

Cytotoxic Activity of Essential Oil from Leaves of *Myrcia splendens* against A549 Lung Cancer Cells

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Research Article

Keywords: *Myrcia splendens*, essential oil, cytotoxicity, apoptosis, clonogenic assay, cell migration.

Posted Date: March 8th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2550885/v1>

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Abstract

Background: Plants of the *Myrcia* genus have been widely used in folk medicine to treat various diseases. The *Myrcia splendens* species has a diverse chemical constitution, but the biological activities of its essential oil have not been well investigated.

Objective: To carry out the chemistry characterization and evaluate the *in vitro* antitumor effect of the essential oil from the leaves of the species *Myrcia splendens* (MSEO) in different tumor lines.

Methods: The essential oil obtained from *M. splendens* (MSEO) leaves was analyzed by GC/MS. The cytotoxic activity of the MSEO was evaluated *in vitro* using the MTT assay. Clonogenic and wound healing assays were used to analyze colony formation and migratory capacity of lung adenocarcinoma cells, respectively. Morphological changes were observed by fluorescence using Phalloidin/FITC and DAPI stains.

Results: Chemical analysis identified 22 compounds, corresponding to 88% of the sample. Major compounds were the sesquiterpenic hydrocarbons bicyclogermacrene (15.4%), germacrene D (8.9%) and E-caryophyllene (10.1%). MSEO was highly cytotoxic in A549, B16-F10 and THP-1 cancer cells. A549 human lung adenocarcinoma cell line was chosen to continue the experiments, due to the high incidence and mortality rates of this type of cancer. MSEO reduced colony formation of at all concentrations and inhibited the migratory capacity of A549 cells at concentrations of 20 and 40 µg/mL. Furthermore, the MSEO induced apoptosis in A549 cells at all concentrations tested.

Conclusion: MSEO may be a promising natural agent in anti-cancer applications.

Background

Cancer is a growing health problem in the world and there are still limitations in the treatment with chemotherapy, which in general cause several toxic effects [1]. Recently there was growth in the studies of biological properties of natural products due its potential to treat several diseases, as cancer [2, 3]. Among these products, essential oils stand out, secondary metabolites that constitute a source of several bioactive compounds with anticancer potential, with their antitumor effects reported in *in vitro* and *in vivo* studies [4, 5]. Tropical plants are known to have plenty of compounds with biological properties, as the *Myrcia splendens* [6]. *Myrcia splendens* (SW.) DC. (Myrtaceae) is a tree plant with a wide distribution from Mexico to the South of Brazil [7]. In Brazil it is also known as “guamirim-da-folha-miúda” and in traditional medicine it is used to treat diarrhea, diabetes and hypertension [8, 9]. Some studies have demonstrated the biological properties of MSEO including antibacterial [10], antifungal [11] and cytotoxic effects in tumor cells [8]. However, little is known about the antitumor activities of MSEO. We hypothesize that MSEO has high *in vitro* antitumor activity. Thus, the present study aims to explore the antitumor effect of MSEO on human tumor cell lines *in vitro*.

Methods

Plant Material

Fresh leaves of *Myrcia splendens* (MS) were collected in Ibura Flora, N. S. do Socorro, Sergipe, Brazil, and identified by Dr. Adauto de Souza Ribeiro, Federal University of Sergipe. A voucher specimen (ASE 33399) is deposited at the Herbarium of the Department of Biology, Federal University of Sergipe, Brazil.

Essential Oil Isolation

Plant material was coarsely divided submitted to hydrodistillation, during 3 hours, using a modified Clevenger apparatus and the procedure described in the European Pharmacopoeia [11]. The essential oil was obtained with a yield of 0.3% (w/w), dried over anhydrous sodium sulfate and kept in the dark at 4°C prior to use.

