0.21 µg/mL).

In conclusion, due to the characteristics that the formulations would present, those with OV2 were thought to be indicated for inflammation or wounds caused by hits. The cream formulation is remniniscent of commercial remedies such as Voltaren ® or Fisiocrem ®, whereas the stick could be comparable to Arnidol ®. On the other hand, the results obtained for OV3 lead us to think about formulations to relief heavy legs and favour blood–returning flow of veins (venotonic effect). Thus, a gel with menthol in the formulation would help with the symptomatology by refreshing and fluid body milk would have an optimal texture for a little massage to relieve pain. Quality control assays would objectively determine those characteristics related to the texture requested by the patient. Most of the topical formulations commercialised with *O. vulgare* L. are composed of essential oils, so not many of the published results can be compared with those presented here.

Moreover, this study must be considered as a first approach, being needed further studies on bioactivity and permeability of the topical formulations. Further studies with the topical formulations proposed should consider the use of pig skin, a low cost *in vitro* model with epidermal similarities to human skin [372], before starting with human *in vivo* studies.

DISCUSIÓN

Historicamente, el tratamiento farmacológico de las enfermedades y la producción de medicamentos comenzó con las plantas medicinales. Inicialmente estas plantas se utilizaban siguiendo criterios mágico–religiosos, y paulatinamente, a lo largo de los siglos se ha ido adquiriendo un conocimiento empírico, llegando a lo que se conoce actualmente como fitoterapia [238].

La fitoterapia es la ciencia que estudia la utilización de productos de origen vegetal con finalidad terapéutica, ya sea para prevenir, atenuar o curar un estado patológico. Constituye, por tanto, una parte del arsenal terapéutico del que se dispone en la actualidad para el tratamiento de ciertas patologías leves o crónicas. Esta integración de la fitoterapia en la terapéutica no solo tiene una base histórica, sino también una base química, fundamentada en la estructura de los principios activos.

Los productos fitoterápicos, obtenidos a partir de las drogas vegetales y/o extractos, han estado relegados durante años a un segundo plano por el gran avance de la química de síntesis para la obtención de moléculas químicamente puras, pero, sin embargo, en las últimas décadas, su presencia es cada vez mayor en terapéutica. Se calcula que entre el 60–80 % de la población mundial utiliza fitoterapia en algún momento de su vida y que, en los países desarrollados, el porcentaje de población que lo hace de forma habitual sigue un ritmo creciente. En Europa, la dispensación de plantas medicinales representa el 25 % del mercado global de los medicamentos publicitarios, con un crecimiento anual estimado del 10 %.

Además, no hay que olvidar que una parte importante de los fármacos empleados actualmente en la medicina convencional derivan, de manera directa o indirecta, de moléculas químicamente puras que inicialmente se aislaron de plantas (morfina, colchicina, reserpina, quininida, paclitaxel, entre otros). Muchos de estos principios activos aislados ejercen una acción farmacológica potente y producen efectos inmediatos con un margen terapéutico relativamente estrecho por lo que, por definición, no se pueden considerar medicamentos fitoterápicos.

Por todo lo expuesto, es evidente que la fitoterapia necesita interactuar con otras ciencias que le son más o menos afines. Entre estas se encuentran la botánica, etnofarmacología, farmacognosia, farmacología, química orgánica y tecnología farmacéutica, abarcando desde la obtención de la planta medicinal hasta la forma farmacéutica final, estudiando tanto la composición química como su actividad farmacológica. En este sentido, es importante hacer énfasis en la etnofarmacología, ciencia que estudia los remedios tradicionales basados en plantas medicinales. Esta representa un punto de partida muy importante para el desarrollo de nuevos agentes fitoterápicos y medicamentos, al cual la

farmacognosia contribuirá, principalmente aportando un major conocimiento de sus características morfológicas y/o químicas, la definición de los principios activos y el establecimiento de los métodos y especificaciones para su identificación y control de calidad. Finalmente, corresponde a la fitoterapia el establecimiento de las pautas para un adecuado uso terapéutico a partir, sobre todo, de la investigación clínica.

Si durante el siglo XX se produjo un incremento importante del interés por los productos fitoterápicos desencadenando el importante avance comentado anteriormente de la fitoterapia, en el siglo XXI se está asistiendo al desarrollo de un nuevo concepto denominado fitoterapia racional. Su objetivo es transformar las formas tradicionales de uso (tisanas, infusiones, cocimientos, etc) en productos fitoterápicos con formas farmacéuticas bien diseñadas en las que exista una estandarización de los principios activos y que cumplan todos los requisitos de calidad, seguridad y eficacia como cualquier medicamento comercializado convencional.

Para conseguir esta transformación, existen varios organismos internacionales trabajando, entre los que destacan la *European Scientific Cooperative On Phytotherapy* (ESCOP) [373], el *Committee on Herbal Medicinal Products* (HMPC) de la Agencia Europea del Medicamento (EMA) [155], la Comisión E del *Bundesinstitut für Arzneicimittel und Medizinprodukte* (BfArM) de Alemania [374] y la Organización Mundial de la Salud (OMS) que están realizando un gran esfuerzo en evaluar toda la información disponible de cada droga vegetal y editar los resultados obtenidos en forma de monografías. De todas ellas, actualmente las que mayor importancia tienen son las publicadas por la Agencia Europea del Medicamento (EMA) porque desarrolla monografías consensuadas entre varios países de la UE sobre drogas y preparados vegetales. Distinguen claramente entre las indicaciones tradicionales y las basadas en un uso médico bien establecido, especificando qué preparados pueden acogerse a una u otra categoría. Además, incluyen datos sobre la composición cuali y cuantitativa de la droga vegetal, forma farmacéutica, indicaciones terapéuticas, posología y forma de administración, contraindicaciones, precauciones especiales, interacciones con otros medicamentos o productos, precauciones en el embarazo y la lactancia, precauciones en el manejo de maquinaria y conducción, efectos secudarios y sobredosis.

La EMA avala el empleo en terapeútica de *Origanum dictamnus* L. desde abril de 2013 [322] y el de *Origanum majorana* L. desde mayo de 2016 [239]. Para *O. vulgare* L., sin embargo, hasta el momento, ningún organismo internacional ha publicado una monografía avalando su seguridad y eficacia a pesar de ser una especie muy empleada en la medicina tradicional [29,238,285]. Una de las razones de esta falta de validación puede ser el reducido

número de estudios farmacológicos y clínicos publicados con esta especie. Por esta razón, en este trabajo se ha planteado el estudio de esta especie desde el punto de vista químico, farmacológico y galénico, con el fin de poder contribuir al conocimiento científico de la misma.

El primer paso de una investigación de estas características es la **obtención** de la droga vegetal. Las partes aéreas floridas de *Origanum vulgare* L. subsp. *vulgare* se recolectaron en Santacara (Navarra, España) por la botánica Dra. RY. Cavero. La recolección por un especialista asegura que la identidad de la especie objeto de estudio es la adecuada. Tras la recolección, el siguiente paso es su **conservación** hasta el momento de su empleo como materia prima. Hay que tener en cuenta, que cualquier planta una vez recolectada experimenta rápidamente alteraciones enzimáticas (hidrólisis, oxidaciones, polimerizaciones, etc.) por la presencia de agua, que influyen directamente en la composición química y, en consecuencia, en su actividad terapéutica. Por ello, uno de los objetivos para conseguir una conservación óptima es la eliminación lo más rápidamente posible del agua por diversos procesos de desecación. En este trabajo la droga vegetal se desecó a temperatura ambiente y protegida de la luz hasta que su contenido en agua fue inferior al 10% establecido por la Farmacopea [375].

El siguiente paso en una investigación fitoquímica y/o farmacológica con plantas medicinales es el **proceso de extracción**. La Farmacopea define los extractos vegetales como preparados de consistencia líquida, semisólida o sólida obtenidos a partir de drogas vegetales usando los disolventes adecuados y procesos de concentración posteriores [375]. Se obtienen por maceración, percolación, decocción o infusion de la droga vegetal. En este trabajo, a partir de las partes áereas floridas de orégano se prepararon 6 extractos combinando dos parámetros: la temperatura de extracción (maceración en frío o caliente) y la polaridad del disolvente de extracción (agua, etanol 50 % v/v y etanol) [161,166,376]. Los extractos brutos posteriormente se concentraron en un rotavapor y, finalmente, se liofilizaron para obtener los extractos secos correspondientes. El rendimiento de extracción obtenido demostró que, para esta droga vegetal, no hay una relación entre la temperatura y el disolvente empleado y la cantidad de extracto obtenido. Los extractos acuosos (OV1 y OV2) y los hidroalcohólicos (OV3 y OV4) mostraron una relación droga–extracto (RDE) 3:1, es decir, que a partir de 3 g de droga vegetal se obtiene 1 g de extracto seco. Sin embargo, los extractos etanólicos (OV5 y OV6) presentaron una RDE de 7:1 y 6:1, respectivamente. Aunque desde el punto de vista de rendimiento de extracción los extractos alcohólicos fueron los menos rentables, es necesario comparar estos datos con el perfil químico de los mismos para seleccionar el/los más interesantes química y farmacológicamente.

1 Caracterización de los extractos

La **caracterización química cualitativa** de los seis extractos se realizó por tres técnicas cromatográficas diferentes: cromatografía en capa fina (TLC), cromatografía líquida de alta resolución con detector de diodo array (HPLC–DAD) y cromatografía líquida acoplada a un espectrómetro de masas (LC–MS).

La **TLC** es una técnica sencilla pero muy utilizada para la detección de distintos tipos de productos naturales [174] y figura en todas las monografías de drogas vegetales de la Farmacopea como ensayo de control de calidad cualitativo. En todos los extractos se empleó como fase estacionaria Silcagel ® con indicador de fluorescencia a 254 nm. Se ensayaron dos fases móviles diferentes, una general formada por acetato de etilo:metanol:agua (65:15:15 v/v/v) y otra más específica para compuestos fenólicos (principalmente para detectar flavonoides y ácidos fenólicos) compuesta por acetato de etilo:ácido acético glacial:ácido fórmico:agua (100:11:11:26 v/v/v/v). Todas las placas cromatográficas se revelaron con el reactivo de Godin y con el reactivo *Natural Products*. Los trabajos previos publicados con orégano indicaban la presencia de numerosos compuestos fenólicos [13,19,52,93,195,377]. El análisis de las placas cromatográficas realizado en este trabajo lo confirmó. Sin embargo, no se detectaron aceites esenciales, también descritos en esta especie [11] porque son compuestos apolares y los disolventes empleados en este trabajo para la extracción tienen carácter polar. El extracto acuoso OV1 fue el extracto que presentó un menor número de compuestos y el que mostró mayores diferencias cualitativas con el resto de los extractos desde un punto de vista químico. En todos los extractos se detectaron manchas amarillas correspondientes a flavonoides [21,174], con la diferencia que en OV1 son muy poco intensas. En todos los extractos, excepto en OV1, se detectaron ácidos fenólicos como manchas con fluorescencia blanca–azulada, que podrían corresponder a ácidos hidroxicinámicos descritos en varias especies de orégano, como el ácido 3,4–dihidroxibenzoico, el ácido rosmarinico, el ácido cafeico y sus derivados [13,21,52,128,176,177]. Finalmente, en OV1 se detectó una intensa banda de color marrón a un valor de R_f = 0,00 ausente en el resto de extractos y que podría corresponder a taninos [174], su cuya presencia en el extracto acuoso podría justificarse por su alta polaridad.

El análisis de los extractos por **HPLC–DAD** permitió detectar 39 picos cromatográficos que se clasificaron en 6 tipos de compuestos químicos diferentes en función de su espectro UV: ácidos dihidroxibenzoicos (λ_{max} = 259.4, 293.7 nm), ácidos siríngicos (260–280 nm), aceites esenciales (254 nm), ácidos dihidroxicinámicos (325–329 nm), flavonoides (254, 348 nm) y ácidos salvianólicos (289, 323 nm).

El análisis posterior por **LC–MS** confirmó la naturaleza de estos grupos de principios activos y, además, permitió la identificación de 27 de ellos. El extracto OV1 fue el que presentó un menor número de compuestos químicos, destacando la presencia en exclusiva del ácido 2,5–dihidroxibenzoico y del ácido 3–*O*–cafeoilquínico–metil–éster, junto con algunos flavonoides (oxalato de quercetina, kaempferol–3–galactósido–6–ramnósido–3–ramnósido, eriodictiol–7–O–glucósido y orientina) presentes también en otros extractos. Los otros cinco extractos presentaron una composición química similar, destacando la presencia del ácido 3,4–dihidroxibenzoico y del ácido rosmarínico, ambos identificados en varias especies de la familia *Lamiaceae* [186,211]. Las caracterizaciones químicas previas descritas en esta familia puede servir como guía para realizar el perfil químico, aunque no de manera decisiva ya que dentro de una misma subespecie y extracto algunos compuestos pueden variar dependiendo de la posición del azúcar conjugado, apareciendo nuevas moléculas anteriormente no descritas [194]. Por ejemplo, la presencia de orientina y homoorientina (ambos derivados de luteolina) no fue la misma en todos los extractos, ya que en OV2 ambas no se encontraban. Además, ciertos compuestos pueden sufrir ligeras modificaciones químicas durante el proceso de extracción [209,378,379], como se pudo observar en los ácidos hidroxicinámicos: OV2, OV3, OV4 y OV5 mostraron ácido clorogénico, mientras que en OV6 se pudo formar un tetrámero de ácido cafeico. Por los resultados expuestos hasta el momento, la maceración acuosa en frío (OV1) extrae compuestos de diferente naturaleza química, hecho que coincide con estudios previos realizados en otras especies como el café [208,379]. La polaridad del disolvente y la temperatura del método de extracción pueden modificar la composición química del extracto obtenido, dotando de diferentes aromas a sustancias como el café o quizás cambios en la bioactividad, si el fin del producto es medicinal [127,200].

La **cuantificación** de los compuestos polifenólicos presentes en los seis extractos se realizó por dos métodos. El primero de ellos fue el método de Folin–Ciocalteu [124] para la determinación de polifenoles totales expresados en ácido gálico. Los valores obtenidos confirmaron los resultados cualitativos anteriores. El extracto OV1 fue el que presentó una menor cantidad de polifenoles totales (93,65 ± 0,22 µg /mg extracto), seguido de OV5 (207,64 ± 0,69 µg /mg). Los extractos OV2, OV3, OV4 y OV6 son los que presentaron contenidos mayores y muy similares entre sí (285,25 ± 0,45 – 298,86 ± 0,90 µg /mg). Estos resultados no coinciden con los publicados por otros autores en los que el extracto acuoso muestra un contenido mayor en polifenoles totales [126]. Estas diferencias pueden ser debidas a la variabilidad del material de partida, diferente clima, suelo, desarrollo vegetativo de la planta, proceso de secado, etc, todos ellos factores que influyen directamente en la composición química de la misma.

La variabilidad de cada tipo de compuesto se ha podido establecer gracias a la **semicuantificación de los compuestos individuales por HPLC–DAD**. Para ello se construyeron curvas de calibrado con diferentes estándares en función de la naturaleza química de los compuestos. Para cuantificar los flavonoides se empleó luteolina, ácido cafeico para los ácidos dihidroxicinámicos y salvanólicos, y ácido 3,4–dihidroxibenzoico para los ácidos DHBAs y siríngicos. El ácido rosmarínico se cuantificó de manera independiente por ser el compuesto mayoritario en cinco de los seis extractos analizados y estar su estándar disponible en el mercado. En la **Figura 96** se muestra la distribución de los principales grupos de compuestos en cada uno de los extractos.

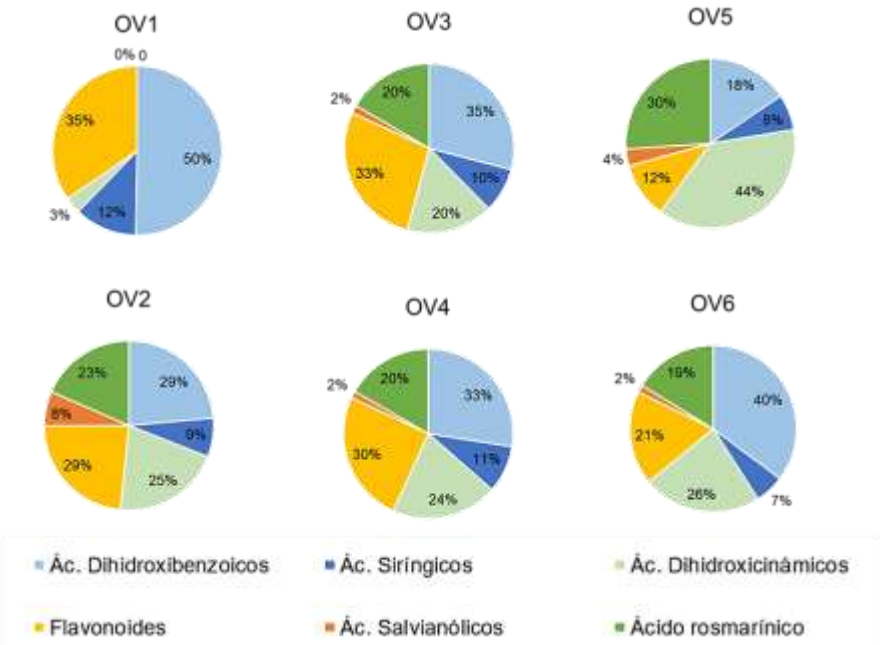


Figura 96. Porcentaje de los grupos de principios activos en cada uno de los extractos.

El análisis de los resultados obtenidos permite clasificar los extractos en tres grupos:

1. Extracto OV1: con alto porcentaje de ácidos dihidroxibenzoicos (46,42 %) y flavonoides (31,56 %). No se pudo cuantificar ni el ácido rosmarínico ni los ácidos salvianólicos.

2. Extractos OV2, OV3, OV4 y OV6: con alto porcentaje de ácidos dihidroxibenzoicos (32 – 33 %) y de flavonoides (15 – 19 %), ácido rosmarínico (30 – 36 %) y bajo porcentaje de ácidos hidroxicinámicos (2 – 8 %).

3. Extracto OV5: con alto porcentaje de ácido rosmarínico (39 %), de derivados de ácidos hidroxicinámicos (18 %) y de ácidos dihidroxibenzoicos (18 %) y bajo porcentaje de flavonoides (12 %).

El alto porcentaje de ácido rosmarínico en cinco de los seis extractos está en consonancia con lo publicado por otros autores [126].

