

Anti-proliferative effect of *Melissa officinalis* on Human Colon Cancer Cell Line

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Abstract

Melissa officinalis L. (*Lamiaceae*) is consumed as a traditional herbal tea in the Mediterranean region. The cytotoxic effect of the 50% ethanolic and aqueous extract, determined by the MTT and NR assays, was evaluated *in vitro* on Human Colon Cancer Cell Line (HCT-116), using Triton 10% as positive control. The 50 % ethanolic extract showed significant differences after 72 hours of treatment, reducing cell proliferation to values close to 40%, even the lowest dose tested (5 µg/ml). In the MTT assay, the same extract caused the lowest cell viability with 13% at a concentration of 1000 µg/ml after 72 h of treatment, being a value lower than triton 10%. The antioxidant activity was also confirmed evaluating the capacity of the extracts to scavenge ABTS and DPPH radicals, and IC50 values were highly correlated with the total phenolic and flavonoid content. Bioassay guided fractionation led to the isolation of an anti-proliferative compound, rosmarinic acid. Its structural elucidation was performed by HPLC/DAD/ESI/MS analysis. High dose of rosmarinic acid (1000 µg/ml) was clearly cytotoxic against HCT-116 cells, with a significant decrease in cell number since the earliest time point (24 h).

KeyWords: Lemon balm; Lamiaceae; Antioxidant; Cytotoxicity; HCT-116; RA.

Abbreviations:

AQE: Aqueous extract

HAE: 50% ethanolic extract

RA: Rosmarinic acid

TFC: Total flavonoid content

TPC: Total phenolic content

Introduction

Cancer is one of the major human diseases and causes considerable suffering and economic loss worldwide. Colorectal cancer (CRC) with 655,000 deaths worldwide per year, it is the fourth most common form of cancer in the United States and the third leading cause of cancer-related death in the Western world [1]. Therefore, further research is needed for the development of safe products for the prevention and treatment of all human cancers. Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products [2] and the plant kingdom has been the most significant source. These include *Vinca* alkaloids, *Taxus* diterpenes, *Camptotheca* alkaloids, and *Podophyllum* lignans. Currently, there are 16 new plant-derived compounds being tested in clinical trials and of these 13 are being tested in phase I or II, and 3 are in phase III. Among these compounds, flavopiridol, isolated from the Indian tree *Dysoxylum binectariferum*, and mesoindigo, isolated from the Chinese plant *Indigofera tinctoria*, have been shown to exhibit anti-cancer effects with lesser toxicity than conventional drugs [3].

Epidemiological studies, experiments on laboratory animals and investigations of humans show that consumption of a diet rich in vegetables and fruits is associated with a low risk of some diseases including cardiovascular diseases and cancer. This beneficial effect has been attributed to the bioactive compounds of these natural products and first of all to phenolic compounds, due to their ability to act as efficient free radical scavengers. Many plants possess antioxidant activities and their consumption was recommended [4, 5]. Therefore, the phytochemicals present in herbal products, marketed as dietary supplements, may act as preventative or therapeutic agents similar to over-the counter (OTC) and prescription drugs.

Lemon balm, *M. officinalis* L. is one of the most used medicinal plants in Europe and the Mediterranean region, as a herbal tea for their aromatic, digestive and antispasmodic properties in nervous disturbance of sleep and functional gastrointestinal disorders [6]. The leaves emit a distinct fragrant lemon odour when bruised. The chemical composition is essential oil, polyphenolic compounds: caffeic acid derivatives in large proportions, such as RA, trimeric compounds, and also some flavonoids such as luteolin-7-0-glucoside. Some pharmacological properties have been attributed

to the principal constituents. Essential oil is considered to be the therapeutic principle mainly responsible for most of the activities mentioned, spasmolytic, antimicrobial, antitumor and antioxidant, mainly [7]; but plant phenolics, especially RA, are involved too. Enriched extracts containing RA are used as a virostatic against herpes viruses, alcohol extracts as sedatives and the essential oil as a digestive aid in pharmaceutical preparations [8]. Moreover, its neuroprotective properties have been demonstrated using an *in vitro* cellular model with the PC12 (rat pheochromocytoma) cell line, as well as its neurological activities with methanolic extracts being more effective than aqueous extracts [9].