GC/MS Analysis

Composition of the oil was accessed by gas chromatography (GC) and gas chromatography-mass spectroscopy (GC/MS). Analytical GC was carried out in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with HP GC Chem-Station Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detection (FID) systems. A graphpack divider (Agilent Technologies, part no. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco, Bellefonte, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m x 0.20 mm i.d., film thickness 0.20 µm) and Supelcowax-10 (polyethylene glycol 30 m x 0.20 mm i.d., film thickness 0.20 µm). Oven temperature program is 70–220°C (3°C.min⁻¹), 220°C (15 min), with injector temperature: 250°C, carrier gas: helium, adjusted to a linear velocity of 30 cm.s⁻¹; splitting ratio 1:40; detectors temperature: 250°C. GC-MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with an HP1-fused silica column (polydimethylsiloxane 30 m x 0.25 mm i.d., 0.25 µm film thickness) interfaced with a Hewlett-Packard 5973 mass selective detector (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters are described above, with interface temperature: 250°C, MS source temperature: 230°C, MS quadrupole temperature: 150°C, ionization energy: 70 eV, ionization current: 60 mA, scan range: 35–350 units, and scans.s⁻¹: 4.51. Essential oil components were identified by their retention indices on both SPB-1 and Supelcowax-10 columns and from their mass spectra. Retention indices, calculated by linear interpolation to retention times of C8–C23 n-alkanes, were compared with those of reference samples included in the Faculty of Pharmacy, University of Coimbra database. Acquired mass spectra were compared with reference spectra from laboratory database, Wiley/NIST library and literature data [12]. Relative amounts of individual components were calculated based on GC raw data areas without FID response factor correction.

Cells

The cytotoxicity of the MSEO was tested against lung adenocarcinoma (A549), melanoma (B16-F10) and acute monocytic leukemia (THP-1) human cancer cell lines, all obtained from American Type Culture

Collection (ATCC). Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium or in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat-inactivated Fetal Bovine Serum (FBS, 10%) and 100 U/mL penicillin with 100 µg/mL streptomycin, and incubated at 37°C with a 5% CO₂ atmosphere.

MTT Assay

The cytotoxicity activity was quantified using the MTT (3 - [4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay, as previously described by [13]. For all experiments, cells were seeded in 96-well plates (2x10⁴ cells/mL in 200 µL of medium) in incubated overnight to permit attachment. After 24 h, the MSEO (2,5 – 100 µg/mL), dissolved in Dimethylsulfoxide (DMSO) or Doxorubicin (25 µg/mL) was added to each well and incubated for 24 h. Subsequently, 200 µL of MTT (5 mg/mL in PBS) was added to each well and further incubated for 3 h later, the formazan product was dissolved in 150 µL of DMSO, and the absorbance was read in a microplate reader (Synergy H1, Biotek, VT, EUA) at 570 nm. Cytotoxicity was expressed as the concentration of oil inhibiting cell viability by 50% (IC₅₀). All measurements were performed in triplicate and the means and standard errors were calculated.

Clonogenic Assay

The procedure of Franken et al. (2006) was employed with some modifications [14]. A549 cells were seeded in 6-well plate (300 cells/well) in RPMI medium containing 10% FBS and 1% antibiotic (penicillin 10,000 U/ml; streptomycin 10,000 mg/ml). After 24 h of incubation conditions in an oven with an atmosphere of 5% CO₂ at 37 °C, the cells were treated with MSEO at concentrations of 10, 20 and 40 µg/mL, which correspond to the values of 0.5xIC₅₀, 1xIC₅₀ and 2xIC₅₀, respectively. DMSO 0.1% and Doxorubicin 25 µg/mL were used as a negative control and a positive death control, respectively. After the treatment time, the media were removed and complete DMEM medium was added to the wells, and the cells were incubated in an oven with a 5% CO₂ atmosphere at 37 °C for 10 days. After aqueous solution with acid + water (3:5 min) stained with crystal violet, 5% in 30 min. At the end of the experiment, the growth pattern in number of colonies of cells was observed and counted, with the aid of Image J software.

Wound Healing Assay

A549 cells were seeded in 12-well plate at a density of 3 x 10⁵ cells per well. After 24 h, the cell monolayer was scratched with a tip of p200 pipette creating a straight-line wound, the debris were removed by washing with PBS and the cells were treated with 10, 20 and 40 µg/mL the MSEO for 24 h. DMSO 0.1% was used as vehicle control and Doxorubicin 10 µM was used as positive control. The images were acquired 0, 24 and 48 h of the scratch using a microscopy Olympus. The percentage of wound closure was calculated for each treatment and controls comparing the time points 24 and 48 h with the time point zero, using the equation proposed by Yarrow et al. (2004) [15]. The images were analyzed using ImageJ 1.46 software.

