Journal of Research in Pharmacy

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Cytotoxic and Antimicrobial Activities of Turkish Rose Oil and its Major Metabolite *S*-(-)-Citronellol

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Received: 01 December 2023 / Revised: 21 February 2024 / Accepted: 23 February 2024

ABSTRACT: Oil Rose (*Rosa x damascena* Mill.) has a great economic value in food, pharmaceutical and cosmetic industries with its essential oil obtained by hydrodistillation. Essential oils are usually volatile at room temperature and they have strong biological effects. These aromatic natural extracts have been used for centuries for their therapeutic properties. Turkish Rose oil has great economic value for Türkiye and is widely used in current aromatherapy applications with its sedative and relaxing effects, besides its usage in cosmetic and fragrance industries. Furthermore, rose oil is one of the preferred aromatherapy oils for increasing the quality of life of cancer patients during chemotherapy. In this study, the cytotoxic, proapoptotic and antibacterial effects of rose oil and its major component citronellol were studied on A549 human lung cancer cells and Beas-2B healthy human lung epithelial cells. Antibacterial tests were performed with CLSI methods. For cytotoxicity test MTT technique was used. Results showed, cytotoxicity (IC_{50} value of 16.9 µg/mL), antiproliferative, antibacterial (MIC value of 750 µg/mL) and proapoptotic effects of citronellol. Additionally, morphological and ultrastructural changes indicating apoptosis were detected. Based on the findings, structural alterations in mitochondria and cell membranes, along with chromatin condensation and apoptotic alterations, were observed in A549 cells. Rose oil and citronellol induced apoptosis in both cell lines at different concentrations.

KEYWORDS: *Rosa* x *damascena* Mill., Rose Oil, Cytotoxicity, Lung cancer, Citronellol

A. INTRODUCTION

Essential oils are natural mixtures, which are mainly composed of mono and sesquiterpenes, volatile at room temperature, and have been used for centuries due to their remarkable biological effects [1]. Being one of the important values of our country, the oil obtained is known worldwide as "Turkish Rose Oil" due to the methods of obtaining it and the originality of the growing conditions [2, 3]. Today, rose oil is widely used in modern aromatherapy approaches for relaxing and against insomnia [4-6]. In recent years, rose oil has been used as an important aromatherapeutic in reducing the side effects of chemotherapeutics on cancer patients and to support symptomatic treatment [5-7]. Pre-clinical studies, reported that essential oils and their components are detected in lungs and blood plasm shortly after inhalation and can easily enter the system. Essential oils or their components are used as auxiliary therapeutics in upper respiratory tract infections by showing anti-inflammatory and antimicrobial properties on the bronchi. As a hybrid of *Rosa gallica* L. and *R. phoenicia* Boiss., *Rosa* x *damascena* Mill. (Rosaceae) is widely harvested in Isparta-Türkiye, for its essential oil. It is known as "Pink oil rose", "Damask rose", "Damascus rose", "Isparta rose", "Kazanlak rose" and "Muhammedi rose". *R. damascena* oil obtained by water distillation from fresh flowers and known as "Turkish Rose Oil" all over the World [8, 9]. It has a great economic value in food, pharmaceutical and cosmetics industries. Türkiye is the largest manufacturer in the world and meets half of its oil rose demand. The other manufacturers are Bulgaria, India, Morocco, Afghanistan and Iran [10]. Beside of its pleasant flavor and fragrance properties, several pharmacological effects such as antimicrobial, antioxidant, hypnotic, antidiabetic, antitussive and relaxant properties have been reported previously [11]. Additionally, it is one of the important essential oils used by inhalation in clinical aromatherapy [6, 10-12].

VEJSELOVA SEZER C, İZGÖRDÜ H, İŞCAN G, İŞCAN A, KUTLU HM, Cytotoxic and Antimicrobial Activities of Turkish Rose Oil and its Major Metabolite S-(-)-Citronellol Res Pharm. 2024; 28(6): X-XX.

© 2023 Marmara University Press
https://dx.doi.org/10.29228/jrp.2022.00
ISSN: 2630-6344

Main components of Turkish rose oil are alcohols (citronellol, nerol and geraniol), and long-chain hydrocarbon derivatives (nonadecane, nonadecene, heneicosane), and a small amount of phenyl ethyl alcohol and methyl eugenol [13, 14]. Citronellol and geraniol are found as main constituents in rose oil and studies have shown that *S*-(-) isomer of citronellol is naturally contained in Turkish and Bulgarian type oils [15, 16].

The aim of this study is to reveal the local anticancer and antibacterial properties of Turkish Rose Oil and its main compound *S*-(-)-Citronellol on non-small cell lung cancer (A549) and healthy lung epithelial (Beas-2B) cells.

B. RESULTS

2.1. Major Constituents of the Rose Oil

According to GC/FID and GC/MS analysis the main compounds (>5%) of the oil were determined as nerol (8%), citronellol (36%), geraniol (19%), nonadecane (16%) and heneicosane (6%) (Figure 1).