Al igual que en la determinación de los polifenoles totales, los extractos OV2, OV3, OV4 y OV6 presentaron un alto contenido en polifenoles, seguido por OV5 y finalmente OV1, manteniendo una buena correlación entre las dos técnicas de cuantificación empleadas (R^2 = 0,9615, **Figura 97**). Evaluando por grupos de compuestos, se podría decir que el ácido rosmarínico fue el dominante no sólo por su alto contenido en los extractos sino por presentar una muy buena correlación de resultados en las dos técnicas de cuantificación (R^2 = 0,9742, **Figura 97**). Es por ello que podría considerarse como el compuesto de referencia para los extractos, seguido de los flavonoides (R^2 = 0,8148, **Figura 97**), con un alto porcentaje en la semi–cuantificación (más del 20 %, excepto 12 % en OV5, **Figura 96**). En cuanto a los compuestos derivados del ácido dihidroxibenzoico, cuya presencia fue la mayor en porcentaje (**Figura 96**), mostraron una ligera correlación positiva entre las dos técnicas de cuantificación utilizadas (R^2 = 0,5577, **Figura 97**). Sin embargo, tanto los flavonoides como los derivados de DHBA engloban más de un único compuesto individual, a diferencia del ácido rosmarínico que fue aislado en un principio debido a su alta prevalencia individual. En un balance meramente químico, el resto de compuestos quedarían en un segundo plano (R^2 < 0,100, **Figura 97**), aunque sin descartar su posible importancia en el plano farmacológico.

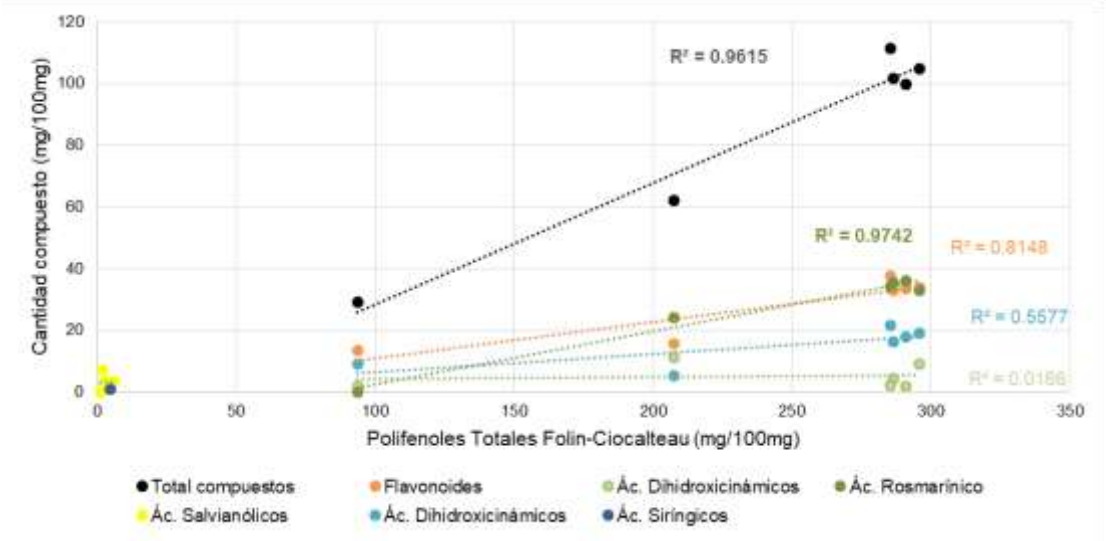


Figura 97. Correlación entre la cantidad de polifenoles totales en los extractos de *O. vulgare* L. determinado por el ensayo Folin–Ciocaletau y la cantidad de compuestos semi–cuantificados por HPLC–DAD.

Las principales acciones farmacológicas descritas en la medicina tradicional para *O. vulgare* se producen después de una administración por vía oral. Esta vía es, desde el punto de vista farmacéutico, la más fácil de diseñar y de administrar y que consigue fácilmente un efecto sistémico cuando los principios activos llegan al torrente sanguíneo. Sin embargo, las condiciones fisiológicas a lo largo del tracto digestivo (pH gástrico, enzimas digestivas, etc) pueden modificar la compleja composición química de los

extractos y, por lo tanto, su biodisponiblidad. Como consecuencia su bioactividad también podría verse afectada dentro del organismo [177,192,380]. En este sentido, la industria farmacéutica está desarrollando nuevas formulaciones galénicas más complejas, como microencapsulaciones y nanopartículas entre otras [177,192,217], que protejan los compuestos químicos responsables de la actividad hasta el momento de su absorción o alcancen el órgano diana correspondiente. Teniendo en cuenta estas consideraciones, el siguiente paso de la investigación aquí desarrollada fue estudiar la **estabilidad química** de los compuestos mayoritarios presentes en los extractos **tras un proceso de digestión gastrointestinal *in vitro***. Para este estudio se seleccionaron tres extractos, OV1 por presentar una composición química muy diferente a los demás, OV2 por ser la forma equivalente a la empleada en la medicina tradicional y OV6 como extracto representativo de la composición química general del orégano.

Aunque se redujo la concentración de algunos de los constituyentes químicos, durante el proceso de digestión *in vitro* no se observó la degradación completa de ninguno de ellos. Los cambios observados en los índices de bioaccesibilidad intestinal se pudieron deber principalmente a procesos de isomerización, hidrólisis, y baja solubilidad en agua, entre otros [381].

Los tres extractos presentaron una bioaccesibilidad del 100 % en la etapa bucal y estomacal. A nivel intestinal también se obtuvieron resultados muy interesantes, alcanzando valores superiores al 80 % en todos los casos, siendo superior en los extractos OV1 y OV2 (94,60 % y 94,85 %) que en el extracto OV6 (80,48 %).

Sin embargo, analizando la bioaccesibilidad de los distintos grupos en principios activos, no se obtuvieron resultados similares entre los tres extractos (**Figura 98**).

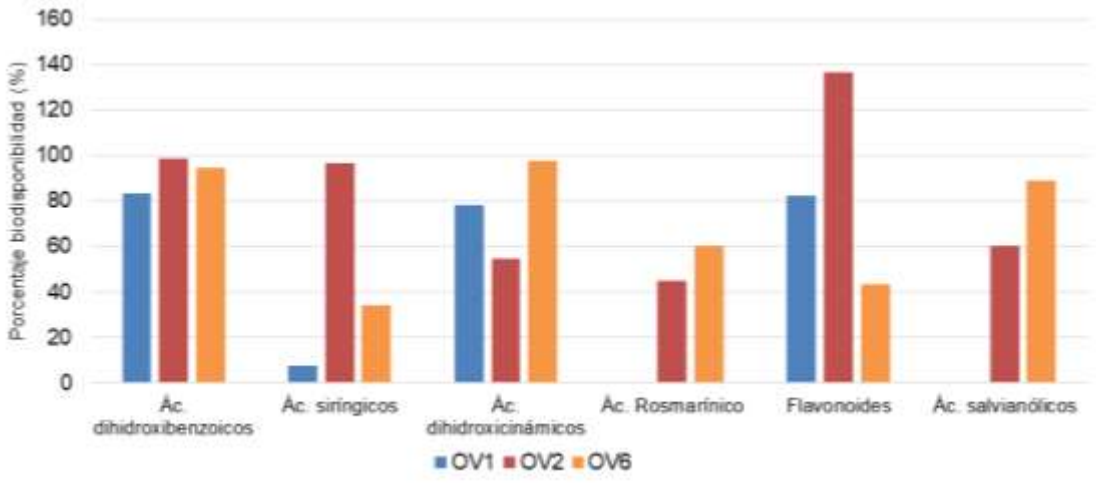


Figura 98. Porcentaje de bioaccesibilidad de cada uno de los grupos de compuestos identificados por el extracto digerido. En azul se muestran los resultados para la fracción intestinal de OV1, en rojo para OV2 y en naranja para OV6.

Los ácidos dihidroxibenzoicos fueron los únicos en los que el porcentaje fue superior al 80 % tras la digestión intestinal en los tres extractos, alcanzando casi el 100 % de biodisponiblidad en OV2 y OV6. Los ácidos siríngicos en el extracto OV2 y los dihidroxicinámicos en OV6 también alcanzaron esos valores.

Los resultados tan variables encontrados en los flavonoides pueden ser debidos a la gran variabilidad de estructuras químicas detectadas. Está descrito que la conservación de los flavonoides después de la digestión puede estar relacionada con el enlace β entre los carbohidratos y la aglicona, que no se puede hidrolizar por las enzimas empleadas habitualmente en el modelo de digestión simulada [125]. Además, se ha demostrado que la degradación de los flavonoides durante el proceso de digestión intestinal es diferente cuando se incuban como compuestos puros o integrados en un extracto. Las interacciones con otros compuestos en la matriz del extracto pueden influir y alterar la estabilidad durante la digestión. En este sentido y considerando la complejidad química de cada uno de los extractos analizados, se puede explicar fácilmente la diferencia obtenida entre ellos en lo que a porcentajes de bioaccesibilidad se refiere. Estos resultados coinciden con los obtenidos con otros autores [11] en los que observan la reducción del contenido en compuestos fenólicos (flavonoides principalmente) tras el proceso de digestion *in vitro* en tres especies denominadas comúnmente orégano pero pertenecientes a dos familias diferentes del Orden *Lamiales*: *Lamiaceae* (*Hedeoma patens,* orégano chiquito) y *Verbenaceae* (*Lippia graveolens* y *L. palmeri,* orégano mexicano). En estos trabajos se vieron afectados los fenoles mayoritarios (apigenín–7–glucósido, escutellareína, luteolina, luteolín–7–glucósido, floridzina y ácido clorogénico) y se atribuyó la reducción en su bioaccesibilidad a los cambios de pH durante el proceso. Estas condiciones fisiológicas también afectaron al ácido rosmarínico, presentando un 40 % de bioaccesibilidad en OV2 y 60 % en OV6. En otro estudio de bioaccesibilidad [192], los autores observaron valores similares (34 – 37 %) tras la digestión intestinal del ácido rosmarínico por tres métodos de digestión diferentes, coincidiendo uno de ellos con el llevado a cabo en el presente trabajo.

2 Estudios farmacológicos *in vitro*

El siguiente paso que nos planteamos fue el **estudio farmacológico *in vitro*** de los extractos obtenidos a partir de las partes aéreas floridas de *Origanum vulgare* L. subsp. *vulgare*. Según Hamburguer y Hostettmann [237], en la investigación de plantas medicinales para la búsqueda de nuevas acciones farmacológicas existen una serie de premisas que garantizan un mayor éxito en los resultados y que la especie contenga compuestos

farmacológicamente activos: i) que la planta tenga un uso tradicional demostrado, ii) que existan estudios previos con especies similares y iii) que la información bibliográfica sobre esa especie sea escasa. En este sentido, *O. vulgare* cumple las tres premisas anteriores, por lo que el objetivo del capítulo II de este trabajo es realizar un cribado sistemático farmacológico buscando su posible actividad antioxidante [243], hipolipemiante [241], hipoglucemiante [242], antiacetilcolinesterasa y anti–inflamatoria, todas ellas enmarcadas dentro de lo que se puede encontrar en el síndrome metabólico y diversas patologías relacionadas con el envejecimiento [244]. Como complemento a estos estudios, se añadieron ensayos de citotoxicidad en líneas celulares intestinales, hepáticas y tópicas.

Cualquier sustancia que en concentraciones bajas retrasa o previene significativamente la oxidación de un sustrato oxidable se denomina antioxidante. Los antioxidantes juegan un papel vital en la preservación de la calidad de los alimentos y el mantenimiento de la salud del ser humano. En el cuerpo humano, aproximadamente el 5 % del oxígeno inhalado se convierte en especies reactivas de oxígeno (ROS), que incluyen el radical hidroxilo, el radical anión superóxido, el peróxido de hidrógeno, el oxígeno singlete, el radical óxido nítrico, el radical hipoclorito y varios peróxidos lipídicos. Todas estas especies químicas son capaces de reaccionar con lípidos de membrana, ácidos nucleicos, proteínas y enzimas, y otras moléculas pequeñas provocando daño celular. La producción excesiva de ROS conduce a estrés oxidativo, que puede causar varias enfermedades. En el cuerpo humano hay una variedad de antioxidantes endógenos (sistema inmunológico) que ayudan a combatirlos. Sin embargo, en muchos casos es necesaria una suplementación externa con antioxidantes para ayudar al cuerpo a neutralizarlos y eliminar los efectos nocivos del estrés oxidativo que puede derivar en multiples patologías [45,246,247,251,382,383]. Se sabe que las frutas, verduras, cereales y plantas medicinales contienen numerosos compuestos (fenólicos principalmente) con una fuerte actividad antioxidante [384].

Por este motivo, en los últimos años la industria se ha centrado en la búsqueda de moléculas antioxidantes [385]. Siguiendo esta linea de investigación ¿los extractos preparados de *O. vulgare* serían lo suficientemente efectivos para combatir el estrés oxidativo relacionado con el síndrome metabólico?

2.1 Actividad antioxidante

Para valorar si un compuesto/extracto se puede considerar antioxidante es imprescindible evaluar su potencial, tanto *in vitro* como *in vivo*. Generalmente, los ensayos *in vitro* suelen implicar reacciones de radicales libres que pueden cuantificarse mediante espectrofotometría. En este trabajo se han utilizado dos ensayos diferentes basados en

la transferencia de electrones que involucran una reacción redox con el oxidante como indicador del punto final de reacción, DPPH• (2,2–difenil–1–picrilhidrazilo) y ABTS• (ácido 2,2'–azino–bis–3–etilbenzotiazolin–6–sulfónico).

Entre los ensayos de captación de radicales libres, el **método DPPH•** es el más rápido, simple, reproducible y de menor coste en comparación con otros. Este radical, que tiene un electrón desapareado, es de color azul–violeta y se decolora a amarillo pálido por la presencia de una sustancia antioxidante. Este cambio de color se puede cuantificar espectrofotométricamente a 517 nm. Los resultados se expresan en porcentaje de actividad antioxidante, en valores de EC_{50} (concentración capaz de reducir al menos el 50 % del radical libre DPPH•). Con este método, frecuentemente los resultados no se pueden comparar con otros estudios realizados con extractos vegetales y productos de origen natural debido a la gran variabilidad de concentraciones de DPPH• que se emplean y los tiempos a los que se mide la reacción. Para evitar esto, en los últimos años se ha establecido el índice de actividad antioxidante (IAA) [253], que es un valor constante e independiente de la concentración de DPPH• empleada. En este trabajo se cuantificó la actividad antioxidante de los seis extractos y de dos compuestos empleados como controles positivos: BHT (butilhidroxitolueno, un antioxidante sintético ampliamente empleado) y ácido rosmarínico (compuesto mayoritario en *O. vulgare* y descrito como agente antioxidante).

Esta actividad se midió a seis tiempos diferentes con el objetivo de establecer la cinética de la reacción en cada muestra y establecer el momento en el que la reacción se estabiliza. Los resultados obtenidos mostraron que el orden de actividad antioxidante de los extractos era OV6 > OV2 ≥ OV4 >OV5 > OV3 > OV1 (**Figura 99**). Los dos compuestos empleados como controles positivos mostraron resultados muy diferentes. El antioxidante sintético BHT presentó una actividad muy inferior a cualquiera de los extractos analizados y, por el contrario, el ácido rosmarínico presentó la actividad mayor. Todos los extractos, excepto OV4, tenían una cinética intermedia alcanzando la estabilidad de la reacción entre los 30 – 60 minutos. El comportamiento de OV4 fue similar al BHT, con una cinética lenta (más de 60 minutos). El ácido rosmarínico tenía la cinética más rápida de todos (15 – 30 minutos).

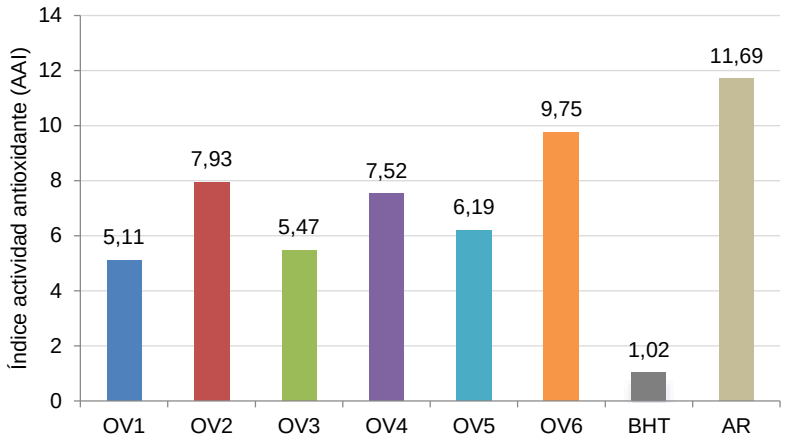


Figura 99. Índice de actividad antioxidante frente al radical libre DPPH. BHT: butilhidroxitolueno; AR: ácido rosmarínico.

Una vez estudiada la actividad antioxidante de los extractos frente al radical libre DPPH, se analizaron los productos de la digestión de los extractos OV1, OV2 y OV6. Tal y como puede observarse en la **Figura 100**, la actividad antioxidante (expresada como índice de actividad antioxidante, IAA) aumentó tras el proceso de digestion en OV1, OV2 y en el ácido rosmarínico. Sin embargo, disminuyó en el extracto OV6. El compuesto BHT mostró un porcentaje de actividad antioxidante inferior al 20 % después del proceso de digestión gastrointestinal por lo que no se consideró antioxidante y no se calculó su valor IAA correspondiente.

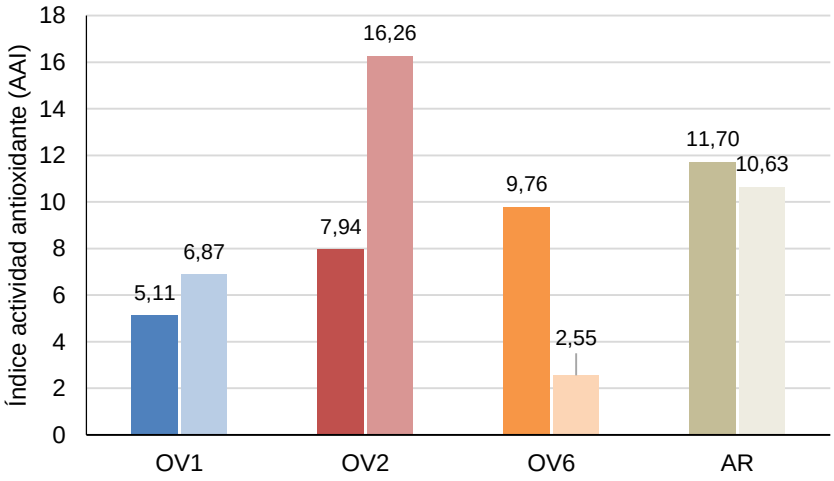


Figura 100. Índice de actividad antioxidante frente al radical libre DPPH. Color oscuro: extracto bruto; color claro: fracción intestinal. AR: ácido rosmarínico.

En todos los productos digeridos el IAA se mantuvo por encima de 2, lo que significa que siguen manteniendo una fuerte actividad antioxidante después del proceso de digestión gastrointestinal. Los extractos acuosos mostraron una mejora del IAA tras el proceso de digestión, mientras que en el extracto etanólico disminuyó. El aumento de la temperatura del agua al preparar el extracto también mejoró la actividad antioxidante, que resultó ser

dos veces más alta después de la digestión que en el extracto bruto. Químicamente se extrajeron más compuestos como flavonoides, DHBAs y derivados del ácido siríngico (**Figura 96**).