Cell Morphology Assay

A549 cells were seeded at a concentration of 1×10^4 in 48-well plates, in RPMI medium containing 10% FBS and 1% antibiotic (penicillin 10,000 U/mL; streptomycin 10,000 mg/mL). After 24 h of incubation, the cells were exposed to concentrations of 10, 20 and 40 $\mu\text{g/mL}$ the MSEO and incubated for 24 h in an oven. DMSO 0.1% and Doxorubicin 25 $\mu\text{g/mL}$ were used as negative control and positive control, respectively. Culture medium from all wells of the plate was removed and the cells washed three times with 1X PBS. After washing, cells were fixed with 4% formaldehyde at room temperature for 15 min and then washed again with 1X PBS. Cells were permeabilized using 0.2% Triton X-100 solution diluted in PBS for 15 min and then 1% Bovine Serum Albumin (BSA) also diluted in PBS was used for 30 min. To visualize the cytoskeleton, cells were stained with Phalloidin/FITC (25 $\mu\text{g/mL}$) for 30 min, in the dark, followed by 2 washes with PBS. The cell nucleus was counterstained with DAPI (1 $\mu\text{g/mL}$) for 10 min, in the absence of light. Images were captured at 200x and 400x magnification under a fluorescence microscope (Olympus, USA).

Statistical Analysis

For all experiments, a 95% confidence interval was used and $p < 0.05$ values considered statistically significant. Analyzes and graphs as well as IC_{50} were obtained using the GraphPad Prism 8. Shapiro-Wilk normality test was applied to assess the normal distribution of the data. For comparison between groups, ANOVA was used, followed by Dunnett post-test.

Results

Chemical Composition of the *Myrcia splendens* Essential Oil (MSEO)

The analysis of the essential oil from the leaves of *M. splendens*, resulted in the identification of 22 constituents that represent 88% of the total composition (Table 1). The oil is composed of hydrocarbon sesquiterpenes (48.5%) and oxygen containing sesquiterpenes (39.6%), being bicyclogermacrene (15.4%), E-caryophyllene (10.1%) and germacrene D (8.9%) the major constituents.

Table 1
Composition of the essential oil from de the leaves of *M. splendens*

RI ^a	RI ^b	Compounds*	Relative amount in sample (%)
			MS
1329	1467	δ -Elemene	2.9
1342	1455	α -Cubebene	0.5
1369	1487	α -Copaene	2.2
1380	1536	β -Cubebene	0.7
1376	1517	β -Bourbonene	1.7
1408	1590	<i>E</i> -Caryophyllene	10.1
1422	2130	γ -Elemene	0.5
1427	1600	Aromadendrene	0.3
1442	1662	α -Humulene	2.4
1466	1699	Germacrene D	8.9
1470	1715	β -Selinene	0.2
1479	1726	Bicyclogermacrene	15.4
1508	1751	δ -Cadinene	2.7
1526	2070	Elemol	7.5
1553	2113	Spathulenol	1.6
1557	1975	Caryophyllene oxide	0.6
1562	2063	Globulol	0.6
1576	2079	Guaiol	7.7
1607	2453	γ -Eudesmol	3.7
1622	2215	β -Eudesmol	5.0
1628	2208	α -Eudesmol	5.1
1639	2196	Bulnesol	7.8
Sesquiterpene hydrocarbons			48.5
Oxygen containing sesquiterpenes			39.6
Total identified			88.0

(*) - Compounds listed in order of their elution on the SPB-1 column.

RI^a - Retention index determined on the non-polar SPB-1 column relative to a series of n-alkanes (C8–C23).

RI^b - Retention index determined on a SupelcoWax-10 column relative to a series of n-alkanes (C8–C23)

Mseo Reduce The Viability Of Cancer Cell Lines

In order to assess the effects of MSEO on the viability of cancer cell lines, after the treatment of MSEO at varied concentrations for 24h, the cytotoxic activities were evaluated in three tumor cell lines: A549 (lung cancer), THP-1 (acute monocytic leukemia) and B16-F10 (melanoma), using the MTT assay. IC₅₀ values and confidence interval are shown in Table 2. MSEO was cytotoxic in all tested tumor lines, presenting IC₅₀ values of 5.37 µg/mL (2.17–13.30) in THP-1 cells, of 17.76 µg/mL (9.48–33.26) in B16-F10 cells and 20.14 µg/mL (16.46–24.64) in the A549 cells. Doxorubicin was used as a positive control and showed an IC₅₀ value of 25.51 µg/mL in A549 cells. Considering the high rates of incidence and mortality of lung cancer, the A549 strain was chosen to continue the experiments.

Table 2
IC₅₀ (µg/mL) values for *M. splendens* essential oil (MSEO) in cancer cell lines

Cells	MSEO
	IC ₅₀ values (µg/mL)
A549	20,14
	16,46–24,64
B16-F10	17,76
	9,48–33,26
THP-1	5,37
	2,17–13,30

Data are presented as IC₅₀ values in µg/mL and their 95% confidence interval obtained by non-linear regression from three independent experiments performed quadruplicate by MTT assay after 24 h of incubation.

