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Melissa officinalis L. ethanolic extract inhibits the growth of a lung cancer cell line by interfering with the cell cycle and inducing apoptosis

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Melissa officinalis is a plant from the family Lamiaceae, native in Europe particularly in the Mediterranean region. Given our interest in identifying extracts and compounds capable of inhibiting tumor cell growth, and given the antioxidant content and the high consumption of *Melissa officinalis* in Portugal, this study aimed to test the tumor cell growth inhibitory activity of five different extracts of this plant (aqueous, methanolic, ethanolic, hydromethanolic and hydroethanolic) in three human tumor cell lines: MCF-7, AGS and NCI-H460. All extracts decreased cell growth in all cell lines in a concentration-dependent manner. The ethanolic extract was the most potent one, presenting a GI_{50} concentration of approximately 100.9 μg mL^{-1} in the NCI-H460 lung cancer cells. This extract was characterized by LC-DAD-ESI/MS regarding its phenolic composition, revealing rosmarinic acid as the most abundant compound. The GI_{75} concentration of this extract affected the cell cycle profile of these cells. In addition, both the GI_{50} and the GI_{75} concentrations of the extract induced cellular apoptosis. Moreover, treatment of NCI-H460 cells with this extract caused a decrease in pro-caspase 3 and an increase in p53 levels. This study emphasizes the relevance of the study of natural products as inhibitors of tumor cell growth.

Introduction

Cancer is a heterogeneous disease caused by genetic alterations in cells. The incidence of cancer is increasing globally, encouraging the search for new and more effective therapies. It is known that many of the FDA-approved antitumor drugs are derived from natural products.[1] Therefore, the search for compounds from natural products with potential antitumor activity has received increased attention in the last few years.[2] Many studies with plant extracts revealed that some of these plants had anti-tumor activity, thus being a source of compounds for successful development of anticancer drugs.[3,4] One example is paclitaxel (Taxol), derived from *Taxus brevifolia* (from the family Taxaceae) and currently clinically used in the treatment of breast cancer.[5,6]

Melissa officinalis is one of the most used medicinal plants in Europe and in the Mediterranean region, where it is known as lemon balm or, in Portugal, as "erva-cidreira" or just "cidreira".[7] The leaves of this plant are used to reduce anxiety, promote sleep and improve symptoms of indigestion.[8] Some studies have also reported antibacterial, antifungal and anti-inflammatory activities,[9] while others have reported antitumoral effects for some *Melissa officinalis* extracts on some human tumor cell lines.[10–12] In order to understand the antitumor activity of this plant, several studies have been conducted to learn more about its composition. *M. officinalis* is composed of polyphenolic compounds such as caffeic acid derivatives (mostly rosmarinic acid), flavonoids and essential oil. In addition, it is constituted of glucose (the most abundant sugar) and vitamins E and C, which have important activity as inactivators of free radicals.[13]

The cell growth inhibitory potential of *Melissa officinalis* was previously studied in some human tumor cell lines. However, the cellular mechanism responsible for its cell growth inhibitory potential has never been studied. Therefore, the aim of this work was to study the effect of different extracts of *Melissa officinalis* (ethanolic, methanolic, hydromethanolic,

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hydroethanolic and aqueous extracts) on the growth of three cell lines representative of different types of cancer: the NCI-H460 cell line from non-small cell lung cancer, the MCF-7 cell line from breast adenocarcinoma and the AGS cell line from gastric adenocarcinoma. The most potent extract was further studied in the most sensitive cell line regarding the effect on cell morphology, cell cycle profile and apoptosis.

Materials and methods

Samples and preparation of *Melissa officinalis* extracts

Melissa officinalis L. dry leaves were collected in Alfândega da Fé (Trás-os-Montes, Northeastern Portugal) in February 2016.

Preparation of the infusion extract

The preparation of the infusion extracts was carried out according to the previously described protocol,[14] by extracting 1 g of the dry leaves with 200 mL of boiled water (heating plate, VELP Scientific) and allowing the extract to stand at room temperature for 5 minutes. After filtration through Whatman no. 4 filter paper, the obtained infusions were frozen and lyophilized.

Preparation of the hydromethanolic, hydroethanolic, methanolic and alcoholic extracts

To obtain the hydromethanolic, hydroethanolic, methanolic and ethanolic extracts, 1 g of the dry leaves was extracted twice with 30 mL of methanol : water and ethanol : water (80 : 20, v/v) and with 30 mL methanol or ethanol, respectively, in a stirring plate (at 25 °C and 150 rpm) for 1 hour. After filtration through Whatman no. 4 filter paper, the final residue of each extract was then evaporated at 40 °C under reduced pressure, using a rotary evaporator (Büchi R-210, Flawil, Switzerland).

