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Original Scientific Paper

Biological activities of *Liquidambar orientalis*: antibiofilm, cytotoxicity, apoptosis, and miRNA expressions

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ABSTRACT:
Due to its strong biological, pharmacological, and medical activities and rich chemical content, *Liquidambar orientalis*, known for its resinous exudate storax, has a widespread and well-established ethnopharmacological use. Although it is known that storax has anticancer, antimicrobial, antioxidant, wound-healing and other ethnomedicinal properties, the number of existing scientific studies is very limited. In this context, the aims of this study were to determine the antibiofilm activity of storax and its cytotoxic and apoptotic effects in A549 lung cancer cells. In addition, with this study, it is also possible to make a very comprehensive biological evaluation by determining the effect of storax on certain microRNA expressions. According to our results, *L. orientalis* storax decreases cell proliferation in A549 lung cancer cells and the IC50 value was determined at 31.5 µg/mL at 24h. Storax also induces apoptosis via upregulating *CASP3, 8, 9,* and *Bax* gene expression and downregulating *Bcl-2* expressions in A549 cells. Furthermore, storax decreases the expression of *miR-146a*, *miR-21*, and *miR-223,* while increasing the expression of *miR-155*. Storax inhibits biofilm formation and reduces the preformed biofilm of microbial strains including *Staphylococcus aureus* (ATCC 33862), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 64548). The results suggest that storax has strong cytotoxic, apoptotic and antibiofilm properties and thus promising potential in medicine.

Keywords:
antibiofilm activity, A549 cells, cytotoxicity, *Liquidambar orientalis*, microRNA.

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INTRODUCTION

The components of natural herbal resources have been studied due to their strong pharmacological activities for the treatment of many diseases, especially influenza, diabetes, cancer, and neurodegenerative and metabolic diseases (Dong *et al.* 2019). Most of the drugs developed against cancer on the market are herbal-based agents (Cragg & Newman 2005). Medicinal plants are widely used because of their rich biological properties (such as antioxidant, antidiabetic, antimicrobial, and anticancer activity etc.). The biological activities of herbal plants offer the potential to be important pharmacological agents (Okmen *et al.* 2014; Zhang *et al.* 2017). The natural compounds whose therapeutic activities are investigated, especially in cancer treatment, should be well defined. For the strategic uses of natural components, it is important to discover their comprehensive biological effects, including any side effects, under *in vitro* and *in vivo* conditions (Greenwell & Rahman 2015).

Liquidambar orientalis Mill. is a tertiary period relict endemic tree in the Altingiaceae family which grows in the southwestern regions of Turkey, especially in locations such as Köyceğiz, Fethiye, and Marmaris. *Liquidambar orientalis* Mill. is commonly known as the Anatolian sweetgum tree and is popularly called "Sığla Ağacı" and "Günlük Ağacı" (Karadeniz *et al.* 2013; Okmen *et al.* 2014, 2017). It is recognised for its resinous exudate and the resin secreted by damaging the tree's outer bark is called oriental sweetgum or storax. In the Mediterranean Basin, storax components are used in ethnomedicine, particularly in the treatment of dermatological diseases such as wounds and acne, gastrointestinal diseases such as ulcers and stomach ache, and parasitic infections (Sagdic *et al.* 2005; Karadeniz *et al.* 2013).

To date, available research in the literature has demonstrated the significant importance of gum extract from *L. orientalis*. It has been demonstrated that the components of *L. orientalis* exhibit strong antioxidant and antimicrobial effects (Okmen *et al.* 2014; Sarac & Sen 2014; Sicak *et al.* 2018; Ulusoy *et al.* 2021). While previous studies have shown the antibacterial properties of this endemic species, cytotoxic and anticancer studies are very scarce (Nalbantsoy *et al.* 2016; Atmaca *et al.* 2022). In a study carried out by Cetinkaya *et al.* (2022), the phytochemical composition, and antiproliferative and apoptotic effects of *L. orientalis* in HT-29 and HCT-116 colon cancer cells were demonstrated. In addition, in another *in vitro* study, it was reported that *L. orientalis* storax inhibited cell viability and induced autophagy on the PI3K/Akt/mTOR signalling pathway in PC-3 and DU-145 prostate cancer cells (Atmaca *et al.* 2022).

The number of studies in the literature showing the effects of *L. orientalis* and its ethnopharmacological properties at the mechanistic and molecular levels is quite limited. In this study, we aim to discover the cytotoxic and apoptotic effects of *L. orientalis* storax in A549 lung cancer cells, to determine its effect on the expression of microRNAs, and to investigate its antibiofilm activity. Thus, by determining the biological activities of *L. orientalis* storax at the molecular level with different biological research models, this study contributes to the determination of its ethnopharmacological properties.