Mseo Reduces The Ability Of A549 Lung Cancer Cells To Form Colonies

To assess whether MSEO has the ability to inhibit the formation of colonies of A549 cells, a clonogenic assay was performed. Cells were treated for 24h with MSEO at concentrations of 10, 20 and 40 µg/mL, which correspond to the values of 0.5xIC₅₀, 1xIC₅₀ and 2xIC₅₀, respectively. After the treatment, the cells were kept growing for 10 days, and then the analyzes were performed. The results obtained are shown in Fig. 1. A significant reduction in the number of colonies ($p < 0.0001$) was observed in the treatment with MSEO at all concentrations tested, compared to the negative control. Treatment with Doxorubicin (25 µg/mL) also caused a significant reduction ($p < 0.0001$) compared to untreated cells. The data are represented in Fig. 1B.

Mseo Inhibits The Migratory And Invasive Ability Of A549 Cells

The migration assay, using the Wound Healing technique, was performed to verify the effects of MSEO treatment on the migratory capacity of A549 cells. MSEO concentrations of 10, 20 and 40 µg/mL were used, and the analyzes were performed at 0, 24 and 48 h. Figure 2 shows the wound closure process in the cell monolayer, with representative images of the wells. According to the data obtained, it was possible to observe a significant effect on the migratory capacity (Fig. 2) of A549 cells at a concentration of 40 µg/mL after 24 h of treatment.

Mseo Induces Apoptosis In A549 Cells

The apoptotic morphological changes in the nucleus and cytoplasm of A549 cells after 24 h of treatment with MSEO were evaluated using DAPI and Phalloidin/FITC dyes. Figure 3 shows the morphological changes caused by increasing concentrations of MSEO. According to the data presented, a reduction in cytoplasmic volume was observed in A549 cells at a concentration of 10 µg/mL of MSEO. At a concentration of 20 µg/mL, in addition to the reduction in cytoplasmic volume, cells with greater accumulation of DAPI in the nucleus were also observed, indicating DNA fragmentation and chromatin condensation. In cells treated at a concentration of 40 µg/mL, rounding and cytoplasmic and nuclear shrinkage were observed, in addition to chromatin condensation and DNA fragmentation. Treatment with Doxorubicin caused important cellular changes such as reduced cytoplasmic volume, chromatin condensation and DNA fragmentation. Overall, the most observed changes after treatment with EOMS concentrations were: decrease in cell number, cell rounding and shrinkage, and reduction of cytoplasmic volume, characteristics of programmed cell death induction. For the same treatment time, the observed changes were more intense as the MSEO concentration was increased.

Discussion

In this study, we demonstrated the cytotoxicity of essential oil from *M. splendens* leaves (MSEO) against the human lung adenocarcinoma cell line (A549). A previous study investigated the chemical characterization and biological activities, including cytotoxicity, of the EO from the leaves of *M. splendens* collected in Amazonian Ecuador. The authors found trans-nerolidol and α -bisabolol as the

major EO compounds [8]. Interestingly, in the present study we observed a different chemical composition of the MSEO, in which the major compounds found were bicyclogermacrene, E-caryophyllene and germacrene D. This difference in the chemical composition of the *M. splendens* EOs may have occurred mainly due to the variation of the place of collection of the leaves, since the samples used in the present study were collected in the northeast of Brazil. Previous studies have reported that factors such as high genetic diversity can influence the variability of essential oils from species of the Myrtaceae family [16]. Despite the difference between the major MSEO compounds found in our study and that of Scalvenzi et al. (2017), the chemical composition of the MSEO evaluated in both studies is compatible with regard to the predominance of sesquiterpenes.

Previous studies suggest that for an EO to be considered promising for cancer drug development, its IC₅₀ values need to be less than 30 µg/mL [17]. In the present study, we observed that MSEO caused cytotoxicity in THP-1 (acute monocytic leukemia), B16-F10 (melanoma) and A549 (human lung adenocarcinoma) cell lines, with IC₅₀ values less than 21 µg/mL. A study by Mohamed et al. (2018) reported cytotoxic activity in A549 cells treated with essential oil of *Pistacia lentiscus* L., which contained sesquiterpene germacrene D as one of the main compounds [18]. Another study reported that germacrene D and bicyclogermacrene, in synergism, were responsible for the high cytotoxic activity of essential oil from *Porcelia macrocarpa* leaves against leukemic cells (HL-60) [19]. In research carried out by The et al. (2021), the cytotoxic potential of the essential oil of the leaves of *Polyalthia suberosa* was evaluated, whose major constituents were bicyclogermacrene, E-caryophyllene and β-pinene. The results obtained showed cytotoxic activity against tumor cell lines of hepatocellular carcinoma (HepG2), breast cancer (MCF-7) and human lung adenocarcinoma (A549) [20]. These studies demonstrate that the sesquiterpenes bicyclogermacrene, E-caryophyllene and germacrene D are compounds that have antitumor action. The fact that these are the major constituents found in MSEO suggests that they may be the main components responsible for the antitumor action of MSEO. However, studies are needed to investigate the action of these isolated compounds, for a definition of their antitumor activity.

