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Research Article

Anti-quorum sensing and cytotoxic activity of elemi essential oil

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Abstract: Essential oils have several biological activities such as antimicrobial, antioxidant, proliferative, and anti-inflammatory. This study aimed identification of bioactive compounds found in Elemi essential oil (EO) and to determine the anti-quorum sensing and cytotoxic activities of EO. In this study, bioactive compounds of EO were analyzed using GC-MS, and the antibacterial activity of elemi was screened against *Staphylococcus aureus* ATCC 25923, Methicillin-Resistant *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* PAO1. Anti-biofilm activity and pyocyanin production on *P. aeruginosa* PAO1 were also investigated. The effect of EO on cell viability was also analyzed by thiazolyl blue tetrazolium bromide (MTT) and neutral red uptake (NR) assay in fibroblast cells. According to GC results, the major component of EO was determined as limonene (55%). A sub-MIC of elemi essential oil inhibited biofilm formation and pyocyanin production by 43% and 56%, respectively. On the other hand, EO also had an acute effect on the mitochondrial and lysosomal activities of fibroblast cell lines. Mitochondrial and lysosomal activities were significantly decreased when EO concentrations were applied for 24 and 48 hours ($p<0.05$). In conclusion, EO has inhibitory activity on biofilm formation and pyocyanin production, and also the lower doses of oil have no toxic effects on fibroblast cells. However, higher doses of EO have more cytotoxic effects on mitochondrial activity rather than the lysosomal activity of fibroblast cell lines. It is thought that EO exhibits these activities due to the amount of limonene in its content.

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1. INTRODUCTION

The history of therapeutics usage of plants dates back to ancient times and still maintains its popularity today. Essential oils are obtained from different parts of the plant such as resin, bark, flower, leaf, seed, root, and woody parts, and have a wide range of uses for many years, especially in cosmetics, medicine, food industry, aromatherapy and phytotherapy (Bhuiyan *et al*., 2020; Hyldgaard *et al*., 2012). The main components of essential oils are mono and

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sesquiterpenes and these components have some biological activities such as antimicrobial, antioxidant, proliferative, and anti-inflammatory (Bilenler & Gökbulut, 2013; Prabuseenivasan *et al*., 2006; Tahir *et al*., 2020). Numerous studies have been conducted on the antimicrobial effects of essential oil components. In addition to the antibacterial activity of the essential oil, anti-quorum sensing activity has also been reported in many studies (Onem *et al*., 2021; Sobrinho *et al*., 2020). In recent years, anti-quorum sensing activity is an approach that is thought to be effective in combating infectious diseases due to antibiotic resistance (Algburi *et al*., 2017; Alva *et al*., 2019; Millezi *et al*., 2016; Roy *et al*., 2018). This system provides communication between bacteria through signal molecules called autoinducers and enables many behaviors to be exhibited (Gürağaç *et al*., 2022).

P. aeruginosa is an opportunistic pathogen that causes chronic infections, especially in severe hospital infections. These diseases are caused by virulence factors whose release occurs under the control of the quorum sensing (QS) system (Abisado *et al*., 2018). Inhibition of this system is seen as a promising new approach to combating bacteria. Synthetic and natural molecules are being researched for this purpose (John *et al*., 2017; Langeveld *et al*., 2014; Morohoshi *et al*., 2007). Elemi oil (EO) which has been used in the form of an ointment as a stomach stimulant and as an expectorant (Mogana & Wiart, 2011), is a collective term used for oleoresin obtained from the bark of the *Canarium luzonicum* (Blume) A Gray (Nikolic *et al*., 2016). *C. luzonicum* is commonly known in the Philippines as 'pisa' and 'basiad' and is used for its oleoresin (known locally as 'sahing') that flows from the stem. When processed, it is called 'brea blanca' (white pitch) and exported as Manila elemi. Anti-bacterial, anti-fungal, and hepatoprotective activities of *Canarium* L. species are generally known and it is demonstrated that *Canarium patentinervium* Miq. extracts had antitumor activity in cancer cells lines (Mogana & Wiart, 2011). In a study conducted with *Canarium album* Raeusch, it was found that scopoletin and isocorilagin, which is one of its phytochemical components, showed strong inhibition on influenza A and their IC50 values were 22.9 ± 3.7 and 5.42 ± 0.97 μg/ml, respectively (Yang *et al*., 2018).