MATERIAL AND METHODS

Plant material and chemical analysis of storax. *Liquidambar orientalis* Mill. storax was obtained from trees growing naturally in the Muğla-Köyceğiz (Turkey) region and purchased from a local shop through the Ministry of Agriculture and Forestry's authorised standard supplier. The production stages of the storax were described in detail in another study (Charehsaz *et al.* 2016). For the determination of the chemical constituents of storax, gas chromatography analysis was performed by Hewlett-Packard GC type 7820A, MSD 5975 (Hewlett-Packard, Wilmington, DE, USA) using a 30-m long HP-5MS (ID 0.25 mm, film thickness 0.25 mm, Hewlett Packard) capillary column at Pamukkale University's Chemical Ecology Laboratory. The chromatographic conditions were set as follows: the flow rate was 1.2 mL min^{-1} and helium was chosen as the carrier gas; the terpene temperature programme ranged from 50°C to 280°C; the heating rate was 5°C min^{-1}, the SCAN technique (mass numbers from m/z 30 to 350 were recorded; signal ions in monitoring; 93, 133, 136, 161, and 204 m/z) was used; the injected volume was 1 µL (diluted 1:100 in hexane). The chemical components were identified using the Wiley 7 MS library (Wiley, New York, NY, USA) and the NIST02 (Gaithersburg, MD, USA) mass spectral databases as previously described (Semiz *et al.* 2018). The component percentages were calculated from the peak areas using the normalisation method. The component percentages were computed as the mean value of duplicate GC and GC-MS analyses.

Cell culture. The human lung adenocarcinoma (A549) and human embryonic kidney (HEK293) cell line were obtained from the European Collection of Cell Cultures (ECACC, UK) and cultured in Dulbecco's Modified Eagle Medium (DMEM-high glucose, Sigma) supplemented with 10% fetal bovine serum (FBS, Capricorn, Germany), 1% penicillin/streptomycin mixture (Gibco, USA) at 37°C and 5% CO_2, changing the media every 2–3 days. The cells were harvested by trypsinisation using 0.25% Trypsin-EDTA (Sigma) at 90% culture confluence as previously described (Kurt-Kizildogan *et al.* 2021).

MTT assay. The MTT (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide, ROTH, Germany) assay was used to evaluate the cytotoxicity of *L. orientalis* storax in the A549 and HEK293 cell line. The cells (2.5 ×

10^3 cells/well) were seeded in a 96-well plate and incubated for 24 h for attachment. Then, different concentrations (6.25, 12.5, 25, 50, 100, 125, and 250 µg/mL) of storax were dissolved in dimethyl sulfoxide (DMSO, not exceeding 0.5%). After 24 h of treatment, the medium was carefully removed and 10 µL MTT reagent (5 mg/mL, in 100 µL fresh medium) was added to each well for 4 h. Afterwards, the medium was removed and 50 µl of DMSO was added to each well. The colour intensity was measured at 590 nm (Epoch, microplate reader, BioTek, USA). The percentage of living cells was calculated using the following formula, as previously described (Yilmaz *et al.* 2021):

Cell viability (%) = (Absorbance of treatment/ Absorbance of control) × 100

Annexin-V/PI staining. An Annexin V/FITC Apoptosis Kit (BioVision, USA) was used to determine the percentage of apoptotic cells as previously described (Arslan *et al.* 2021). Briefly, A549 cells (1×10^5 cells) were treated with storax oil at an IC50 concentration for 24 h. The same concentration of DMSO was also applied to the control cells. The cells were then collected and washed twice with phosphate buffered saline (PBS). The resuspended cells (100 µL of binding buffer) were treated with 5 µL Annexin-V and 1 µL propidium iodide (PI) dye. The cells were then incubated in the dark for 15 min at room temperature. After that, the cells were loaded onto an image cytometer (Arthur, NanoEnTek, USA) and the percentage of apoptotic, necrotic, and live cells was determined. The apoptotic cell percentages were presented as the mean ± SE of triplicate experimental setups. Hydrogen peroxide (H_2O_2, 0.2 mM) was used as the positive control.

RNA isolation, cDNA synthesis and real time PCR. A549 cells (1×10^7 cells) were treated with IC50 values of extracts for 24 h. The DMSO was also applied to the control cells (v/v). The total RNA was then isolated using the innuPREP RNA Mini Kit 2.0 (Analytik Jena, Germany). The RNA was quantified spectrophotometrically at 260/280 nm and subsequently 2.5 µg of total RNA was reverse-transcribed into cDNA using the OneScript® Plus cDNA Synthesis Kit (ABM, USA). Quantitative Real-time PCR for the determination of changes in the *Bax, Bcl-2, CASP3, 8,* and *9* mRNA levels were performed using the Applied Biosystems™ StepOnePlus™ Real-Time PCR System (Thermo, USA) as previously described (Mutlu *et al.* 2022). Reactions were performed using ABM KiloGreen 2X qPCR MasterMix (ABM, USA). GAPDH was used as a housekeeping gene. The primer sequences are listed in Table 1.