In addition, MSEO was shown to significantly reduce ($p < 0.05$) the percentage of A549 cell colonies at all concentrations evaluated. Our results were similar to those obtained in the study by Dahham et al. (2015), who evaluated the anticlonogenic activity of sesquiterpene β-caryophyllene in colon carcinoma cells (HCT 116) [21]. A study by Toyang et al. (2013) reported the anticlonogenic activity of two sesquiterpenes isolated from *Vernonia guineensis* leaves, Vernopicrin and Vernomelitsin, which reduced the number of colonies in the prostate adenocarcinoma cell line (PC-3), during the nine-day exposure period, in a period similar to that used in the present study [22].

Among the mechanisms involved in the development and establishment of cancer is the process of tumor cell migration. During this process, tumor cells migrate from the primary site to a secondary organ, being a critical process of invasion, which allows the adaptation of primary tumors to metastasis [23, 24]. Our results indicate that MSEO causes a significant reduction in the migration capacity of A549 cells at the highest concentration (40 µg/mL), after 24 h of treatment. Studies show that essential oils have the ability to reduce tumor cell migration [25, 26]. The study by Chen et al. (2018), the essential oil of

Eupatorium adenophorum Spreng., consisting mainly of sesquiterpenes, reduced the migration of hepatocellular carcinoma (HepG2) cells [27].

Furthermore, MSEO was able to induce apoptosis in A549 cells, a cell death mechanism observed through changes in the nucleus and cytoplasm of A549 cells [28]. All concentrations promoted a reduction in cytoplasmic volume, while DNA fragmentation and chromatin condensation, evidenced by the accumulation of DAPI present in the cell nucleus, were only observed from a concentration of 20 µg/mL. This apoptotic effect was also observed in the study by Pereira et al. (2017), when evaluating the essential oil of *Baccharis milleflora* (Less.) DC against non-Hodgkin's lymphoma (Raji) cells. The authors found bicyclogermacrene, germacrene D, E-caryophyllene and α-humulene as major compounds in the essential oil of *Baccharis milleflora* (Less.) DC [29].

Conclusion

In summary, MSEO has antitumor activity in human lung adenocarcinoma cells, observed through its cytotoxic action, reducing colony formation and migratory capacity and inducing apoptosis of these tumor cells. These activities probably occur due to the action of sesquiterpenes, which have antitumor activity well described in the literature. The findings of this work indicate that OEMS can be considered an effective antitumor agent, of great interest to the pharmaceutical industry for the development of new substances for the treatment of cancer. Future studies should be directed to clarify its mechanism of action and prove its effectiveness.

Abbreviations

A549: Human Lung Carcinoma Epithelial Cell Line; B16-F10: Melanoma Cell Line; CDK: Cyclin Dependent Kinases; DAPI: 6-Diamidine-2-phenylindole dihydrochloride; DMSO: Dimethylsulfoxide; DOX: Doxorubicin; EDTA: Ethylenediaminetetraacetic acid; FBS: Fetal Bovine Serum; GC-MS: Gas Chromatography Coupled with Mass Spectrometry; IC₅₀: Inhibitory concentration for 50% of cells; INCA: José Alencar da Silva National Cancer Institute; MCF-7: Adenocarcinoma Cell Line; MSEO: *Myrcia splendens* Essential Oil; PHALOIDIN/FITC: Fluorescein Isothiocyanate; PSB: Phosphate Saline Buffer; THP-1: Leukemia Cell Line; WHO: World Health Organization

Declarations

Ethics approval and consent to participate

Fresh leaves of *Myrcia splendens* (MS) were collected in Ibura Flora, N. S. do Socorro, Sergipe, Brazil (collection authorization No. 68163/2019 – SISBIO - ICMBio), and identified by Dr. Adauto de Souza Ribeiro, Federal University of Sergipe. A voucher specimen (ASE 33399) is deposited at the Herbarium of the Department of Biology, Federal University of Sergipe, Brazil. The experimental research and field

studies on plants, including the collection of plant material, comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated and/or analyzed in this study are available from the corresponding author on reasonable request.