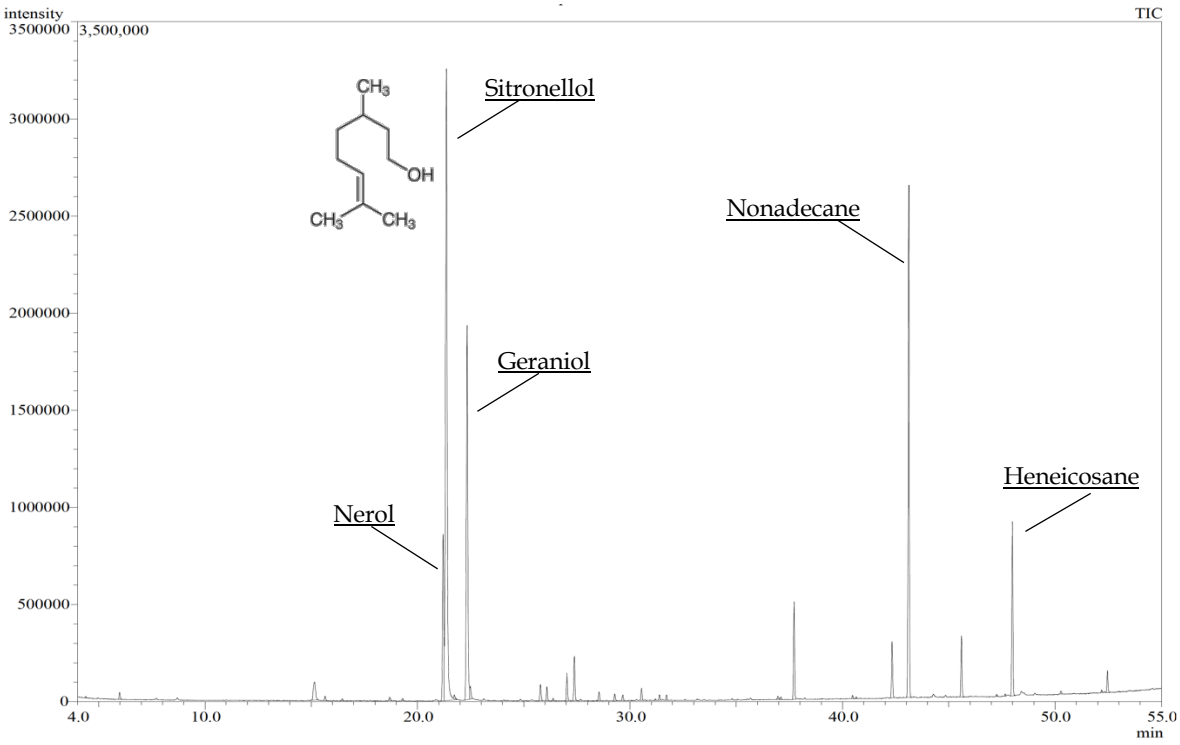

Figure 1. The GC/MS chromatogram of Rose oil

2.2. MTT results

As a result of the application in Figure 2-A, it was determined that the cell viability decreased in concentration-dependent manner. The IC_{50} value in A549 cells was 16.6 µg/mL and 33.7 µg/mL for Beas-2B cells. After 48 hours of rose oil application, the IC_{50} value was determined as 11.5 µg/mL in A549 cells and 69.8 µg/mL in Beas-2B cells (Figure 2-B). Rose oil exposure for 72 hours resulted in IC_{50} values 7.35 µg/mL for A549 and 52.1 µg/mL for Beas-2B (Figure 2-C).

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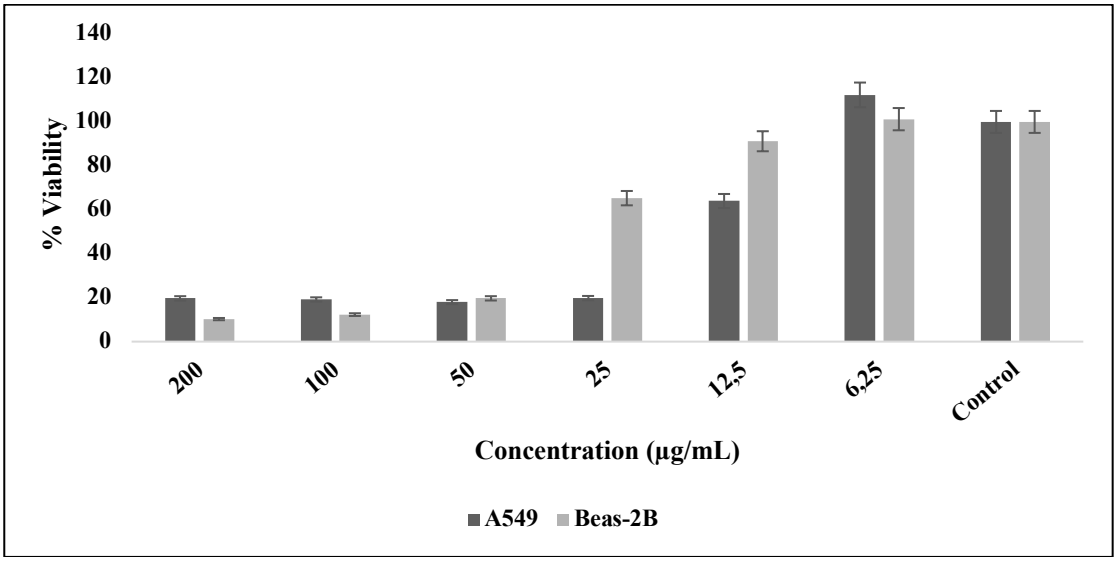

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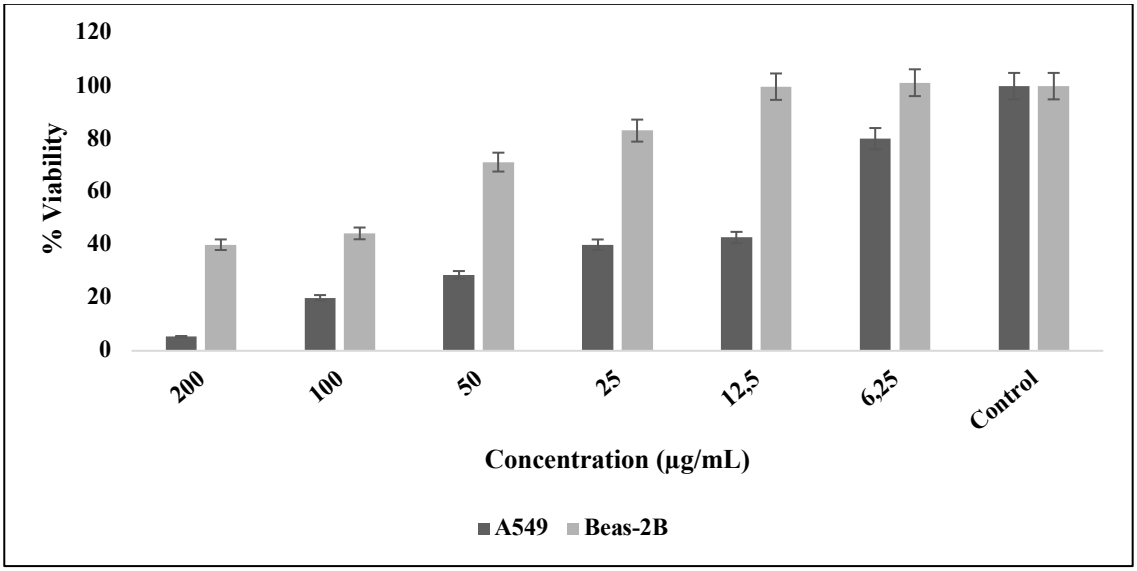