In the course of our interest in biological and industrial applications from this plant [9-12], this paper focuses on the analysis of the anti-proliferative activity of *M. officinalis* extracts, fractions and RA. *In-vitro* cytotoxicity analysis, using MTT and NR assays, indicated that this plant was very effective against human colon cancer cell line (HCT-116). These results pointed to the potential use of *M. officinalis* as an anti-cancer agent.

Material and Methods

Chemicals

Cell culture products as well a media were obtained from Gibco (Barcelona, Spain). Chemical reagents were purchased from Sigma-Aldrich (Spain). RA (purity $\geq 99\%$) was obtained form Extrasynthèse (Genay, France).

Preparation of Extracts

M. officinalis dried leaves were purchased from Plantarom S.L. (Barcelona, Spain). 50% ethanolic (HAE) and aqueous (AQE) extracts of plant were prepared [11]. Both extracts presented a very similar percentage of extraction (HAE: 23.01%; AQE: 21.76% dry weight base).

Determination of the scavenging activity, total phenolic (TPC) and flavonoid (TFC) content

Two different methods were used for the evaluation of the antioxidant activity: DPPH radical scavenging assay [13], and 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay [14]. The results were compared with BHT and BHA, two of the most usually used synthetic antioxidants. The antioxidant activity was expressed as IC₅₀ values, which were calculated by non-linear regression with a one phase exponential association equation using GraphPad Prism version 4.0.

TPC and TFC of samples were quantified spectrophotometrically according to the published procedures [15, 16]. All determinations were carried out in triplicate and the mean values were used.

Determination of the cytotoxicity in HCT-116 cells (Human Colorectal Carcinoma Cells)

Cell Culture

HCT-116 cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (10 U/ml), streptomycin (10 µg/ml) and 0.2 mM sodium pyruvate. Cultures were incubated in the presence of 5% CO₂ at 37 °C and 100% relative humidified atmosphere.

Determination of Cell Survival by the MTT Reduction Assay

HCT-116 cells were seeded in 96-well microplates at a density of 10×10³ cells/well and grown for 24 h at 37 °C in 5% CO₂ prior to the addition of test samples. Cells were treated with various concentrations of samples (5-1000 µg/ml) dissolved in Dulbecco's phosphate buffered saline (PBS). After 24, 48 and 72 h of incubation, cell viability was determined using the colorimetric MTT assay. MTT solution at 5 mg/ml was dissolved in 1 ml of PBS, and 200 µL of it was added to each of the 96 wells. The wells were wrapped with aluminium foil and incubated at 37 °C for 90 minutes. The solution in each well containing media, unbound MTT and dead cells were removed by suction and 100 µL of DMSO was added to each well. Then the plates were shaken and cell survival (%) was measured as reduction of MTT into formazan at 550 nm. 10 µL of 10% Tritón® X-100 solution was used as positive control, because in biological research, this detergent is used for lysing cells. Untreated cells (at 0 µg/ml, vehicle alone) were chosen as the negative control Controls and samples

were assayed in triplicate for each concentration and replicated three times. The absorbance values were converted into percentages of cell viability using the following formula: Cell viability (%) = $(\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$

Neutral Red (NR) Assay

5×10^3 cells were plated in 96-well microplates and incubated at 37 °C in 5% CO₂. After 24 h, cells were treated with various concentrations of samples (5-1000 µg/ml) dissolved in PBS. After 24, 48 and 72 h of incubation, cell viability was determined using the colorimetric NR assay. Neutral red solution at a final concentration of 50 µg/ml in culture medium and 200 µL of it was added to each of the 96 wells. The wells were wrapped with aluminium foil and incubated at 37 °C for 90 minutes. The solution in each well containing media, unbound NR and dead cells were removed by suction and with 100 µL of a mixture with 1% formaldehyde-1% CaCl₂ and the dye extracted with 0.2 ml of 1% acetic acid in 50% ethanol was added to each well. Then the plates were shaken and cell survival (%) was measured at 540 nm. The absorbance values were converted into percentages of cell viability using the same formula as for the MTT assay.