Competing interests

The authors report no conflicts of interest of this work.

Funding

Research partially funded by Fundação para a Ciência e Tecnologia, Portugal, ref. UIDP/00102/2020.

Authors contributions

MMM carried out the research and wrote the first draft of the manuscript. FFB and EWPS assisted in the research work and revised the manuscript. JFS helped in the clonogenic assay and wound healing experiments. WLJ contributed to the research work. ASR collected and identified the leaves of *Myrcia splendens*. ASF and SMFM prepared *Myrcia splendens* essential oils. CC revised the manuscript. RS helped with data interpretation and revised the manuscript. CBC guided the research, reviewed and submitted the final manuscript.

Acknowledgements

Declared none.

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Figures

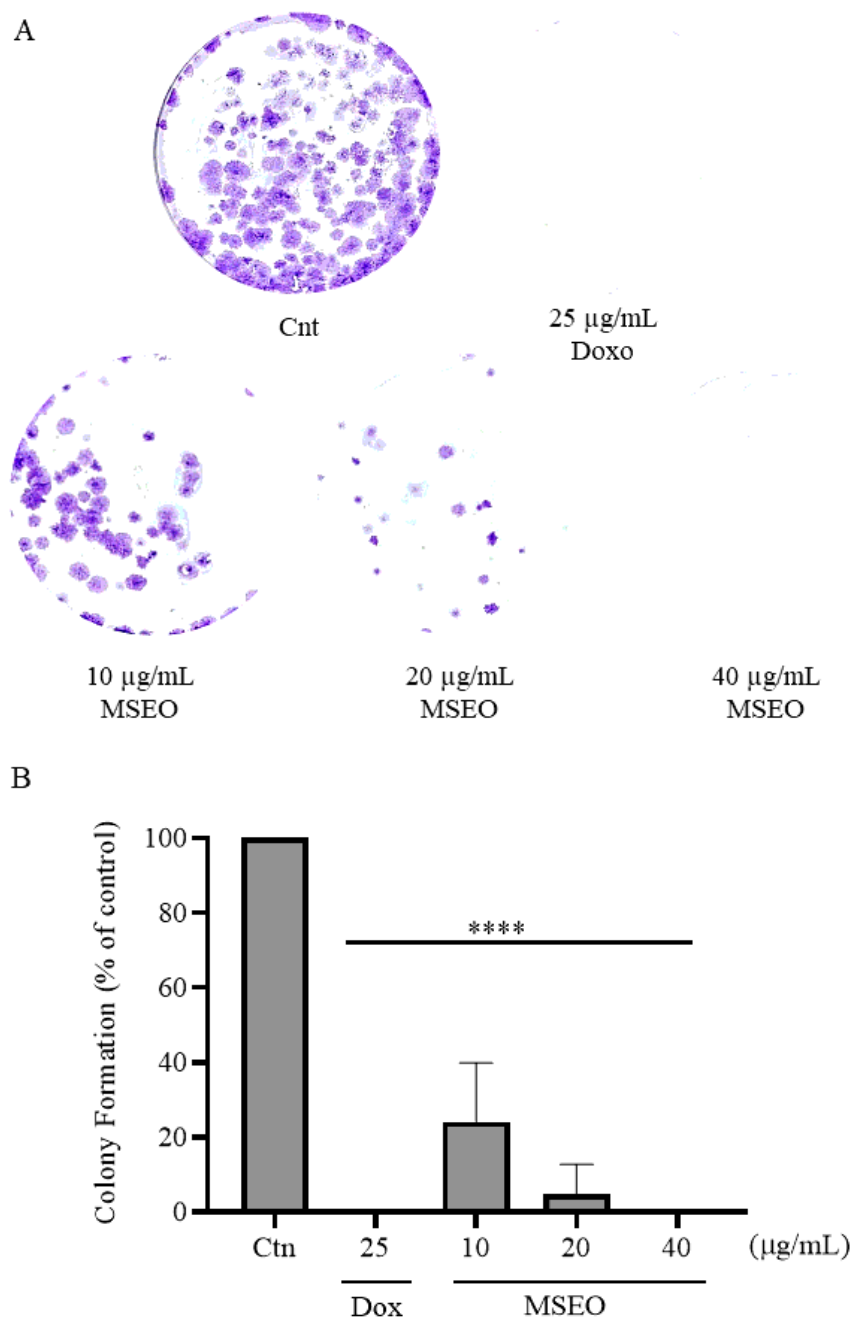