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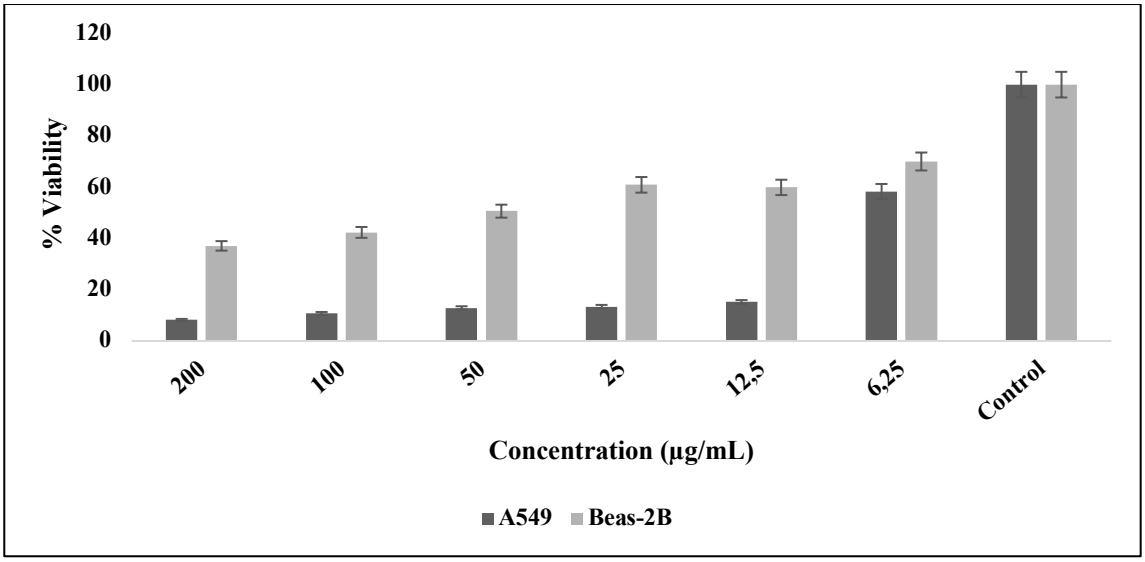

C)

Figure 2. Cytotoxicity values of rose oil on A549 and Beas-2B cells for A) 24 hours, B) 48 hours and C) 72 hours.

https://dx.doi.org/10.29228/jrp.2022.00
J Res Pharm 2024; 26(6): 1-XX

The IC_{50} value of citronellol for 24 hours was determined as 7.9 µg/mL for A549 and 16.93 µg/mL for Beas-2B cells. The same values for 48 hours were 10.6 µg/mL and 53.77 µg/mL for A549 and Beas-2B cells, respectively. Citronellol exerted cytotoxicity on A549 and Beas-2B cells with IC_{50} values of 9.53 µg/mL and 8.50 µg/mL (Figure 3 A, B and C).

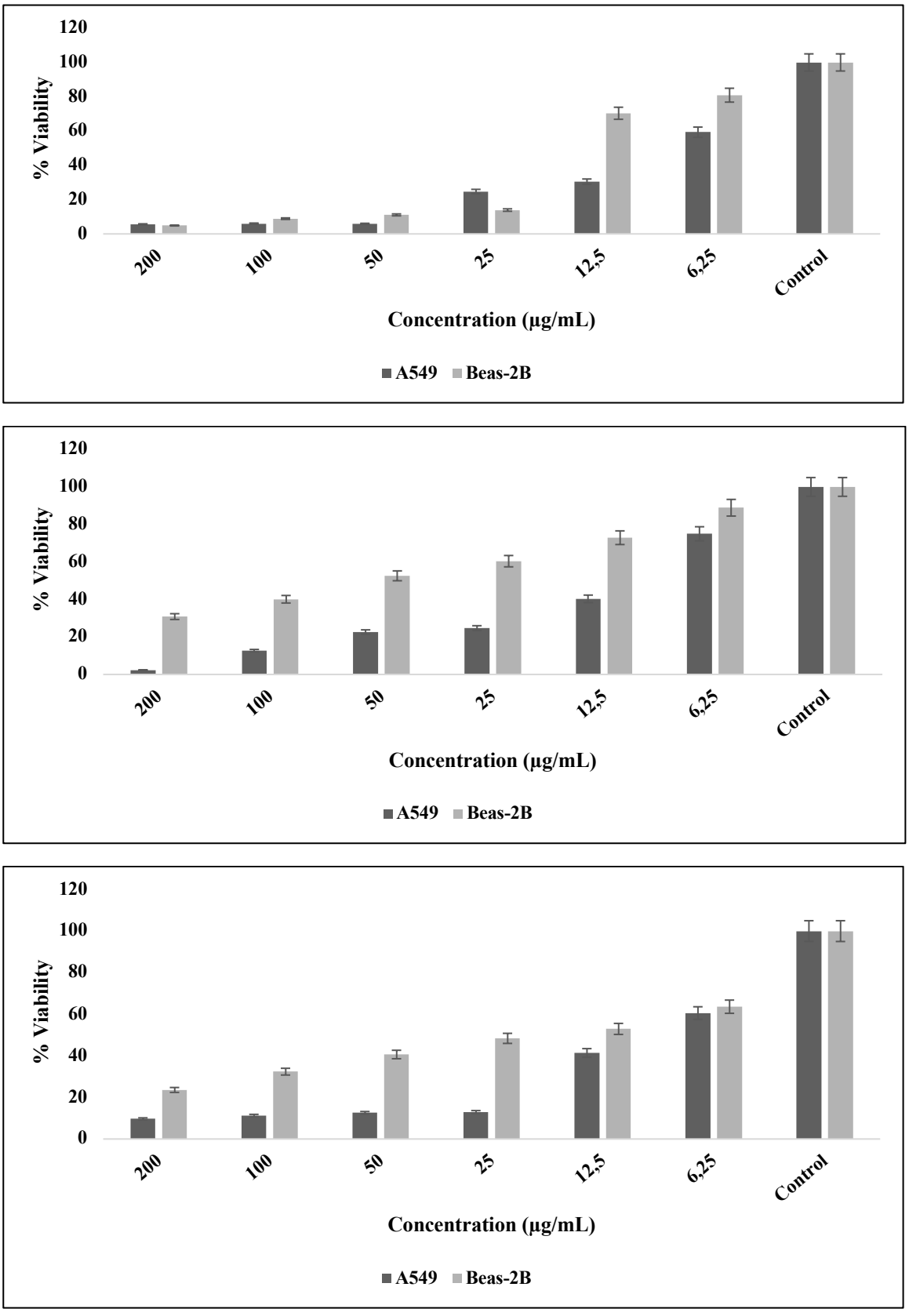

Figure 3. Cytotoxicity values of citronellol on A549 and Beas-2B cells for A) 24, B) 48 and C) 72 hours.

2.3. ATP test results

S-(-)-citronellol inhibited the proliferation of test cells with IC_{50} values of 40.46 µg/mL and 85.56 µg/mL for A549 and Beas-2B cells for 24 hours, respectively. IC_{50} values for 48 hours were 5.02 µg/mL and 35 µg/mL, and 4.81 µg/mL and 20.14 µg/mL for 72 hours, respectively fot A549 and Beas-2B cells (Figure 4). The results indicated for rose oil that A549 cells were inhibited for 24 hours with 32.6 µg/mL and 42.76 µg/mL for a549 and Beas-2B cells. For Application period of 48 hours IC_{50} values were detected to be 6.88 µg/mL and 22.50 µg/mL and 37.15 µg/mL and 13.32 µg/mL for A549 and Beas-2B cells (Figure 5).