El otro método empleado para determinar la actividad antioxidante fue el ensayo de ABTS•, que se puede emplear tanto para sustancias hidrofílicas como lipofílicas [386]. Este ensayo se basa en una técnica espectrofotométrica en que un agente antioxidante provoca la inhibición de la absorbancia del catión radical de ABTS (2,2'– azinobis (3–etilbenzotiazolina–6–sulfonato), que es un cromóforo azul–verde y estable con un espectro de absorción de longitud de onda larga característico [387]. Así, en primer lugar se generan cationes radicales de ABTS a partir de peróxido de hidrogeno H_2O_2 u otros oxidantes fuertes como persulfato potásico en presencia de metamioglobina. Esto genera un radical intermedio ferrilmioglobina que posteriormente reacciona con ABTS para formar el catión radical ABTS. En este punto, los antioxidantes presentes en la muestra pueden neutralizar estos cationes radicales mediante la transferencia de electrones u átomos de hidrogeno. Esto hace que el catión radical (cromóforo) vaya perdiendo coloración y, por tanto, se produzca una disminución de la absorbancia. Los resultados se expresan en porcentaje de inhibición en un punto de tiempo fijo, expresados en equivalentes de Trolox.

Los resultados obtenidos mostraron que los extractos más polares, OV1 y OV2, fueron los que presentaban una menor actividad antioxidante (19,64 ± 0,10 mg y 22,59 ± 0,18 mg, respectivamente), y los más apolares (OV3 – OV6, en los que el disolvente de extracción fue etanol 50 % o solo etanol) los de mayor actividad y muy similar en todos ellos (33,75 – 34,28 mg).

El análisis de las fracciones digeridas mostró que la actividad antioxidante aumentó considerablemente en los extractos acuosos OV1y OV2 alcanzando valores similares a los de los extractos apolares (32,41 ± 0,10 y 33,87± 0,14 mg, respectivamente), mientras que en el etanólico (OV6) se mantuvo igual que en el extracto bruto.

A la vista de los resultados obtenidos por ambos métodos, se deduce que en cuanto a la actividad antioxidante frente al radical libre DPPH influye más el método de extracción que el disolvente empleado, ya que se obtienen mejores resultados para OV1, OV3 y OV5. Sin embargo, en la determinación por ABTS•, la actividad está relacionada con la polaridad del disolvente de extracción, obteniendo mejores resultados cuando se emplea etanol o mezclas hidroalcohólicas al 50% que agua.

La diferente relación encontrada entre las condiciones de extracción y el radical libre empleado para determinar la actividad antioxidante conlleva también una baja correlación no significativa (–0,3271) entre ambos métodos. Por separado, los dos métodos

mostraron correlaciones similares con la cantidad de polifenoles totales (TPC), 0,6696 para el radical libre DPPH y 0,6081 para ABTS. Además, atendiendo a la composición química, de todos los compuestos mayoritarios cuantificados, el ácido rosmarínico es el que mejor correlación presenta en ambos métodos de actividad antioxidante (DPPH• – 0,7081 y ABTS• 0,6211). Sin embargo, cuando se tienen en cuenta el resto de compuestos, existe una muy buena correlación entre el sumatorio de los compuestos mayoritarios y DPPH• (–0,912), pero no con ABTS• (0,141). El método con DPPH• es el más frecuente cuando se estudia la actividad antioxidante pero puede resultar conveniente la elección de otro método complementario para obtener resultados más aproximables a la realidad.

Existen una serie de patologías que están relacionadas con procesos de oxidación en el organismo y la formación de radicales libres. La diabetes tipo 2 es una resistencia a la insulina provocada generalmente por la obesidad y la acumulación de lípidos [257]. La causa de la dislipidemia puede implicar cambios en la oxidación de los lípidos por los radicales libres en los tejidos grasos o el hígado [246,271]. El Alzheimer es una enfermedad neurodegenerativa que implica inflamación [75,121,278]. Por este motivo, en este trabajo, se estudió la actividad antidiabética, hipolipemiante, antiacetilcolinesterasa y antiinflamatoria de los extractos y sus productos digeridos.

2.2 Actividad hipoglucemiante e hipolipemiante

La **diabetes mellitus** es una enfermedad crónica que, de no ser tratada de forma adecuada, genera complicaciones graves e invalidantes, reduciendo la calidad de vida de quienes la padecen y aumentando el coste de su atención. Es la enfermedad endocrina más común y su prevalencia aumenta de forma constante en todo el mundo. De los distintos tipos de diabetes, la tipo 2 es la más frecuente. Se caracteriza por una transición gradual, donde las alteraciones de la masa y de la función de las células β–pancreáticas están precedidas por una disminución de la respuesta de los tejidos periféricos a la insulina. Estos pacientes, inicialmente, no necesitan tratamiento con insulina y el control glucémico puede lograrse mediante pérdida de peso, actividad física o agentes hipoglucemiantes orales. La administración de hipoglucemiantes orales, como biguanidas, tiazolidinedionas y sulfonilureas, puede regular meritoriamente la hiperglucemia. Sin embargo, tienen efectos secundarios notables, que incluyen hipoglucemia y problemas gastrointestinales. Así, estas terapias pueden provocar efectos secundarios y complicaciones de larga duración provocando unos costes sanitarios extraordinarios con una alta morbilidad y mortalidad [388]. Por lo tanto, existe un requisito urgente de sustituciones eficaces para reducir las complicaciones de la diabetes con

menores efectos secundarios. En los últimos años, la búsqueda de medicamentos alternativos para el tratamiento de la diabetes ha atraído una gran atención. En este sentido, los productos naturales están jugando un papel fundamental. Se sabe que hay más de 400 plantas medicinales tradicionales cuyos sus compuestos bioactivos poseen propiedades antidiabéticas. A pesar de ello, solo un pequeño número de ellas han recibido una adecuada evaluación científica y médica para establecer su eficacia [389].

Los polisacáridos de origen natural son metabolitos primarios presentes en todos los vegetales y, por tanto, esenciales para su desarrollo. Estos compuestos, generalmente extraídos de plantas medicinales, cereales, frutas, verduras, hongos comestibles y alimentos, se han estudiado mucho los últimos años por su baja toxicidad y numerosas actividades farmacológicas, entre ellas la antidiabética [195,390]. Están formados por unidades de monosacárido unidas entre sí por enlaces glicosídicos. Varios estudios han demostrado que los polisacáridos purificados de calabaza, pepino de mar, baya de goji, hongos, judías verdes, té y avena ejercen efectos favorables sobre la homeostasis de la glucosa, reduciendo las complicaciones de la diabetes a través del mecanismo defensivo contra la lesión por estrés oxidativo y mejorando eventualmente la sensibilidad a la insulina [391–393].

Los carbohidratos se ingieren en forma de polisacáridos y, a lo largo del tracto gastrointestinal y gracias a las enzimas digestivas α–amilasa (saliva o páncreas) y α–glucosidasa (intestino delgado), se transforman en monosacáridos que se pueden absorber fácilmente por el intestino delgado y alcanzar el torrente circulatorio. Por lo tanto, la inhibición de estas enzimas hace que no sea posible esta conversion y sirva como un paso eficaz para controlar la glucosa en sangre en pacientes diabéticos [394]. Los hidratos de carbono no digeridos en el intestino delgado se metabolizan en el colon por las bacterias intestinales, lo que explica los efectos secundarios frecuentes de estos fármacos (meteorismo, flatulencia, diarreas, etc.).

Por todo lo expuesto, encontrar extractos/compuestos que inhiban estas enzimas puede ser una estrategia eficaz para combatir esta patología. En este trabajo se ensayó la actividad inhibidora de la enzima α–glucosidasa en los seis extractos. Matsui *et al.* [264] describieron un método por el que es posible determinar por medio de espectrofotometría la inhibición de esta enzima gracias a un sustrato que, tras la acción de α–glucosidasa, se descompone en un compuesto amarillo y glucosa. En el análisis se empleó como control positivo la acarbosa, uno de los fármacos comercializados como tratamiento oral de la diabetes no insulino ependiente (diabetes tipo 2), porque interfiere en la absorción intestinal de los hidratos de carbono y disminuye la hiperglucemia postprandial al inhibir de forma competitiva la acción de la α–glucosidasa intestinal que hidroliza los

oligosacáridos y polisacáridos. Rodriguez–Solana *et al.* [296] también utilizaron como control positivo este compuesto (1 mg/mL), mostrando un 78.34 % de inhibición de la enzima α–glucosidasa y un 78.33 % de inhibición para α–amilasa. Las muestras de los extractos analizadas presentaron una eficacia superior al 50 % para inhibir la actividad de la enzima α–glucosidasa.

El extracto OV1 extraído por maceración en frío con agua, a pesar de ser el extracto menos antioxidante de todos y con menor número de compuestos químicos detectados, fue el que presentó mejores resultados como agente inhibidor de la α–glucosidasa, con una actividad 7,72 veces mayor que la acarbosa (IC_{50} = 18.31 ± 0.30 y 141.47 ± 3.57 µg/mL, respectivamente). Dos extractos, OV3, macerado en frío con etanol 50 %, y OV2, macerado en caliente con agua, presentaron una actividad similar al control positivo (149.40 ± 3.36 y 205.17 ± 7.36 µg/mL, respectivamente). Durante el proceso de digestion gastrointestinal, la actividad inhibidora de la α–glucosidasa de la acarbosa aumentó un 77,82 % (141.47 ± 3.57 → 110,10 ± 1,15 µg/mL). En el caso del extracto OV1, la actividad aumentó un 57,02 % (18.31 ± 0.30 → 10,44 ± 0,53 µg/mL). Aunque el aumento en el caso del extracto es menor, los valores de IC_{50} muestran que la actividad del extracto bruto después del proceso de digestión gastrointestinal es 10,55 veces superior a la de acarbosa.

Las bioactividades dominantes encontradas en los extractos y las diferencias entre unos y otros deberían poder justificarse mediante algún factor o compuesto químico. OV1 fue significativamente más hipoglucémico que OV2. El extracto acuoso en caliente extrajo el mismo porcentaje de DHBA que de flavonoides (siendo en cantidad 17.03 ± 0.12 mg / 100 mg y 38.83 ± 0.41 mg / 100 mg), mientras que el obtenido por maceración en frío está compuesto por 50 % de DHBA (9.20 ± 0.09 mg / 100 mg) y 35 % de flavonoides (13,53 ± 0,47 mg / 100 mg). En esta actividad, OV5 presentó el valor de IC_{50} estadísticamente más alto (503,80 ± 2,69 µg / mL, con $p < 0,001$) siendo el porcentaje más bajo tanto para los flavonoides (12 %) como para DHBA (18 %), por lo que estos compuestos podrían estar relacionados con la capacidad de inhibir la α–glucosidasa reductasa *in vitro*. OV3 y OV4 presentaron porcentajes de DHBA y flavonoides similares a OV2 (OV3 = 33 % y 35 %, OV4 = 33 % y 30 %, respectivamente), pero sus actividades hipoglucémicas fueron significativamente diferentes (IC_{50} = 149,40 ± 3,36 µg/ml y 339,56 ± 1,06 µg/ml, respectivamente con $p < 0,001$).

En este sentido, el DHBA podría no ser el menor de los compuestos capaces de inhibir esta enzima por su presencia en OV1 (9,20 ± 0,09 mg / 100 mg) y porque OV5 tiene 6,73 ± 0,23 mg / 100 mg. En otras palabras, un alto contenido de ácidos fenólicos (ácido rosmarínico, cafeico y DHBA) y derivados podría estar relacionado con una actividad

antioxidante potencialmente alta, pero estos compuestos no podrían considerarse tan buenos inhibidores de la α–glucosidasa. No hay correlación entre esta actividad y los compuestos polifenólicos, excepto con los derivados del ácido cinámico (0,7578), actividad previamente demostrada [395], pero podría haber otro tipo de compuestos no detectados en la HPLC–DAD responsables de esta actividad. En realidad, la acarbosa es un azúcar, por lo que esta actividad podría estar relacionada con los azúcares adheridos a los compuestos principales. Algunos de estos azúcares podrían haberse retenido en primera línea de HPLC y no se detectan mediante esta técnica, ya que el sedimento de OV1 presentaba un perfil en la HPLC simple y pobre, igual que la fracción intestinal de OV1. Sin embargo, ambas fueron las muestras más hipoglucémicas probadas *in vitro* incluido el extracto crudo OV1 completo.

Varios estudios muestran que los polisacáridos de *Ribes nigrum*, *Coreopsis tinctoria*; *Turbinaria conoides*, *T. adornado*, *Armeniaca sibirica*, *Plantago asiatica*, *Fucus vesiculosus* y *Sargassum wightii* presentan inhibición significativa de las enzimas α–glucosidasa y α–amilasa *in vitro* de forma dosis–dependiente [13,213,268,269,396–400]. Olennikov y Tankhaeva [401], describieron la presencia de polisacáridos solubles en el agua presente en las partes aéreas de *O. vulgare*, lo que justificaría la actividad detectada en OV1, OV2 y OV3.

Además, los resultados también están en concordancia con los publicados por Ma *et al*. [402], donde se analizaron varios parámetros físicos de extracción de polisacáridos, entre los que se encuentran el disolvente y la temperatura de extracción. Los autores establecieron que: i) el agua es un disolvente óptimo para su extracción, ii) la proporción de extracción de polisacáridos aumenta con el tiempo de extracción, y que iii) temperaturas de extracción superiores a 80 ºC provocan un menor rendimiento de la extracción. Estos hechos confirman que el extracto OV1, obtenido por maceración en frío con agua, sea el que presente una mejor actividad. Para el extracto OV2, aunque también se empleó agua como disolvente, el método de extracción usado empleó altas temperaturas. En cuanto al extracto OV3, aunque se realizó por maceración en frío, el disolvente empleado fue etanol 50 % en el que los polisacáridos no son totalmente solubles. Los extractos alcohólicos OV5 y OV6 fueron los que peores resultados mostraron porque el alcohol generalmente precipita los polisacáridos y, por tanto, no pueden estar presentes en el extracto crudo. El alto rendimiento de extracción obtenido en el extracto OV1 (31,24 %) junto con la baja cantidad de compuestos detectados en la TLC y la HPLC–DAD corrobora, en cierta manera, una alta concentración de polisacáridos solubles en agua en este extracto.

La diabetes tipo II forma parte de un cuadro clínico de síndrome metabólico y, a diferencia de la tipo I que es inmunológica, la edad es uno de los factores de riesgo. Los resultados hipoglucemiantes de los extractos mostraron correlaciones relevantes con la actividad antioxidante: con ABTS• (0,7314) y con DPPH• (–0,5354). De igual manera, numerosos estudios han demostrado la estrecha relación entre la **dislipidemia** y la diabetes tipo 2 [228,389,390,403,404]. El mecanismo actualmente aceptado es que la resistencia a la insulina en la diabetes tipo 2 puede provocar trastornos del metabolismo de los lípidos [295,405,406].

Los principales medicamentos que están disponibles en el mercado para controlar la hiperlipidemia, fundalmente el colesterol, son las estatinas. Su mecanismo de acción se basa en la inhibición de la enzima HMG–CoA reductasa (3–hidroxi–3–metil–glutaril–CoA reductasa o HMGR). Esta enzima controla la velocidad de la vía del mevalonato, vía metabólica que produce colesterol a partir de acetil–CoA, en una reacción dependiente de NADPH. La HMG–CoA reductasa reduce la HMG–CoA para generar mevalonato y CoA e induce la expresión de los receptores de LDL en el hígado, lo que reduce la concentración plasmática de colesterol. Es importante destacar que las estatinas están contraindicads en aproximadamente el 40 % de los pacientes debido a los efectos adversos que pueden provocar (mialgias, miopatías, problemas hepáticos, y rabdomiolisis, entre otros) [407]. Estas limitaciones provocan que se esté buscando nuevas moléculas con este espectro de actividad.

En este trabajo, para la determinación de la actividad hipolipemiante se empleó un sistema comercial de ensayo que permite medir la actividad de la HMG–CoA reductasa y, en consecuencia, detectar inhibidores/activadores de dicha enzima. Se basa en el consumo de NADPH por la enzima, que puede cuantificarse mediante la disminución de la absorbancia a 340 nm. Debido al precio elevado de este sistema comercial y a la falta de muestra se ensayaron únicamente los extractos intestinales OV1 (alta actividad α–glucosidasa) y OV6 (baja actividad α–glucosidasa), para comprobar si tenían alguna relación con la actividad de la HMG–CoA reductasa. Los resultados obtenidos mostraron una inhibición enzimática de 54,84 ± 0,11 % para el extracto OV1 y una activación enzimática de 56,91 ± 0,30 % para OV6.

Estos resultados, al igual que en la actividad α–glucosidasa, pueden ser debidos a la presencia de polisacáridos solubles en agua presentes en *O. vulgare*. Aunque no existen datos científicos con esta especie, varios estudios con *Auricularia auricular* [408] y *Copernicia prunifera* [409] han demostrado una relación positiva entre los polisacáridos y sus extractos acuosos con la reducción del colesterol sérico y, por tanto, un menor riesgo de enfermedad cardiovascular [410].

Debido a la falta de datos, esta actividad no se incluyó en la matriz de correlación, aunque su implicación en el resto de enfermedades relacionadas con el síndrome metabólico está altamente respaldada por numerosos estudios y publicaciones clínicas [294,411,412]. De hecho, en la prevención primaria que tiene en cuenta los factores de riesgo como la edad, tabaquismo, diabetes y/o co–adyuvante de la hipertensión arterial, es frecuente (y a veces inadecuada) la prescripción de estatinas [413] .

2.3 Actividad inhibidora de la enzima acetilcolinesterasa

La **enfermedad de Alzheimer** (EA) es una enfermedad neurológica que, según los datos de la Sociedad Española de Neurología (SEN), padecen actualmente unas 800.000 personas en España y alrededor de 47 millones en el mundo. Se estima que en el año 2050 habrá alrededor de 130 millones de personas afectadas. En España, entre un 3 – 4 % de la población de entre 75 – 79 años está diagnosticada con esta enfermedad, unas cifras que aumentan hasta el 34 % en mayores de 85 años. El Alzheimer no es sólo la principal causa de demencia en todo el mundo, sino también la enfermedad que mayor discapacidad genera en personas mayores, generando un problema sanitario de primer orden y uno de los mayores gastos. Se calcula que en España el tratamiento supone el 1,5 % del producto interior bruto nacional.

Se estima que la mitad de los casos de la enfermedad de Alzheimer se puede atribuir a nueve factores de riesgo potencialmente modificables: diabetes mellitus, hipertensión arterial en la edad media de la vida, obesidad en la edad media de la vida, tabaquismo, inactividad física, depresión, inactividad cognitiva o bajo nivel educativo, hipoacusia y aislamiento social por lo que una reducción de entre un 10 – 25 % en dichos factores de riesgo podrían potencialmente prevenir entre 1 y 3 millones de casos de Alzheimer en el mundo [277].