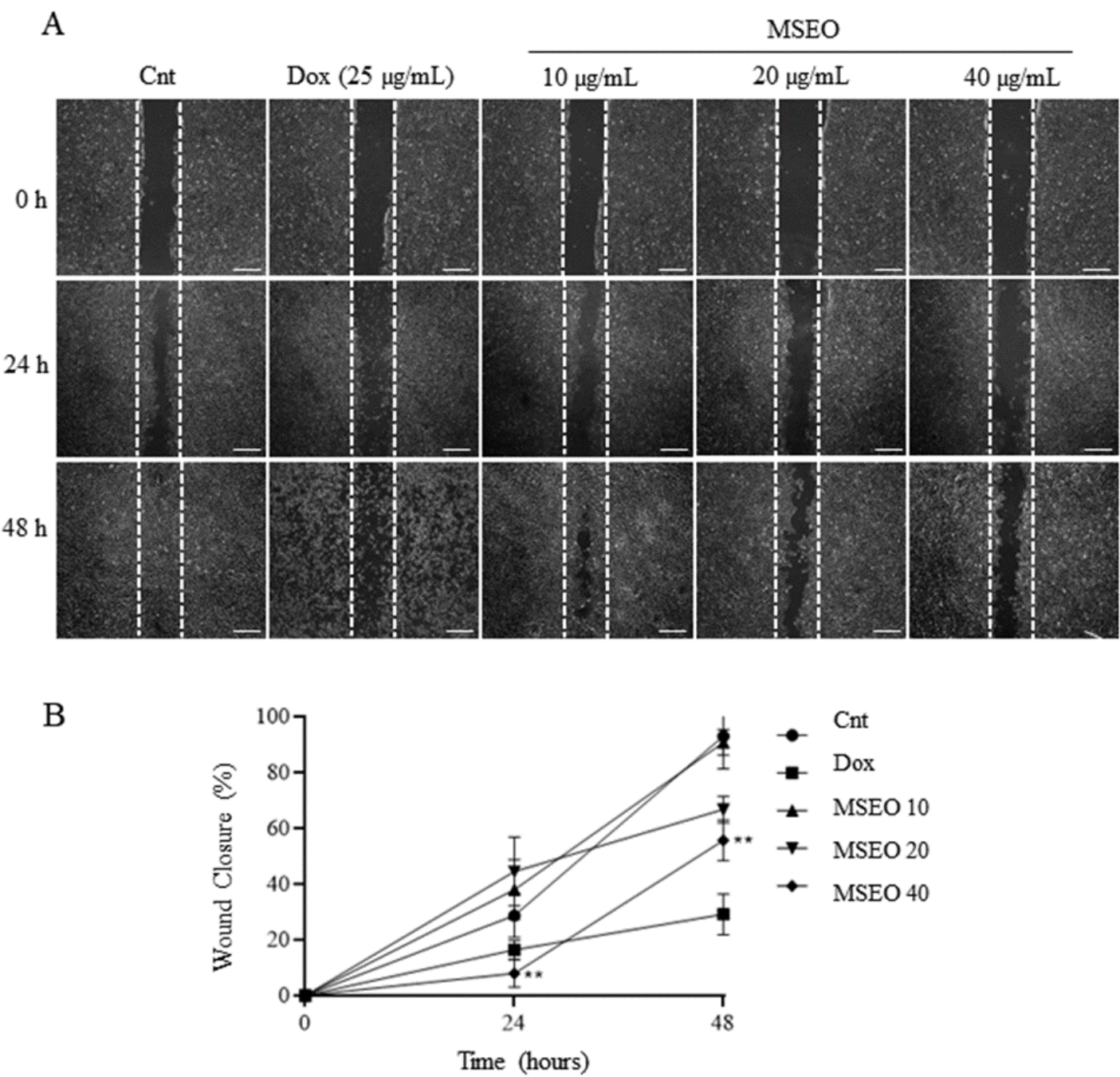


Figure 1

Inhibitory effect of the MSEO on the formation of A549 lung cancer cells colonies. **(A)** Colonies formed after treatment with increasing concentrations (10, 20 and 40 µg/mL) for 24h of *M. splendens* essential oil (MSEO) and 10 days of growth. **(B)** Percentage of colony formation after treatment compared to

control cells. Doxorubicin (25 µg/mL) was used as a positive control. Data show are mean ± SD obtained from three independent experiments. Statistical differences, compared to untreated control cells, were assessed by a one-way ANOVA with Dunnett post-test (****) $p < 0.0001$



are mean $\pm$ SD obtained from three independent experiments. Statistical differences, compared to untreated control cells, were assessed by a Two-way ANOVA with Dunnett post-test (**) $p < 0.05$