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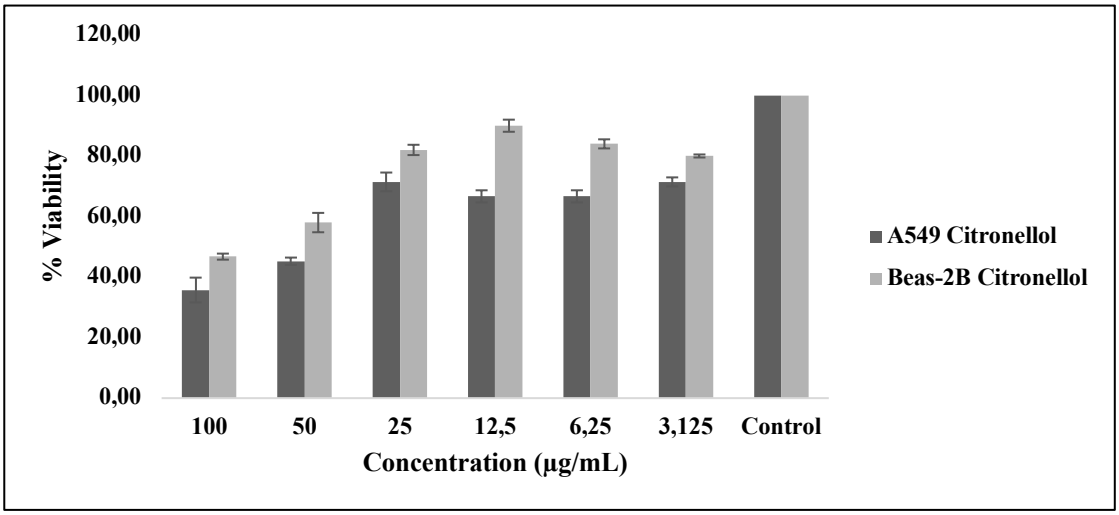

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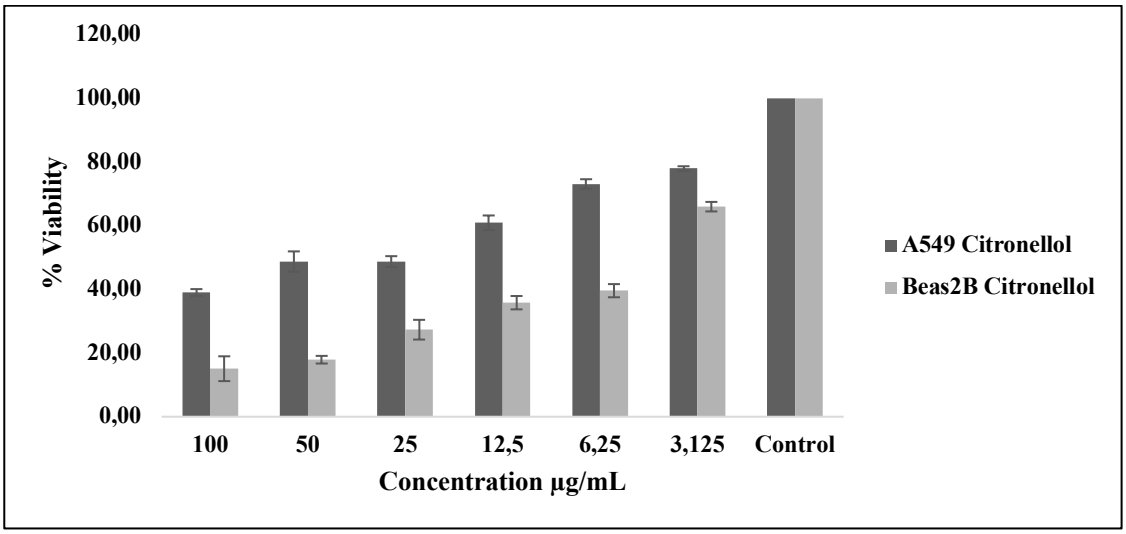

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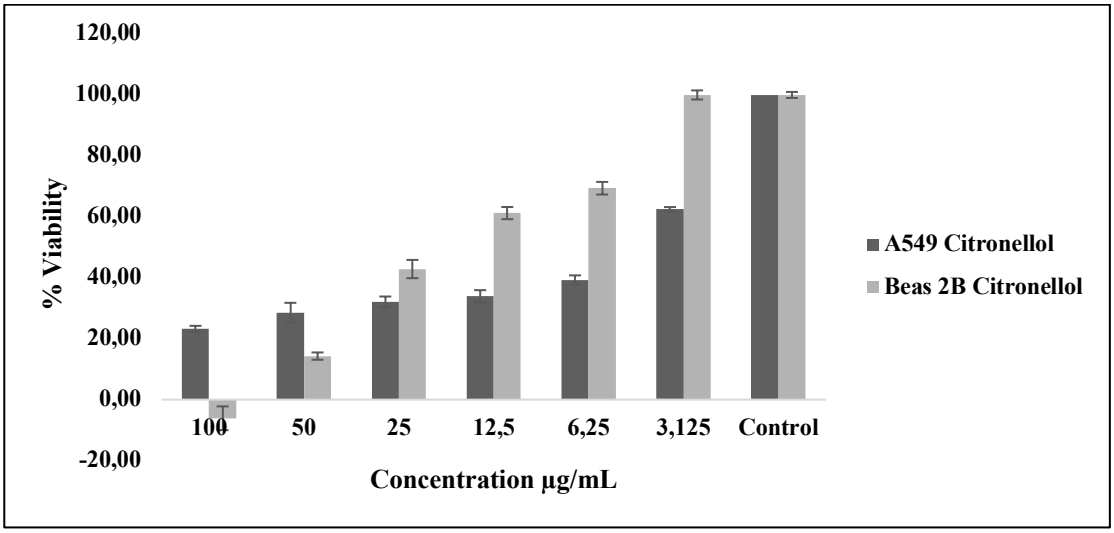
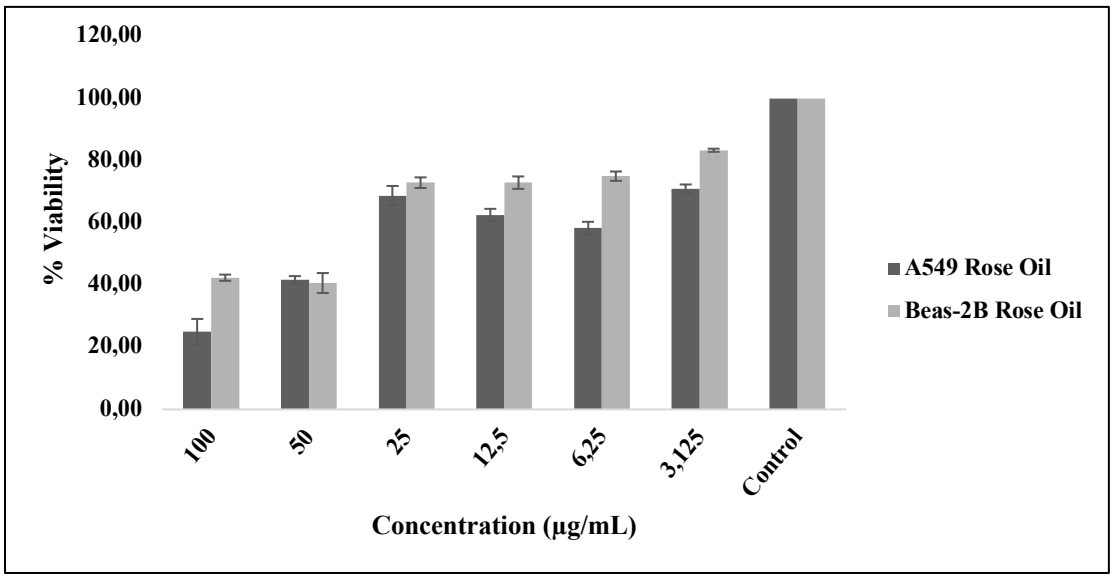