Similarly, miRNAs were isolated from the total RNAs using the miRNA cDNA Synthesis Kit with the Poly(A) Polymerase Tailing Kit (ABM, Canada) according to the manufacturer's instructions. The changes in *hsa-miR-146a-5p, hsa-miR-21-5p, hsa-miR-155-5p,* and *hsa-miR-223-5p* expression were determined as described above. *GAPDH* was used as a housekeeping gene for mRNA normalisation and *U6-2* was used for miRNA normalisation processes. The $2^{-\Delta\Delta Ct}$ method was used to determine the changes in the levels of mRNAs and miRNAs. The data were analysed using the PCR Array Data Analysis QIAGEN web portal at GeneGlobe. The microRNA primer sequences are listed in Table 2.

Antibiofilm activity. Gram-positive and gram-negative bacteria and yeast strains were used as the indicator pathogens in this study. *Staphylococcus aureus* ATCC 33862 and *Pseudomonas aeruginosa* ATCC 27853 were grown at 37°C in Mueller-Hinton Broth (MHB, BD, Heidelberg/Germany), and *Candida albicans* ATCC 64548 was also grown at 30°C in Potato Dextrose Broth (PDB, Biolife, Italy). The antibiofilm effect of storax was evaluated using the method previously described with minor modifications (Nostro *et al.* 2019). First, each culture was adjusted to 0.5 McFarland (equivalent to 1.5×10^8 colony-forming unit (CFU)/mL). The cell suspension (5%), medium and different concentrations of storax (ranging from 6.25 to 250 µg/mL) were added to each well of sterile 96-well microplates. The plates were further incubated to form biofilm for 24 hours. At the end of the incubation period, the planktonic cells were removed, and the wells were washed several times with 0.01 M sterile PBS (phosphate-buffered saline, pH 7.2). The cleaned wells were stained with 0.1% crystal violet for 15 minutes. The wells were then washed several times with sterile distilled water. 200 µL of 20% glacial acetic acid was added to the wells. The optical density of the solution was measured at 570 nm with a microplate spectrophotometer (Epoch, Biotek). All the tests were performed in triplicate and the biofilm reduction percentage was calculated using the following formula, as previously described (Daikh *et al.* 2020). Both the positive (Culture + growth media) and negative controls (DMSO + culture + growth media) were taken into account in the calculations.

Antibiofilm activity (%) = [(Control OD–Sample OD)/Control OD] × 100

A cell viability assay was applied with the storax concentrations used in the antibiofilm method. The cells were inoculated into a medium containing storax at different concentrations and incubated for 24 hours. At the end of the period, serial dilutions were made using tubes containing SFS (containing 0.85% NaCl) and incubated for 24 hours by spreading on the agar plate. The colonies formed were counted and the results were determined as CFU/ml.

Fluoresence microscopy analysis. A 12-mm coverslip was placed on the cell culture with a maximum sub-inhibitory concentration of storax and incubated for 24 hours for biofilm formation. The coverslip was sub-

Table 1. The sequences of the primers used in this study.

Genes	Forward primer 5'-3'	Reverse primer 3'-5'
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
Bcl-2	ATCGCCCTGTGGATGACTGAGT	GTCTCCTCTGACTTCAACAGCG
Bax	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
CASP3	GGAAGCGAATCAATGGACTCTGG	GCATCGACATCTGTACCAGACC
CASP8	AGAAGAGGGTCATCCTGGGAGA	TCAGGACTTCCTTCAAGGCTGC
CASP9	GTTTGAGGACCTTCGACCAGCT	CAACGTACCAGGAGCCACTCTT

Table 2. Information regarding the microRNA primers used in the study.

microRNA	Catalog no	microRNA ID	microRNA Accession no
U6-2	MPH00001	NR_002752	NR_002752
hsa-miR-146a-5p	MPH02204	MIMAT0000449	MIMAT0000449
hsa-miR-21-5p	MPH02337	MIMAT0000076	MIMAT0000076
hsa-miR-155-5p	MPH02225	MIMAT0000646	MIMAT0000646
hsa-miR-223-5p	MPH02354	MIMAT0000280	MIMAT0000280

sequently washed once with sterile distilled water. A mixture of SYBR Green and propidium iodide (PI) was added to cover the coverslip (SYBR Green/PI = 1:3). The coverslips were kept in the dark for 20 minutes in the dye solution and examined under a fluorescence microscope at 100× magnification.

Statistical analysis. GraphPad Prism 9.0 software was used for the statistical analyses. The results were expressed as means ± standard deviation (SD). The statistical differences between the groups were analysed by One-way ANOVA.

RESULTS

Chemical composition. Based on the GC-MS analysis, the components of the essential oils obtained from storax are shown in Table 3. A total of 21 compounds, representing 98.2% of the total volatile composition of storax, were identified. The main components were ethyl cinnamate (56.06%), cinnamyl cinnamate (35.51%), methyl cinnamate (2.73%), and cinnamaldehyde (1.75%). Two major components represented 91.57% of the composition (Fig. 1). The total percentage of the other remaining compounds was below 5%.

