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ARAŞTIRMA / RESEARCH

Evaluation of the cytotoxic and membrane damaging effects of mountain tea (Sideritis stricta Boiss & Heldr.) essential oil on parental and epirubicin-HCl resistant H1299 cells

Parental ve epirubicin-HCl dirençli H1299 hücrelerinde dağ çayı (Sideritis stricta Boiss & Heldr.) uçucu yağının sitotoksik ve membran hasar verici etkilerinin değerlendirilmesi

Ayşe Erdoğan[1], Aysun Özkan[2], Orhan Ünal[2], Canan Dülgeroğlu[2]

[1]Alanya Alaaddin Keykubat University, Faculty of Engineering, Dept of Genetic and Bioengineering, Antalya, Turkey
[2]Akdeniz University Science Faculty, Department of Biology, Antalya, Turkey

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Abstract

Purpose: In this study, we evaluated Sideritis stricta (S. stricta), as potential-oxidative agents against parental and epirubicin-HCl resistant H1299 cells.

Material and Methods: Oxidative stress biomarkers such as malondialdehyde level determined in cell lysates. Assessment of cell viability was made by CellTiter-Blue® Cell Viability Assay and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay after 20-600 µg/mL essential oil concentrations treated to parental and epirubicin-HCl (drug) resistant H1299 cells for 24, 48 and 72 h. Malondialdehyde levels were assayed for determining the membrane damaging effects.

Results: Parental H1299 cells were found to be more sensitive to cytotoxic effect of the essential oil. Essential oil showed cytotoxic and more selective effects depend on time and concentration. Essential oil caused increasing malondialdehyde level on both parental and drug resistant H1299 cells. The highest concentration of the essential oil (IC70) treatment caused the highest membrane damage on both parental and drug resistant H1299 cells.

Conclusions: Parental and epirubicin-HCl resistant H1299 cells showed different cellular response against potential antitumour and pro-oxidative effects of essential oil.

Key words: Sideritis stricta, essential oil, membrane damage, parental and epirubicin- resistant H1299 cell line,

Öz

Amaç:. Bu çalışmada, Sideritis stricta (S. stricta) parental ve epirubicin-HCl dirençli H1299 hücrelerine karşı potansiyel oksidatif ajan olarak değerlendirilmiştir.

Gereç ve Yöntem: Hücre lisatlarında malondialdehit seviyesi gibi oksidatif stres biyobelirteçleri saptanmıştır. Hücre sitotoksisitesinin değerlendirilmesi, 20-600 µg/mL konsantrasyonlarında 24, 48 ve 72 saat parental ve epirubicin-HCl (ilaç) dirençli H1299 hücrelerine uçucu yağ uygulamasından sonra CellTiter-Blue® Hücre Canlılık Testi ve 3- (4,5-dimetil-2-tiazolil) -2,5-difenil-2H-tetrazolyumbromid (MTT) testi ile yapılmıştır. Malondialdehid seviyeleri membran hasar etkisini ortaya koymak için belirlenmiştir.

Bulgular: Parental H1299 hücreleri uçucu yağın sitotoksik etkisine karşı daha duyarlı bulunmuştur. Uçucu yağ, zaman ve konsantrasyona bağlı olarak sitotoksik ve daha seçici etkiler göstermiştir. Uçucu yağ, hem parental hem de ilaca dirençli H1299 hücrelerinde malondialdehit seviyesinde artışa neden olmuştur. Uçucu yağın (IC70) en yüksek konsantrasyonu, hem parental hem de ilaca dirençli H1299 hücrelerinde en yüksek membran hasarına neden olmuştur.

Sonuç: Parental ve epirubicin-HCl dirençli H1299 hücreleri uçucu yağın potansiyel antitümör ve pro-oksidatif etkilerine karşı farklı hücresel tepkiler göstermiştir.