In this study, activity of EO obtained from *C. luzonicum* was examined on Gram-positive and Gram-negative bacteria. Biofilm inhibitory effect and pyocyanin production of *P. aeruginosa* PAO1 were also investigated. On the other hand, its effect on the cellular activity of normal fibroblast cells was examined with thiazolyl blue tetrazolium bromide (MTT) and neutral red uptake (NR). MTT and NR assays are the most commonly used for the detection of cytotoxicity or cell viability following exposure to toxic substances (Fotakis & Timbrell, 2006). The reduction of tetrazolium salts as MTT enables them to transform into a structure called formazan and brings about a color change (Tokur & Aksoy, 2017). The tetrazolium ring can only be broken by active mitochondria so that only living cells can produce the purple color (Mosmann, 1983; Perez *et al*., 2017). On the other hand, dead cells lose their ability to reduce tetrazolium compounds and do not cause any color change (Riss & Moravec, 2006). NR is a compound that interacts with the lysosomes of living and uninjured cells. Both assays are based on different physiological endpoints (Borenfreund *et al*., 1988). In this study, the effect of EO on L929 cell viability was evaluated in a controlled manner through these assays that measure cytotoxicity by two different pathways. In the literature review, no similar studies were found on the anti-quorum sensing activity in PAO1 and cytotoxicity of elemi oil.

2. MATERIAL and METHODS

2.1. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Compounds of EO (commercially available) were analyzed by Shimadzu GC-MS QP 5050 (Kyoto, Japan) gas chromatograph-mass spectrometer system and the results of the analysis are shown in Table 1.

Table 1. GC/MS analysis conditions.

Column	Cp WAX 52 CB capillary column (50 m x 0.32 mm ID, df:1.2 µm)
Carrier gas	Helium (99.999%)
Flow rate	10 p.s.i.
Injection volume	1 µL
Oven temperature	60°C raised 220°C'at 2°C/min -220ºC 20 min
Injection block	240ºC-250ºC

2.2. Anti-bacterial activity

The anti-bacterial effect of EO on *Staphylococcus aureus* ATCC 25923, Methicillin-Resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 was evaluated and *P. aeruginosa* PAO1 strains were tested using the agar well method (Holder & Boyce, 1994) and microdilution method was used to determine the minimum inhibitory concentration (MIC) values. According to the method, 100 µL of EO was added to 96-well microplates containing 100 µL of medium and serial dilutions were made in two folds, respectively. The microplates were incubated overnight at 37 °C by adding the bacteria suspension prepared according to 0.5 McFarland (108/mL). Following the incubation, microplates were evaluated and the lowest concentration without growth was determined as the minimum inhibitory concentration (MIC).

2.3. Pyocyanin Assay

Pyocyanin assay was carried out as described before (Essar *et al*., 1990). PAO1 culture was incubated overnight in Luria Bertani Broth. Pyocyanin was extracted from the culture with 3 mL chloroform and separated organic phase fixed with 1mL of 0.2 N HCl. The absorbance of the solution was measured at 520 nm.

2.4. Biofilm Formation Assay

Biofilm formation was conducted by crystal violet (CV) assay (O'Toole, 2011). A volume of 20 µL of EO, 180 µL of medium, and 10 µL of PAO1 culture were added to each well of 96-well microplate. After 48 hours of incubation, the plate was poured and washed 3-5 times with distilled water. Then the crystal violet at 0.1% concentration is added to the wells for 30 minutes. The plate was again washed 3-5 times with distilled water. After the washing process 200 µL of 95% ethanol was added to each well and after 15 minutes resolving CV read at 570 nm (Biotek-Epoch 2-Microplate Spectrophotometer).

2.5. Cell Culture and Cellular Activity

Mouse fibroblast cells (L929) were maintained in DMEM medium with 10% Fetal Bovine Serum. Cells were trypsinized with 0.05% Trypsin/EDTA solution and cells ($2x10^4$) were seeded in a 96-well plate and cultured for 24 hours. After 24 hours of incubation, EO concentrations were applied to the each well. EO was dissolved in dimethylsulphoxide (DMSO) and diluted in complete DMEM to 630, 420, 210, and 105 µg/mL concentrations. The final DMSO concentration was below 1%. Concentrations of EO were removed after 24 and 48 h, and 100 µL of MTT in 5 mg/mL was added to the wells. The formazan crystals after 3 hours of incubation were dissolved with 100 µL DMSO and absorbances at 570 nm were measured by a microtiter plate reader spectrophotometer (Multiskan GO-Thermo).

After EO application for 24 and 48 h, 100 µL neutral red medium was added, and the plate was washed after 3 h incubation at 37 °C. The dye was extracted with 100 µL acidified ethanol solution (Repetto *et al*., 2008). The optical density (OD) of neutral red extract at 540 nm was measured in a microtiter plate reader spectrophotometer (Multiskan GO-Thermo).