Cytotoxicity assay. The effect of storax according to varying doses and times is shown in Fig. 2. The cell viability was decreased with increasing doses of storax and the IC50 values were 31.58 μg/mL and 85.63 μg/mL for the A549 and HEK293 cells, respectively.

Apoptosis analysis. 22% of the A549 cell population was apoptotic, 65% of the storax-treated cells were viable, and 13% were classified as dead, respectively. Sto-

Table 3. The relative percentage composition of the constituents in *Liquidambar orientalis* storax.

No	Compounds[a]	RT[b]	RI[c]	RI[d]	%
1	Styrene	7.56	890	890	0.83
2	*α*-Pinene	9.64	937	936	0.13
3	Benzaldehyde	9.78	962	962	0.02
4	*β*-Pinene	11.25	978	977	0.03
5	*β*-Myrcene	12.11	990	989	0.01
6	Benzyl alcohol	12.32	1038	1036	0.07
7	Cinnamaldehyde	16.45	1215	1215	1.75
8	Indole	19.70	1294	1298	0.01
9	*α*-Cubebene	22.10	1351	1351	0.02
10	Ethyl cinnamate	23.50	1372	1374	**56.06**
11	Methyl cinnamate	23.60	1378	1376	**2.73**
12	*β*-Gurjunene	25.84	1429	1431	0.04
13	*β*-Copaene	26.00	1432	1433	0.10
14	Aromadendrene	26.37	1442	1440	0.01
15	*γ*-Muurolene	27.99	1473	1476	0.08
16	Germacrene D	28.17	1478	1480	0.02
17	*α*-Amorphene	28.20	1482	1482	0.03
18	*α*-Muurolene	28.89	1490	1498	0.01
19	*γ*-Cadinene	29.30	1514	1513	0.01
20	Isophytol	35.24	1946	1946	0.74
21	Cinnamyl cinnamate	37.11	2447	2446	**35.51**
				TOTAL	**98.20**

[a] The compounds are listed in order of elution. [b] RT: Retention time. [c] RI: Retention indices measured relative to n-alkanes on the HP-5MS column. [d] Retention indices from the literature (Adams 2007; Babushok *et al*. 2011; Nist 2018). Bold values indicate the highest compounds of the essential oils.

rax treatment caused an approximately 7-fold induction of apoptosis compared to the control cells (Fig. 3). Our results clearly showed that storax induces apoptosis in A549 cells.

The effects of storax on changes in apoptotic biomarkers: *Bax*, *Bcl-2*, *CASP3*, *CASP8*, and *CASP9*. The changes in the *Bax, Bcl-2, CASP3, CASP8,* and *CASP9* mRNA levels due to storax treatment were determined in the A549 cells by using qRT-PCR. The qRT-PCR results for the mRNA levels are given in Fig. 4a. The *CASP3* mRNA levels were increased 5.99-fold as a result of storax treatment. Similarly, the *Bax, CASP8,* and *CASP9* mRNA levels were increased 1.08, 1.58, and 2.18-fold, respectively ($p < 0.05$). On the other hand, storax caused a 1.57-fold decrease in the level of *Bcl-2* mRNA.

***miR-146a*, *miR-223*, *miR-21*, and *miR-155* expression in the A549 cells.** When the results of the microRNA expression changes were examined, a 3.70, 3.64 and 1.77-fold decrease was observed in *miR-146a, miR-223* and *miR-21* expressions, respectively, in the dose group treated with storax compared to the control group. A 9.65-fold increase in *miR-155* expression was detected in the dose group. However, these expression changes were not statistically significant. The fold regulation of the microRNAs is given in Fig. 4b.

Antibiofilm activity. The antibiofilm effect of different concentrations of storax is shown in Table 4. The results showed that all the concentrations of storax have varying antibiofilm effects on each microorganism. Accordingly, the highest antibiofilm activity was observed against *P. aeruginosa* ATCC 27853, while the lowest was observed against *S. aureus* ATCC 33862. Interestingly, only two of the lower concentrations (12.5 and 25 µg/mL) exhibited inhibitory activity in *S. aureus* ATCC 33862. The antibiofilm effect was observed against *P. aeruginosa* ATCC 27853 for all the tested concentrations of storax (except 6.25 µg/mL). In addition, an increase in concentration-dependent activity was observed up to 100 µg/mL (74.13%), while higher concentrations resulted in an inverse correlation between the sample concentration and antibiofilm activity. The effect of storax on *C. albicans*, one of the eukaryotic microorganisms, was also investigated. Among the concentrations studied, the highest activity was 60.16% at 250 µg/mL and the lowest activity was 16.85% at 50 µg/mL. The obtained antibiofilm results for all the microorganisms were confirmed by CFU counting (Fig. 5). The reduction in the CFU counts under conditions of high antibiofilm activity was noteworthy.