Bioassay-guided fractionation and isolation of cytotoxic and antioxidant compound

A multi-step fractionation procedure was carried out in order to separate the different constituents of the HAE, using Sephadex LH-20 as stationary phase. About 3.5 g of HAE, was dissolved in 2 ml of 50% ethanol and chromatographic separation was achieved with 50% ethanol as mobile phase, yielding six fractions (F1: 0.45 g; F2: 1.74 g; F3: 0.61 g; F4: 0.27 g; F5: 0.47 g; F6: 0.31 g). F5 showed antioxidant and antiproliferative activity and 0.31 mg of the active compound from this fraction (**1**) was isolated by preparative reversed-phase HPLC, using water (85%) and acetonitrile (15%) as eluents. The purity of peak **1** (>95%) was checked by a Diode Array Detector coupled to the HPLC system, and the identification was performed by HPLC-DAD, HPLC-MS comparing the isolated compound with authentic reference sample or data reported in the literature.

HPLC-DAD and HPLC-MS Analysis

The extracts and fractions were analyzed by HPLC using a Waters (Milford, MA, USA) 600E multi-solvent delivery system, a Waters U6K sampler and a Waters 991 photodiode-array detector. Chromatography was performed on a C18 reversed-phase column (Nova-Pak, 150 mm x 3.9 mm., 4 μ m, Waters) at 25°C. Detection was a range between 210-500 nm. The mobile phase was formed by acetonitrile (A) and twice distilled water adjusted to pH 3 with acetic acid (B) in different proportions. The elution system was: 0-1 min, 93-90% of B; 1-5 min, 90% of B; 5-8 min, 90-85% of B; 8-13 min, 85% of B; 13-30 min, 85-40% of B and 30-40 min, 40% of B. The flow rate employed was 1 ml/min. HPLC-MS analysis was performed using a HP 1100L liquid chromatograph linked to a HP 100 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies, Palo Alto, CA, USA). The column, time period and flow rate were similar to those used during the HPLC-DAD analysis. The mass spectrometer operating conditions were: gas temperature, 350 °C; nitrogen flow rate, 10 L/min; nebulizer pressure 30 psi; quadrupole; temperature, 25 °C; capillary voltage, 3500 V. The mass spectrometer was operated in positive and negative mode at 12 eV.

Statistical Analysis

Data are mean \pm SD of three independent experiments. Cell assays were analyzed by ANOVA followed by Dunnett's test whereas IC₅₀ values were analyzed by Student test.

Results and Discussion

Botanicals and plant foods are also a source of antioxidants, which play a special role in chemoprevention. In fact, plant extracts and isolated compounds are formulated in a great variety of dietary supplements regarding antioxidant and protective effects against ROS, of which an excess is associated with ageing, cataracts, cardiovascular and neurodegenerative diseases and certain types of cancer. The antioxidant activity was evaluated in different *in vitro* models (Table 1). The antioxidant capacity is described quantitatively by the concentration of antioxidant needed to scavenge 50% of

either DPPH[•] or ABTS^{•+} which is referred to as the IC₅₀. The IC₅₀ was obtained from the graph of the percentage of scavenged radical versus the concentration of antioxidant. Both DPPH[•] and ABTS^{•+} radicals have been widely used to investigate the ability of plant extracts and fractions and/or pure compounds of those, to act as free radical-scavengers or hydrogen donors. Both extracts tested in this work were capable of scavenging DPPH[•] radicals. Activity of HAE (IC₅₀=11.04±0.72 µg/ml) and AQE (IC₅₀=17.11±1.70 µg/ml) demonstrated a higher scavenging capacity compared to other medicinal plants previously analyzed [10, 13]. The ABTS^{•+} assay, revealed similar results as the DPPH[•] assay (8.60±1.48 and 9.22±1.28 µg/ml, respectively). Extremely high and negative correlation (*P<0.01) was observed between TPC and IC₅₀ for DPPH[•] radical scavenging activity ($r^2=-0.955$), and ABTS^{•+} radical scavenging activity ($r^2=-0.967$). Results also showed that TFC had high and statistically significant negative correlation (**P<0.05) with DPPH[•] radical scavenging activity ($r^2=-0.885$) and ABTS^{•+} radical scavenging activity ($r^2=-0.863$). Therefore, good correlation suggests that phenolic compounds, not only flavonoid type, play an important role as antioxidants. In the same way, various studies correlating lemon balm's medicinal properties with its antioxidant activity and phenolic profile have been published. Despite, the usage of various methods for the evaluation of antioxidant capacity makes comparison even more difficult, the same results have been found by other authors; it was previously reported that aqueous methanolic extract of *M. officinalis* caused a concentration-dependent inhibition of lipid peroxidation, and phenolic components present demonstrated antioxidant activity [17]. Hydroalcoholic extracts have also shown significant antioxidative activities, by free radical scavenger effect on DPPH, partly in relation to their RA content [18].