Anahtar kelimeler: Sideritis stricta, uçucu yağ, membran hasarı, parental ve epirubicin dirençli H1299 hücre dizisi

Yazışma Adresi/Address for Correspondence: Dr. Ayşe Erdoğan. Alanya Alaaddin Keykubat University, Faculty of Engineering, Genetic and Bioengineering Department, Alanya, Antalya, Turkey e-mail: ayse.erdogan@alanya.edu.tr
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INTRODUCTION

Herbal decoctions are among the most commonly consumed beverages in the world and are well-known for their high bioactive content. Functional beverages are the fastest growing segment of functional food with strong consumer interest[1]. Herbal teas are usually rich in bioactive compounds such as purines, polyphenols, carotenoids, as well as vitamin C, vitamin E, and chlorophylls, which have been shown to possess health-promoting properties, such as antioxidative, antimicrobial, anticarcinogenic, antihypertensive, antimutagenic and antiangiogenic effects[2-5].

The genus Sideritis, a member of the Lamiaceae family, is widely distributed in Mediterranean area represented by more than 150 species and of which 46 found in Turkey[6,7]. In Turkey 12 subspecies, and 2 varieties grow, among which 36 species, 10 subspecies, and 2 varieties are endemic[8-10]. The genus Sideritis is characterized by its essential oil constituents6, diterpenoids11-14 and flavonoids[15,16].

Aerial parts of Sideritis species are widely used in folk medicine and are traditionally known for their anti-inflammatory, antimicrobial, antibacterial, antirheumatic and gastroprotective properties[17,18]. Sideritis stricta Boiss & Heldr. (S. stricta), called locally as "dag çayı or yayla çayı (mountain tea)" in Anatolia[19], is commonly being consumed as a herbal tea almost all over Turkey for medicinal purposes i.e. in the treatment of gastrointestinal disorders and common colds[20]. In general consumer practice for the preparation of mountain tea, the aerial part (stalk and leaves) of the dried plant is infused in boiled water for 3-5 min and the resulting extract is to drink as hot after filtering the plant.

Overproduction of reactive oxygen species (ROS) can result in an oxidative stress situation, damaging cellular structures including proteins, lipids and DNA. ROS are derived from molecular oxygen, but with an increased reactivity such as hydrogen peroxide, superoxide anion, hydroxyl radical and peroxyl radical[21]. Any compound able to increase the damage caused by ROS may be a potential agent for the therapy of cancer[22].

The antracycline analogue epirubicin-HCl (EPI) is an intensely potent cytotoxic compound. It causes less cardiac injury than doxorubicin derivatives at doses producing equal antitumor activity. The proposed mechanism for its cytotoxic effects involves the formation of intracellular free radicals caused by the quinine group of antracycline[23].

Tumours are heterogeneous in many respects, including chemotherapeutic susceptibility[24]. Resistance to chemotherapeutic agents is a major problem in the treatment of patients with small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Acquired multidrug resistance is the main obstacle for the cure of SCLC. Increased antioxidant mechanisms enzymes amounts in drug resistance cells can be one of the reasons of acquired multidrug resistance[25]. A group of drug resistance cells can occur in tumours during the chemotherapy. We thus focused on comparison of cytotoxic effect and malondialdehyde (MDA) levels (the end product of lipid peroxidation) of Sideritis stricta essential oil on the parental and epirubicin-HCl resistant H1299 cells.

MATERIALS AND METHODS

S. stricta was collected from Sağırin village in Serik, Antalya (50-100 m), Turkey, in June 2013. The taxonomic identification of plant materials were confirmed by a plant taxonomist, Assistant Professor Dr. Orhan Ünal, in Department of Biology, Akdeniz University, Antalya, Turkey (Voucher no: TR 103 SE).

Isolation of the essential oil

The dried aerial parts of plants (100 g) collected were submitted to water distillation for 3 h using a Clevenger-type apparatus (ILDAM Ltd., Ankara, Turkey) at Molecular Biology Department in Biology in Akdeniz University. The obtained essential oil was dried over anhydrous sodium sulphate and after filtration, stored at +4 °C until tested.