En el cerebro sano, la acetilcolinesterasa (AchE) es la colinesterasa predominante (80 %). Esta enzima es responsable de la hidrólisis del neurotransmisor acetilcolina en colina y un grupo acetilo, y se distribuye principalmente por las sinapsis del cerebro y las uniones neuromusculares. Se ha demostrado que la acumulación de placas de β–amiloide en los tejidos cerebrales de pacientes con EA está asociada con cantidades aumentadas de AchE [296]. Por lo tanto, el principal tratamiento que existe en este momento para esta patología son los inhibidores de la acetilcolinesterasa (galantamina, donepezilo y rivastigmina), que actúan como agentes que provocan un aumento en la actividad colinérgica y, por lo tanto, ayudan a mantener durante un tiempo el estado neuropsicológico y funcional del paciente. En todo caso, estos fármacos sólo consiguen enlentecer la progresión de los síntomas y tienen, además, un uso limitado debido a sus

efectos secundarios adversos, como trastornos gastrointestinales y problemas de bioaccesibilidad. Por todo ello, en estos momentos la investigación está centrada en dos líneas principales. Una de ellas consiste en desarrollar agentes más potentes, ya sean productos naturales o análogos sintéticos, con efectos secundarios mínimos y, la otra, la de disponer de moléculas que sean capaces de modificar la enfermedad reduciendo su gravedad y restaurando la función cognitiva.

En este trabajo se ensayó la actividad de los extractos de orégano como posibles inhibidores de la enzima acetilcolinesterasa. Para que la actividad inhibitioria pueda ser cuantificable la reacción tiene lugar en dos pasos. En primer lugar, la enzima actúa sobre el sustrato ATCI dando lugar a acetato y el anion tiocolín iodado. En segundo lugar, éste último reacciona con el reactivo de Ellman (a quien se atribuye el protocolo del ensayo [283]) dando lugar a un compuesto amarillo cuantificable por espectrofotometría. Los extractos que presentaron mejores resultados en este ensayo fueron OV6 (etanol, maceración en caliente), OV1 (agua, maceración en frío) y OV5 (etanol, maceración en frío), con valores de IC_{50} 2,5, 1,4 y 1,2 veces inferiores a la galantamina empleada como control positivo.

Existen muchos trabajos de investigación centrados en detectar nuevos agentes anti–EA, pero son muy escasos los que trabajan con plantas de la familia *Lamiaceae* y, en concreto, con especies del género *Origanum*. En un estudio realizado por Chung *et al*. [414] se analizaron 139 especies y, de todas ellas, el extracto etanólico de *O. majorana* L. fue el que mostró mayor efecto inhibitorio sobre la AChE *in vitro*. Mediante el fraccionamiento guiado por actividad se identificó al ácido ursólico, compuesto triterpénico, como principal responsable de esta actividad. Otros estudios posteriores realizados con *Salvia neriifolia*, *Origanum ehrenbergii* y *O. syriacum* establecieron una relación entre la actividad inhibidora de las enzimas acetilcolinesterasa y butirilcolinesterasa con los aceites esenciales y otros terpenos presentes en estas especies [415]. Los autores justifican esta actividad por el pequeño tamaño molecular y lipofilia de estos compuestos, que hace que atraviesen fácilmente la barrera hematoencefálica. Estos compuestos, aunque también están descritos en *O. vulgare*, no se detectaron en el análisis químico realizado en este trabajo por dos razones: i) se trabajó con disolventes polares y este compuesto es apolar, e ii) su λ_{max} = 210 nm es difícil de detectar por HPLC–DAD.

Más recientemente, en otro estudio realizado por Vladimir–Knežević *et al*. [288] se evaluó la actividad antioxidante y antiacetilcolinesterasa de 26 plantas medicinales de esta familia y su relación con su composición química. Algunos extractos etanólicos mostraron una capacidad de inhibir la enzima acetilcolinesterasa de forma dosis dependiente; sin embargo, el extracto etanólico de *Origanum vulgare*no alcanzó el 50 % de inhibición

enzimática. Esto podría justificar los resultados obtenidos en extractos anteriores, que atribuye este espectro de actividad a compuestos lipofílicos.

Sin embargo, la alta actividad detectada en los extractos OV6 (etanol, maceración en caliente), OV1 (agua, maceración en frío) y OV5 (etanol, maceración en frío), sugiere que debe de haber otros compuestos químicos no lipofílicos que también posean este espectro de actividad farmacológica y que no sean polifenoles, dado que no se encontró ninguna correlación con los compuestos mayoritarios cuantificados de carácter fenólico (ligera correlación con flavonoides (0,438) y derivados del ácido salvianólico (0,506)).

Los resultados obtenidos tras el proceso de digestion gastrointestinal a la que se sometió a los extractos OV1 y OV6 (los de major actividad) mostraron que esta actividad diminuyó significamente en ambos casos, siendo mucho más en el caso de OV6 (4,41 veces menor que el extracto crudo) que en OV1 (1,8 veces). En este sentido, Rodriguez–Solana *et al.* [296] han publicado resultados positivos de extractos obtenidos por maceración, destilación, percolación o infusion acuosa e hidroalcohólica del licor de algarrobo como agentes inhibidores de las enzimas acetilcolinesterasa, tirosinasa, α–glucosidasa y α–amilasa, relacionándolos con su contenido en compuestos fenólicos. Estos autores también han observado una disminución de la actividad después del proceso de digestion.

2.4 Actividad antiinflamatoria

Entre las defensas del cuerpo humano se encuentra el fenómeno de la **inflamación**, que puede ser aguda (desencadenado por un agente en concreto) o crónica (como consecuencia de enfermedades no controladas, por ejemplo las patologías cardiovasculares). Como se ha explicado anteriormente, existen numerosas rutas y células involucradas en éste fenómeno, desde las plaquetas en primera línea ante una agresión en la piel hasta los macrófagos que liberan la cascada de citoquinas, responsables de la reparación de los tejidos dañados. Contrariamente a otros estudios que centran la actividad anti–inflamatoria en la ruta de inhibición de la enzima ciclooxigenasa (COX) [74,298], en el presente trabajo se optó por el análisis del comportamiento de los extractos ante dos de los agentes presentes en la sangre que se consideraron motores del proceso de inflamación: la albúmina y los eritrocitos (glóbulos rojos).

Recordando el proceso farmacocinético LADME (liberación, absorción, distribución, metabolismo y excreción) de cualquier fármaco o sustancia administrada, tras la absorción en el intestino (si es administrado por vía oral), tiene lugar la distribución por el cuerpo hasta llegar al lugar de acción. La mayoría de las sustancias son transportadas

por la sangre de la mano de la albúmina, una proteína transportadora responsable también del equilibrio electrolítico del organismo. Existen estudios de diferente naturaleza que relacionan un déficit de albúmina en pacientes con el proceso inflamatorio, entre otras explicaciones por la desnaturalización de la proteína transportadora [83,416]. Debido a la importancia de esta proteína en el transporte de compuestos hasta el lugar de acción y su implicación en el proceso de reparación en el proceso inflamatorio, se consideró oportuno estudiar el comportamiento de los extractos frente a la albúmina. El método consistió en determinar el grado de desnaturalización de la misma ante un aumento de la temperatura cuantificando mediante espectrofotometría su grado de preservación en presencia de los extractos a diferentes concentraciones.

Tal y como mostraron los resultados, el control positivo seleccionado presentó un porcentaje dosis–dependiente de desnaturalización de la albúmina superior al 100 %. Las dosis de Aspirina comercial (Bayer ®) y del genérico (A.A.S) seleccionadas fueron de 500 mg, concentración a la que el ácido acetilsalicílico actúa como anti–inflamatorio y no como antiagregante plaquetario (< 300 mg/ dosis). Las dosis seleccionadas (50, 100, 200 y 400 ug/mL) no mostraron una preservación de la albúmina, sino que incrementaron su desnaturalización térmica, efecto que también observaron Reshma *et al*.[292].

A diferencia del efecto dosis–dependiente observado en los controles positivos, los extractos presentaron comportamientos diferentes dependiendo de la dosis, tal y como se muestra en la **Figura 101**.

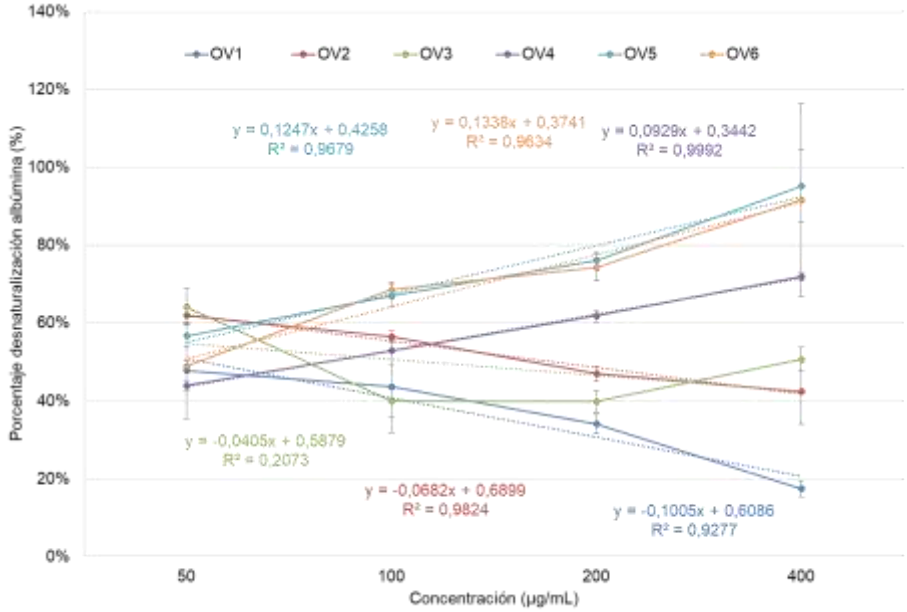


Figura 101. Porcentaje de desnaturalización de la albúmina (media ± DE %) en función de la concentración de cada extracto (50, 100, 200 y 400 µg/mL).

De modo general, se observaron correlaciones muy altas entre las dosis de 100, 200 y 400 μg/mL (> 0.9100), por lo que los resultados obtenidos a dosis de 50 μg/mL resultan claves para determinar el comportamiento de cada extracto. Los extractos acuosos siguieron la tendencia dosis–dependiente, siendo menor el porcentaje de desnaturalización de la albúmina a mayor dosis (pendiente negativa, R^2 = 0,9277 (OV1) y R^2 = 0,9824 (OV2)), mientras que los extractos etanólicos presentaron la tendencia inversa: se obtuvo una mejor preservación a una menor dosis (pendiente positiva, R^2 = 0,9679 (OV5) y R^2 = 0,9634 (OV6)). Una de las hipótesis sostenidas para este comportamiento puede ser la presencia de trazas de etanol que puedan actuar como agente desnaturalizante además del efecto térmico inducido voluntariamente para evaluar la desnaturalización. Sin embargo, la disparidad entre los resultados del grupo de extractos hidroalcohólicos sugiere que estos resultados podrían estar más relacionados con la composición química de cada uno de ellos que con las trazas de disolvente que pudieran estar presentes. Mientras que el extracto OV4 siguió la tendencia de los extractos alcohólicos preservando mejor la albúmina a bajas dosis (pendiente positiva en **Figura 101**, R^2 = 0,9992), en el extracto OV3 se observó una clara ventana terapéutica, siendo menor la desnaturalización en las dosis medias que en las mayores y menores (50 y 400 ug/mL).

Está descrito que los ácidos salvianólicos son un grupo de metabolitos capaces de establecer una unión química con la albúmina en tres de los dominios de la proteína transportadora [417], sin embargo en los resultados obtenidos no se observó una correlación entre el contenido de estos compuestos y el porcentaje de desnaturalización de la albúmina. Además, ninguno de los otros componentes mayoritarios identificados presentó ninguna correlación con este espectro de actividad a ninguna de las dosis ensayadas. La proporción y sinergia química de cada extracto pudiera ser la responsable del comportamiento frente a la albúmina a cada concentración. Dosis de 400 μg/mL presentaron correlaciones significativas con ABTS• (0,8536), mientras que el ensayo antioxidante con DPPH• se correlaciona con dosis de 100 μg/mL (–0,7269).

Es destacable que el extracto con una menor variedad de compuestos químicos (OV1) y sin presencia de ácidos salvianólicos, impidió, a todas las concentraciones ensayadas, el 50 % de la desnaturalización de la albúmina, considerándose así el mejor preservador de la proteína transportadora. Este mismo extracto también fue el que mostró la mejor inhibición de la enzima α–glucosidasa a pesar de su pobreza química – en comparación con el resto de extractos – viéndose así altas correlaciones entre la actividad antidiabética y las dosis de 100 μg/mL (0.8275), 200 μg/mL (0.9554) y 400 μg/mL (0.9517) del ensayo de desnaturalización de albúmina.

Al igual que en el proceso de inflamación se ha observado una disminución de la albúmina [83,416], una rápida tasa de sedimentación eritrocítica también puede ser indicativa de la existencia de inflamación en el organismo [418]. Al inicio del proceso de inflamación, ya sea aguda o crónica, los vasos sanguíneos cercanos a la zona dañada sufren una vasodilatación que permite la llegada de las células que contrarrestarán el daño [228,290,291]. Entre las células presentes, los glóbulos rojos son los encargados de la activación plaquetaria y la activación de los macrófagos gracias a las proteínas ICAM–4 de su membrana. Además, por ser las células transportadoras de oxígeno por excelencia, si existe un aumento de glóbulos rojos en la zona de inflamación, el tejido logrará repararse más rápidamente si el oxígeno se encuentra en forma O_2 en lugar de en su forma oxidada. La liberación de citoquinas en el lugar de la inflamación puede generar un aumento de las especies reactivas de oxígeno (ROS) [291,292].

Para el estudio, los glóbulos rojos procedieron de muestras de sangre de cinco sujetos caucásicos que no habían tomado ningún tipo de anti–inflamatorio en los 10 días anteriores. Tras el aislamiento de la fracción de eritrocitos de cada muestra, se sometieron a condiciones de hemólisis a fin de determinar por espectrofotometría la capacidad de los extractos de preservar este tipo de células.

Todos los extractos de orégano del presente trabajo mostraron una actividad antioxidante potencialmente muy fuerte (AAI > 2); por lo que, caso de preservar la membrana de los glóbulos rojos, podrían también preservar la forma óptima del oxígeno. Las dosis más altas (400 µg/mL) se correlacionan significativamente con la actividad antoxidante frente al radical libre ABTS (0,9688) y las dosis intermedias de 100 µg/mL tienen buena correlación con la actividad antioxidante frente a DPPH• (–0,7233), al igual que en el ensayo de desnaturalización de albúmina. Sin embargo, *a priori* no existe una relación directa entre la concentración, la actividad antioxidante y la preservación de la membrana eritrocítica, sino que los resultados tendrían más relación con el disolvente de extracción (**Figura 102**).

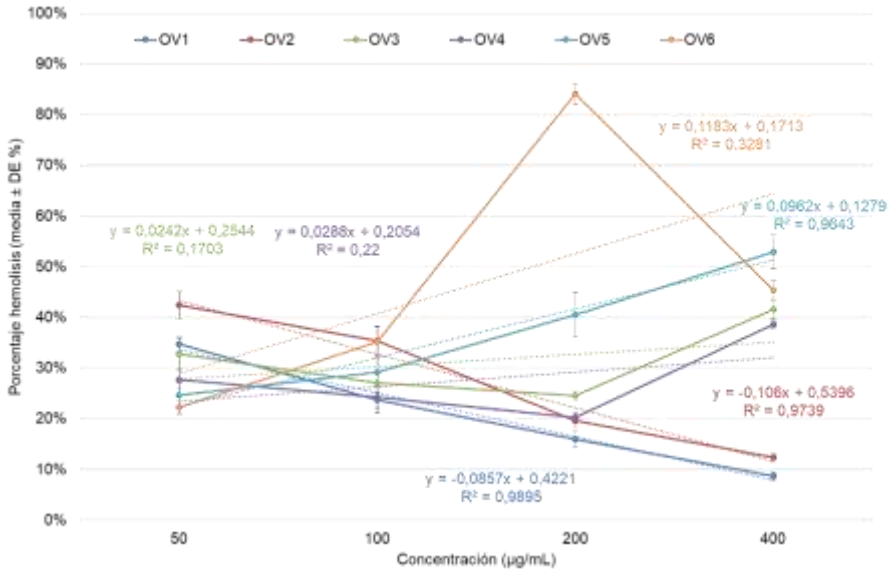


Figura 102. Porcentaje de hemólisis (media ± DE %) en función de la concentración de cada extracto (50, 100, 200 y 400 µg/mL).

De nuevo, los extractos acuosos mostraron una alta relación dosis–dependiente (R^2 = 0,9895 (OV1) y R^2 = 0,9739 (OV2)), de manera similar a los extractos acuosos de *Aegle marmelos* y *Ocimum sanctum* en el estudio de Reshma *et al.* [292], siendo OV1 y OV2 los mejores estabilizadores de membrana, ya que no se alcanzó el 50 % de hemólisis a ninguna de las concentraciones analizadas. En cambio, los extractos hidroalcohólicos tendieron de nuevo a ser mejores estabilizadores a dosis medias, porque no alcanzaron el 50 % de hemólisis.

Entre los compuestos químicos identificados, los derivados del ácido salvianólico presentan propiedades beneficiosas en el sistema cardiovascular a nivel de miocardio y micro y macrovascular: antioxidante, antitrombótica, antifibrótica y antiinflamatoria [419]. Su presencia se vio significativamente correlacionada con en el ensayo de preservación de membrana eritrocítica pero sólo a concentraciones de 100 µg/mL (0,778). Por ello, tampoco se puede atribuir el efecto protector a nivel sanguíneo a ningún compuesto en concreto, sino más bien la composición química del extracto en función del disolvente de extracción utilizado.

Así pues, a la vista de los resultados de los dos análisis – con correlaciones significativas a distintas dosis – y descartando los extractos puramente alcohólicos, el resto de extractos (OV1, OV2, OV3 y OV4) podrían ser buenos coadyuvantes antiinflamatorios.

3 Toxicidad

El cribado farmacológico *in vitro* continuó con un estudio para **evaluar la toxicidad de los extractos en varias líneas celulares**. Dado que en la administración oral intervienen diversos órganos y tejidos su absorción y el primer tejido de paso es clave. Por ello, se seleccionaron las líneas celulares Caco–2 y HepG–2.