Figure 4. Cytotoxicity of citronellol on A549 and Beas-2B cells for A) 24 hours, B) 48 hours and C) 72 hours.

A.



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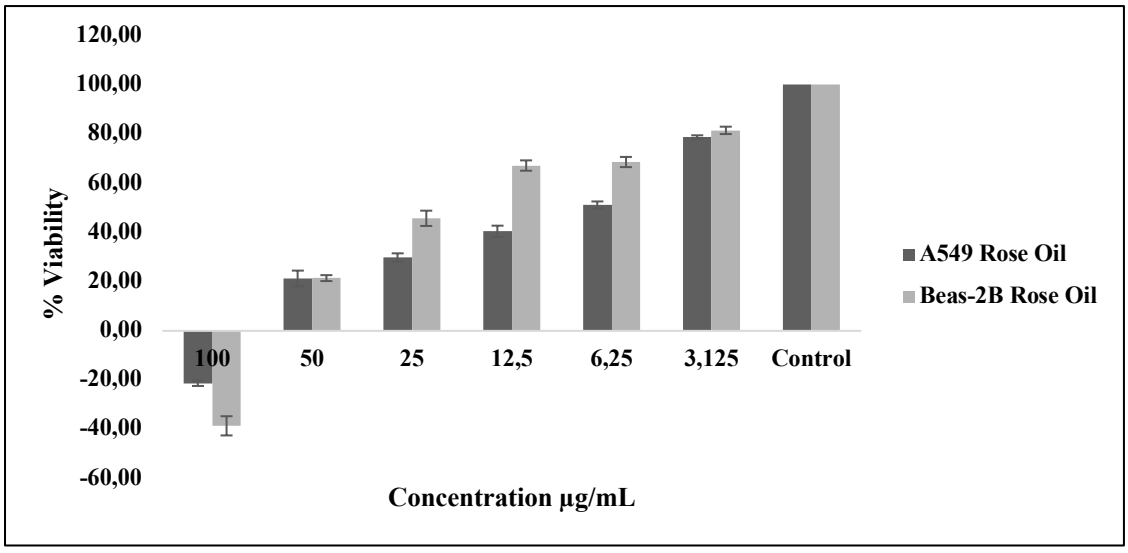

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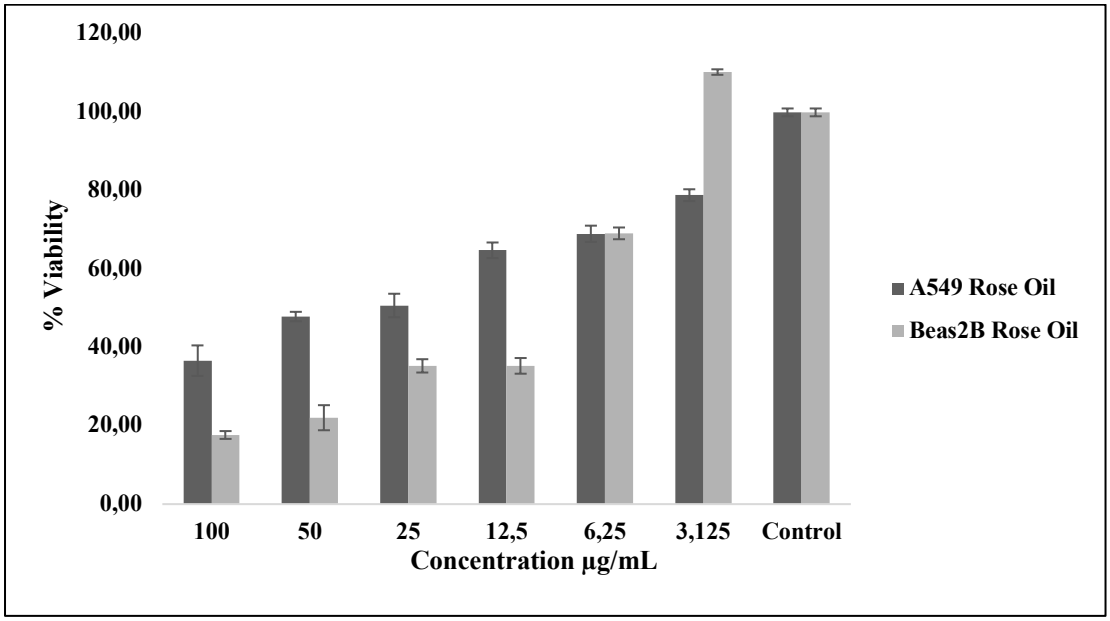

Figure 5. Cytotoxicity of rose oil on A549 and Beas-2B cells for A) 24 hours, B) 48 hours and C) 72 hours

Based on cytotoxicity results it was found that rose oil inhibited the proliferation of A549 cells in low concentrations (IC_{50} 32.6 µg/mL) compared with that of citronellol (IC_{50} 40.46 µg/mL). The selective cytotoxicity was detected both in rose oil and citronellol but test results indicated that rose oil has more effective toxicity on A549 cells being cytotoxic in low concentrations (IC_{50} values 16.93, 11.5 and 7.35 µg/mL for 24, 48 and 72 h, respectively).

When all cytotoxicity test results were evaluated, it was found that citronellol, the active ingredient of rose oil, was found to inhibit cell proliferation on lung cancer cells depending on concentration and time. Rose oil also has cytotoxicity on test cells, but both citronellol and rose oil have low (Citronellol IC_{50} 85.56 µg/mL and Rose oil IC_{50} 42.76 µg/mL) cytotoxicity on healthy lung epithelial cells when compared to their toxicity on cancer cells (Citronellol IC_{50} 40.46 µg/mL and Rose oil IC_{50} 32.6 µg/mL). It has been concluded that citronellol and rose oil may be selective and can be recommended for research on cancer drug development.