Cell lines and culture

The human non-small cell lung cancer (NSCLC) cell line H1299 (purchased from American Type Culture Collection) was used in this study. Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic solution in a humidified atmosphere containing 5% CO2 at 37°C. For subculturing, cells were harvested after trypsin/ethylenediaminetetraacetic acid treatment at 37°C. Cells were used when monolayer confluence had reached 75%. The epirubicin-HCl resistant

(drug resistant) H1299 tumor cells were derived from the parental line by stepwise selection in increasing concentrations of epirubicin-HCl until the cells were capable of propagating in 220 ng/mL drug, as described previously25,26.

Cytotoxicity assays

Parental and drug resistant cells were seeded into 96-well microplates (1×10^4 cells well^{-1}) for 24 h and then treated with different concentrations of the essential oil (20-600 μg/mL) for 24, 48 and 72 h. Cytotoxicities of the essential oil was assayed by CellTiter-Blue® Cell Viability Assay and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay.

The CellTiter-Blue-Cell Viability Assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resofurin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal[27]. Following cellular reduction, fluorescence is recorded at 560 nm excitation/590 nm emissions.

The MTT assay, tetrazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye and are, therefore, useful for the measurement of cytotoxicity. Changes in mitochondrial integrity and activity as cell viability measurement were determined using MTT assay. Test reagents were added to the culture medium. Briefly, 15% volume of dye solution was added to each well after the appropriate incubation time. After 2 h of incubation at 37°C, an equal volume of solubilization/stop solution (dimethylsulfoxide) was added to each well for additional 1 h incubation. The absorbance of the reaction solution at 490 nm was recorded[28].

The data were expressed as average values obtained from eight wells for each concentration. The IC_{50} and IC_{70} values of *S. stricta* were calculated from equation of graph. Essential oil was dissolved in 0.5% dimethyl sulphoxide (DMSO). So we treated 0.5% DMSO alone to parental and drug resistant cells. The reading taken from the wells with cells cultured with only the medium (untreated cells) was used as a 100% viability value.

Determination of malondialdehyde levels

Malondialdehyde (MDA) levels were determined after parental and drug resistant H1299 cells were exposed to different concentrations of essential oil for 24 h (IC_{50} and IC_{70}). They were dissolved in 0.5% DMSO. So we treated 0.5% DMSO alone to parental and drug resistant cells. Parental and drug resistant H1299 cells were plated at a density 15×10^4 cell/100 mm dishes. Cells were scraped off culture plates with culture medium and were centrifuged 600×g for 10 min. The cell pellets were washed with phosphate buffered saline and then sonicated (3×15 sec) in 50 mM potassium phosphate, pH 7.2, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/mL of leupeptin and centrifuged at 150,000×g for 45 min. The supernatant was used for the determination of malondialdehyde level. Malondialdehyde levels in parental and drug resistant H1299 were assayed as described in a previous method[29]. This fluorometric method for measuring thiobarbituric acid-reactive substances (TBARS) in supernatant is based on the reaction between malondialdehyde and thiobarbituric acid. The product of this reaction was extracted into butanol and measured at 525 nm (excitation) and 547 nm (emission) spectrofluorometrically. Protein was determined by the Bradford method[30] with bovine serum as a standard. The experiment was performed in triplicate and mean values were recorded.

Statistical analysis

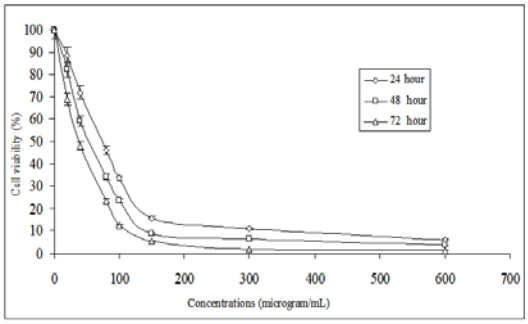
The results of the replicates were pooled and expressed as mean ± standard error. Analysis of variance (ANOVA) was carried out. The ANOVA was used to determine whether there are any significant differences between the means of three or more independent (unrelated) groups on some variable. Tukey multiple comparisons tests were used. Significance was accepted at $p \leq 0.05$[31]. Statistical analyses were performed using the Minitab program Release 13.0.