La línea Caco–2 corresponde a células epiteliales generalmente utilizadas en ensayos de permeabilidad porque son capaces de expresar gran variedad de proteínas transportadoras presentes en varias barreras epiteliales y endoteliales del organismo y determinar la ventana terapéutica para una administración oral. A pesar del uso seguro del orégano como condimento alimenticio, el posible uso terapéutico de los extractos sería a través de una administración oral. Por ello es necesario evaluar su posible toxicidad y a qué concentración, ya que *Dosis sola facit venenum*. Cuando se quiere determinar la citotoxicidad de un compuesto se realizan ensayos en los que se calcula la concentración a la que muere o dejan de proliferar el 50 % de las células (IC_{50}) El método por excelencia para determinar la citotoxicidad de un compuesto es el ensayo de MTT. Se trata de un compuesto amarillo soluble en agua perteneciente a la familia de las sales de tetrazolio que, al reducirse, se convierte en un compuesto insoluble en agua de color violeta (cristales de formazán). Los cambios fisicoquímicos producidos en la reacción posibilitan la cuantificación por medio de espectrofotometría. Su aplicación en líneas celulares se basa principalmente en la capacidad de las deshidrogenasas mitocondriales para reducir el MTT convirtiéndolo en formazano. De este modo, la cantidad de formazán producido será reflejo de la supervivencia celular.

Las muestras intestinales de los extractos previamente sometidos al proceso de digestión *in vitro* no mostraron citotoxicidad alguna en la línea celular intestinal (IC_{50} > 1 mg/mL). El estudio en Caco–2 sólo muestra la toxicidad en esta línea celular quedando pendiente un estudio de permeabilidad que, por motivos ajenos, no pudo finalizarse. Los estudios de permeabilidad en células Caco–2 no sólo cuantifican la cantidad de compuesto capaz de ser absorbido por la membrana basolateral, sino que permiten analizar más en detalle las bioactividades una vez que la muestra ha sido absorbida [297,420,421]. Un estudio realizado con arándano negro comercial evaluó la capacidad antioxidante potencialmente disponible en el torrente sanguíneo, concluyendo que el mantenimiento de su bioactividad tras la administración (digestión y absorción) oral era debido a un alto contenido en polifenoles [297]. Otros trabajos han centrado su estudio en la actividad anti–acetilcolinesterasa relacionada con el Alzheimer [287] o en la capacidad de inhibir la enzima

HMGCo–A [229,273]. Esta última actividad suele ir acompañada también por estudios de citotoxicidad en las células donde es producida, el hígado [53,270,393].

A diario ingerimos sustancias químicas a través de la alimentación o como suplementación/tratamiento que el cuerpo humano elimina. Se define como xenobiótico cualquier sustancia exógena al organismo humano que, dependiendo de la vía de administración y dosis, puede llegar a producir toxicidad según su naturaleza. Según los fundamentos de la ciencia toxicológica [422], cuando un xenobiótico penetra en un organismo pueden ocurrir tres escenarios: ser eliminado directamente, sufrir una transformación estructural que aumente su polaridad facilitando su excreción o experimentar algún tipo de cambio estructural que provoque una modificación en su actividad tóxica. Aunque los sistemas enzimáticos encargados de la biotransformación son ubícuos, el hígado es el principal encargado en la metabolización de los xenobióticos.

La línea celular HepG–2 es una línea de hepatoma humano comúnmente utilizada *in vitro* como una alternativa a los hepatocitos humanos para estudios relacionados con el metabolismo de fármacos y la hepatotoxicidad. De nuevo, el estudio de la viabilidad celular a través del ensayo de MTT fue el primer paso en la determinación de la posible toxicidad de los extractos. Como se ha indicado, es un método que permite ensayar varias concentraciones y es aplicable a distintas líneas celulares. Generalmente, la introducción del xenobiótico en el medio de cultivo se produce durante un tiempo pre–establecido (24 h) una vez que las células han alcanzado su crecimiento. Como novedad a este ensayo, se decidió determinar la citotoxicidad de los extractos variando el diseño experimental durante el tratamiento. Basándose en que la capacidad tóxica de un compuesto no sólo puede deberse a su concentración, sino también al tiempo de exposición a éste. Las toxicidades crónicas se producen generalmente por la absorción repetida a dosis más bajas de la concentración letal media (CL_{50}). Así en el ensayo denominado *normal* de MTT, el extracto se incorporó desde el crecimiento de las células manteniéndose una exposición de 24 h. En la variante denominada *growing* el tratamiento se suspendió tras 72 h de crecimiento de las células. También se estudió el efecto producido ante una exposición crónica en la variante denominada *life*.

Los extractos acuosos, junto con el hidroalcohólico, mostraron una menor toxicidad en las células del hígado que el resto de extractos. Salvo OV4 (con una posible alta hepatotoxicidad en larga exposición) los extractos de orégano mostraron una baja o moderada toxicidad (alrededor de 50 % en altas concentraciones), de manera análoga a estudios previos. En consonancia, las muestras intestinales tampoco mostraron una alta toxicidad en este tipo de células, permitiendo incluso una alta exposición de los extractos acuosos a altas concentraciones. De hecho, el efecto citoprotector de OV2 en las células

intestinales y el hígado (IC_{50} > 1 mg/mL en ambas), podría complementarse con una posible actividad inhibidora de la HMGCo–A reductasa como parte del tratamiento de la dislipidemia cuando se administra por vía oral [50,233,407,423,424]. Además, el efecto respetuoso positivo observado en las células de la sangre [90,230,291] apuntaría a una actividad antioxidante sistémica y el transporte a órganos diana como el hígado, mucho más que a una actividad antiinflamatoria precoz [83,290,327] que debería comprobarse de una manera más amplia dada la complejidad de las reacciones en el proceso de inflamación [75,290,291].

Las líneas celulares evaluadas hasta ahora son útiles para la obtención de información aplicable a la vía de administración más común, la oral. Sin embargo, la piel (Vía tópica) es otra de las posibles vías de administración terapéuticas. Siendo el órgano más extenso del cuerpo humano, se encuentra en contacto con numerosos agentes tóxicos y ejerce de barrera frente a estos para impedir su penetración. Los efectos toxicos que se producen pueden ir desde una erupción a nivel de la epidermis hasta efectos más sistémicos, como ocurre con el Cl_4Cl que puede ocasionar daños hepáticos tras ser absorbido por la piel [422]. La línea A375 corresponde a células de melanoma maligno, generalmente utilizadas para ensayos de tumorigenicidad y toxicidad cutánea. De nuevo, la posible citotoxicidad que puedan producir los extractos sobre este tejido se determinó por medio del ensayo MTT en los tres niveles diferentes de aplicación del tratamiento: 24 h (*normal*), durante las 72 h de crecimiento (*growing*) y exposición crónica (*life).*

El efecto producido en la línea celular A375 mostró diferencias según la exposición y la naturaleza del extracto de *O. vulgare.* Mientras que los extractos acuosos deberían evitarse para largas exposiciones a altas concentraciones por esta vía de administración (IC_{50} = 0,5 y 0,4 mg/mL), el menor efecto tóxico observado se produjo en los extractos hidroalcohólicos, quedando descartados los extractos alcohólicos por sus altos valores de toxicidad incluso a concentración baja–media (0,2 mg/mL). La EMA recomienda otras especies de orégano para su uso cutáneo en forma de extracto hidroalcohólico, mientras que la infusión o decocción queda indicada para una administración oral. No obstante, de acuerdo con los resultados obtenidos y excluyendo los extractos puramente alcohólicos, no habría razones para descartar el uso del extracto hidroalcohólico en frío por vía oral o la aplicación cutánea de los extractos acuosos, siempre y cuando sea como tratamiento agudo.

4 Estudios farmacológicos *in vivo*

Las actividades y comportamiento de los extractos estudiados hasta ahora se han realizado *in vitro*. En 1965, Sydney Brenner comenzó a trabajar con *Caenorhabditis*

elegans como organismo modelo para estudiar el desarrollo y el comportamiento de los animales por razones que actualmente son bien conocidas [305]:

- Es un método económico.
- Tienen un ciclo de vida corto y producen más de 1.000 huevos/día.
- Son fáciles de manipular porque son pequeños y se pueden congelar y descongelar cuando sea necesario.
- Es transparente durante toda su vida. Su anatomía y desarrollo se pueden examinar fácilmente con un microscopio.
- Aunque es un organismo relativamente simple, muchas de las señales moleculares que controlan su desarrollo también se encuentran en organismos más complejos, como los humanos.

C. elegans fue el primer organismo multicelular cuyo genoma fue completamente secuenciado y posee equivalentes funcionales en humanos. En la actualidad, están disponibles más de 3000 cepas mutantes de *C. elegans* para investigación, lo que lo convierte en un modelo extremadamente útil para estudiar diversas enfermedades humanas, realizar cribados rápidos de potenciales fármacos y analizar los efectos del envejecimiento. Debido a que éste iba a ser el primer estudio de *O. vulgare* en *C. elegans*, se eligió la cepa N2 considerada la cepa silvestre sin mutaciones genéticas como comienzo de una posible nueva línea de investigación.

La velocidad de crecimiento de *C. elegans* depende directamente de la temperatura en la que se cultivan. Las tablas de equivalencia para la planificación de experimentos están disponibles *online* [314]. La temperatura habitual de trabajo suele ser de 20 ºC, aunque a veces es necesario acelerar o frenar los tiempos de crecimiento modificando este parámetro. *C. elegans* crece 2,1 veces más rápido a 25 ºC y 1,3 veces más rápido a 20 ºC que a 16 ºC. En este estudio se trabajó a 20 ºC.

A pesar que algunos investigadores utilizan un medio de crecimiento de nematodos en estado líquido [177,425,426], se empleó un medio de crecimiento sólido porque es el medio más común para *C. elegans* y por las ventajas que tiene para contar, medir y detectar la fluorescencia.

C. elegans se alimentó con una cepa bacteriana de *E. coli* OP50 resistente a ampicilina. Esta cepa se utiliza habitualmente como alimento bacteriano en el mantenimiento de laboratorio de *C. elegans* en placas de agar [427].

Antes de comenzar a realizar los distintos experimentos con *C. elegans* y *O. vulgare*, y ante la falta de publicaciones científicas en las que apoyarse de forma teórica se plantearon una serie de cuestiones que fue necesario resolver experimentalmente en el laboratorio.

- La actividad antimicrobiana es una de las principales acciones farmacológicas descritas para el *orégano*. Por ello, la primera de estas cuestiones fue valorar si dicha actividad pudiera interferir en la fuente de alimento proporcionado (*E. coli*) para el crecimiento de *C. elegans*. Para ello, se realizó un antibiograma con el objetivo de descartar posibles efectos antibióticos de *O. vulgare* sobre *E. coli* OP50. Se ensayaron los extractos acuosos de oregano (OV1 y OV2) a seis concentraciones diferentes (0,1 – 50 mg/mL), empleando ampicilina como control positivo. Ninguno de los extractos mostró un efecto antibiótico a las concentraciones ensayadas.
- La segunda cuestión planteada fue la forma de añadir los extractos de orégano al medio. Se diseñaron dos posibilidades para ello: i) sobre la superficie del medio de cultivo solidificado, e ii) añadirlo directamente al medio antes de su solidificación. Se optó por la segunda porque al preparar las placas se observó que con la primera opción el medio no resultaba homogéneo y los gusanos prácticamente no se movían, lo que disminuía mucho el ingesta de alimento y/o extracto.

El movimiento de la faringe es indicativo de que *C. elegans* está ingiriendo el alimento y/o la sustancia a ensayar [318]. Sin embargo, está descrito que una suplementación adicional a la dieta de *E. coli* podría modificar la ingesta y el crecimiento normales de los gusanos [428]. Estudios previos con nanopartículas administradas a *C. elegans* mostraron diferencias significativas en la tasa de bombeo faríngeo y, por tanto, en la ingesta al agregar el tratamiento [426]. Por ello, el siguiente paso, fue comprobar que *C. elegans* seguía ingiriendo el medio aunque tuviera una adición de extracto y estudiar si dicha adición alteraba su crecimiento en las distintas fases. Para este ensayo se emplearon los extractos acuosos (OV1 y OV2) a dos concentraciones (1 y 10 mg/mL), no observándose diferencias significativas respecto al control respecto al número de movimientos orofaríngeos/minuto. A pesar de los valores más bajos de OV1, los gusanos mantenían una tasa normal de aproximadamente 200 movimientos faríngeos por minuto. Es muy pronto para establecer conclusiones sobre qué podría afectar la funcionalidad, pero en el perfil químico OV1 presentó compuestos que podrían considerarse más grandes y/o polisacáridos no detectados en HPLC–DAD por polaridad pero presentes en el extracto. Estos compuestos

podrían afectar el volumen de partículas y, por lo tanto, al tiempo y/o dificultad para la ingesta [318,426].

Los resultados obtenidos en el estudio de la longitud de los nematodos muestran valores entre 1137,72 ± 2,21 – 1146,10 ± 2,13 µm, rango que entra dentro de lo que se considera fase adulta (1110 – 1150 µm) según Parada *et al*. [429]. Mörck y Pilon [430] estudiaron la cepa N2 junto con cepas mutantes, mostrando N2 una longitud promedio de 1200 ± 100 µm. Por otro lado, Knigth *et al*. [431] estudiaron el crecimiento en la cepa N2 midiendo al nematodo en cada una de sus etapas larvarias y relacionándolo con el proceso de división y diferenciación celular que tiene durante estas etapas. En ese análisis encontraron que el nematodo adulto presentaba una longitud promedio de 1500 ± 400 µm, un valor algo superior a lo observado por nosotros. Esta discrepancia puede deberse a las diversas metodologías empleadas, cuyo fundamento y proceso difieren entre sí. Como en todo organismo vivo, un normal crecimiento y desarrollo suelen deberse a una dieta adecuada. Los resultados muestran que los gusanos comen y crecen dentro de la normalidad de su especie, tanto en el caso de los no tratados como los no tratados. Esta normalidad descarta las dudas sobre si los gusanos están comiendo o no.

Cuando se trabaja con un único principio activo o determinadas formas farmacéuticas, como las nanopartículas, la trazabilidad del compuesto es posible gracias a la incorporación de marcas o etiquetas fluorescentes [432]. En este caso, la compleja composición química de los extractos dificulta este proceso. Por ello, se desarrolló y puso a punto un método sencillo para poder determinar la capacidad antioxidante dentro del gusano y así, poder relacionar directamente los resultados obtenidos con la ingesta del tratamiento. Los detalles del método se publicaron con un compuesto estándar como referencia (el ácido rosmarínico) y un extracto de té verde comercial, preparado como maceración acuosa en frío y en caliente [254]. Este método de análisis demostró una elevada reproducibilidad (*reliable method*) así como una preservación de la actividad antioxidante observada *in vitro*.

4.1 Actividad antioxidante

De forma general, la relación matemática entre los resultados *in vitro* e *in vivo* siempre es compleja de establecer o predecir. Los extractos acuosos demostraron ser muy antioxidantes frente al radical DPPH *in vitro.* Este es el método más frecuente y reproducible para determinar la actividad antioxidante *in vitro*, por lo que para comparar y verificar dicha actividad *in vivo* las condiciones de análisis fueron las mismas a las de las muestras *in vivo*. Para ello, se trataron los gusanos adultos con diferentes concentraciones de OV1 y OV2. El nuevo método permitió la extracción del material

interno del gusano, cuya comparación frente al control (gusanos no tratados con extracto) serviría para cuantificar por espectrofotometría el efecto antioxidante dentro del mismo. Generalmente, los estudios llevados a cabo con extractos de plantas medicinales determinan la actividad antioxidante *in vitro* y la asocian *a posteriori* con el efecto fisiológico observado *in vivo*, sin llegar a determinar si esta actividad se mantiene con la ingesta. Como ocurría con el BHT tras el proceso de digestión, puede que algunos compuestos pierdan la capacidad observada *in vitro* cuando se exponen a alteraciones bio–fisicoquímicas en el ambiente fisiológico. Gracias al nuevo método pudimos verificar *in vivo* la capacidad antioxidante de los extractos observada *in vitro.* La **Figura 103** recopila los resultados de actividad antioxidante de los extractos OV1 y OV2 *in vitro* e *in vivo*, comparados con el control positivo (ácido rosmarínico).

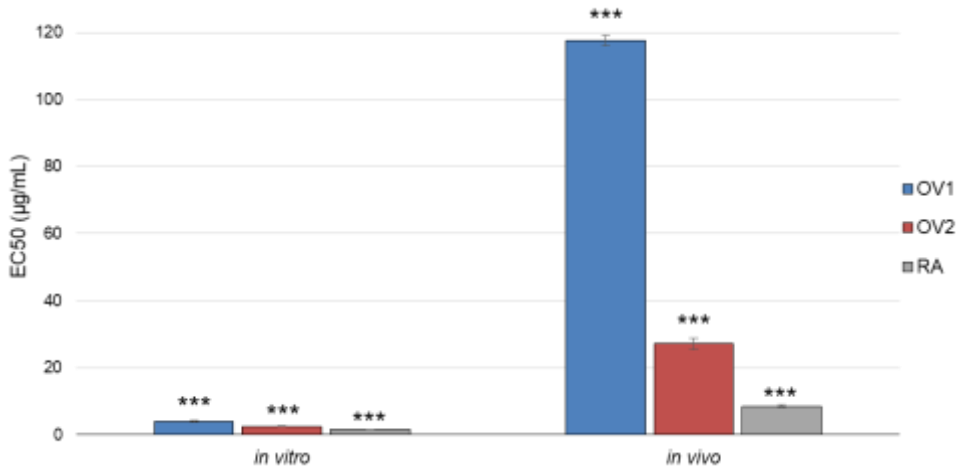


Figura 103. Actividad antioxidante frente al radical libre DPPH *in vitro* e *in vivo* de OV1 (azul) y OV2 (rojo) comparado con ácido rosmarínico (RA, gris). Resultados correspondientes a los valores de IC_{50} (media ± DE µg/mL) en el punto de estabilización. ***: Diferencias significativas, $p < 0{,}001$.

Al igual que los resultados obtenidos previamente *in vitro*, se observó que OV2 tiene una mayor capacidad antioxidante frente al radical libre DPPH que OV1. Además, si OV2 resultó ser el doble de activo que OV1 *in vitro*, esta relación se duplica *in vivo* ya que muestra una actividad cuatro veces superior. *In vitro*, ambos extractos fueron catalogados como antioxidantes muy potentes con una cinética intermedia. Sin embargo, con los resultados *in vivo*, los valores de AAI fueron 0,17 (OV1) y 0,74 (OV2), considerándose antioxidantes pobre y moderado, respectivamente. Tal y como se ha comentado anteriormente, el propósito del índice AAI es la estandarización de los resultados de los ensayos de actividad antioxidante frente al radical libre DPPH, ya que muchas veces es necesaria una conversión o no son equiparables debido a las diferentes condiciones del ensayo. Sin embargo, hay que tener en cuenta que este índice está pensado para su cálculo a partir de los resultados obtenidos *in vitro*, por lo que se desconoce cuál podría ser su aplicabilidad a los resultados *in vivo* con este nuevo método, si permitiría su

comparación entre distintos modelos *in vivo* o cuál sería el rango de diferencias entre los resultados *in vitro* e *in vivo*. En un futuro, si el uso de este método se extiende se podría proponer el cálculo de un índice adaptado a las variables *in vivo* – donde, por ejemplo, se debería tener en cuenta el número de individuos en la medida. En este trabajo se realizaron ensayos con diferentes cantidades de gusanos y no se pudo encontrar una relación lineal ni proporcional entre la actividad antioxidante y la cantidad de éstos. Por ello, para una primera aproximación se decidió controlar esta variable estableciendo una cantidad de gusanos fija y fácil de manejar, que además diera resultados reproducibles y fiables.