2.6. Statistical Analysis

The data obtained from the study were evaluated with IBM SPSS 21 package program. The compliance of the data to normal distribution was determined by the Shapiro-Wilk test. Group comparisons were made using the independent sample tests one-way ANOVA. Results are given as mean ± standard deviation. The statistical significance level was accepted as p <0.05.

3. RESULTS

3.1 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The compounds of EO determined by GC-FID and GC/MS techniques are shown in Table 2, and 38 compounds were determined in different percentages. It was seen that the highest value belongs to limonene with 55.88%; followed by elemol with 17.54%, phellandren alfa with 10.96%, and beta phellandrene with 4.51% rate.

Table 2. GC–MS analysis of extracts % major components and their retention times.

No	Name of compound	rt (min)	%
1	alpha pinene	6.689	0.55
2	beta phellandrene	8.112	4.51
3	pinene beta	8.314	0.20
4	beta myrcene	8.758	0.90
5	phellandrene alpha	9.544	10.96
6	delta 3-carene	9.636	0.05
7	alpha terpinene	10.002	0.25
8	p-cymene	10.386	2.84
9	limonene	10.781	55.88
10	cis-ocimene	10.929	0.20
11	beta ocimene y	11.432	0.20
12	gamma terpinene	12.023	0.19
13	trans sabinene hydrate	12.669	0.05
14	alpha terpinolene	13.481	1.38
15	1 -methyl-4-isopropenylbenzene	13.697	0.10
16	linalool	14.316	0.04
17	*	15.697	0.04
18	cis p-mentha 2,8-dien 1 ol	16.424	0.04
19	*	16.788	0.04
20	4-terpineol	19.098	0.48
21	cis p-mentha-1 8-dien-2-ol	19.335	0.01
22	dmbca	19.513	0.26
23	beta fenchyl alcohol	20.055	1.72
24	a-phellandrene epoxide	20.536	0.26
25	trans-carveol	21.594	0.04
26	cis-sabinol	22.930	0.05
27	d-carvone	23.126	0.06
28	piperitone	23.783	0.08
29	alpha cubebene	29.910	0.03
30	copaene alpha	31.700	0.15
31	beta elemene	32.655	0.11
32	methyleugenol	33.451	0.37
33	caryophyllene	34.432	0.19
34	alpha humulene	36.675	0.10
35	spathulenol	37.663	0.03
36	germacrene-d	38.310	0.08
37	alpha muurolene	39.535	0.02
38	elemol	42.726	17.54
Total			100

*Unknown, rt: retention time

3.2. Antibacterial Activity and Minimum Inhibitory Concentration

According to agar well diffusion results, different inhibition zone has detected at the test concentration, which was statistically significant compared to a positive control (gentamicin 40 µg/disc) (p<0.001) (see Table 3). The lowest MIC value was determined against *P. aeruginosa* (MIC = 2.7 µg/mL) and MIC values of elemi were in the range of 2.7 µg/mL to 21.85 µg/mL (MRSA 5.4 µg/mL; *S. aureus* 21.85 µg/mL; *E. coli* 5.4 µg/mL).

Table 3. Zone diameter of Elemi on strains.

	S. aureus ATCC 25923	MRSA ATCC 43300	*E. coli* ATCC 25922	P. *aeruginosa* ATCC 27853	*P. aeruginosa* PAO1
Control (Gentamicin)	15.00±0.00 ^{ns}	15.00±0.00 ^{ns}	14.00±0.00 a*	16.00±0.00 a*	16.00±0.00 a**
Elemi	14.67±1.53	18.00±2.00	11.00±1.00 b	10.33±1.15 b	9.33±0.58 b

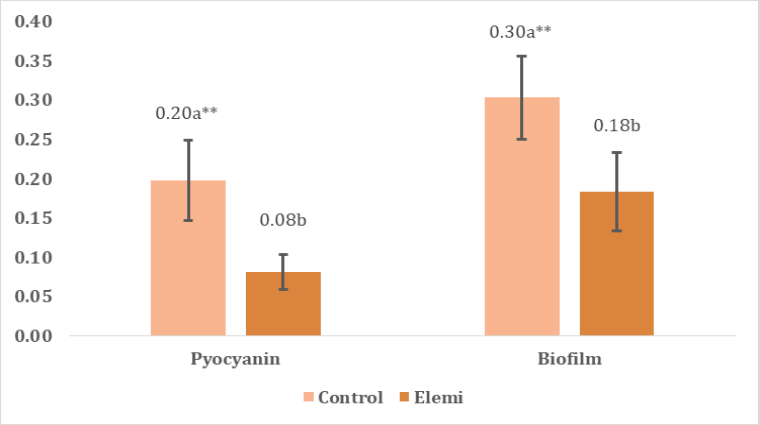
*The differences within columns signed with different letters are significant (p<0.05); **The differences within columns signed with different letters are significant (p<0.01); SD ± mean

3.3. Pyocyanin and Biofilm Inhibition

The discovery of bacterial communication and especially the knowledge that some microorganisms cause diseases by using this QS system has led to an increase in studies on the inhibition of the system (Banu & Mary, 2016).