2.4. Antibacterial effect

According to the microdilution tests, the results (Table 1) rose oil and its main conponent *S*-(-)-citronellol had the same effect, except for *Pseudomonas aeruginosa*. MIC values were found between 180 µg/mL to 3000 µg/mL. Unlike rose oil, citronellol showed an inhibitory effect at the concentration of 750 µg/mL.

Table 1. MIC values of rose oil and *S*-(-)-citronellol.

Test strains	Rose Oil µg/ml	*S*-(-)-Citronellol µg/ml	S1 µg/ml	S2 µg/ml
***Escherichia coli* NRRL B-3008**	180	180	2	8
***Staphylococcus aureus* ATCC 6538**	750	750	2	1
***Pseudomonas aeruginosa* ATCC 27853**	3000	750	16	64
***Salmonella typhimurium* ATCC 13311**	370	370	8	2
***Serratia marcescens* NRRL B-2544**	750	750	8	8
***Klebsiella pneumoniae* NCTC 9633**	750	750	4	32

S1: Chloramphenicol; **S2: Ampicillin**

2.5. Enzyme activity assay results

Table 2. Ceramide-1-phosphate and sphingomyelinase levels on A549 and Beas-2B cells treated with rose oil and citronellol

Beas-2B			Quantification	A549		
Control	**Rose Oil**	**Citronellol**		**Control**	**Rose Oil**	**Citronellol**
5.1	4.6	4.7	**Sphingomyelinase mu/mL**	5.9	6.1	5.5
0.27	1.56	-0.53	**Ceramide-1-phosphate pg/mL**	-0,34	1.01	1.72

Rose oil applied to the cells caused significant increase in ceramide-1-phosphate levels both in A549 and Beas-2B cells, while the sphingomyelinase level in Beas-2B cells was decreased but increased in A549 cells. While citronellol decreased the sphingomyelinase levels of both A549 and Beas-2B cells. It increased the ceramide-1-phosphate level in A549, whereas decreased in Beas-2B cells**.**

2.6. Confocal microscopy findings

In confocal microscopic images of A549 control cells, it was determined that the cell morphology did not change, and the integrity of the nucleus, cell membrane and skeleton was preserved (Figure 6-A). Confocal microscope images of A549 cells treated with citronellol show that holes are formed in the cytoskeleton, the chromatin was condensed, and nuclei were fragmented (Figure 6-B). In addition, shrinkage of the cells, membrane blebs were detected.

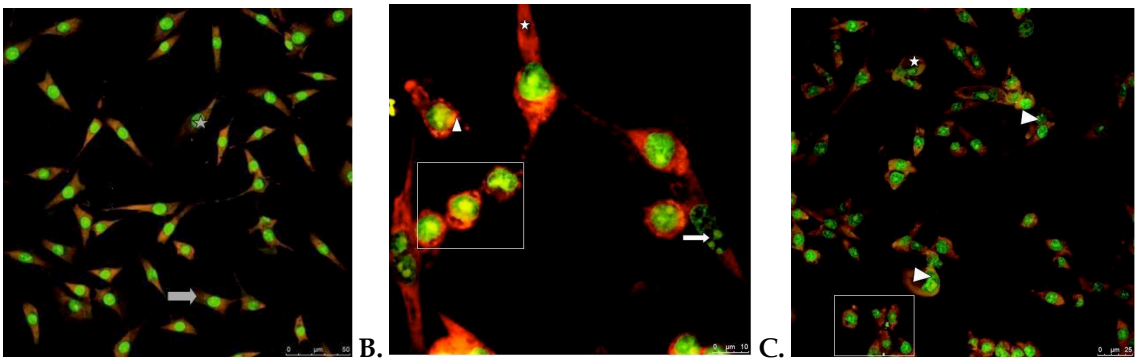

Figure 6. Confocal microscopic images of A) A549 cells, (Asterisk: Cell nucleus, Arrow: Cytoskeleton), B) A549 cells treated with Citronellol, (Asterisk: Hole in the cytoskeleton, Arrow: Chromatin condensation and nuclear fragmentation, Square: Shrunken and rounded cells, Arrowhead: Membrane blebbing), C) A549 cells incubated with rose oil (Asterisk: Cytoskeletal fragmentation, Arrowhead: Chromatin condensation and nuclear fragmentation, Square: Fragmented and circular cells).

It was determined that large holes were formed in the cytoskeleton, advanced chromatin condensation and nuclear fragmentation occurred in the confocal microscope in A549 cells to which the effective concentration of rose oil was applied (Figure 6-C). In confocal microscopic images of Beas-2B control cells, it was determined that the cell morphology remain compact, cell membrane and skeleton are not changed (Figure 7-A. In Beas-2B cells treated with citronellol, it was observed that the cytoskeleton and nuclei were completely disintegrated (Figure 7-B). In the healthy lung cells applied with rose oil (Figure 7-A), cells were undamaged compared to Beas-2B cells applied with citronellol (Figure 7-B). However, in this group, holes were formed in the cytoskeleton and signs of chromatin condensation and shrinkage were detected.

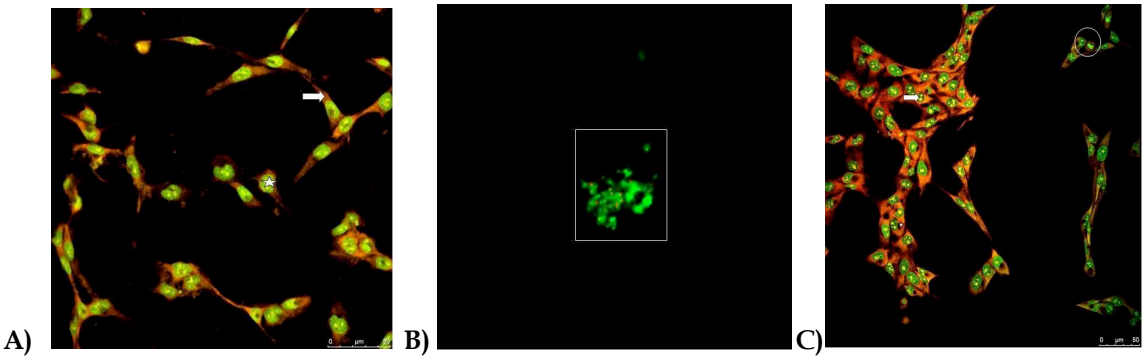

Figure 7. Confocal microscopic image of A) Beas-2B cells, (Asterisk: Cell nucleus, Arrow: Cytoskeleton), B) Beas-2B cells treated with citronellol (Square: Completely fragmented Beas-2B cell), C) Beas-2B cells treated with rose oil (Asterisk: Hole formation in the cytoskeleton, Arrow; Chromatin condensation, Circle; Shrunken rounded cells).

2.7. TEM findings

It was determined that no significant change occurred in the control group A549 cells. It was determined that the mitochondria and nuclear membrane were preserved (Figure 8).