De cualquier manera, tras la publicación del método [254], su aplicación a los extractos de este estudio verificó la presencia de compuestos en el interior de los gusanos. Por lo tanto, los efectos funcionales podrían atribuirse al tratamiento *in vivo*. Así, el procedimiento se adaptó para poder analizar el perfil químico de los compuestos ingeridos por los gusanos. El tratamiento de los gusanos con los extractos y la toma de muestra del interior del gusano fueron las mismas, pero en lugar de realizar el ensayo de DPPH•, el sobrenadante del triturado filtrado se analizó químicamente mediante HPLC–DAD.

En el interior del gusano tratado con OV1 se detectó la presencia del ácido 2,5–dihidroxibenzoico (**9**), compuesto de referencia en el extracto OV1, y en menor cantidad, un flavonoide (**15**). Siendo el pico **9** (2,5–DHBA) el compuesto referente para OV1, se encontró al menos un 50 % de la concentración del extracto crudo dentro de los gusanos tratados con la mayor dosis (50 mg/mL). A pesar de presentarse en menor cantidad, el flavonoide fue detectado dentro del gusano en un 64,08 %, 83,10 % y 95,77 %, respectivamente a la dosis y con respecto a la semi–cuantificación *in vitro* del extracto crudo.

En cuanto al extracto acuoso en caliente (OV2), se identificaron dos picos relevantes: el ácido rosmarínico (**32**), compuesto mayoritario de los extractos (salvo de OV1), y el mismo flavonoide identificado en OV1 *in vivo* (**15**), aunque con menor presencia dentro del gusano (23,32 %, 30,57 % y 34,72 %). La cantidad detectada por HPLC–DAD de los cuatro compuestos fue proporcional a la dosis de extracto (10, 20 y 50 mg/mL). La referencia se determinó en base al ácido rosmarínico, que se utilizó como control positivo. En los gusanos tratados con 50 mg/mL de ácido rosmarínico, la presencia en el producto digerido fue del 37,33 ± 0,02 %, mientras que en los tratados con OV2 la presencia fue de 23,60 ± 0,02 % (74,14 % del extracto crudo). Tras el proceso de digestión *in vitro* de OV2, el porcentaje de bioaccesibilidad del ácido rosmarínico fue del 45,05 %, mientras que la digestión del ácido rosmarínico preserva un 79,12 % biodisponible en el intestino. Aunque sirve de referencia, el comportamiento del ácido rosmarínico utilizado como

control positivo no puede considerarse igual al que se encuentra en un extracto, donde hay más compuestos también expuestos a los cambios bio–fisicoquímicos del ambiente y sufren modificaciones. Si bien es cierto que la presencia de ácido rosmarínico en ambos casos fue dosis–dependiente dentro del gusano, la dieta del gusano fue más variada en el caso de OV2 que los que sólo recibieron ácido rosmarínico y *E. coli* OP50. De cualquier modo, esta adaptación del método para cuantificar la actividad antioxidante *in vivo*, ha servido para corroborar la presencia de compuestos químicos bioactivos dentro del gusano y así, poder atribuir los efectos que se observen tras los tratamientos a la composición química de los extractos acuosos.

Por ser el extracto más antioxidante entre los dos acuosos, la capacidad antioxidante de la fracción absorbible de OV2 tras una simulación gastrointestinal *in vitro* se determinó *in vivo* siguiendo el mismo procedimiento descrito anteriormente. Mientras que el ácido rosmarínico no mostró diferencias significativas en su actividad antioxidante tras la digestión en ninguno de los dos escenarios (*in vitro* e *in vivo,* **Figura 104**), los resultados para el extracto incluso mostraron una mejoría del 40,73 % de la actividad antioxidante *in vivo* (**Figura 104)**, por lo que en condiciones *in vivo*, el extracto podría ser más antioxidante una vez digerido.

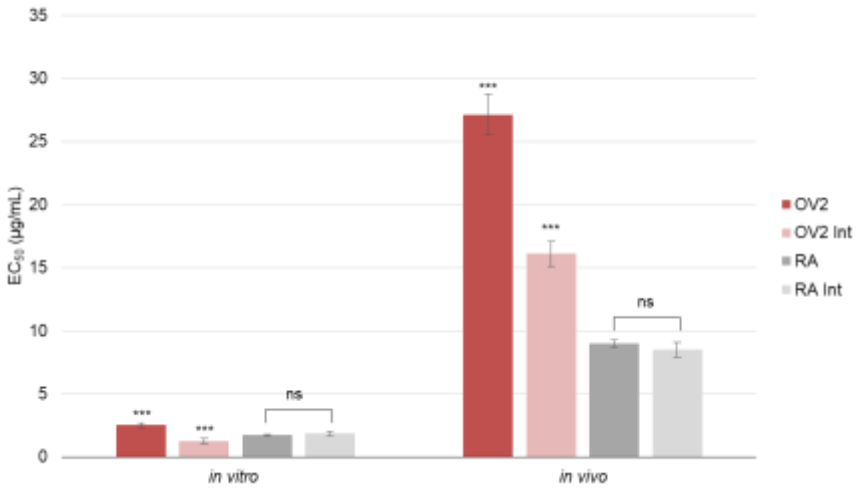


Figura 104. Actividad antioxidante frente al radical libre DPPH *in vitro* e *in vivo* de OV2 (rojo) comparado con ácido rosmarínico (RA, gris). Resultados correspondientes a los valores de IC_{50} (media ± DE µg/mL) en el punto de estabilización. ***: diferencias significativas, $p < 0{,}001$; ns: no diferencias sifnicifativas, $p > 0{,}05$.

Otro método para determinar la actividad antioxidante en el *modelo C. elegans* es el efecto protector que ejercen los extractos frente a un agente que provoca estrés oxidativo. Dependiendo de la dosis y del tiempo de exposición del xenobiótico, el efecto tóxico en los gusanos será agudo y letal, subagudo o crónico [177,329]. Por ejemplo, en el ser humano, los síntomas de intoxicación por paraquat (agente pro–oxidante) no aparecen de modo aparente y claro, sino que son intoxicaciones subagudas que aparecen a lo largo del

tiempo [422]. En este estudio se empleó juglona como agente pro–oxidante porque es capaz de generar especies reactivas de oxígeno (ROS) [329]. En *C. elegans*, la juglona se puede aplicar como un acelerador del envejecimiento con el fin de evaluar genes y vías con funciones protectoras y muchos estudios evalúan la letalidad de los gusanos a lo largo del tiempo después de dosis bajas de exposición a juglona [325,329]. Todos esos estudios se centran principalmente en los gusanos y sus propios mecanismos naturales protectores de ROS. Sin embargo, en este trabajo, al elegir una dosis letal de juglona (80 mM) a las 24 h, el interés del estudio se traslada a determinar la supervivencia de los gusanos con respecto al tratamiento recibido [177,405]. Este ensayo se realizó en las mismas condiciones que las descritas para determinar la actividad antioxidante *in vivo* frente al radical libre DPPH (48 h de tratamiento, de L1 a L4–edad adulta), para determinar si la actividad antioxidante observada en los extractos OV1 y OV2 actúa como protectora frente a ROS, evaluando la tasa de supervivencia de los gusanos como parámetro de sensibilidad al estrés oxidativo. Para la realización de este ensayo, dado que la juglona se disuelve en etanol, fue necesario realizar un estudio previo para determinar si el disolvente afectaba de alguna manera al desarrollo de *C. elegans*. Tras 24 h se observó que a todas las concentraciones ensayadas de extracto OV1 y OV2 (0,1 – 50 mg/mL) los gusanos tenían una supervivencia superior al 90 %. Posteriormente, en el ensayo definitivo, se sustituyó el etanol por la juglona (80 mM). Se observaron diferencias entre ambos extractos a dosis bajas de 0,1 y 10 mg/mL. Sin embargo, a partir de 10 mg/mL ambos presentaron una supervivencia de los gusanos superior al 70 %. En todos los casos se observó una respuesta dosis–dependiente, siendo siempre superior la del extracto OV2. La supervivencia de los gusanos a una dosis letal de un compuesto pro–oxidante como es la juglona quedaría justificada por la actividad antioxidante observada en los ensayos *in vitro* e *in vivo* expuestos anteriormente.

El objetivo principal de los compuestos antioxidantes es contrarrestar el desequilibrio producido en la generación de ROS. En *C .elegans* es posible la medición de ROS tras la administración de diferentes tratamientos, a fin de observar si existe un efecto protector que se pueda relacionar con una actividad antioxidante funcional y anti–edad. A nivel de laboratorio, el compuesto H_2DCFDA es un compuesto permeable que, en presencia de ROS, es oxidado dando lugar a un compuesto altamente fluorescente cuantificable (DCF). De este modo, el tratamiento previo de los gusanos con compuestos antioxidantes (como los extractos acuosos, cuya actividad antioxidante interna y protectora ante un xenobiótico pro–oxidante ya han quedado demostradas) propiciará una menor generación de ROS y, por tanto, una menor fluorescencia con respecto al control. Contrariamente al pensamiento general de que más es mejor, los gusanos tratados con ácido rosmarínico

mostraron una sobreexcitación siendo significativamente mayor la cantidad de ROS en el organismo vivo tratado que en ausencia de tratamiento. Esto podría justificarse por una posible toxicidad, tal y como también se observó en las células cuando este compuesto se administraba puro y a altas dosis. Otros autores han apuntado también a una activación de rutas metabólicas mitocondriales. Una alta bioactividad puede llevar a un aumento del estrés oxidativo de la célula o el organismo que se traduce en un aumento de la generación de ROS. A diferencia del compuesto control, los extractos OV1 y OV2 (con capacidad antioxidante dentro del gusano) mostraron el efecto esperado reduciendo significativamente la cantidad de ROS respecto al control. Mientras que OV1 no mostró una relación dosis–dependiente reduciendo la cantidad en un 18 %, OV2 fue capaz de reducir a la mitad el efecto respecto del control cuando se administra a 5 mg/mL en vez de a 1 mg/mL. Sin embargo, la alta actividad antioxidante de la fracción intestinal de OV2 posiciona esta dosis al mismo nivel que 5 mg/mL; es decir, la capacidad antioxidante capaz de contrarestar la acumulación de ROS en el gusano es cinco veces mayor en el extracto digerido. Lo que es lo mismo, el extracto digerido *in vitro*, mantiene sus actividad antioxidante *in vivo*. Viendo que la actividad antioxidante de los extractos acuosos se mantiene *in vivo,* puede que el resto de actividades determinadas *in vitro* también lo hagan en este modelo.

4.2 Actividad hipoglucemiante

En los estudios realizados previamente *in vitro*, el extracto OV1 extraído por maceración en frío con agua fue el que presentó mejores resultados como agente inhibidor de α–glucosidasa, con una actividad 7,72 veces mayor que la acarbosa. Para comprobarlo *in vivo*, se puso a punto un método con *C. elegans* similar al realizado para la actividad antioxidante. Las condiciones de crecimiento y tratamiento de los gusanos fueron las mismas que para la determinación de actividad antioxidante directa del nuevo método propuesto en el presente trabajo. Después, la cuantificación de la actividad hipoglucemiante de la muestra obtenida del interior del gusano se determinó por espectrofotometría de la misma manera que *in vitro*. Se ensayaron las mismas concentraciones de extractos OV1 y OV2, entre 0,1 – 50 mg/mL. Sólo se pudo calcular el valor IC_{50} (13,78 mg/mL) del extracto OV1, mostrando una actividad 3,51 veces superior a la acarbosa. Este ensayo permitió corroborar los resultados obtenidos *in vitro*. Para confirmar definitivamente estos resultados sería interesante trabajar en un futuro con concentraciones mayores de extracto o con muestras más concentradas.

4.3 Esperanza de vida

Todas estas actividades analizadas hasta el momento tanto *in vitro* como *in vivo* tienen una finalidad común, encontrar un posible compuesto preventivo de enfermedades metabólicas relacionadas con la edad que conduzca a una mejora de la calidad de vida y un alargamiento de la esperanza de vida. En humanos resultaría prácticamente imposible realizar este tipo de estudios, pero en el caso de *C. elegans* su corto ciclo de vida de está permitiendo realizar ensayos de este tipo (*lifespan*). Muchos estudios de este tipo se realizan con la cepa BA17 de *C. elegans* porque no produce huevos y así, la inexistencia de descendientes permite analizar la supervivencia sin el elemnto confusor de la aparición constante de individuos nuevos en el cultivo [426]. En este caso, se ha trabajado con la misma cepa que en el resto de ensayos *C. elegans* N2 a la que se añadió 5–fluro–2'–desoxiuridina (FuDR) en el medio de cultivo para evitar la eclosión de los huevos y la aparición de nuevos descendientes [433].

En el diseño de este experimento se tuvieron en cuenta varios factores que podrían condicionar el desarrollo uniforme de los gusanos:

- Incorporación de los extractos. En los estudios expuestos anterioremente se descarta que la adición de los OV1 y OV2 alteren el comportamiento de los gusanos.
- Fuente de alimento limitada que podría modificar el crecimiento de los gusanos. En este estudio los gusanos permanecieron en la misma placa hasta su muerte. Por ello, la fuente de alimento natural (*E. coli* OP50) se aumentó a una cantidad inicial de 200 µL en lugar de los 100 µL estándar. Esta cantidad era suficiente según estudios previos [315].

En un tratamiento farmacológico no solo es importante la dosis, sino también la duración del mismo. En un estudio convencional de *lifespan*, se puede considerar que la adición de un compuesto al medio es análogo a la realización de un tratamiento crónico en edad adulta, ya que los gusanos tienen contacto con ese tratamiento desde la etapa L4–adulto hasta su muerte [328]. Sin embargo, ¿qué pasaría si ese tratamietno se administrara durante toda la vida incluyendo el desarrollo o se considerara como un suplemento sólo durante su crecimiento? Por este motivo, como novedad en este experimento, se diseñaron dos variantes, denominadas *life* y *growing*. Para la primera, los gusanos estaban en contacto los extractos desde desde la fase L1 hasta su muerte. En la segunda, los extractos se administraron a los gusanos durante su periodo de crecimiento (L1–L4–adulto) y se les retiró a partir de la fase adulta.

Para controlar que el desarrollo y crecimiento fueran los adecuados, durante los primeros días se vigiló la longitud y la velocidad de bombeo faríngeo. A lo largo del estudio, se fueron contando el número de gusanos muertos mediante un toque suave con un alambre esterilizado cada dos días, y se fueron retirando de la placa [315,328]. Generalmente, los resultados brutos se expresan como porcentaje de supervivencia para cada día, con los cuales se calcula la esperanza de vida para el 25, 50, 75 y 90 % de los gusanos. En el ser humano se acostumbra a escuchar el dato de esperanza de vida para determinadas poblaciones (por condición geográfica, económica, de raza o sexo) en años: las mujeres españolas viven de media 85,06 años, frente a los 79,59 de los hombres españoles. Estos datos suelen ser calculados cada año y han ido creciendo con el avance de la ciencia y tecnología, aunque la presente pandemia vaya a disminuir dichos valores en los próximos años. De igual manera, pero en días, fueron expresados los resultados para el estudio de esperanza de vida en *C. elegans* según el tratamiento aplicado con respecto a la ausencia de tratamiento (control).

El experimento se diseñó con tres réplicas, de las que dos de ellas se tuvieron que interrumpir por la pandemia el día 25. En ese día los diferentes tratamientos ya estaban mostrando un mayor porcentaje de supervivencia que el control sin tratamiento aplicado. La **Figura 105** recopila dichos porcentajes, donde se puede observar que, a grandes rasgos, el tratamiento con extracto OV2 mostró mayor supervivencia que el tratamiento con OV1; aunque también presentó una menor reproducibilidad de los datos quizás por encontrarse en el declive de supervivencia (la mayor parte de los gusanos empiezan a morir).

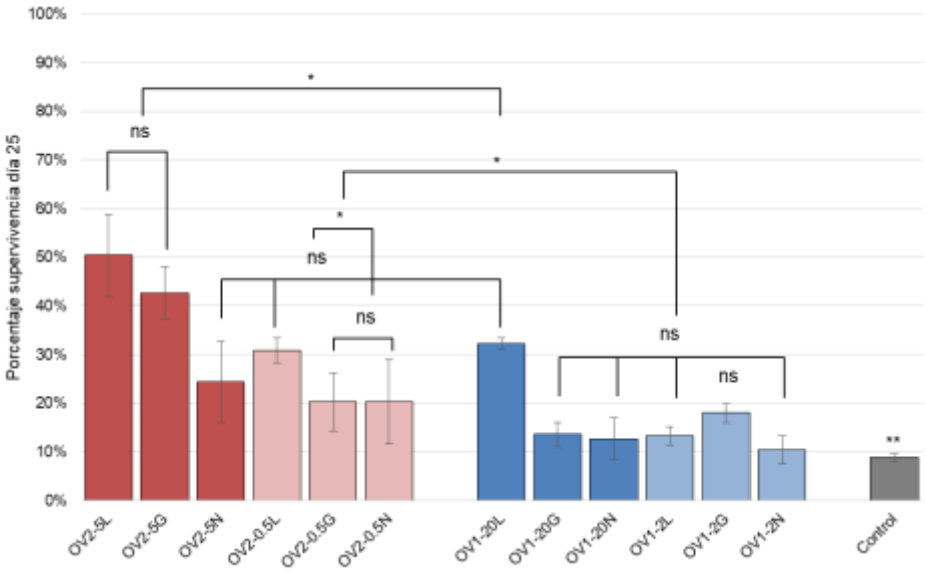


Figura 105. Porcentaje de supervivencia de las tres réplicas de l experimento de *lifespan* a día 25 de conteo (media ± DE). L:, experimento *life*; G:, experimento *growing*; N: experimento *normal*. El valor que acompaña al extracto indica la dosis en mg/mL. ***, $p < 0,001$; **, $p < 0,01$; * $p < 0,05$; ns: diferencias no significativas.

La falta de estudios publicados evaluando los diferentes tipos de tratamiento (*life*, *growing* y *normal*) dificulta la comparación con los resultados de otros autores. Sin embargo, independientemente de la duración del tratamiento, la presencia de OV2 durante las fases de crecimiento (*life* y *growing)* parece mejorar la esperanza de vida, sobre todo en el caso de la dosis a 5 mg/mL. El extracto acuoso en frío no mostró diferencias significativas a día 25 entre los tratamientos, salvo para una dosis alta de por vida (OV1–20L, **Figura 105**) y una dosis de 2 mg/mL durante el crecimiento (OV1–2G, **Figura 105**). De cualquier manera, las tres réplicas coinciden en que los extractos de orégano alargan la vida del gusano frente a la ausencia de tratamiento.