Stock solutions of all extracts were prepared in 100% sterile DMSO (methanolic and ethanolic extract) or water (aqueous, hydromethanolic and hydroethanolic) at a final concentration of 100 mg mL^{-1} and further stored at −20 °C.

Cell culture

The three human tumor cell lines used in this study were MCF-7 (breast adenocarcinoma, from ECACC), NCI-H460 (non-small cell lung cancer, a kind gift from NCI, Bethesda, MD, USA) and AGS (gastric adenocarcinoma, from ATCC). These adherent cell lines were maintained in RPMI-1640 medium with Ultra-glutamine I (Lonza, Basel, Switzerland) supplemented with heat-inactivated bovine fetal serum (FBS, Biowest, Riverside, MO, USA). The FBS concentration used was 5% for the cell growth inhibitory assay and 10% for the remaining assays. Cultures were incubated in the presence of 5% CO_2 at 37 °C. Cells were routinely observed with an inverted microscope (Nikon eclipse TS100 microscope) to analyse their number and morphology. Passages were made when confluence reached 80–90% by trypsinization with TrypLE™ Express (Life Technologies, Carlsbad, CA, USA) and gentle mechanical detachment. The viable cell number was regularly determined with the trypan blue (1 : 1) exclusion assay. All the experiments were performed only when exponentially growing cells presented more than 90% viability.

Phenolic characterization

The phenolic profile was analysed through a LC-DAD-ESI/MS system (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA). Detection was performed using a diode array detector (DAD, 280, 330 and 370 nm as preference wavelengths) and coupled to a mass spectrometer (Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA), following a procedure previously described by Bessada, Barreira, Barros, Ferreira & Oliveira (2016).[15] Phenolic compound quantification was performed through the calibration curves of phenolic standards (caffeic acid, ferulic acid, rosmarinic acid, Sigma-Aldrich, St Louis, MO, USA) and the results were expressed in mg per g of extract.

Cell growth inhibition assay

The sulforhodamine B (SRB) assay was performed according to the *in vitro* anti-cancer drug screening procedure of the National Center Institute (NCI).[16] Cells were plated in 96-well plates at the previously determined optimal cell concentration. In the case of MCF-7 and NCI-H460 cells, the appropriate cellular density was 5.0×10^4 cells per well[17,18] and for the AGS cells the cellular density was 7.5×10^4 cells per well.[19] Two plates were prepared, one to be analysed at the time of addition of the extracts to cells (T0 plate) and the other to be analysed 48 hours after treating the cells with the extracts. Cells were incubated for 24 hours to allow cells to adhere to the plate. In the T48 plate (TPP® Tissue Culture Plates), cells were then treated with five serial dilutions of each extract, ranging from 400 μg mL^{-1} to 25 μg mL^{-1}. Doxorubicin (DOXO) was used as a positive control, in successive 1 : 2 dilution ranging from 150 nM to 9.37 nM. The effect of the solvents of the extracts, DMSO or water, on the growth of the cell lines was also evaluated (as controls) by treating cells with the maximum concentration used. After 48 hours of incubation with the extracts (or immediately for the T0 plate), cells were fixed by adding ice-cold 10% trichloroacetic acid (TCA, w/v, Panreac, Barcelona, Spain) and allowed to air-dry. Cells were then stained with 0.4% sulforhodamine B reagent (SRB, Sigma Aldrich, St Louis, MO, USA) in 1% (v/v) acetic acid. The bound dye was then solubilized by adding 10 mM of Tris-base solution (pH 10.5) (Sigma Aldrich, St Louis, MO, USA). The absorbance was measured at 510 nm in a microplate reader (BioTek® Synergy HT, Winooski, VT, USA). All the SRB experiments used medium supplemented with 5% of FBS (Biowest) and 1% of antibiotic antimycotic solution (100×, Sigma-Aldrich, St-Louis, MO, USA). The cell number was determined as a percentage, compared to untreated cells. The GI_{50} concentration (concentration that inhibits 50% of net cell growth) was assessed from the dose–response curves and determined for each extract and each cell line.

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Preparation of cells for other analysis

For the analysis of the cell cycle, apoptosis or western blot, the NCI-H460 cells were plated at the density of 1.5×10^5 cells per well in 6-well plates and incubated for 24 hours. Then, the cells were treated with *Melissa officinalis* ethanolic extract at different concentrations: 100.9 μg mL^{-1} (approximately the GI_{50} concentration) and 180 μg mL^{-1} (approximately the GI_{75} concentration). Blank cells (treated with medium) and control cells (treated with the same concentrations of DMSO as used in treatments with the extract: corresponding to the GI_{50} treatment – control 1 – and corresponding to the GI_{75} treatment – control 2) were also included. Cells were then centrifuged at 1200 rpm for 5 minutes at 4 °C. For each treatment, the cell number and viability were confirmed by the trypan blue exclusion assay (cells were counted using a hemocytometer and trypan blue reagent; trypan blue penetrates the membrane of dead cells allowing to distinguish them from the alive ones) and analyzed in relation to blank cells.