Fluorescence microscopy analysis. The biofilm structure formed by the three studied organisms in media treated and untreated with storax was examined under a fluorescent microscope in different lights (green and blue). The concentrations of the storax samples demonstrating maximum antibiofilm activity were used in the fluorescence microscope analysis. The resulting images are shown in Fig. 6. According to the results, it is clearly seen that the biofilm structures of the organisms which were not treated with storax were compact and densely aggregated. In contrast to the control group, a significant reduction in biofilm formation was detected in the storax-treated organisms. In addition, disruption was

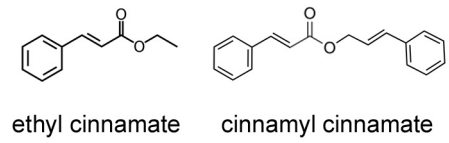

Fig. 1. The chemical structure of two major components of storax.

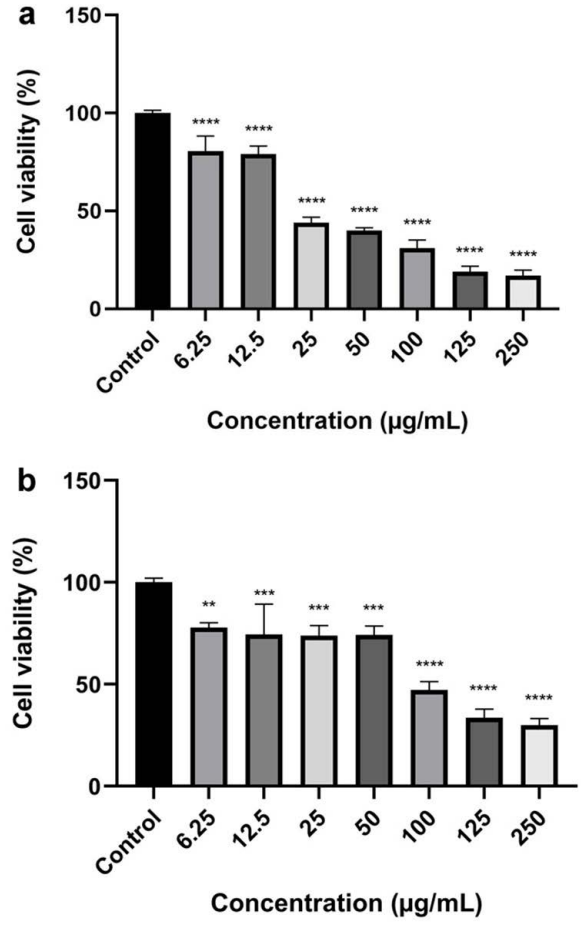

Fig. 2. The effect of different concentrations of storax on A549 cell viability, represented as the percentage reduction compared to the control. The data are the means of two independent replicates. ** = $p < 0.005$, *** = $p < 0.001$, **** = $p < 0.0001$ compared to the control group.

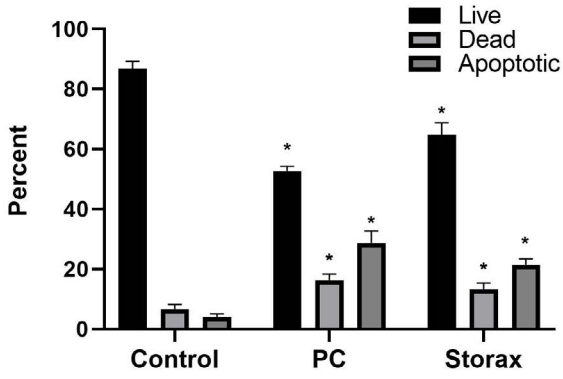

Fig. 3. Apoptosis assay by Image-based cytometer using an Annexin V/FITC kit. Following a 24 h incubation period with storax, the cell populations were assessed for apoptosis. PC: Positive control (0.2 mM H_2O_2). * = $p < 0.05$ compared to the control group.

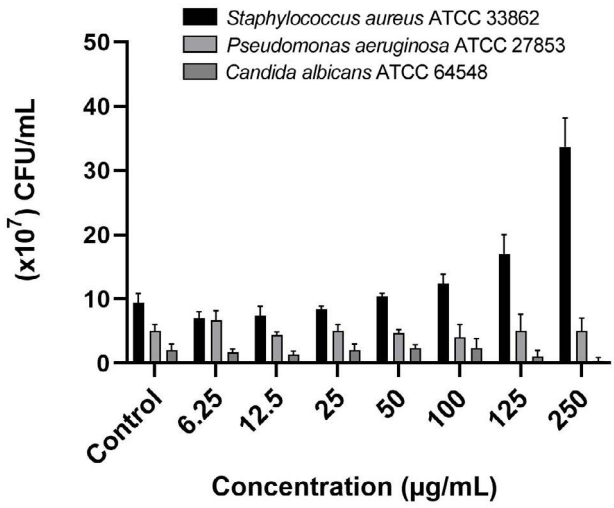

Fig. 5. CFU results for biofilm inhibition by storax (x 10^7 CFU/ml).