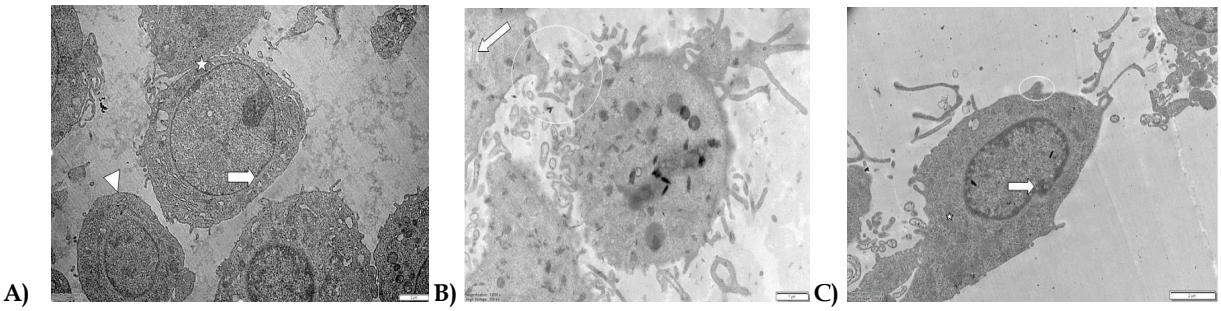

Figure 8. TEM image of A) A549 control cells, (Asterisk: Mitochondria, Arrowhead: Cell membrane, Arrow: Nuclear membrane), B) A549 cells ultrastructure after citronellol application, (Circle: Membrane blebbing, Arrow: Loss of cristae), C) A549 cell ultrastructure after application of rose oil (Rectangle: Membrane blebbing, Arrow: Chromatin condensation, Asterisk: Mitochondrial disruptions and swelling, Circle: Membrane blebbings).

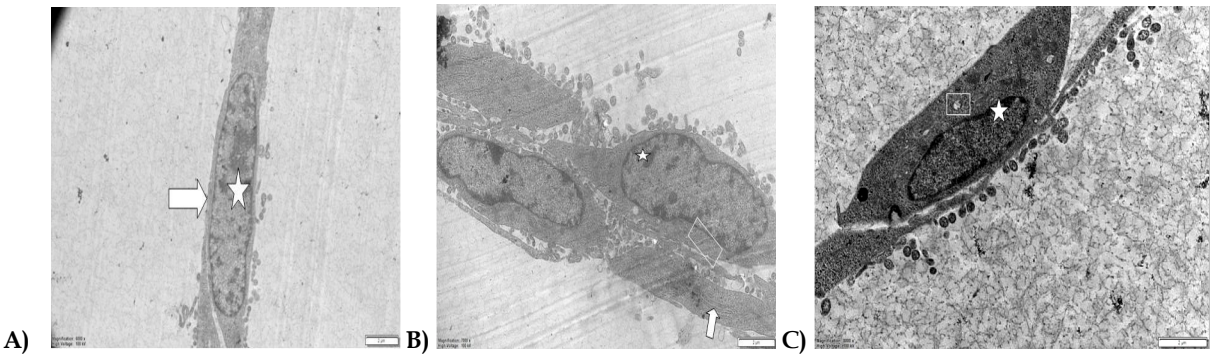

Figure 9. TEM image of A) Beas-2B control cells (Asterisk: Nucleus, Arrow: Cell membrane), B) Beas-2B cell treated with citronellol (Asterisk: Chromatin condensation, Square: Loss of cristae, Arrow: Swelling in ER cisterns), C) Beas-2B cell treated with rose oil (Asterisk: Chromatin condensation, Square: Empty mitochondria).

3.DISCUSSION

The use of medicinal plants containing essential oils can serve as a promising resource for managing certain diseases, potentially offering an effective therapeutic alternative. Essential oils consist of natural volatile components derived from aromatic plants and various classes of compounds, including esters of fatty acids, mono- and sesquiterpenes, phenylpropanoids, aldehyde alcohols, and occasionally, aliphatic hydrocarbons.

The use of essential oils for medicinal purposes dates back several centuries, with records from Ancient Egypt. With this perspective in mind, the current study seeks to explore the biological properties of essential oils, encompassing their antioxidant, anticancer, antifungal, antibacterial, and anti-inflammatory activities across diverse plant sources abundant in essential oils [11, 19]. With the increasing demand for additional anticancer treatment options, traditional herbs are now being investigated to determine whether they can serve as more effective anticancer drugs. Available information indicates that these herbs may target the same molecules and pathways as modern medicines used in cancer treatment. Therefore, it is crucial to dedicate efforts to comprehensively studying the mechanism of action and other aspects of these herbs to develop them into potential anticancer drugs [20-21].

In the study conducted with *Rosa damascena* Miller var. *trigintipetala* Dieck (Taif rose), different components of Taif rose essential oil were characterized using GC-MS technique. Additionally, the *in vitro* cytotoxic, genotoxic and anticancer effects of the oil were investigated in HepG2 and MCF-7 cell lines. The results proved that it showed anticancer activity against HepG2 and MCF-7 with IC_{50} = 13.03 ± 0.8 and 16.44 ± 1.4 µg/mL in the MTT assay. As a result, after more toxicological *in vitro* and *in vivo* studies, it was concluded that Taif rose essential oil can be used as an effective therapeutic natural agent [22].

Similarly with our research, in a study [23] the cytotoxicity potential of essential oils of *Rosa* x *damascena* was investigated A549, PC3, MCF-7 and HEK 293 cells and 473 g/mL, 412 g/mL and 105 g/mL were found to be IC_{50} values for A549, PC3 and MCF-7 cells, respectively. The essential oils were reported to be non-toxic on healthy HEK 293 cells [23].

Within the scope of this study, the cytotoxicity, anti-inflammatory, pro-apoptotic and antimicrobial effects of rose oil, which is one of the important export products of Türkiye, and its main component, citronellol, on non-small cell lung cancer and healthy lung cells were investigated. Antibacterial activity tests of rose oil and citronellol were performed against standard strains of *E. coli, S. aureus, P. aeruginosa, S. typhimurium, S. marcescens and K. pneumoniae*. The oil and its main compounds have demonstrated similar antimicrobial effects.

It was showed that after exposure to citronellol ultrastructural changes as loss and swellings in crystae and membranous organelles on A549 and Beas-2B were detected. It was found horseshoe-shaped nuclei as a result of chromatin condensation are present as well as circular cell shape and membrane blebbings are showed. These ultrastructural changes also indicate the cytotoxicity on A549 cells when compared to the untreated cells, and the results are evaluated as apoptotic markers in the accordance with confocal microscopy findings. Since, the apoptosis indicators are not clear evident in Beas-2B cells, citronellol and rose oil are recommended for further research for use in pharmaceutical agent synthesis and anticancer research. Rose oil increased ceramide-1-phosphate levels both in A549 and Beas-2B cells, while decreased the sphingomyelinase level in Beas-2B cells. The level of sphingomyelinase was increased in A549 cells after exposure to rose oil. Our findings can be interpreted as initial steps for intracellular accumulation of ceramide in turn as sparks of apoptosis. Also, citronellol, changed the intracellular lipid levels of Beas-2B and A549 cells that means triggering cell death. Based on all findings, cytotoxicity, antiproliferative, antibacterial and proapoptotic effects of citronellol and rose oil were underlined for human non-small cell lung cancer cells together with all morphological and ultrastructural changes indicating apoptosis.