Analysis of the cell cycle profile

Following trypsinization and centrifugation, cells were fixed with ice-cold 70% ethanol and stored at 4 °C overnight. Prior to analysis, the cells were centrifuged for 5 minutes at 1200 rpm and re-suspended in PBS containing propidium iodide (5 μg mL^{-1}; Sigma Aldrich, St Louis, MO, USA) and RNase A (0.1 mg mL^{-1}; Sigma Aldrich, St Louis, MO, USA). Samples were kept on ice for at least 30 minutes. Cellular DNA content was analysed using the flow cytometry (BD Accuri™ C6 Flow cytometry, USA) by analysing at least 20 000 events per sample.[20] The percentage of cells in the G0/G1, S and G2/M phases of the cell cycle were determined using the FlowJo software (version 7.6.0, Tree Star, Inc., Ashland, USA) after cell debris and aggregate exclusion.

Analysis of apoptosis

For the analysis of apoptosis, after trypsinisation and centrifugation, the pellet of the cells was re-suspended in binding buffer solution from the Annexin V-FITC Apoptosis Detection Kit (eBioscience, Bender MedSystems), as indicated by the manufacturer. The cells were then incubated for 10 minutes with 5 μL of human Annexin V-FICT and further incubated for 5 minutes with 10 μL of propidium iodide (in the dark and on ice). Samples were then analysed by flow cytometry using the BD Accuri™ C6 flow cytometry and respective software, plotting at least 20 000 events per sample.

Analysis of protein expression

Proteins from the different cellular treatments were extracted by centrifugation of the cells at 1000 rpm for 5 minutes followed by lysis of the pellet in Winman's buffer (1% NP-40, 0.1 M Tris-HCl pH 8.0, 0.15 M NaCl and 5 mM EDTA) with an EDTA-free protease inhibitor cocktail (Roche, Indianopolis, USA) for 30 minutes at 4 °C (with agitation). The supernatants (corresponding to cellular proteins) were obtained after centrifugation at 13 000 rpm, at 4 °C for 10 minutes. The total cell protein was quantified using a modified Lowry assay (Bio-Rad DC Protein Assay, Hercules, CA, USA).[21,22] Bovine serum albumin (BSA, Sigma Aldrich, St Louis, MO, USA) was used as a protein standard. After quantification, 20 μg of each cell extract was separated on 12% Bis-Tris SDS-Page gel at 70 V (for 30 minutes) and then 100 V (for 1 hour) and transferred to a nitrocellulose membrane (GE Healthcare, Wisconsin, USA) at 100 V (for 1 hour). Membranes were then blocked in Tris-buffered saline solution with 0.1% Tween-20 (Promega, Fitchburg, USA) – TBS-T – containing 5% (w/v) of non-fat dry milk, for at least 30 minutes with agitation at room temperature. Membranes were then incubated with the following primary and secondary antibodies for 1 hour and 30 minutes or 1 hour, respectively, at room temperature: mouse anti-p53 (Sc-126) (1 : 5000), mouse anti-caspase 3 (sc-7272) (1 : 1000) and goat anti-mouse IgG-HRP (1 : 2000) from Santa Cruz Biotechnology (Heidelberg, Germany) and mouse anti-α-tubulin (1 : 10 000) from Sigma Aldrich (St Louis, MO, USA). The signal from the membranes was detected using the ECL Western Blot Detection Reagents (GE Healthcare, Wisconsin, USA), the Amersham Hyperfilm ECL (GE Healthcare, Wisconsin, USA) and the Kodak GBX developer and fixer (Sigma Aldrich, St Louis, MO, USA). The intensity of the bands of each film was analysed using the software Quantity One – ID Analysis (Bio-Rad), as previously described.[23]

Statistical analysis

The results of three independent experiments from each ethanolic extract were statistically analyzed using the two-tailed paired Student's *t*-test.

Results and discussion

Effect of *Melissa officinalis* on the growth of several human tumor cell lines

The different extracts (ethanolic, methanolic, hydromethanolic, hydroethanolic and aqueous extracts) of *Melissa officinalis* were tested for cell growth inhibitory activity in MCF-7, AGS and NCI-H460 tumor cell lines, using the sulforhodamine B (SRB) assay. All the extracts tested presented similar activities in the three studied cell lines, suggesting that they have a non-specific effect (Fig. 1). Additionally, it is possible to observe that higher concentrations of the ethanolic, the hydroethanolic (only in the NCI-H460 and MCF-7 cells) and the hydromethanolic extracts reduced the cell number to values lower than the number of cells plated at the beginning of the experiment (*i.e.* the T48 plate had fewer cells than the T0 plate), suggesting that these extracts induce some degree of cell death on these cell lines (Fig. 1).