The initial screenings for plants used for cancer treatment are cell-based assays using established cell lines, in which the toxic effects of plant extracts or isolated compounds can be measured. Cytotoxic screening models supply important preliminary data to select plant extracts or compounds with potential antineoplastic properties. In addition, cytotoxic assays do not provide false negative results since they consider plant extracts or compounds which affect cell viability. In this paper, the HAE and AQE were tested on HCT-116 tumour cell lines by the tetrazolium MTT and the

neutral red (NR) assays. The NR assay is based on the uptake of neutral red, a supravital dye, and its accumulation in the liposome of viable uninjured cells, and it is based on the reduction of the soluble yellow MTT tetrazolium salt (purple compound) to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase [19]. Various studies demonstrate that both the NR and MTT *in vitro* cytotoxicity assays can be applied to screening the acute toxicities of compounds and to identifying interactions when combinations of them are used [20]. The good agreement ($r=0.939$) between the ranking of the values of both methods suggests that although each test has a different physiological endpoint, the assays yield comparable data. Conversely, the differential sensitivities between the NR and MTT assays for some antineoplastic drugs might prompt the inclusion of both assays in a battery of test methods. The cell proliferation inhibition was registered at six different doses and three times (Fig. 1). The profile of results obtained by the MTT and NR assays for both extracts was similar. In the case of AQE, significant ($P<0.05$) or very significant ($P<0.01$) differences were found in comparison to negative control at higher doses tested (1000 $\mu\text{g/ml}$), reaching values similar to triton (positive control), after 24, 48 and 72 h of treatments (Fig. 1a-b). Figure 1d shows that HAE showed significant differences after 72 hours of treatment, reducing cell proliferation to values close to 40%, even the lowest dose (5 $\mu\text{g/ml}$). In the MTT and NR assays; the HAE extract caused the lowest cell viability with 13% at a concentration of 1000 $\mu\text{g/ml}$ after 72 h of treatment, being a value lower than triton 10% (Fig. 1c-d). Recently, many plant extracts and natural products, especially phenolics, with high antioxidant activity have shown antiproliferative effects in different cell lines including NCI-H82 (human, small cell lung, carcinoma), DU-145 (human, prostate, carcinoma), Hep-3B (human, black, liver, carcinoma, hepatocellular), K-562 (human chronic myeloid leukemia), MCF-7 (human, breast, adenocarcinoma), PC-3 (human, prostate, adenocarcinoma) and MDA-MB-231 (human, breast, adenocarcinoma) [21, 22]. Fractionation of the HAE by Sephadex LH-20 CC yielded six fractions. The antioxidant activity evaluation tests of the fractions showed that, the most active fractions were F5 against DPPH radical and ABTS^{•+} assay (1.84 \pm 0.06 and 4.44 \pm 0.25 $\mu\text{g/ml}$, respectively) (Table 1). The same correlations were also obtained between TPC and TFC with antioxidant activity. In cell viability

assays, fraction F5 was also the most active against the HCT-116 cell line at 1000 µg/ml, 24, 48 and 72 hours after treatment (Fig. 2). As can be seen the percentages obtained are similar to triton.