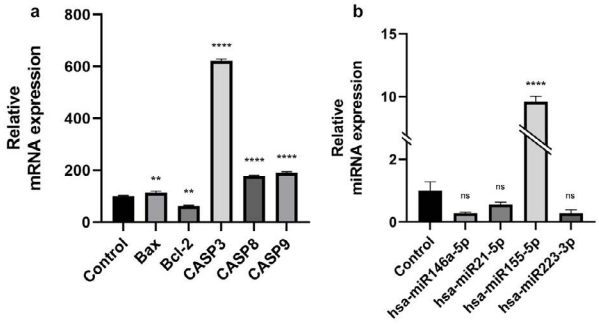

Fig. 4. The effects of storax on the mRNA and miRNA levels **a.** The effect of the IC50 concentration of storax on the mRNA level in the human liver cancer (A549) cell line. GAPDH was used as a housekeeping gene. **b.** The relative expression of target miRNA compared to U6-2 control miRNA. ns = not significant, ** = $p < 0.01$, **** = $p < 0.0001$ compared to the control values.

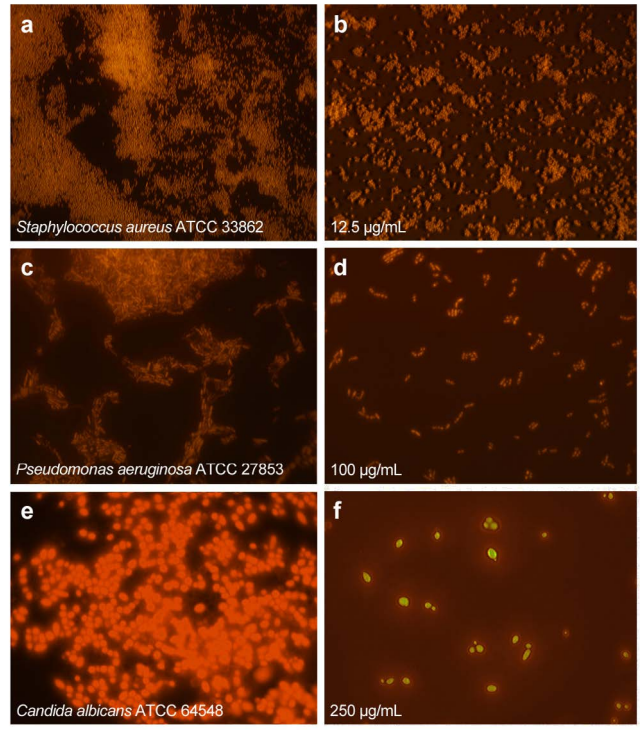

Fig. 6. Images of biofilm structure under a fluorescence microscope (**a.** control of *Staphylococcus aureus* ATCC 33862, **b.** *Staphylococcus aureus* ATCC 33862 treated with 12.5 µg/mL storax, **c.** control of *Pseudomonas aeruginosa* ATCC 27853, **d.** *Pseudomonas aeruginosa* ATCC 27853 treated with 100 µg/mL storax, **e.** control of *Candida albicans* ATCC 64548, **f.** *Candida albicans* ATCC 64548 treated with 250 µg/mL storax).

observed within the biofilm communities, with a decrease in the number of living cells (glossy cells) and an increase in the number of dead cells (matte cells) (Nostro *et al.* 2012). The formation of micro-colonies and the presence of individual cells are further evidence of the deterioration of the biofilm structure (Sahin *et al.* 2021). Also, cell deformations and changes in cell morphology (extensions in the shape of a shuttle) were observed (Fig. 6f). The fluorescent microscope images and the antibiofilm activity studies are in agreement.

DISCUSSION

Liquidambar orientalis is a medicinal plant which has been used since ancient times in the geographies where

Table 4. The percentage of reduction of biofilm formation by different storax concentrations.

µg/mL	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Candida albicans*
	ATCC 33862	**ATCC 27853**	**ATCC 64548**
6.25	ND	ND	30.70 ± 1.91
12.5	19.66 ± 4.67	28.20 ± 3.39	51.72 ± 4.10
25	13.06 ± 7.88	39.62 ± 4.37	42.37 ± 1.64
50	ND	67.76 ± 4.02	16.85 ± 8.75
100	ND	74.13 ± 0.54	17.04 ± 9.21
125	ND	52.05 ± 1.61	41.28 ± 7.75
250	ND	31.57 ± 3.21	60.16 ± 2.19

ND: No activity detected. The data were expressed as means ± SD.

it grows due to its antibacterial, antifungal, antiparasitic and other pharmacological effects (Feng *et al.* 2018; Ozbek & Bilek 2018). Previous studies have demonstrated the chemical composition and content of storax. It was determined that the major compound in the content of storax is cinnamyl cinnamate (Gurdal & Kultur 2013). In another study, Askun and colleagues (2021) determined the main compounds of the essential oils of *L. orientalis* balsam as (*E*)-ethyl cinnamate, torreyol and cinnamyl alcohol. Other content analysis studies have identified other important components in storax, including cinnamic acid, cinnamyl cinnamate, 3-phenyl propyl cinnamate, benzyl cinnamate, styrene, triterpenic acids (oleanolic and 3-epioleanolic acids), and vanillin (Hafizoglu 1982; Fernandez *et al.* 2005; Gurbuz *et al.* 2013; Gurdal & Kultur 2013). In our study, according to the GC-MS results, the two compounds with the highest levels of storax content were determined to be ethyl cinnamate and cinnamyl cinnamate, respectively. The remaining ingredient components were similar to those reported in the studies in the literature.