RESULTS

Effect of *S. stricta* essential oil on parental and drug resistant H1299 cells viability

To investigate the cytotoxic activity of *S. stricta* essential oil, we evaluated its effects on parental and drug resistant H1299 cells by CellTiter-Blue® Cell Viability and MTT tests. Parental and drug resistant H1299 cells were submitted to increasing concentrations of *S. stricta* essential oil for 24, 48 and 72 h. The concentrations of a compound needed to

reduce growth by 50% and 70%, respectively (IC_{50} and IC_{70}) were calculated using the Linear functions (The equation of a straight line) (Table 1).



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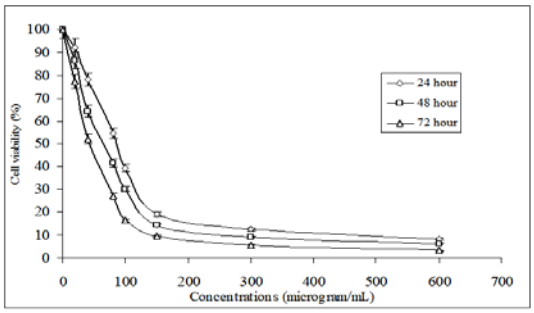

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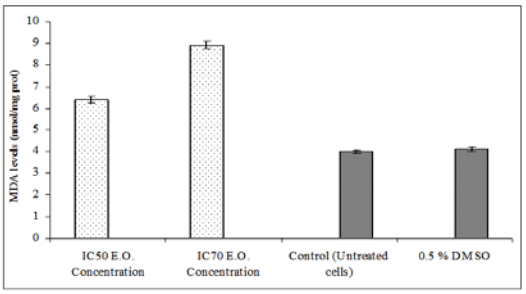
Figure 1. The cytotoxic effects of S. stricta essential oil on parental-H1299 after 24, 48 and 72 h measured by (A) The CellTiter-Blue-Cell Viability Assay; (B) MTT Assay.

Results are presented as viability ratio compared with the control group (treated with with only the medium-untreated cells). Values were expressed as the mean of three separate experiments ± S.E.

The essential oil from *S. stricta* was found cytotoxic in concentration and time dependent manners in parental and drug resistant H1299 cells according to both cytotoxicity assays (Figure 1, 2). After 24, 48 and 72 hours incubations IC_{50} values were calculated respectively from MTT test results, for essential oil on parental cells, 90, 76 and 60 µg/mL, for essential oil on drug resistant cells 115, 92 and 68 µg/mL (Table 1). Also, after 24, 48 and 72 hours incubations IC_{50} values were calculated respectively from CellTiter-Blue® Cell Viability test results, for essential oil on parental cells, 75, 50 and 37 µg/mL, for essential oil on drug resistant cells 106, 84 and 69 µg/mL (Table 1). The CellTiter-Blue-Cell Viability Assay was found to be more sensitive than MTT assay. So we studied other parameters according to CellTiter-Blue-Cell Viability assay results.



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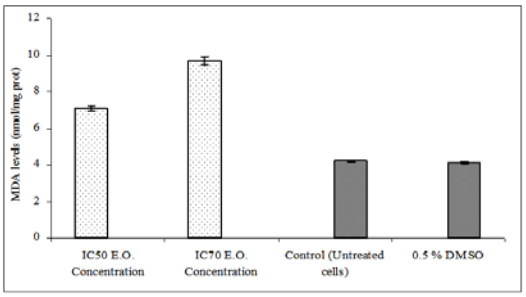