The most potent extract was the *Melissa officinalis* ethanolic extract and the most potent effect was observed in the NCI-H460 cells, with a GI_{50} concentration of 100.9 μg mL^{-1} (Table 1). On the other hand, the aqueous extract was the least potent extract in this study, with values of GI_{50} always higher than 200 μg mL^{-1}. Saraydin and colleagues tested an aqueous extract of *Melissa officinalis* and verified that the extract was very potent,

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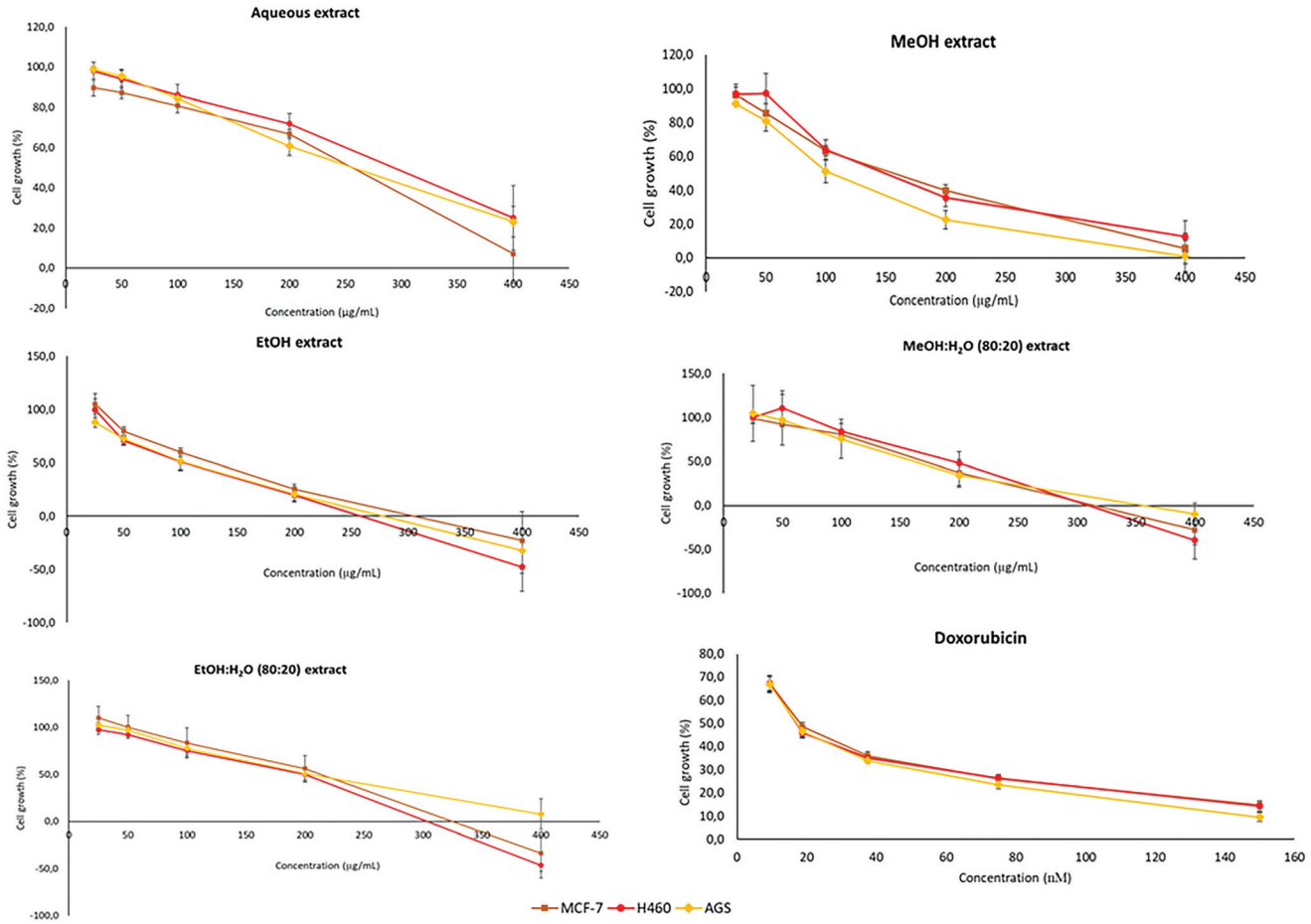

Fig. 1 Dose–response curves of different extracts of *Melissa officinalis* and doxorubicin (as a positive control). Results were determined with the SRB assay following 48 h treatment of MCF-7, NCI-H460 or AGS cells. The extracts were tested at 5 concentrations ranging from 400 μg mL^{-1} to 25 μg mL^{-1}. Doxorubicin was tested in successive dilutions ranging from 150 nM to 9.37 nM. Results are presented as percentage (%) of cell growth when compared to blank (untreated cells). Percentage (%) of growth below zero indicates a decrease in the cell number to values lower than the ones plated at the beginning of the experiment. Results are the mean ± S.E. of at least 3 independent experiments.