EO was tested for the inhibition effect of pyocyanin production and biofilm formation which were QS-related virulence factors in *P. aeruginosa* PAO1. The sub-MIC concentration of EO inhibited pyocyanin production by 56%. (p<0.01) (Figure 1).

Figure 1. Inhibition effect of EO on pyocyanin production and biofilm formation in PAO1.

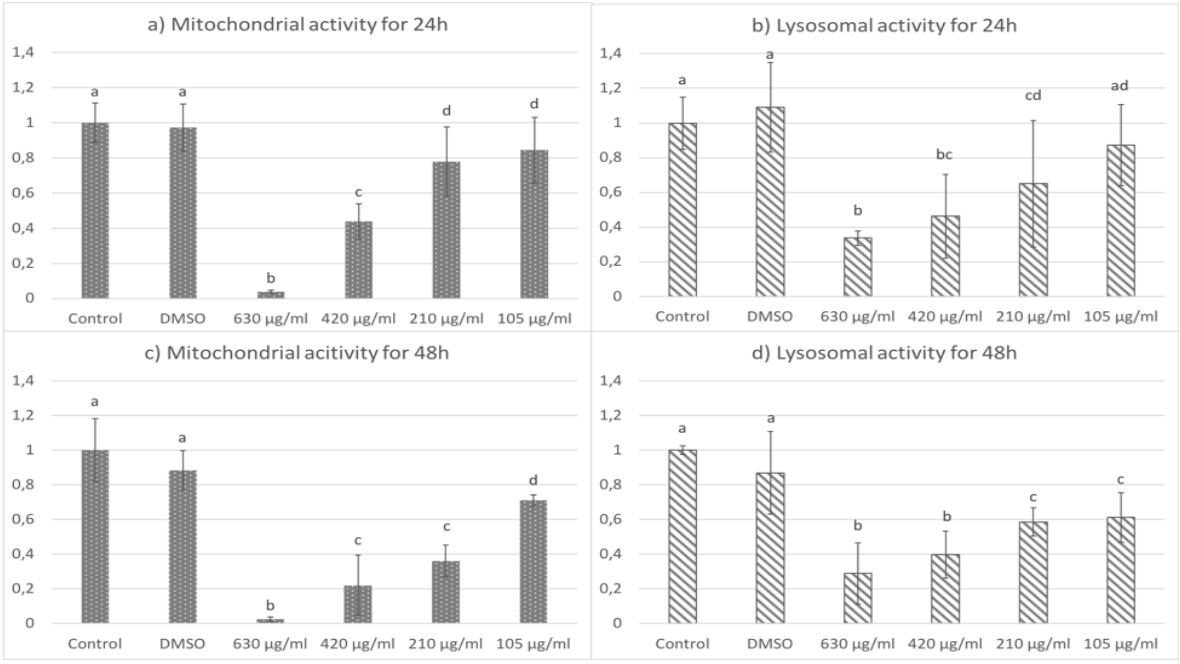


**The differences signed different letters are significant (p<0.01); SD ± mean

3.4. Effects of Elemi oil on Cellular Activity of Fibroblast Cells

As seen in Figure 2a, the mitochondrial activities of the cells decreased significantly compared to the control at the end of 24 hours at all the concentrations applied (p<0.05). The concentration leading the greatest reduction was 630 µg/mL. Concentrations of 210 and 105 µg/mL caused inhibition of mitochondrial activity, similarly. When the effects of the concentrations applied at the end of 24 and 48 hours on the lysosomal activities of the cells were examined, it was determined that the 630 and 420 µg/mL had similar effects, and also the 210 and 105 µg/mL doses had similar effects. All concentrations suppressed lysosomal activity relative to control (p<0.05) (Figures 2b and 2d). In Figure 2c, all concentrations caused suppression of mitochondrial activities of cells compared to control (p<0.05), while doses of 420 and 210 µg/mL had similar effects in manner time-dependent. With the longer exposure to concentrations, all of them exhibited more inhibitory activity.