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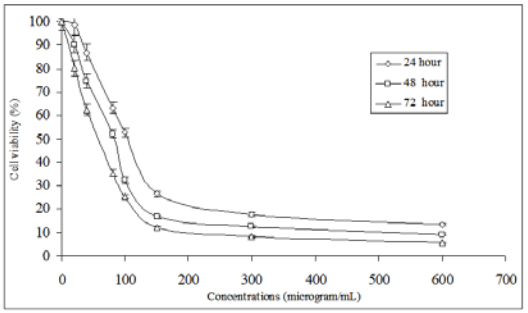
Figure 2. The cytotoxic effects of S. stricta essential oil on drug resistant H1299 cells after 24, 48 and 72 h measured by (A) The CellTiter-Blue-Cell Viability Assay; (B) MTT Assay.

Results are presented as viability ratio compared with the control group (treated with with only the medium-untreated cells). Values were expressed as the mean of three separate experiments ± S.E.

Effect of *S. stricta* essential oil on parental and epirubicin-HCl resistant H1299 cell membrane

The induction of cytotoxic cell death can be accompanied by membrane and DNA damage. Essential oil induced membrane damage at IC_{50} and IC_{70} concentrations (Figure 3) than those that mediate its anticancer activities. The results of membrane damage effects of the essential oil (IC_{50} and IC_{70}) on parental and epirubicin-resistant H1299 cells after 24 h exposure was shown in Figure 3. Essential oil caused increasing malondialdehyde level (MDA) on both parental and drug resistant H1299 cells, an end product of lipid peroxidation of membrane. The highest concentration of the essential oil (IC_{70}) treatment caused the highest membrane damage on both parental and drug resistant H1299 cells. The MDA amounts in IC_{50} and IC_{70} essential oil concentrations exposed both parental and drug resistant H1299 cells were found to be statistically different from the control and only 0.5% DMSO treated cells ($p \leq 0.05$) (Figure 3).



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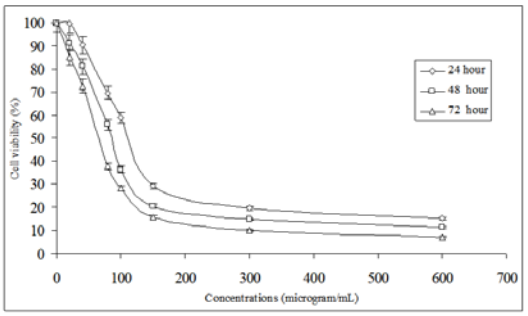

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Figure 3. Dose-dependent membrane damaging effects of the essential oil from S. stricta on parental and drug resistant H1299 cells (A) Parental cells; (B) Drug resistant cells.

Values represent mean ± S.E. from three independent experiments.

* Significantly different from control (untreated cells) and 0.5% DMSO control; $ Significantly different from IC50 essential oil treatment in parental cells; # Significantly different from IC50 essential oil treatments in drug resistant cells (ANOVA, $p \leq 0.05$)

The amounts of MDA increased almost 1.6 fold in IC_{50} concentrations of the essential oil treated parental cells while 1.7 fold increased in drug resistant H1299 cells compared to control (untreated parental cells) cells. Parental cells membrane was more sensitive to the essential oil than drug resistant cells. Biochemical changes like membrane structure in drug resistant cells will be the reason for this. Also having different antioxidant enzymes and amounts will be other reasons. Essential oil showed membrane damaging effects on both parental and drug resistant cells depending on concentrations.

In our study, the essential oil from *S. stricta* induced membrane damage and cytotoxicity in parental and drug resistant H1299 cells at higher concentrations and this can mediate its anticancer activity. The induction of cytotoxic cell death can be accompanied by membrane damage.