Table 1 GI_{50} concentrations of different extracts of *Melissa officinalis* in three human tumor cell lines

	GI_{50} concentrations (μg mL^{-1}) in different tumor cell lines		
Melissa officinalis extracts	MCF-7	NCI-H460	AGS
Aqueous	270.0 ± 38.7	260.3 ± 21.6	251.7 ± 27.4
MeOH	151.6 ± 15.3	161.3 ± 2.3	104.0 ± 14.3
EtOH	122.5 ± 9.0	100.9 ± 16.2	108.1 ± 20.6
MeOH·H_2O	181.7 ± 18.9	197.0 ± 30.7	166.4 ± 24.5
EtOH·H_2O	178.8 ± 28.7	189.6 ± 22.5	204.4 ± 29.5

GI_{50} refers to the concentration that inhibits 50% of net cell growth. GI_{50} concentrations represented in this table correspond to the mean ± S.E. of at least three independent experiments. Doxorubicin was used as a positive control: 17.9 ± 14.5 nM in MCF-7 cells, 16.7 ± 1.8 nM in NCI-H460 cells and 16.7 ± 1.6 nM in AGS cells.

with IC_{50} concentrations of 18 μg mL^{-1} in MCF-7 cancer cells.[12] The difference between our results and this study may be explained by the geographic variability of *Melissa officinalis* – while the plant used in this work was collected in Portugal, the plant used in the work of Saraydin *et al.* was collected in Turkey – which may influence the natural concentration of the constituents responsible for the antitumor activity. Indeed, the percentage of the constituents in a plant extract is highly influenced by exogenous factors such as temperature, climate, soil quality or the time of the year when the plant is harvested.[24] Additionally, Saraydin *et al.* measured the IC_{50} concentration which represents the concentration that inhibits 50% of a desired activity, while the GI_{50} concentration (used in this study) represents the concentration that inhibits 50% of cell growth.

Given the obtained GI_{50} concentrations of the different *Melissa officinalis* extracts on the three human tumor cell lines, the following studies were performed with the most potent extract (ethanolic extract, EtOH) in the most sensitive cell line (NCI-H460).

Phenolic characterization of *Melissa officinalis*

The phenolic profile characteristics of the ethanol extract (final concentration of 5 mg mL^{-1}) performed by LC-DAD-ESI/MS

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is shown in Table 2. A total of 20 compounds were identified taking into account the identification pattern previously performed by Pereira *et al.*,[25] using an infusion of *M. officinalis*. Hydroxycinnamic acid derivatives were the main class of phenolic compounds present in the extract, specifically rosmarinic acid (caffeic acid dimer) was the most abundant compound present. The high levels of this compound in *M. officinalis* have already been mentioned in the literature.[26,27]

Effect of *Melissa officinalis* on the viability of NCI-H460 cells

The effect of two concentrations (100.9 μg mL^{-1} – corresponding to the GI_{50} concentration – and 180 μg mL^{-1} – corresponding to the GI_{75} concentration) of the ethanolic extract of *Melissa officinalis* was analysed in terms of cell viability using the trypan blue exclusion assay. Results (Fig. 2) showed that the ethanolic extract (EtOH), at either of the concentrations used, significantly decreased NCI-H460 cell viability. Treatment with 100.9 μg mL^{-1} of ethanolic extract reduced cell viability to approximately 35% while treatment with 180 μg mL^{-1} decreased the cell viability to approximately 20% (when compared to the "blank" cells, Fig. 2G). Neither of the controls (CTR 50 and CTR 75 of DMSO) had a significant effect on cell viability in contrast with the treatment with doxorubicin, the positive control used, which significantly reduced cell viability. In addition, no significant morphological alterations were detected on cells treated with the extract when compared to blank cells (Fig. 2A–F).