Figure 2. Effects of EO concentrations on mitochondrial (a and c) and lysosomal activity (b and d) of L929 cell lines for 24 and 48 hours. Different letters show statistically significant differences between groups.



4. DISCUSSION and CONCLUSION

The use of plants in health has a history of many years. They are used in different forms such as extraction, boiling, and obtaining essential oil. Essential oils are obtained from different parts of plants and are known to have therapeutic effects. In this study, we evaluated the antibacterial, antibiofilm, and cytotoxic activities of EO. In addition to these, the phytochemicals have tried to be determined. The results showed that the major component of the Elemi is limonene (55.88%) and the similar studies with elemi, limonene is found major component but it was observed at 36.40 % and 36.38 % rates (Galovičová *et al*., 2020; Kačániová *et al*., 2020). The composition of essential oils may differ depending on the region where they are grown, the time of harvest, and the procedure used to extract the oils (Nannapaneni *et al*., 2009; Paibon *et al*., 2011).

Major components of essential oils are in the group of hydrocarbons and refer to the terpenes consisting only of carbon and hydrogen. Limonene is also one of the monoterpenes included in hydrocarbons. In a study conducted with limonene, α-pinene, β-pinene, *p*-cymene, it was observed that the antibacterial effects of these monoterpenes were at different rates (Koutsoudaki *et al*., 2005). This can be explained by the fact that the contents of essential oils have a synergistic effect and thus show more antibacterial properties (Hyldgaard *et al*., 2012). The antibacterial activity of essential oils has been linked to their hydrophobicity. This feature permits the EO to enter the bacterial cell membrane, damaging it and making it more permeable (Dănilă *et al*., 2018). In a similar study, the essential oil extracted from the resin of *Canarium strictum* Roxb. showed antimicrobial activity against *S. aureus, P. aeruginosa, E. coli, Klebsiella pneumonia*. The most effective results were seen on MR*SA* and MDR *E. coli* strains with >0.66 mg/mL MIC value (Tahir *et al*., 2020).

Pyocyanin is one of the important virulence factors produced by *Pseudomonas* for the control of the QS system. Another factor that plays an important role in virulence, biofilm, is the 3-dimensional structure that bacteria build by exhibiting coordinated behavior (Millezi *et al*., 2016). The biofilm formation is important for the treatment of infectious diseases because more than 60% of persistent and chronic infections are known to involve biofilm and it is more

difficult to treat with antibiotics than planktonic form (Ceylan *et al*., 2014; Lewis, 2001). EO reduced biofilm formation 43% rate (p<0.01) (Figure 1) and no similar research on *Pseudomonas* was found in the literature review.

In this study, it was determined that cytotoxic activities of EO on L929 cells. Similar activity was observed in HT-29 epithelial cells exposed to increasing concentrations of essential oils which include EO (25–200 g/mL) and it was determined that EO exhibited strong cytotoxicity on the mitochondrial activity of cells (Senthil Kumar *et al*., 2020). According to GC-MS analysis, it was determined that limonene was the most abundant compound (%55.88) in EO. Since limonene is the major constituent of EO, limonene inevitably contributes to EO's cytotoxicity. In human neuroblastoma cells, limonene caused cytotoxicity and mitochondrial damage in a previous study (Russo *et al*., 2013). Limonene also reduced cell viability and triggered mitochondrial-dependent apoptosis in human colon cancer cells LS174T in a dose-dependent manner (ranging from 0.4 to 3.2 mol/L) (Mukhtar *et al*., 2018; Vieira *et al*., 2018). Limonene extracted from *Citrus sinensis* also decreased cell viabilities of human adenocarcinoma (SW480 and HT-29) cells (Murthy *et al*., 2012). Limonene also exhibited cytotoxic activity by inducing the autophagy-lysosomal pathway (Russo *et al*., 2014; Yu *et al*., 2018). In this study, it was seen that EO had a more suppressive effect on mitochondrial activity than lysosomal activity in fibroblast cells.

In conclusion, EO has inhibitory activity on biofilm formation and pyocyanin production, and also lower doses of oil have no toxic effects on fibroblast cells. However, higher doses of EO have more cytotoxic effects on mitochondrial activity rather than lysosomal activity of fibroblast cell lines. It is thought that EO exhibits these activities due to the excessive amount of limonene in its content. More research is needed to determine the efficacy of EO *in vivo* for dermatological applications.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Ahu Soyocak, Ebru Onem: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft. **Ayse Ak**: Investigation, Writing, Methodology, Supervision and Validation.

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