DISCUSSION

Lung cancer is the leading cause of cancer-related death among women and men. The search for new chemical entities against cancer using natural plant products has attracted considerable attention nowadays. Treatments against cancer which is the one of the most important disease of our age and to investigate the possibilities of these methods include treatment with plant-derived chemicals is a current issue. Essential oils are used for a variety of purposes, especially in scientific and commercial areas. At the beginning of these usage areas are cosmetics, medicine, food industry, aromatherapy and phytotherapy. Today, many anticancer drugs such as vinblastine, irinotecan, topotecan, vincristine have been obtained from plants. Therefore, it is very important to obtain and evaluate purely the medical oils and essential oils belonging to these plants, both scientifically and economically. The plant-derived products are expected to induce lesser side effects compared to synthetic drugs. *Sideritis* species have been used in folk medicine in Turkey and Europe for their antinflammatory, antirheumatic, digestive and antimicrobial properties[45,46].

Drug-resistant cell groups may be present in every tumor[47]. Therefore, when studying in vitro cell lines, it is necessary to study the cell lines of resistant cells together with the parental cells in consideration of the presence of resistant cells in tumor formation in vivo. Parental and epirubicin-HCl resistant lung cancer cells have been shown to have different responses to cytotoxic agents by our previous works[33,34,48].

The widespread use and interest in natural products, such as essential oils, familiar to us with their smells, makes it important to understand the biological activity of these substances for new uses in human health, agriculture and the environment[39]. The lipophilic nature of essential oils enables them to easily cross the membranes of the cells and reach inside the cell. Essential oils are described as strong antioxidants[49,50] and antimicrobial[51] and are in use for themanagement of severe diseases like cardiovascular[39], diabetes[52], Alzheimer's[53], cancer[54], and others. In our study, the essential oil was found less cytotoxic on drug resistant cells than parental cells. The activities of detoxification and antioxidant mechanisms enzymes were found higher in epirubicin-HCl resistant H1299 cells than parental cells[25]. This will be the reason for essential oil's less

cytotoxicity on drug resistant cells than parental cells. Also, extrusion of the drug by cell membrane pumps, increased DNA damage repair, redistribution of intracellular accumulation of drugs, modification of drug target molecules, suppression of drug-induced apoptosis, up-regulation of lipids and other biochemical changes will be the others reasons[32].

Essential oil of *Origanum acutidens* demonstrated strong cytotoxic effect in HeLa cells [inhibitor concentration (IC_{50}) <10 µg/ml] using the method xCELLigence[55]. *O. majorana* essential oil's and its oxygenated monoterpene component linalool were found more cytotoxic in parental H1299 cells than epirubicin-HCl resistant H1299 cells[22]. Also, in another studies, eugenol, eucalyptol, terpinen-4-ol, camphor, carvacrol and thymol which were found in many essential oils as components, were showed more cytotoxicity in parental H1299 cells than epirubicin-HCl resistant H1299 cells[33,34]. The essential oils from wild and cultivated form of *Salvia pisidica* showed cytotoxicity on H1299 cells[35]. Juniperus excels essential oil was effective against multidrug resistance (MDR) P-glycoprotein-expressing CEM/ADR5000 leukemia cells and reversed their resistance indicating the use of essential oil in MDR treatment in cancer[56]. *Melaleuca alternifolia* tea tree oil, can ameliorate adriamycin resistance in human melanoma cells and terpinen-1-ol is responsible for this activity[57]. Studies showed that some plant essential oils and extracts found to be cytotoxic in dose dependent on cancer cells[22,36,37].

Table 1. Summary of the cytotoxic effects of S. stricta essential oil on parental and drug-resistant H1299 cells.