Effect of the ethanolic extract of *Melissa officinalis* on the cell cycle profile and apoptosis of NCI-H460 cells

In order to understand the effect of the ethanolic extract of *Melissa officinalis* on the NCI-H460 cell cycle profile, the nuclear DNA content of the cells was analyzed by flow cytometry following 48 h treatment with the extract. Doxorubicin treatment was used as a positive control for the assay. Results demonstrated that in cells treated with the GI_{50} concentration of the extract, there were no major alterations in the percen-

Table 2 Phenolic characterization and quantification of *M. officinalis* ethanol extract

Peaks	t_R (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	Tentative identification	Quantification (mg g^{-1} ethanol extract)
1	4.9	279	197	179(995), 135(100)	3-(3,4-Dihydroxyphenyl)-lactic acid[1]	8.9 ± 0.5
2	5.5	328	311	179(100), 149(93), 135(25)	Caftaric acid[1]	11.4 ± 0.2
3	7.4	324	341	179(100), 149(5), 135(36)	Caffeic acid hexoside[1]	0.36 ± 0.01
4	8.6	326	325	193(100), 149(8), 145(22), 134(40)	Fertaric acid[2]	0.29 ± 0.06
5	11.7	328	179	135(100)	Caffeic acid[1]	2.3 ± 0.1
6	13.3	328	439	359(100), 179(10), 161(38), 135(25)	Sulphated rosmarinic acid[3]	2.6 ± 0.3
7	13.5	272	571	527(16), 483(65), 439(50), 329(21), 259(25), 241(47), 197(100), 179(72), 135(85)	Yunnaneic acid E[3]	7.5 ± 0.5
8	14.7	275, 322sh	537	493(55), 359(10), 313(25), 295 (100), 269(25), 197(15), 179(72), 135(40)	Lithospermic acid A isomer[3]	90 ± 2
9	15.5	328	473	311(15), 293(16), 179(66), 149(100), 135(30)	Chicoric acid[1]	4.5 ± 0.3
10	17.4	273, 332sh	597	359(30), 295(25), 197(12), 179(11), 135(10)	Yunnaneic acid F[3]	6.2 ± 0.1
11	18.6	265, 335sh	553	491(8), 359(3), 311(5), 197(3), 179(18), 161(10), 135(100)	Salvianolic acid C derivative I[3]	11.5 ± 0.4
12	18.9	264, 335sh	553	491(10), 359(3), 311(5), 197(3), 179(18), 161(11), 135(100)	Salvianolic acid C derivative II[3]	4.7 ± 0.3
13	19.7	326	521	359(100), 197(19), 179(27), 161(68), 135(19)	Rosmarinic acid hexoside[3]	14.3 ± 0.7
14	21.8	284, 328sh	719	539(18), 521(14), 359(100), 197(21), 179(24), 161(89), 135(8)	Sagerinic acid[3]	13.5 ± 0.8
15	24.7	328	359	197(79), 179(68), 161(100), 135(21)	Rosmarinic acid[3]	184.4 ± 0.3
16	27.9	326	493	359(72), 313(5), 295(50), 269(5), 197(30), 179(40)	Salvianolic acid A[3]	25.9 ± 0.8
17	28.9	328	829	667(89), 535(100), 491(20), 311(35), 293(10), 179(8)	Salvianolic acid C derivative III[3]	20 ± 1
18	30.7	288, 326sh	537	493(50), 359(100), 313(5), 295(16), 269(3), 197(38), 179(55)	Lithospermic acid A[3]	60 ± 2
19	31.4	322	493	359(100), 313(5), 295(5), 269(5), 197(13), 179(39)	Salvianolic acid A isomer[3]	4.8 ± 0.9
20	35.1	288, 322sh	715	535(100), 491(34), 311(63), 293(5), 179(5), 135(18)	Salvianolic acid C derivative IV[3]	6.9 ± 0.2
					Total phenolic compounds	480 ± 5

Phenolic compound standards used for the quantification: 1 – caffeic acid, 2 – ferulic acid and 3 – rosmarinic acid.

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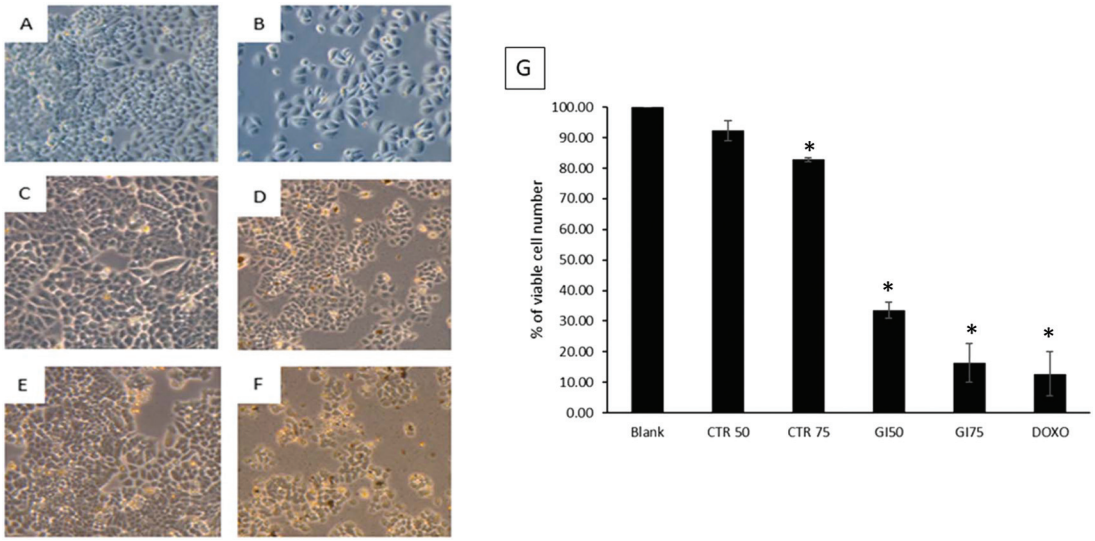