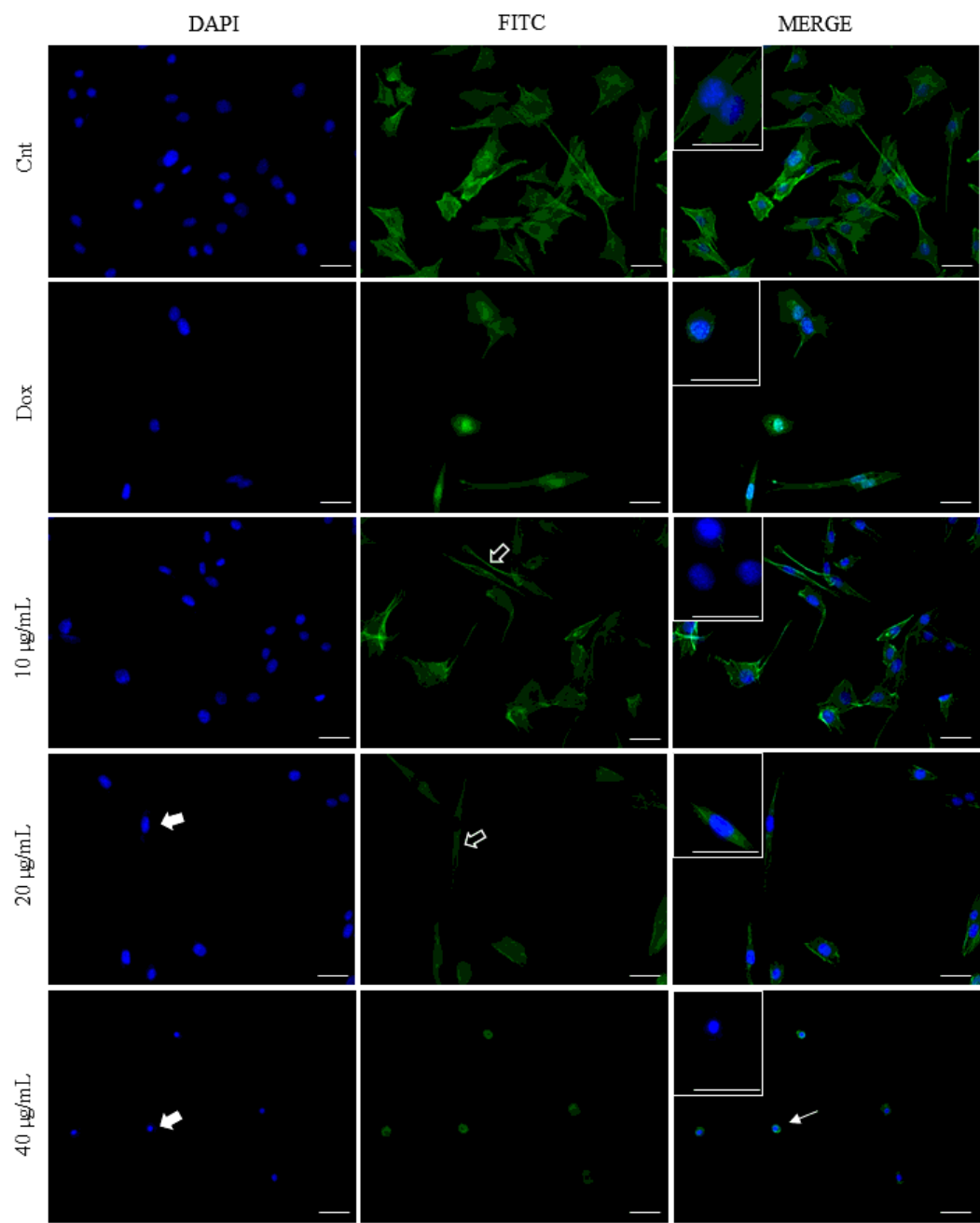


Figure 3

Morphological changes in the cytoskeleton and nucleus of A549 cells, observed with DAPI and Phalloidin/FITC staining, after treatment for 24 h with OEMS at concentrations of 10, 20 and 40 µg/mL.

Doxorubicin (25 µg/mL) was used as a positive control. Filled arrows represent chromatin condensation and DNA fragmentation; hollow arrows represent reduction in cytoplasmic volume and thin arrows, rounding and cell shrinkage. Bar: 20 µm