Storax is commonly used as a stimulant, antiseptic, antibacterial, and anti-inflammatory in Turkish ethnomedicine. Specifically, it is a well-known expectorant in Anatolia. Previous studies on storax have emphasised it antimicrobial activity (Sagdic *et al.* 2005; Oskay & Sari 2007; Lee *et al.* 2009; Basim & Basim 2013; Gurbuz *et al.* 2013). For example, Keyvan & Savas (2021) reported that *Styrax* liquids reduced the population of *S. aureus*. In contrast to this finding, our study showed an increase in the cell growth of *S. aureus* ATCC 33862 at 100–250 µg/mL concentrations of storax. We hypothesised that *S. aureus* may use the investigated storax as a carbon source and growth factor. In another study, Lee *et al.* (2009) tested storax components and essential oils against three phytopathogenic fungi. They found that some of the storax components inhibited the growth of fungi by up to 100%. The results of the current study are the first to show the antibiofilm activity of *L. orientalis* storax. It was observed that the storax inhibited 67.76% (50 µg/mL) and 74.13% (100 µg/mL) of the *P. aeruginosa* biofilm. *Pseudomonas* is a type of bacterium known to cause lung infections. Patients with bronchiectasis are particularly susceptible to *P. aeruginosa* infections. Moreover, *P. aeruginosa* has been shown to be the primary cause of mortality in cystic fibrosis patients (Ciofu *et al.* 2015). Pathogen microorganisms, thanks to their biofilms, exhibit resistance against various chemical (for example, antibiotic resistance) and environmental conditions. In other words, *Pseudomonas* biofilms are largely resistant to various known antibiotics, thus presenting the major cause of increased antibiotic resistance (Masák *et al.* 2014). Similarly, storax demonstrated a strong antibiofilm effect on *C. albicans* biofilm formation. *Candida albicans* is another causative agent of hospital-acquired infections which usually forms biofilm on implanted devices, where it acts as a reservoir for other pathogenic cells (Gulati & Nobile 2016). In short, it is important to find antibiofilm agents for the inhibition of *Pseudomonas* and *Candida* biofilms. There are no scientific reports on the antibiofilm effect of storax in the current literature.

In this study, the IC50 value of *L. orientalis* storax was found to be 31.5 µg/mL at 24 h in the A549 lung cancer cells. In addition, it was also shown that the viability of A549 cells gradually decreases depending on the increasing dose. The MTT results revealed that *L. orientalis* storax decreases proliferation of A549 cells in a dose-dependent manner. According to our Annexin-V results, this reduction in cell proliferation may be attributed to the induction of apoptosis, since an apoptotic cell rate of 22% was observed in the A549 cells treated with storax (IC50 dose) compared to the control. Furthermore, the upregulation of *Bax, CASP3, 8,* and *9* mRNA expression and the downregulation of *Bcl-2* mRNA expression in the storax-treated group of cells compared to the control, show us that the apoptosis mechanism may be triggered by affecting the expression of these genes.

The cytotoxic effects of *L. orientalis* resin extract on different cancer cells, such as human cervical cancer cells (HeLa), human breast cancer cells (MCF-7), human colon cancer cells (Caco-2), human pancreatic cancer cells (mPANC96), human prostate cancer cell (PC-3), human glioblastoma cells (U87MG) and normal cells such as human embryonic kidney cells (HEK293) and African Green Monkey Kidney Epithelial Cells (Vero) were previously determined (Nalbantsoy *et al.* 2016). It was shown that *L. orientalis* extract applied at concentrations of 0.5, 5, and 50 μg/mL exhibits antiproliferative activity in the specified cells depending on dosage and time. The same study also showed that *L. orientalis* extract caused iNOS inhibition in RAW 264.7 cells (Nalbantsoy *et al.* 2016). In another study, *L. orientalis* gum extract was found to suppress cell proliferation in PC-3 and DU145 prostate cancer cell lines, and the IC50 values were found to be 37.8 μg/mL in the PC-3 cells and 25.9 μg/mL in the DU145 cells at 72 hours, respectively (Atmaca *et al.* 2022). In the same study, it was also shown that 24-hour treatment with *L. orientalis* extract was not cytotoxic in normal Wi38 lung fibroblast cells, however, high doses reduced the viability of fibroblast cells after 72 hours. The IC50 value in the fibroblast cells was reported to be higher than the concentration in the prostate cancer cells and it was determined to be 135 μg/mL at the 72nd hour. The same study revealed that *L. orientalis* gum extracts induced autophagy in PC-3 and DU145 prostate cancer cells through the PI3K/Akt/mTOR signalling pathway. A further study reported that *L. orientalis* extract induces NF-κB-mediated apoptosis in HT29 and HCT116 colon cancer cells under *in vitro* conditions. This study demonstrated a significant increase in *CASP3, 7* and *Bax* gene expressions in both cell lines, and a reduction in *Bcl-2* gene expression in the *L. orientalis* dose group compared to the control (Cetinkaya *et al.* 2022). All of these results are consistent with our findings.