	CellTiter-Blue Cell Viability Assay		MTT Assay	
	IC50 (µg/mL) ± S.E	IC70 (µg/mL) ± S.E	IC50 (µg/mL) ± S.E	IC70 (µg/mL) ± S.E
24 hour P-H1299	75 ± 1.07 ef	105 ± 0.04 hı	90 ± 0.68 g	125 ± 0.05 jk
48 hour P-H1299	50 ± 0.45 c	70 ± 0.93 e	76 ± 1.01 ef	110 ± 0.08 ı
72 hour P-H1299	37 ± 0.96 a	53 ± 0.52 c	60 ± 0.42 d	93 ± 0.65 g
24 hour R-H1299	106 ± 0.08 hı	145 ± 0.04 lm	115 ± 0.02 ıj	156 ± 0.06 mn
48 hour R-H1299	84 ± 1.02 f	118 ± 0.06 ıj	92 ± 0.72 g	128 ± 0.09 jk
72 hour R-H1299	69 ± 0.46 de	103 ± 0.09 h	68 ± 0.57 de	95 ± 0.65 gh

Values followed by different letters within a column are significantly different ($p \leq 0.05$); SE, standard error. P-1299, Parental H1299; R-H1299, drug-resistant H1299

Free radicals cause cytotoxicity and lipid peroxidation associated with chronic diseases such as cell senescence and cancer. *Reactive oxygen species (*ROS) are generated inside the cells in response to external stimuli or stress under normal conditions[58]. ROS interacts with the double bonds of the polyunsaturated fatty acids to form lipid hydroperoxide. One of the major secondary oxidation products of peroxidized polyunsaturated fatty acids is malondialdehyde (MDA), which has a mutagenic and cytotoxic effect. The lipid peroxidation caused by free radicals causes changes in the structure, permeability and fluidity of the membrane, impairment of lysosomal balance and induction of apoptosis. In our one of previous study*,* we showed that *O.majorana* essential oil's and its oxygenated monoterpene component linalool had membrane damaging effects on both parental and and epirubicin-HCl resistant H1299 cells. Parental cell's membrane was found more sensitive to the essential oil than epirubicin-resistant H1299 cells[33]. Also, eugenol, eucalyptol, terpinen-4-ol, camphor, carvacrol and thymol which were found in many essential oils as components, were showed membrane damaging effects in parental and epirubicin-HCl resistant H1299 cells[33,34]. The essential oil of *Origanum onites* (Lamiaceae) and its two phenolic components, thymol and carvacrol increased MDA levels according to controls on Hep G2 cells[38]. Also in another study *T. revolutus* C. essential oil caused to increase MDA levels in Hep G2 cells according to control cells[37].

Reaction mechanism for the essential oils can be envisaged: essential oils by penetrating through the cell wall and cytoplasmic membrane disrupt and permeabilize them and especially damage mitochondrial membranes. The mitochondria, by changes in electron flow through the electron transport chain, produce free radicals which oxidize and damage lipids, proteins and DNA. Moreover, some phenolic components of essential oils are oxidized by contact with ROS producing very reactive phenoxyl radicals which add to the ROS

released by mitochondria. The essential oils were not mutagenic clearly shows that the nuclear DNA lesions induced arose through reactive species such as phenoxyl, thiyl, hydroxyl, peroxyl and superoxide radicals which rather induce base modifications and single strand breaks which are repairable without giving rise to significant nuclear mutagenic effects[39].

Also, in eukaryotic cells, essential oils can provoke depolarisation of the mitochondrial membranes by decreasing the membrane potential, affect ionic Ca^{++} cycling[40-42] and other ionic channels and reduce the pH gradient, affecting (as in bacteria) the proton pump and the ATP pool. They change the fluidity of membranes, which become abnormally permeable resulting in leakage of radicals, cytochrome C, calcium ions and proteins, as in the case of oxidative stress and bioenergetic failure. Permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis[43,44].

At the end of our work due to reasons of essential oils obtained from *S. stricta* cytotoxic effects and membrane damage in parental and epirubicin-HCl resistant H1299 cell to be offered as a natural plant sources in the production of new anticancer drugs will provide significantly contribute to the national economy. Further studies concerning the anticancer activities of *S. stricta* essential oil against lung cancer may have the potential to develop anticancer therapeutic drugs. We believe that a better understanding of the intracellular mechanisms of essential oils obtained from other plants, such as the essential oil obtained from *S. stricta*, will bring new strategies for the production of drugs used in cancer treatments.

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