4. CONCLUSION

Consequently, rose oil and citronellol as its major component were confirmed to trigger apoptosis effectively in human lung cancer cells and have potency as natural candidates for designing anticancer agents with therapeutic properties after deeper *in vitro* and *in vivo* pharmacokinetic studies. Rose oil and citronellol were showed cytotoxicity at the similar concentration, however rose oil was less toxic on healthy cell lines compared to its major component, which is support the holistic and synergistic effects of essential oils as natural mixtures.

Conflict of interest: Authors claim no conflicts of interest for this study.

Data Availability: Not applicable.

Acknowledgement: This study was supported by Project Office of Anadolu University (No 1905S076).

5. MATERIALS AND METHODS

5.1 Materials

Roswell Park Memorial Institute medium (RPMI-1640), Dimethyl sulfoxide (DMSO), Fetal Bovine Serum (FBS), Trypsin-Ethylenediaminotetraacetic acid (Trypsin/EDTA), Dulbecco's Phosphate Buffered Saline (PBS), Penicillin-Streptomycin Solution (10000 U/mL penicillin, 10mg/mL streptomycin), Rose oil (Gülbirlik, Izmir, Türkiye) and S-(-)-citronellol (Aldrich, ≥99%) were used as test materials in this study.

5.2 Methods

5.2.1. Gas Chromatography and Gas Chromatography/Mass Spectrometry Analysis

Shimadzu QP2010 Plus system was used for GC/MS analysis. CPSil-5CB column (25m x 0.25mm Ø, 0.25mm film thickness) was used with helium as carrier gas (1 ml/min), column temperature was kept 10 min at 60 °C, and programmed to 260 °C at a rate of 4 °C /min, and kept at 260 °C for 5 minutes. The split ratio was 50:1. The injection port temperature was set at 260 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 35 to 450. The same column used in the Shimadzu GC2010 system. FID temperature was 300 °C, and the temperature program given above was used to obtain the same elution order with GC-MS. Computer matching against commercial libraries (Wiley GC/MS Library, MassFinder 3 Library) was used for the identification.

5.2.2. Antibacterial activity

The antibacterial activity of rose oil and the *S*-(-)-citronellol were screened against *Escherichia coli* NRRL B-3008, *S. aureus* ATCC-6538, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 1331, *Serratia marcescens* NRRL B-2544, and *Klebsiella pneumoniae* NCTC 9633 by using CLSI (formerly NCCLS) M7-A7 dilution antimicrobial susceptibility test protocol. Chloramphenicol and ampicillin-Na were used as the standard antibacterial agents [17].

5.2.3. Cell culture

Lung cancer cells A549 and healthy lung epithelial cells Beas-2B were incubated in RPMI-1640 medium containing 10% (Fetal Bovine Serum/FBS) serum, 1% Penicillin-Streptomycin at 37°C, under incubator conditions with %5 CO_2 and appropriate humidity standards. Flask with 85% of confluency were used for all tests.

5.2.4. Cytotoxicity assessment

A549 and Beas-2B cells were seeded in 96-well cell culture plates at a density of $5x10^3$ cells/well. Cells were incubated for 24, 48, 72 hours with different concentrations of citronellol and rose oil (3.13-200 µg/mL). After incubation, 20µL of MTT (5mg/mL) was added to the wells and incubated at 37°C for 2 hours. The liquid part in each well was removed and 200 µL DMSO/well was added then absorbances were read on 570 nm wavelength in HTX-Sinergy (Bio-Tek, USA) plate reader. Viability percentages were calculated according to the absorbances of control group. The IC_{50} concentrations were determined based on the obtained viability values [18].

5.2.5. ATP analysis

For this purpose, A549 and Beas-2B cells were incubated in 96-well plates at a density of $5x10^3$ cells/well. Citronellol and rose oil were applied for 24, 48 and 72 hours. ATP content in treated cells and control cells was measured using a luminometer (HTX-Sinergy (Bio-Tek, USA) using the luciferin-luciferase bioluminescence reaction shown below. The results are taken as a relative light unit (Relative Light Unit, RLU).

$$ATP + Luciferine + O_2 \xrightarrow{Luciferase} AMP + 2Pi + CO_2 + Photon\ (RLU)$$

Percentages of viability of the samples was calculated according to the RLU values to be obtained from the control cells. Viability calculation was made as follows.

$$\text{Viability (\%)} = [100 \times (\text{Sample RLU}) / (\text{Control RLU})]$$

5.2.6. Determination of morphological changes of cells by confocal microscopy

Morphological changes caused by citronellol on A549 and Beas-2B cells were investigated by confocal microscopy method. In brief, A549 and Beas-2B cells incubated in 6-well plates ($3x10^5$ cells/well) and treated with IC_{50} concentrations of citronellol and rose oil. For staining, cells were firstly washed in phosphate buffer (PBS) and fixed in glutaraldehyde and stained with acridine orange and phalloidin. The morphological changes in the cells were imaged using the Leica Confocal Software Version 2.00 on a confocal microscope (Leica TCS-SP5 II).

5.2.7. TEM Analysis

In order to examine ultrastructural changes under transmission electron microscope (TEM), A549 and Beas-2B cells ($1x10^6$ / mL) were incubated with IC_{50} concentrations of citronellol and rose oil for 24 hours. After the incubation, cells were fixed with glutaraldehyde and osmium tetraoxide. All cell groups were dehydrated in a series of ethyl alcohol (50%, 70%, 90%, 96% and absolute ethyl alcohol). Dehydrated cells were exposed to propylene oxide and embedded in resin. After polymerization for 48 hours at 60 ^oC the prepared blocks were sectioned (80-100 nm). Prepared thin sections were taken on copper grids, stained in lead citrate and uranyl acetate, and imaged under a TEM at 120 kV (Biotwin FEI, USA).

5.2.8. Enzyme activity assay

In order to determine whether Turkish rose oil induce apoptosis by lipid related pathway the IC_{50} concentration of citronellol and rose oil were applied to A549 and Beas-2B cells for 24 hours. Ceramide-1-phosphate (C1P) levels and sphingomyelinase (SMase) activity levels in the cells were determined by the instructions of kit manufacturer.

5.2.9. Statistics

Cytotoxicity test results were tested with one-way analysis of variance (ANOVA) using the GraphPad 6.0 program. The statistical value of $p<0.05$ was considered as significant.

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