Fig. 2 Representative photographs of NCI-H460 human tumor cells treated for 48 h with the ethanolic extract of *Melissa officinalis* or with a positive control (doxorubicin). (A) Blank; (B) positive control – doxorubicin; (C) control of the GI_{50} concentration of the extract; (D) treatment with the GI_{50} concentration of the extract; (E) control of the GI_{75} concentration of the extract and (F) treatment with the GI_{75} concentrations of the extract. The graph (G) represents the effect of *Melissa officinalis* ethanolic extract on NCI-H460 cellular viability following 48 h of treatment, determined with the trypan blue exclusion assay. The % of viable cells was calculated in relation to blank. Conditions tested were: complete medium (blank); GI_{50} and GI_{75} concentrations of the ethanolic extract (EtOH) and the solvent (DMSO) concentration corresponding to the one used in each extract concentration (CTR 50 and CTR 75). Doxorubicin (DOXO) 17 nM was used as a positive control. Results are the mean ± S.E. of three independent experiments. * indicates $p \leq 0.05$ between the extract treatment and blank.

tage of cells in the different phases of the cell cycle. However, in cells treated with the GI_{75} concentration of the ethanolic extract of *Melissa officinalis* there was a statistically significant decrease in the % of cells in the G2/M phase of the cell cycle (Fig. 3).

In order to verify whether the extract has an effect on cellular apoptosis, the Annexin V-FITC/PI assay was performed by flow cytometry. The ethanolic extract of *Melissa officinalis* induced apoptosis on NCI-H460 cells in both tested concentrations when compared with blank and the respective controls (CTR 50 and CTR 75). When the cells were treated with the GI_{50} concentration of this extract, the % of cells undergoing apoptosis increases from 4.1% in blank cells to 19.1%. When the cells were treated with the GI_{75} concentration of the extract they presented 28.3% of cells in apoptosis (Table 3). These results show that the ethanolic extract of *Melissa officinalis* has a potent and dose-dependent effect on cellular apoptosis in NCI-H460 cells.

Other studies have previously shown the apoptotic activity of other types of *Melissa officinalis* extracts in human breast cancer cells.[12] Nevertheless, the effect of the ethanolic extract of this plant on the apoptosis of NCI-H460 cells has, to our knowledge, never been previously reported.

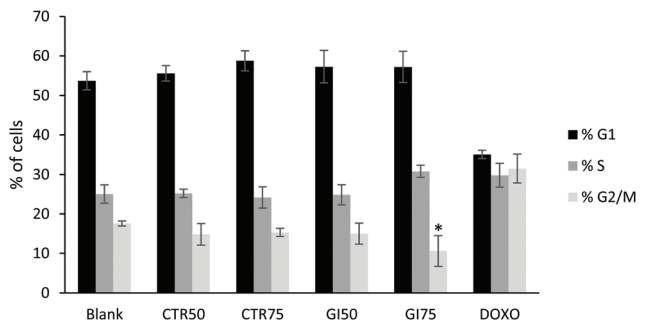

Fig. 3 Effect of *Melissa officinalis* ethanolic extract on the NCI-H460 cell cycle profile. Cells were treated for 48 h with complete medium (blank); with the extract at a concentration that inhibited cell growth by 50% (GI_{50}, 100.9 μg mL^{-1}) or 75% (GI_{75}, 180.0 μg mL^{-1}) or with DMSO at concentrations corresponding to the extract GI_{50} (CTR 50) or GI_{75} (CTR 75). Cells were also treated with 17 nM of doxorubicin (DOXO), used as a positive control. Results are the mean ± S.E. of three independent experiments. * indicates $p \leq 0.05$ between the extract treatment and blank.