Los valores obtenidos reflejan la esperanza de vida de una población y no de un individuo sólo. Cuando en el ser humano se habla de esperanza de vida, el dato tiene en cuenta a la persona más longeva del mundo, pero su edad no es representativa de la edad media estimada que vivirá el resto de la población. Por este motivo, los resultados que deberían ser comparados no deberían referirse a cuántos días vivió el último gusano en la placa sino, la edad media de vida de los gusanos representativa, correspondiente al 50 – 75 % de la población. Los resultados una vez terminado el estudio (**Figura 106**), aunque sólo correspondan a los valores de la primera réplica, muestran que los gusanos que recibieron OV2 a una dosis de 5 mg/mL durante al menos el crecimiento vivieron significativamente de media más días que el resto de gustanos, un 67 % más que el control. El resto de tratamientos – dosis bajas de OV2 o ausentes en el crecimiento, así como OV1 en cualquier etapa y a cualquier edad – alargan significativamente la vida de los gusanos en torno a un 40 %.

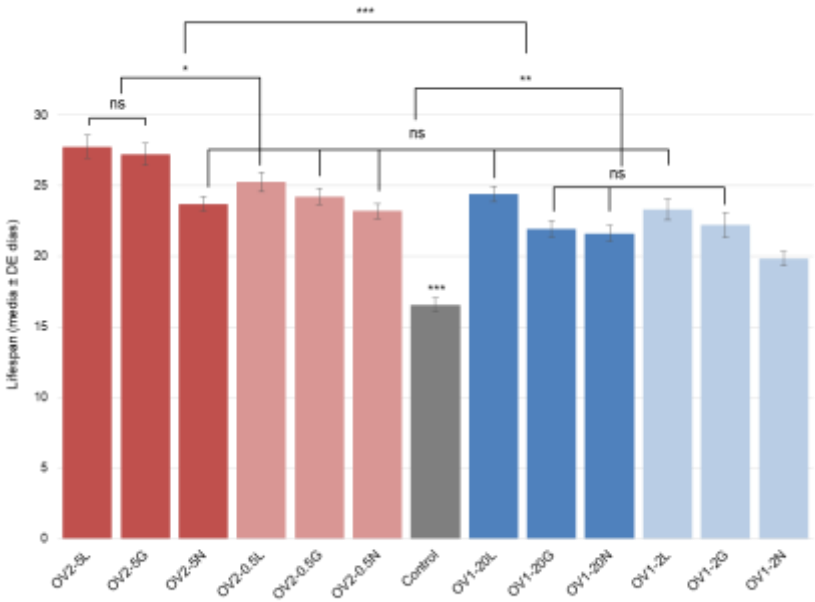


Figura 106. Vida media de supervivencia (media ± DE días). L: experimento *life*; G: experimento *growing*; N: experimento *normal*. El valor que acompaña al extracto indica la dosis en mg/mL. ***, $p < 0{,}001$; **, $p < 0{,}01$; * $p < 0{,}05$; ns: diferencias no significativas.

Anteriormente se determinó el efecto protector frente a dosis letales de un xenobiótico pro–oxidante, donde la tasa de supervivencia fue mayor para las poblaciones que recibieron el extracto acuoso como tratamiento [177,405], siendo OV2 más potente a dosis bajas. Los análisis de *lifespan* también estudian el efecto de supervivencia de un compuesto en gusanos donde el desafío a superar es el envejecimiento [339]. Los gusanos vivieron más tiempo cuando reciben orégano de por vida pero, en cualquier caso, todos los tratamientos prolongaron la esperanza de vida de los gusanos. Sumado al resto de actividades (la actividad antioxidante e hipoglucemiante interna, la disminución de la acumulación de ROS y la capacidad protectora frente a agentes tóxicos) estos resultados apuntarían a la posibilidad de uso de los extractos acuosos de manera preventiva, ya que podría aparecer menor daño oxidativo en el organismo humano envejecido.

5 Formas farmacéuticas

El ultimo paso de este trabajo fue **diseñar varios productos fitoterápicos con distintas formas farmacéuticas para uso oral y tópico** en base a los resultados fitoquímicos y farmacológicos obtenidos en los capítulos anteriores Se denominan “productos fitoterápicos” aquellos productos elaborados a partir de drogas vegetales o sus derivados listos para su administración y destinados a la utilización en pacientes o personas sanas con finalidad terapéutica, preventiva o para el mantenimiento de la salud.

Desde un punto de vista regulatorio e independientemente de su forma de comercialización (medicamentos a base de plantas, medicamentos tradicionales a base de plantas, complementos alimenticios, productos sanitarios, etc.), todos ellos presentan una estructura similar formada por dos partes: los ingredientes activos y la forma farmacéutica.

Los ingredientes activos de los productos fitoterápicos son principalmente preparados vegetales, que se definen como productos homogéneos que se obtienen sometiendo las drogas vegetales a tratamientos como extracción, destilación, prensado, fraccionamiento, purificación, concentración o fermentación. Su composición química es compleja, lo que representa una dificultad para el establecimiento de especificaciones de calidad a nivel de su estandarización y comercialización.

Al diseñar la forma farmacéutica de un producto fitoterápico se debe seleccionar el preparado vegetal más adecuado de acuerdo con sus características farmacológicas, objetivo terapéutico, características del paciente, dosificación y duración del tratamiento y vía de administración. El objetivo terapéutico de la planta medicinal condiciona la forma y la vía de administración. Si la enfermedad está localizada en determinados órganos o tejidos puede ser mejor una administración local, por ejemplo sobre la piel, la nariz, la garganta o los ojos. De ese modo el principio activo, en general, no accede a la circulación sistémica y se evita la aparición de efectos secundarios no deseados. Si se necesita una acción sistémica, la vía oral es fácil de utilizar y muy cómoda para el paciente, pues es la entrada fisiológica de sustancias al organismo. Por ello, la mayor parte de las plantas medicinales se administran en preparados de uso oral y sobre todo cápsulas. No obstante, no siempre se puede utilizar la vía oral ya que algunos principios activos no se absorben por esta vía o experimentan un efecto de primer paso muy importante. Además, algunos pacientes pueden presentar problemas de deglución que hacen que esta vía de administración sea poco práctica.

La administración de plantas medicinales se puede realizar utilizando desde formulaciones galénicas muy sencillas (infusiones, decocciones, tinturas, etc.) hasta formas farmacéuticas complejas con extractos vegetales. La utilización de extractos en fitoterapia permite la preparación de una mayor variedad de formas farmacéuticas y, en el caso de los extractos secos, disminuir la dosis con respecto a las drogas vegetales de origen. Esto redunda en una mayor facilidad de administración y la elaboración de formas que van a contener una mayor concentración de principios activos que la droga vegetal de partida. Sin olvidar tampoco su mayor estabilidad, eficacia y seguridad y, en general, mejor presentación y comodidad en su administración.

Entre las ***preparaciones orales*** utilizadas en fitoterapia se pueden diseñar formulaciones líquidas o sólidas. Entre las preparaciones líquidas destacan los zumos, macerados, infusiones, cocimientos, tisanas, extractos fluidos, suspensiones integrales de planta fresca, aceites esenciales, aguas aromáticas, licores, vinos medicinales, gotas orales, jarabes, elixires y ampollas bebibles. Sin embargo, las preparaciones sólidas presentan ciertas ventajas frente a las líquidas, entre las que destaca su mayor estabilidad. Las formas sólidas de plantas medicinales más utilizadas para la administración oral son polvos, comprimidos y, sobre todo, las cápsulas.

Dentro de las ***preparaciones para aplicación cutánea***, en función de las características fisicoquímicas de los componentes de la formulación, se pueden encontrar preparaciones líquidas y preparaciones semisólidas. Las formas de administración líquidas pueden prepararse como soluciones, suspensiones o emulsiones. Entre las más frecuentes se encuentran: aceites esenciales, aguas aromáticas, linimentos, lociones y baños. Los preparados de consistencia semisólida constituyen el grupo más amplio dentro de las formulaciones de aplicación cutánea. Están destinadas a su aplicación sobre la piel con el fin de ejercer una acción local o sistémica. Las formas farmacéuticas de consistencia semisólida constan de un excipiente, sencillo o complejo, en cuyo seno se disuelven o se dispersan los principios activos. En la Real Farmacopea Española se distinguen varias categorías de preparados de consistencia semisólida: pomadas, cremas, geles y pastas.

Por lo que se refiere al número de ingredientes activos incluidos dentro de un producto fitoterápico se puede distinguir:

- Productos simples o monodrogas, que son aquellos que llevan como ingrediente activo un único preparado vegetal, ya sea una droga vegetal, un extracto u otro tipo de preparado.
- Combinaciones de preparados vegetales, constituidas por mezclas de un número variable de drogas vegetales o extractos. Lo que se pretende con estas asociaciones es que la actividad de una de las drogas se vea complementada con la de las demás.

5.1 Vía oral

De acuerdo con las propiedades *in vitro* del extracto macerado en frío, se diseñaron **dos formas farmacéuticas orales simples o monodroga** con el liofilizado del extracto hidroalcohólico OV3: preparados o polvos solubles instantáneos y cápsulas de gelatina dura. Ambas dosis diarias se estandarizaron a 500 mg por dosis y día (500 mg de polvo o dos cápsulas de 250 mg cada una) con al menos un 33 % de ácido rosmarínico [355]. Las

fitomedicinas y los complementos alimenticios con plantas tienden a expresar la dosis en porcentaje o cantidad del compuesto principal [235,340,434]. Esta información garantiza la estandarización del extracto elaborado así como un control de seguridad de la eficacia [153,248]. En cuanto al control de calidad, tanto las cápsulas como el polvo para resuspensión en agua satisfacieron los ensayos de preparación (uniformidad de masa y contenido) y disgregación de acuerdo con lo establecido por Real Farmacopea.

Posteriormente, ambas formulaciones se sometieron a un proceso de digestión gastrointestinal *in vitro,* de la misma manera que los extractos OV1, OV2 y OV6 (**capítulo I**). El principal interés en el diseño de las formulaciones orales es la mejora de la eficacia del tratamiento gracias a la preservación de compuestos frente al proceso de digestión, que puede inactivar o digerir ciertos compuestos disminuyendo su bioaccesibilidad. En términos generales, ambas formulaciones presentaron una bioaccesibilidad superior al 80 %, suponiendo que gran parte de los compuestos sobrevivieron a los cambios fisiológicos de pH en las diferentes etapas y la acción de las enzimas digestivas (amilasa, pepsina, pancreatina y bilis). Como primera diferencia entre las dos formulaciones, los compuestos se encuentran más expuestos en el polvo disuelto en agua que en la cápsula, ya que en ésta tienen que liberarse primero en el estómago. De hecho, la encapsulación conservó mejor algunos compuestos relacionados con la actividad antioxidante como los flavonoides, sin embargo la bioaccesibilidad del ácido rosmarínico (compuesto de referencia) no fue significativamente diferente del polvo seco para solución oral. Ante esto, hemos de tener en cuenta que la cantidad biodisponible no implica que sea potencialmente activa. Un ejemplo de esto es el desarrollo de fórmulas galénicas gastrorresistentes o la formulación del principio activo como prodroga que debe modificarse y activarse antes de llegar al lugar de acción. No obstante, la presencia de compuestos bioactivos como los flavonoides, derivados del ácido dihidroxibenzoico o el ácido rosmarínico puede ser considerada una buena premisa de la potencial bioactividad de las dos formulaciones.

Los buenos resultados de biodisponiblidad obtenidos en ambas formulaciones nos llevó a ensayar varias actividades farmacológicas con los productos digeridos: actividad antioxidante frente al radical libre DPPH, hipoglucemiante, hipoglucemiante, hipolipemiante y acetilcolinesterásica *in vitro*, así como, la actividad antioxidante *in vivo*.

Los resultados de los ensayos de actividad antioxidante, tanto *in vitro* como *in vivo*, indicaron que el extracto encapsulado muestra una mayor actividad antioxidante tras el proceso de digestion *in vitro* que el extracto administrado en forma de polvo instantáneo (**Figura 107**).

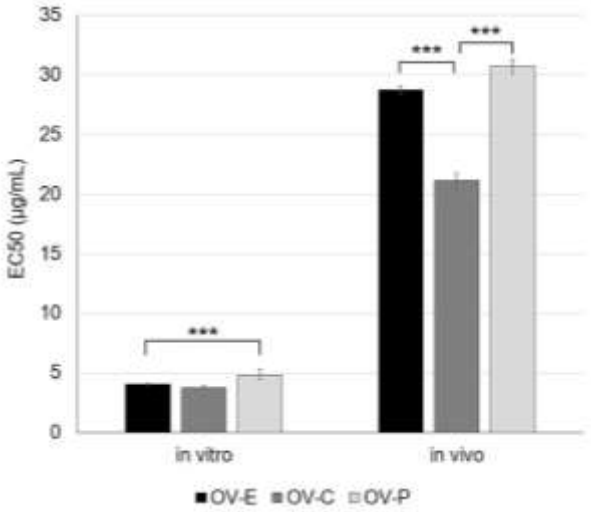


Figure 107. Actividad antioxidante cuantificada por el ensayo DPPH• *in vitro* e *in vivo*. Resultados expresados en EC_{50} (media ± DE µg/mL). Valores mostrados en el punto de estabilización. OV–E: OV3 extracto bruto sin digerir (en negro); OV–C: fracción intestinal absorbible de OV3 encapsulado (gris oscuro); OV–P: fracción intestinal absorbible de OV3 en forma de polvo para suspensión (gris claro). ***, diferencias significativas ($p < 0,001$).

A pesar de no existir un factor de conversión matemático *in vitro – in vivo* entre estos dos ensayos, en la **Figura 107** se observa claramente cómo la cápsula mejora esta actividad. La preservación de los compuestos químicos por parte de la cápsula ya anticipaba los resultados de bioactividad observados aunque, en este caso, la actividad antioxidante fue incluso mayor que en el propio extracto sin digerir (OV3–E, **Figura 107**).

Tal y como se observó en el **capítulo II** con los extractos, una menor cantidad de compuestos no siempre se correlaciona con una menor bioactividad. De hecho, OV1 demostró un efecto inhibitorio de la enzima α–glucosidasa significativamente mayor que el control positivo. En esta actividad, OV3 resultó igual de eficaz que la acarbosa, medicamento comercializado como antidiabético oral usado como control positivo. Hasta ahora, dicha actividad se ha visto potenciada tras el proceso de digestión gastrointestinal cuando se administra como polvo liofilizado (OV1–Int y Acarbosa–Int). El polvo para resuspensión en agua de OV3 no sólo mejoró su actividad tras la digestión sino que la de la acarbosa también. En el caso de la cápsula, esta forma farmacéutica no demostró una mejora de la actividad como con la antioxidante.

No se ha establecido una clara relación entre la composición química y las actividades farmacológicas tras el proceso de digestión entre las dos formas farmacéuticas orales. Si bien es cierto que la encapsulación preservó mejor los flavonoides, lo que podría explicar los resultados obtenidos en las actividades antioxidantes y similares, como la actividad hipolipemiante, donde la cápsula vuelve a ser la formulación de elección.En el caso de la

actividad anti–acetilcolinesterasa ésta resultó beneficiada tanto para la cápsula como para el polvo de resuspensión.

Finalmente se realizaron ensayos de citotoxicidad frente a las líneas celulares Caco–2 y HepG–2. A nivel intestinal no se encontró riesgo de toxicidad, incluso a la dosis más alta testada (toxicidad < 30 % a 1 mg/mL), siendo significativamente menor en forma de polvo para resuspensión que en cápsula. Además, gráficamente los datos tendieron a describir una asíntota horizontal, por lo que se podría esperar que no aumentaran a dosis superiores. A nivel hepático, el extracto encapsulado siguió la tendencia del extracto no digerido con un valor límite de IC_{50} próximo a 0,5 mg/mL. En cambio, la larga exposición a dosis altas sí que produjo toxicidad en el polvo liofilizado con valores superiores al 90 % para concentraciones mayores de 0,5 mg/mL.

En resumen, el tratamiento encapsulado tuvo más éxito en las bioactividades relacionadas con el efecto antioxidante (frente a los radicales libres e inhibición de la acetilcolinesterasa), mientras que el polvo funcionaría mejor para la diabetes por la vía de inhibición de α–glucosidasa. Sin embargo, a pesar de no haberse detectado citotoxicidad en Caco–2, se recomendaría un estudio más profundo de la farmacocinética de ambas formulaciones debido a una posible hepatotoxicidad en tratamientos crónicos de altas dosis.

5.2 Vía tópica

Cambiando la vía de administración, la EMA recopila el uso cutáneo tradicional de otras especies de orégano [239,322]. De hecho, estudios previos propusieron este remedio para trastornos de la piel como el acné [190,303,435]. Como complemento de los ensayos realizados, las propiedades microbiológicas de las formulaciones tópicas también podrían ser parte de interés en estudios posteriores.

Previo al diseño de las formas farmacéuticas tópicas, se realizó un ensayo más exahustivo del efecto tóxico de los extractos en la línea celular A375. Generalmente, el ensayo de MTT se utiliza para determinar la toxicidad producida por un compuesto en una línea celular a una concentración conocida. Cuando el porcentaje de toxicidad supera el 50 %, se puede considerar un efecto tóxico, y por ello es que se testan diferentes concentraciones de muestra a fin de conocer éste límite. No obstante, se desconoce qué cambios ocurren en la célula para que ésta no sea viable en presencia de un xenobiótico a dicha concentración.

Una célula eucariota típica mide entre 10 y 30 µm y, aunque los microscopios han evolucionado desde su origen en 1590, el estudio molecular de una célula puede ser

complejo y costoso. En el campo de la química existen técnicas espectroscópicas capaces de alcanzar niveles moleculares, que se utilizan en la identificación de sustancias químicas o grupos funcionales y análisis de los enlaces químicos. Una de ellas es la espectroscopía infrarroja por transformada de Fourier (FTIR), que permite comprender la estructura de moléculas individuales y composición de las mezclas moleculares. Mediante el uso de energía infrarroja, se determina la absorción, emisión o reflexión a una frecuencia específica, que está directamente relacionada con la energía de enlace vibratrio interátomico de la molécula, lo que permite identificar el tipo de enlace presente en la muestra.

Llevando esta técnica de análisis al campo biológico, sus ventajas en identificación permiten el estudio de los principales grupos moleculares que componen las células. Bandas de absorción en la franja de 3,050 a 2,180 cm^{-1} corresponden a los grupos CH_2 y CH_3, propios de las cadenas aciladas de los lípidos, mientras que las bandas obtenidas entre 1,800 y 900 cm^{-1} corresponden a los enlaces éster de los fosfolípidos (1,725 cm^{-1}) y amidas primarias (1,650 cm^{-1}), grupos amino de las aminas secundarias (1,540 cm^{-1}), ácidos grasos (1,4080 – 1,300 cm^{-1}), enlaces CO simples de los carbohidratos y material genético (1,300 – 900 cm^{-1}).