The HPLC-DAD analysis of fraction F5 revealed the presence of a main compound (**1**) (95% of the composition) and several small peaks in less proportion (5%). The active compound (**1**) of this fraction was isolated by preparative reversed-phase HPLC and the structure was elucidated as an ester of caffeic acid and 3-(3,4-dihydroxyphenyl)lactic acid, rosmarinic acid [23]. Compound **1** was obtained as a finely crystalline light yellow, mp 165-174 °C (dec.), $[\alpha]_D^{20}$ -32.5°(c 0.05, MeOH), $m/z = 360.08$ $[M]^+$. The UV absorbance maximum at 327 nm and a shoulder at 290 nm indicate the nature of phenolic acid with two aromatic rings. These observations were confirmed by the LC-MS analyses of compound **1**, where the most important ion fragmentations were $m/z = 359.07$ $[M-H]^-$, 197.04 $[M-H-162]^-$, 179.03 $[M-H-180]^-$, 161.02 $[M-H-198]^-$, corresponding to the deprotonated form of 3-(3,4-dihydroxyphenyl)lactic and caffeic acids and their dehydrated forms. RA is a polyphenolic compound present in high amount in many herbal plants of the *Lamiaceae* family such as rosemary, lemon balm, sage, oregano or savory. There is consensus that the significant antioxidant properties by lemon balm are mainly due to the large quantities of RA [24]. Several biological activities have been described for RA in relation to its antioxidant activity, the most known being anti-inflammatory, antiviral, antimicrobial, neuroprotective and neurological properties, and anticancer against various cell lines [9, 25-28]. However, this study is the first to establish a relationship between the antioxidant activity of RA and this cell line. HCT-116 cells were treated with RA (5-1000 µg/ml) for 24, 48 and 72 h, and as shown in Table 2, high dose was clearly cytotoxic, with a significant decrease in cell number since the earliest time point (24 h).

In conclusion, the results of this study have shown a high correlation between the phenolic content, antioxidant effect and antiproliferative activity in extracts and fractions. For this reason, RA could contribute, at least in part, induced cytotoxic effect in this cell line. Future research would be needed to determine the chemical nature of other compounds present in lemon balm, and possible synergism of action with RA. Considering our results along with the preliminary studies, we suggest

that RA in combination with the anticancer drugs may offer a good strategy for the treatment of a human colon cancer.

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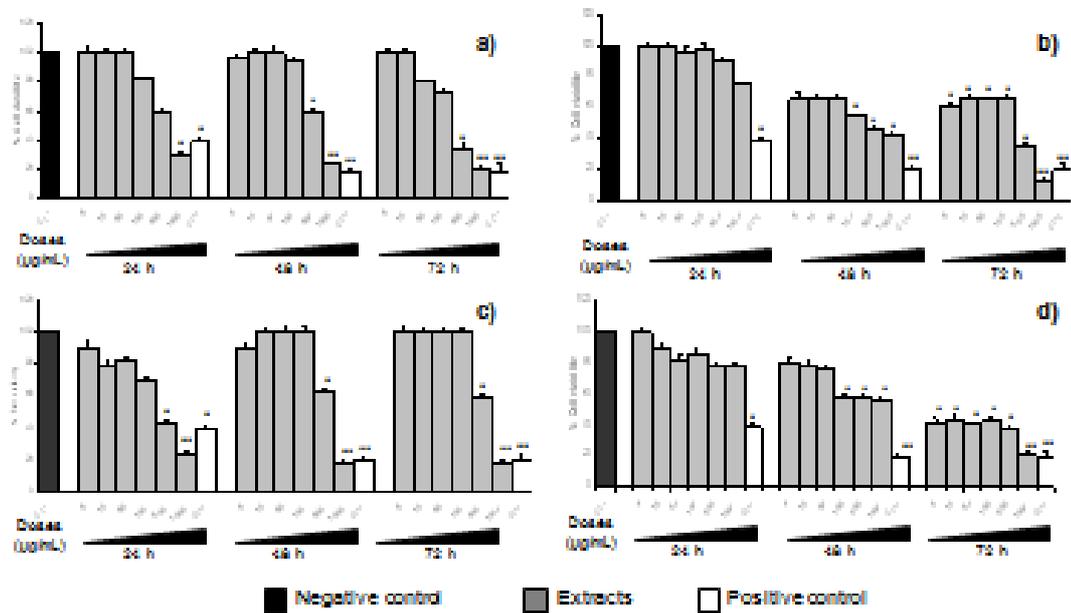
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Table 1. Total phenol and flavonoids, antioxidant activity of *Melissa officinalis* extracts and fractions.