Table 3 Apoptosis levels of NCI-H460 cells treated with *Melissa officinalis* ethanolic extract

Conditions[a]	% Apoptosis
Blank	4.1 ± 1.1
CTR 50	4.1 ± 1.5
CTR 75	6.7 ± 3.8
GI_{50}	19.1 ± 4.0
GI_{75}	28.3 ± 6.5
DOXO	44.7 ± 6.4

[a] Cells were treated for 48 h with complete medium (blank); with the GI_{50} and GI_{75} concentrations of the extract or with DMSO at concentrations corresponding to the extract GI_{50} (CTR 50) and GI_{75} (CTR 75). Doxorubicin (DOXO) at 17 nM was used as a positive control. Results are represented as the mean ± S.E. of three independent experiments.

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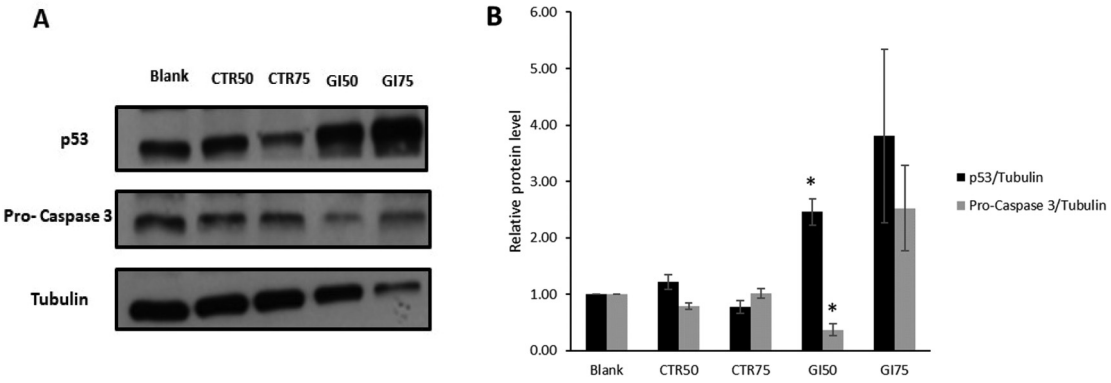

Fig. 4 Expression of apoptosis-related proteins in NCI-H460 cells following 48 h treatment with the ethanolic extract of *Melissa officinalis*. Treatments were performed in complete medium (blank); vehicle of each condition (DMSO; CTR 50 and CTR 75) or with the GI_{50} or GI_{75} concentrations of the ethanolic extract of *Melissa officinalis*. (A) Western Blot images are representative of three independent experiments. Tubulin was used as a loading control. (B) Densitometry analysis of the western Blot. Results are the mean ± S.E. of three independent experiments and are expressed after normalization of the values obtained for each protein with the values obtained for tubulin. * indicates $p \leq 0.05$ between the extract treatment and blank.

The effect of the ethanolic extract on the expression levels of proteins involved in the cell cycle and apoptosis was also assessed. Results obtained from the western blot analysis showed a significant increase in the levels of p53 expression in treatments with the GI_{50} and GI_{75} concentrations of the extract, when compared with the blank and the controls (Fig. 4). This is in agreement with the results obtained in the cell cycle and apoptosis analysis, since it is known that an increase in the levels of p53 expression may be responsible for alterations in the cell cycle and an increase in apoptotic levels.[28–31] In addition, the GI_{50} concentration of the ethanolic extract of *Melissa officinalis* decreased the expression levels of pro-caspase 3 when compared with the blank and the control cells. This is in agreement with the results obtained with the Annexin assay, since a reduction of pro-caspase 3 levels is indicative of the activation of caspase cleavage in the late stages of apoptosis.[32–34] Unfortunately, when treating cells with the GI_{75} concentration of this extract, it was not possible to properly analyze the expression of these proteins since the obtained results varied substantially between experiments (high error bars of Fig. 4B), probably due to the high levels of apoptosis in the cells treated with the extract. Nevertheless, the results obtained with the GI_{50} treatment further confirmed that the ethanolic extract of *Melissa officinalis* induces apoptosis in NCI-H460 cells.

Conclusions

Previous studies have investigated the antitumor effects of the aqueous, ethanolic and hydroalcoholic extracts of *Melissa officinalis*. Nevertheless, the mechanism of action of such extracts was never previously investigated. The results here obtained revealed that the ethanolic extract presented the highest cell growth inhibitory potential in all the human tumor cell lines tested. The most sensitive cells to this extract treatment were the non-small cell lung cancer cells (NCI-H460). Furthermore, results showed that this extract affects the cell cycle profile of NCI-H460 cells and induces cell death by apoptosis. This study highlights the importance of studying the ethanolic extract of *Melissa officinalis* as a source of bioactive compounds with antitumor activity.

Conflicts of interest

The authors declare no conflict of interest.

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