Las células A375 ya cultivadas en los soportes de cristal del equipo de medición de FTIR (espectrómetro Thermo Electron Corporations® Nicolet™ FTIR) se trataron durante 24 h con los extractos a dos concentraciones diferentes. Tras la medición por espectroscopía, las células de la piel sufrieron cambios moleculares diferentes para cada extracto con respecto al control en las diferentes bandas de absorción (**Figura 108**).

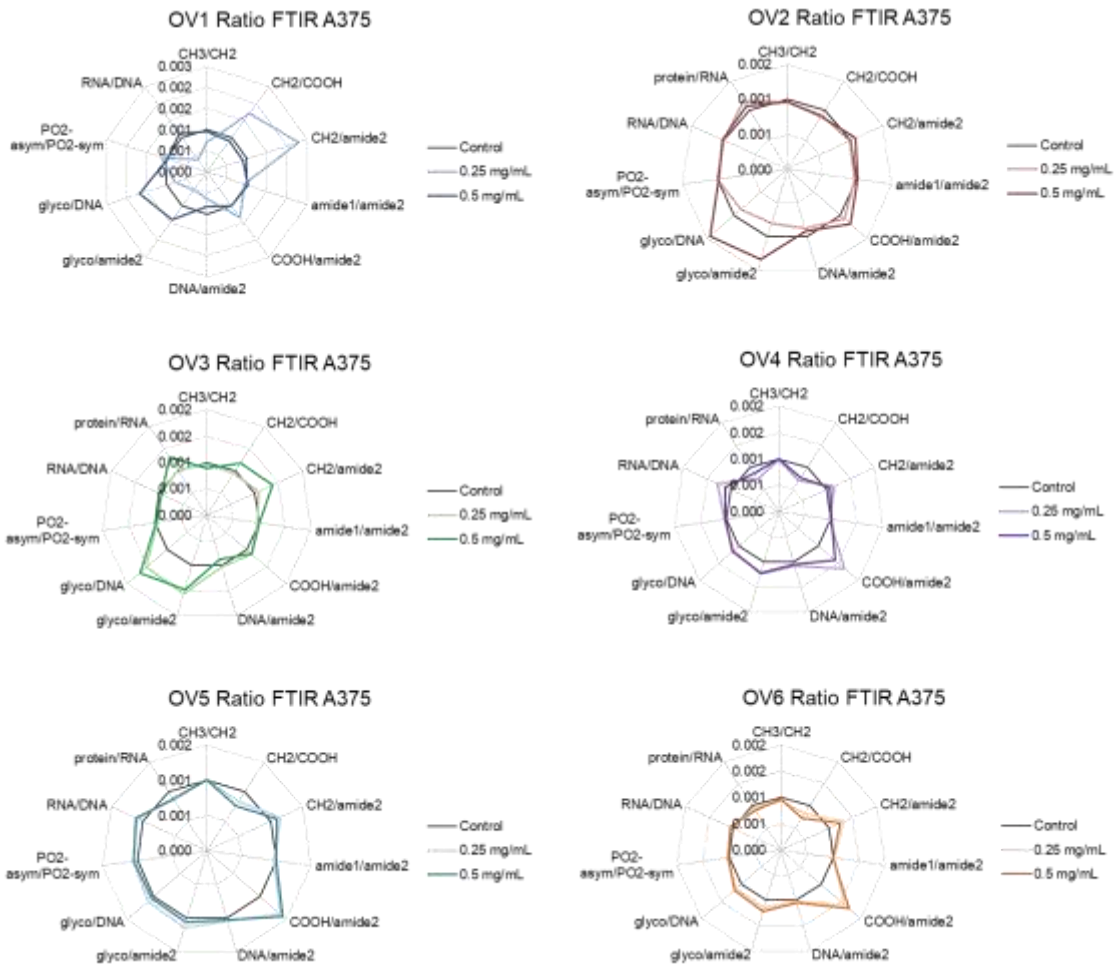


Figura 108. Cambios moleculares en A375 expresados como proporción por grupo analizado mediante la técnica FTIR. Cada gráfico corresponde a cada extracto a diferentes concentraciones dadas a las células (0,25 y 0,50 mg / mL). Azul para OV1, rojo para OV2, verde para OV3, morado para OV4, azul más claro para OV5 y naranja para OV6.

A simple vista, la forma de los gráficos de la **Figura 108** permite resumir los cambios moleculares observados con respecto al control (poliedro regular) en cada una de las bandas, asociadas según la proporción de los tipos de grupos moleculares en la célula. OV4, OV5 y OV6 se pueden agrupar, ya que la forma del gráfico es similar sin diferencias de concentración dentro del mismo extracto (líneas de 0,25 y 0,50 mg mL superpuestas). La proporción COOH/amide2 tiene relación con los fosfolípidos de membrana y las amidas secundarias [229], cuyo valor con respecto al control se mantuvo o incluso dismunuyó en estudios previos con cisplatino y un extracto de *A. adenophora* [302]. El incremento significativo compartido por OV4, OV5 y OV6 podría explicar la toxicidad de estos extractos, siendo menor para OV4.

Junto con la tendencia del perfil químico y ciertas bioactividades, OV1 fue completamente diferente al resto de extractos y al control en cuanto a la forma (**Figura 108**). Además, ambas concentraciones probadas presentaron resultados diferentes y, por lo tanto,

formas en el gráfico. De acuerdo con las desviaciones con respecto al control (sobre todo el aumento en COOH/amida II, como en OV4, OV5 y OV6) y la citotoxicidad encontrada en el ensayo MTT, el uso tópico de estos extractos no sería una opción a desarrollar. En el caso de OV3 se encontró una forma con efectos beneficiosos y ausencia de efecto tóxico sin importar la concentración (0,25 o 0,50 mg/mL) y OV2 a 0,50 mg/mL. Estos tres pefiles comparten un incremento en la proporción de los carbohidratos con amidas secundarias y material genético, que parece estar relacionada con una defensa a situaciones de estrés oxidativo [302]. En cambio, OV2 a 0,25 mg/mL parece ser el extracto más parecido al control, pudiendo significar que la concentración no fue suficiente como para producir cambios moleculares y, por lo tanto, efecto.

Se seleccionó OV2 para las formulaciones farmacéuticas tópicas similares a Arnidol ® (barra) y Voltaren ® (crema), que estarían indicadas para la inflamación aguda [290]. En ocasiones, este tipo de inflamación también está asociada a heridas o daños en la piel como, ya que la causa suele ser un traumatismo. Por ello, la actividad antimicrobiana ampliamente descrita para el orégano podría funcionar como propiedad coadyuvante en el proceso de curación.

Por otro lado, según el comportamiento observado en la línea A375, se pretendía que las formulaciones tópicas con OV3 fueran fluidas y extensibles para el uso diario, y así lo mostraron los ensayos de viscosidad y extensibilidad. Tanto el gel como la emulsión fluida (loción corporal) estarían indicados para las piernas pesadas, gracias al posible efecto venotónico y protector de vasos. Los ensayos de control de calidad corroboraron que las formulaciones farmacéuticas tópicas presentaban las características físico–químicas deseadas. Con un pH óptimo para uso cutáneo, la crema formulada con OV2 era menos extensible y más viscosa que el gel y la emulsión fluida que contenía OV3. En cualquier caso, el extracto recuperado de las cuatro formulaciones aún presentaba una gran actividad antioxidante *in vitro*, lo que anima a realizar más estudios con piel de cerdo.

La ciencia es una disciplina en continuo desarrollo, por lo que este trabajo ha significado la continuación de anteriores estudios y se espera que signifique el inicio de nuevos estudios. El principal objetivo y punto de partida de este trabajo fue la demostración científica del uso tradicional de la especie *O. vulgare* L. que, a pesar de su uso tan extendido no cuenta con monografía en la EMA y, por lo tanto, existen pocas presentaciones en el mercado farmacéutico. Se trata de una planta con muchas propiedades farmacológicas (antioxidante, hipoglucemiante, antiinflamatoria, hipolipemiante, antibacteriana,…), debidas a la combinación de los diferentes compuestos químicos identificados, y los no identificados como los polisacáridos

potencialmente hipoglucemiantes de OV1, que pudiera ser utilizada en un futuro próximo terapéutica con base científica.

La preparación de seis extractos ha demostrado la importancia de escoger un método de extracción óptimo y fundamentado con el fin de aprovechar todas las actividades farmacológicas que posee esta especie. La presencia de un compuesto o una planta medicinal en un producto sanitario o medicamento no implica que éste vaya a producir el efecto descrito en la literatura. La dosis terapéutica es crucial en farmacología, estableciendo una clara ventana terapéutica y posología según la indicación. En fitoterapia se adiciona la elección del material de partida (tipo de extracto), donde es necesario extraer los principios activos en las proporciones idóneas. Así pues, de acuerdo con todos los resultados de actividad farmacológica, los métodos de extracción más recomendables a tener en cuenta para *O. vulgare* L. serían maceración acuosa en frío (OV1) o caliente (OV2) y maceración hidroalcohólica en frío (OV3).

Según indicación, la forma farmacéutica también puede jugar un papel importante, ya que las cápsulas diseñadas con OV3 inhibían menos la enzima alpha–glucosidasa que el polvo para resuspensión en agua. Por muy obvio que un concepto pueda resultar, siempre hay un punto de partida que pretende demostrar, justificar y poner cimiento firme para los próximos avances. En el presente trabajo quedan reflejados varios métodos puestos a punto necesarios para la determinación *in vitro* de la actividad antidiabética y antiinflamatoria, que seguramente sirvan de herramienta para otras especies de plantas medicinales que se vayan a estudiar en el laboratorio de Farmacognosia de la Universidad de Navarra. Además, sin ser uno de los objetivos iniciales, este trabajo recoge un nuevo método para la determinación directa de la actividad antioxidante *in vivo* de compuestos que no puedan ser fluorométricamente trazables en *C. elegans.* El protocolo original –publicado en 2019 [254]– ha sido adaptado a otra actividad farmacológica y análisis químico como ejemplos de las múltiples adaptaciones que pueda llegar a tener este método en un futuro.

CONCLUSIONS

1. The yield of extraction of the chemical compounds present in the aereal flowered parts of *Origanum vulgare* L. depends on the polarity of the solvent used, but not on temperature of extraction. Rosmarinic acid is the majority compound in all the extracts, with the exception of aqueous cold macerated, OV1, where it is not detected. However, OV1 presents a high percentage of dihydroxibenzoic acids. In the six extracts, flavonoids are present in variable quantities.

2. After an *in vitro* gastrointestinal digestion process, aqueous extracts (OV1 and OV2) and the hot ethanolic extract (OV6) show intestinal bioaccesibility values over 80 %, being higher in aqueous extracts. It confirms the potentially access to systemic circulation and, in consequence, the possibility of being pharmacological agents.

3. All the extracts present high antioxidant activity *in vitro*. There is a relation between scavenging activity against DPPH free radical and temperature of extraction, being hot macerated extracts more active. However, scavenging activity against ABTS free radical is related to the polarity of the solvent of extraction, being results better for ethanol or hydroalcoholic solutions. Both activities enhance after digestion process of aqueous extracts.

4. Aqueous extract OV1 best inhibits the enzyme *α–glucosidase*, before and after gastrointestinal digestion process, being higher than acarbose, the oral hypoglycaemic reference drug. Furthermore, OV1 counts with a good lipid–lowering activity against the enzyme HMG–Coa reductase.

5. The extracts with better results as inhibitors of acetyl–cholinesterase enzyme are OV6 and OV1, with higher values than galantamine, reference drug. In spite of this, the activity of both extracts decreases after digestion process.

6. With reference to anti–inflammatory activity, all the extracts show lower percentages of albumin denaturation and haemolysis than acetylsalicylic acid. OV1, OV2 and OV3 afford the most successful results.

7. The study of citoxicity on Caco–2 cell line shows that aqueous extracts provide percentages below 25 % at all concentrations tested.

8. It has been designed two new variants of traditional MTT method, which could be extended to any cell line. The variant *growing* assesses the cytotoxic effect when the xenobiotic is dispensed during cellular growth, whereas the variant *life* contemplates the effect for a continuous exposition.

9. In the hepatic cell line, the extracts OV2, OV3 and OV5 did not overpassed 50 % of toxicity in any of the variants, same as the positive control doxorubicin. These results

are maintained or improved after digestion process. In A375 cell line, OV3 shows low toxic levels at any concentration and time of exposition. Aqueous extract OV2 does not provide toxic effect on short expositions.

10. A new simple and reliable method has been developed to determine pharmacological activities inside *in vivo* model *C. elegans*. Thanks to this method, functional effects can be certainly attributed to the intake of a non–fluorimetrically traceable compound.
11. Bioactivity results found on *in vivo* experiments are correlated to the ones obtained *in vitro*, both before and after the digestion process. Moreover, presence of extract inside the worm has chemically been confirmed, finding compounds such as 2,5–DHBA after treatment with OV1 and rosmarinic acid after treatment with OV2.
12. Aqueous extracts do significantly improve survival rate of *C. elegans* after 24 h treatment with juglone. Furthermore, OV2 demonstrates a high capacity on reducing 50 % of ROS accumulation at a dose of 5 mg/mL. This effect boosts after gastrointestinal digestion process.
13. Two new non-published variants of the conventional *Lifespan* method have been developed to evaluate the effect on growth of the worm (*growing*) or lifelong administration (*life*). Either OV1 or OV2, dispensed at adulthood, expand average life expectancy on a 40 %. Treatments with 5 mg/mL of OV2 during growth or continually provide a 67 % of expansion on the average lifespan.
14. Two oral pharmaceutical forms were designed with OV3 extract, hard gelatine capsules and lyophilised powder to suspend in water. After digestion process, encapsulation better preserves chemical composition and antioxidant activity, both *in vitro* and *in vivo.* By contrast, lyophilised powder shows better hypoglycaemic activity. Citotoxicity assays performed on Caco–2 and HepG–2 support a possible safe use of both formulations.
15. Four pharmaceutical forms for cutaneous use have been designed. A cream and a stick with OV2, indicated for acute anti–inflammatory use, and a gel and body lotion with OV3 for lower extremity venous chronic stasis.

CONCLUSIONES

1. El rendimiento de extracción de los compuestos químicos presentes en las partes áereas floridas de *Origanum vulgare* L. depende de la polaridad del disolvente empleado, pero no de la temperatura de extracción. El ácido rosmarínico es el compuesto mayoritario en todos los extractos, excepto en OV1, el cual presenta un alto porcentaje de ácidos dihidroxibenzoicos. En todos los extractos se pueden cuantificar cantidades variables de flavonoides.
2. Tras un proceso de digestión gastrointestinal *in vitro,* los extractos acuosos (OV1 y OV2) y el extracto etanólico en caliente (OV6), presentan valores de bioaccesibilidad intestinal superiores al 80 %, siendo mayor en los acuosos. Esto apunta que podrían alcanzar la circulación sistémica y ser potenciales agentes farmacológicos.
3. Todos los extractos muestran una alta actividad antioxidante *in vitro*. Frente al radical libre DPPH, los extractos obtenidos en caliente son más activos. Sin embargo, frente al radical libre ABTS, los mejores resultados se obtienen con etanol o mezclas hidroalcohólicas. Dicha actividad aumenta tras el proceso de digestión en los extractos acuosos.
4. El extracto OV1 es el que presenta mejores resultados como agente inhibidor de α–glucosidasa, antes y después del proceso de digestión y superiores a la acarbosa, fármaco hipoglucemiante de referencia. Además, este mismo extracto muestra una buena actividad hipolipemiante.
5. Los extractos que presentan mejores resultados como inhibidores de la enzima acetilcolinesterasa son OV6 y OV1, con valores superiores a la galantamina, fármaco de referencia. En este caso, la actividad de ambos extractos tras el proceso de digestión disminuye.
6. Respecto a la actividad anti–inflamatoria, todos los extractos presentan porcentajes de desnaturalización de albúmina y hemólisis inferiores al ácido acetilsalicílico. Los extractos OV1, OV2 y OV3 son los que presentan mejores resultados.
7. El estudio de citotoxicidad realizado sobre células Caco–2 muestra que los extractos acuosos presentan un porcentaje inferior al 25 % a todas las dosis ensayadas.
8. Se han diseñado dos nuevas variantes al método MTT tradicionalmente empleado, que podrían aplicarse a cualquier estudio con otras líneas celulares. La variante *growing* evalúa el efecto citotóxico cuando se administra el xenobiótico durante el crecimiento celular, mientras que la variante *life* contempla la exposición continua durante todo el ensayo.

9. En la línea celular hepática, los extractos OV2, OV3 y OV5 no superaron el 50 % de toxicidad en ninguno de los ensayos, de manera similar a la doxorrubicina. Estos resultados se mantienen o mejoran tras el proceso de digestión. En la línea celular A375, OV3 muestra niveles bajos a cualquier concentración y tiempo de exposición. El extracto acuoso OV2 no presenta efecto tóxico en exposiciones cortas.

10. Se ha diseñado y puesto a punto un nuevo y sencillo método para cuantificar actividades farmacológicas en el interior de *C. elegans*. Gracias a este método, los efectos funcionales *in vivo* que se testen podrán ser ciertamente atribuidos a la ingesta del tratamiento con un compuesto no trazable por fluorometría.

11. Los resultados de actividad encontrados en los experimentos *in vivo* están correlacionados con los obtenidos *in vitro*, tanto antes como después del proceso de digestión. Asimismo se ha podido corroborar la presencia de extracto en el interior del gusano, encontrándose compuestos como 2,5–DHBA tras el tratamiento con OV1 y ácido rosmarínico tras el tratamiento con OV2.

12. Los extractos acuosos mejoran notablemente la supervivencia de *C. elegans* a las 24 h tras un tratamiento con juglona. Además, el extracto OV2 muestra una alta capacidad de disminuir en un 50 % la acumulación de ROS a una dosis de 5 mg/mL. Este efecto se quintuplica después de un proceso de digestión gastrointestinal.

13. Se han introducido dos variantes al método convencional de *Lifespan* no descritos hasta el momento. Ambas variantes permiten evaluar el efecto de un tratamiento durante el crecimiento del gusano o de manera crónica. Los extractos acuosos administrados durante la vida adulta alargan la esperanza de vida media del gusano un 40 %. Tratamientos con 5 mg/mL de OV2 durante el desarrollo del gusano o durante toda la vida consiguen alargar un 67 % la esperanza de vida media.

14. Se han diseñado dos formulaciones orales con el extracto OV3, cápsulas de gelatina dura y polvo liofilizado para resuspensión en agua. Tras el proceso de digestión, la encapsulación preserva mejor su composición química y su actividad antioxidante, tanto *in vitro* como *in vivo*. Por el contrario, el polvo liofilizado presenta mejor actividad hipoglucemiante. Los ensayos realizados de citotoxicidad en células Caco–2 y HepG–2 confirman su posible uso seguro.

15. Se han diseñado cuatro formas farmacéuticas para uso tópico. Una crema y un stick con extracto OV2 como antiinflamatorio de uso agudo, y un gel y una loción con OV3 para insuficiencia venosa crónica de extremidades inferiores.

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