There are also no studies related to microRNAs and *L. orientalis* in the literature. This study sheds light on how *L. orientalis* treatment impacts on the expression of certain important inflammation- and cancer-associated microRNAs. Specifically, expression changes of *miR-21, miR-223, miR-155,* and *miR-146a* were observed in A549 cells after *L. orientalis* treatment. MicroRNAs are a group of non-coding RNAs involved in the regulation of gene expression and associated with a wide variety of disease groups and pathologies, including cancer, neurological diseases, dermatogenetic diseases, neurodegenerative diseases, and viral infections (Marchi *et al.* 2021). These microRNAs, whose roles in infectious diseases are currently being investigated, exhibit immunological functions by regulating target genes through virus-host interaction and by regulating the expression of immune-related genes (Marchi *et al.* 2021; Yuan *et al.* 2021). Increased expression of *miR-21* has been reported in a wide variety of cancers, especially lung, colon, stomach, and pancreatic cancers. *miR-21* is an *oncomiR* known to trigger cancer progression (Zheng *et al.* 2018). It has also been identified as a potential prognostic biomarker in lung cancer and is associated with increased mortality (Yanaihara *et al.* 2006). Furthermore, it has been reported to be upregulated in immune cells and promote immune-related inflammatory diseases (Zheng *et al.* 2018). Studies have identified the significant roles played by *miR-155* in immune response and immune regulation (Zhou *et al.* 2010; Mahesh & Biswas 2019). It has been reported that *miR-223* is an important regulator of immunopathogenesis in COVID-19 and various inflammatory diseases (Houshmandfar *et al.* 2021). In our study, a 3.70, 3.64, and 1.77-fold decrease was demonstrated in *miR-146a, miR-223* and *miR-21* expressions, respectively, in the storax treatment group compared to the control group. Furthermore, a 9.65-fold increase in *miR-155* expression was demonstrated in the treatment-group cells. When compared among themselves, no significance was observed for miR146a-5p, miR-221-5p, and miR-223-3p ($p > 0.05$). However, miR-155-5p exhibited a significant change, with this alteration being statistically significant ($p < 0.0001$). Thus, in this study, the possible effects of storax application on microRNA expression were shown for the first time.

CONCLUSIONS

The biological properties of *L. orientalis*, especially its anticancer, antibiofilm and antiviral activities were determined for the first time in this study. The obtained results showed that *L. orientalis* storax has strong biological activities and promising therapeutic potential for lung cancer research. Further biological and medical studies are needed to gain more detailed insight into the different molecular mechanisms underlying its effects.

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Biološka aktivnost *Liquidambar orientalis*: antibiofilm, citotoksičnost, apoptoza i miRNK ekspresija

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Zbog snažnih bioloških, farmakoloških, medicinskih aktivnosti i bogatog hemijskog sadržaja, *Liquidambar orientalis*, poznat po smolastom eksudatu po imenu storaks, ima široku i dobro utvrđenu etnofarmakološku upotrebu. Iako je poznato da storaks ima antikancerogena, antimikrobna, antioksidativna, lekovita i druga etnomedicinska svojstva, broj postojećih naučnih studija je veoma ograničen. U tom kontekstu, ciljevi ove studije su bili da se utvrdi antibiofilmska aktivnost storaksa i njegovih citotoksičnih i apoptotičkih efekata u ćelijama raka pluća A549. Pored toga, ovom studijom moguće je napraviti veoma sveobuhvatnu biološku procenu određivanjem efekta storaksa na neke ekspresije mikroRNK. Prema našim rezultatima, storaks *L. orientalis* smanjuje proliferaciju ćelija u ćelijama raka pluća A549, a vrednost IC50 je određena na 31,5 µg/mL u 24 h. Storaks takođe indukuje apoptozu preko regulacije ekspresije gena CASP3, 8, 9 i Bak i smanjenjem ekspresije Bcl-2 u A549 ćelijama. Štaviše, storaks smanjuje ekspresiju miR-146a, miR-21 i miR-223 dok povećava ekspresiju miR-155. Storaks inhibira formiranje biofilma i smanjuje prethodno formirani biofilm sojeva mikroba uključujući *Staphilococcus aureus* (ATCC 33862), *Pseudomonas aeruginosa* (ATCC 27853) i *Candida albicans* (ATCC 64548). Rezultati sugerišu da storaks ima snažna citotoksična, apoptotička i antibiofilmska svojstva i da ima obećavajući potencijal u medicini.

Ključne reči: antibiofilmska aktivnost, A549 ćelije, citotoksičnost, *Liquidambar orientalis*, microRNK.