	% TPC	% TFC	DPPH	ABTS
	(mg/100g)	(mg/100g)	IC ₅₀ (µg/ml)	IC ₅₀ (µg/ml)
AQE	1980.54 ± 162.22	672 ± 0.04	17.11 ± 1.70	9.22 ± 1.28
HAE	3407.32 ± 360.47	927 ± 0,08	11.04 ± 0.72	8.60 ± 1.48
F1	33.72 ± 2.71	<0.5	218.43 ± 0.85	> 500
F2	399.70 ± 10.52	33.48 ± 1.87	37.96 ± 1.27	63.98 ± 5.14
F3	846.42 ± 9.55	78.70 ± 2.34	3.39 ± 0.07	8.30 ± 0.73
F4	196.90 ± 5.01	87.65 ± 3.46	7.13 ± 0.09	13.38 ± 0.81
F5	1423.93 ± 142.33	269.48 ± 9.29	1.84 ± 0.06	4.44 ± 0.25
F6	178.32 ± 2.65	97.58 ± 3.12	8.62 ± 0.14	21.79 ± 2.47
BHA	-	-	3.88 ± 0.72	2.79 ± 0.13
BHT	-	-	5.05 ± 0.63	5.22 ± 0.75

Table 2. Percent cell survival of rosmarinic acid (**1**) against HCT-116 colon human tumour cell lines.

Doses ($\mu\text{g/ml}$)	Method	Time (hour)		
		24 h	48 h	72 h
5	MTT	76.47 ± 5.41	75.24 ± 6.53	65.32 ± 7.51
	NR	75.50 ± 9.74	6.217 ± 5.59	65.19 ± 9.16
500	MTT	70.59 ± 2.97	60.70 ± 5.77	58.87 ± 9.30
	NR	74.17 ± 5.12	45.01 ± 3.26	62.11 ± 7.15
1000	MTT	19.36 ± 5.07	18.82 ± 3.56	18.30 ± 2.98
	NR	14.84 ± 4.15	10.46 ± 1.87	10.17 ± 2.03
C(+)	MTT	20.14 ± 7.09	19.39 ± 3.45	19.55 ± 4.32
	NR	10.40 ± 2.11	10 ± 2.57	10 ± 1.99
C(-)		100 ± 0.00		



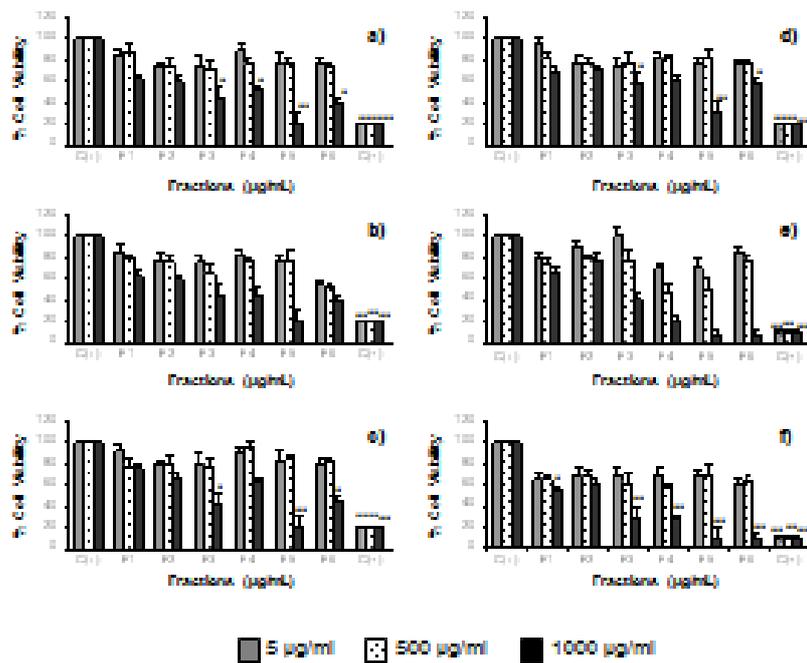


Figure 2. Fractions of 50% ethanolic extract inhibit HCT-116 colorectal cancer cell proliferation. a) 24 h; MTT assay; b) 48 h; MTT assay; c) 72 h; MTT assay; d) 24 h; NR assay; e) 48 h; NR assay; f) 72 h; NR assay.