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Synergic potential of *Pelargonium endlicherianum* Fenzl. Essential oil and antibiotic combinations against *Klebsiella pneumoniae*

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ABSTRACT

In this study we investigated antimicrobial activity against *Klebsiella pneumoniae* and the phagocytic functions of human leukocyte cells as revealed in an in vitro experimental model combining cefepime and gentamicin with *Pelargonium endlicherianum* Fenzl. essential oil treatments. The bactericidal effects of this essential oil and antibiotic combinations were dynamically detected by time-kill assay. To examine the function of this essential oil and antibiotics in permeating outer membrane barriers when used singly or in combination, a UV spectrophotometer was used, and morphologic images were captured by scanning electron microscopy.

The antibacterial activity of the essential oil and antibiotics was assessed using broth microdilution and agar well diffusion. The combined effects of the essential oils of *P. endlicherianum* and gentamicin and cefepime were evaluated by means of the checkerboard method against *K. pneumoniae.* In the assays, fractional inhibitory concentration (FIC) values were calculated to characterize the interactions between the combinations. In the combinations of essential oil and antibiotics, the sensitivity of the bacteria to antibiotics increased and the antibiotics had a synergistic effect, and the antibacterial effect on the microorganisms increased. The cefepime + essential oil pair tested showed a synergistic effect (FIC $\leq$ 0.5), but the gentamicin + essential oil pair did not (FIC $>$ 0.5–4.0). Thus, the cefepime + essential oil pair has been found to exhibit a synergistic effect against *K. pneumoniae* compared to the gentamicin + essential oil pair. According to the results obtained here, the combined use of essential oils with antibiotics can be applied as a treatment strategy to reduce the use of antibiotics, reduce side effects, and possibly reverse antibiotic resistance to these microorganisms in light of the increase in multiple antibiotic resistance.

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1. Introduction

Klebsiella pneumoniae is an opportunistic pathogen that usually affects immunosuppressed patients and inpatients. It causes nosocomial infections in the urinary system and soft tissue, pneumonia, and bacteremia (Cescutti et al., 2016). *K. pneumoniae* has many virulence factors, particularly capsule polysaccharide, hypermuchoviscosis (HV), fimbria, toxins, and iron uptake determinants (Hennequin and Robin, 2016). Capsular polysaccharides are important virulence factors that provide resistance to phagocytosis and inhibit bacterial killing by bactericidal serum factors. The HV phenotype is associated with invasive syndromes of *K. pneumoniae* and is thought to increase the virulence of the bacterium (Fang et al., 2004; Yu et al., 2006). Bacteria experience difficulties in phagocytosing because they are present in clusters and the exopolysaccharide matrix and humoral immune system components are prevented from reaching the bacteria (Hoiby, 2017; Kuş et al., 2017).

During combined antibiotic therapy, the normal flora becomes more inhibited and an increase in colonization of resistant microorganisms is observed, and multiple resistant Gram-negative rods may also occur (Uzun, 1993; Moellering, 1995). Super infections occur during combination therapy at much higher rates than with single agents. Therefore, antibiotic therapy should be as narrow-spectrum as possible (Çolak, 1997). Combined antibiotic therapy increases the risk of toxic side effects of antibacterial agents. As with other drugs used in treatment, antimicrobial agents affect each other's metabolisms. As a result, the indications for treatment with more than one antibiotic are quite limited. When planning treatment with antibiotic combinations, drug-drug and drug-host interactions should be considered, and the potential benefits and harms of such treatments

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should be considered as well. Combined antibiotic therapy will not only increase the cost; when inappropriate combinations of antibiotics are used, the expected therapeutic benefit is not achieved and undesirable side effects are also observed. When such side effects occur under combined antibiotic therapy, it is difficult to link them to a particular antibiotic; therefore, the necessary discontinuation of all antibiotics leads to prolonged treatment and increased patient costs. This is time-consuming and expensive, and it delays the treatment of the patient.

As resistance to antibiotics increases, the importance of infection control also increases. Due to the increased incidence of multidrug-resistant microorganisms, antibiotic combinations do not have sufficient effects on those microorganisms. Thus, the potentials of antibiotics and their combined usage have led to the search for new and natural antimicrobial compounds for reasons such as avoiding high costs and side effects. Essential oils are aromatic mixtures and contain many bioactive compounds in their structures. Therefore, they are also used in health applications due to their various biological activities.

Pelargonium essential oil is known as geranium oil, and all species used in this oil's extraction are of the genus *Pelargonium.* Geranium essential oil is used in aromatherapy, premenstrual tension and menopausal problems, ailments of the lymphatic system and stimulation of the immune system, circulatory problems, hemorrhoids and phlebitis, sore throat, tonsillitis, asthma, and excessive mucus secretion. Geranium essential oil is known to be beneficial in the treatment of skin problems, general infections, acne, burns, scars, shingles, eczema, and dermatitis (Lis-Balchin, 2006).

In this study, the essential oil obtained from this plant was analyzed by gas chromatography-mass spectrometry (GC/MS) using the water distillation and headspace method. Germacrene-D, β-caryophyllene, T-cadinol, α-caryophyllene, α-pinene, and β-pinene were found as the main constituents of the essential oil (Bozan et al., 1999). The essential oil from *P. endlicherianum* exhibited significant antibacterial activity against *K. pneumoniae.* The correlation between this antimicrobial activity and the chemical composition shows that the tested essential oil's activity may be associated with the presence of high concentrations of monoterpene hydrocarbons such as α-pinene and β-pinene. Antimicrobial and antifungal activities of α-pinene and β-pinene hydrocarbons have been previously demonstrated (Alma et al., 2004). In addition, other major components, including sesquiterpenes and monoterpenes, such as β-bourbonene and germacrene D, known to be responsible for bacteriostatic activity against *K. pneumoniae*, also contributed to the antimicrobial activity of the oil (Brehm-Stecher and Johnson, 2003, Pepeljnjak et al., 2005). The bactericidal and antifungal effects of germacrene D were previously detected (Al-Macqtari et al., 2011).

Due to the increase in antibiotic-resistant infections in recent years, studies to investigate new drugs have gained importance in combating these infections. In the present work, we investigate whether the terpenoids, hydrocarbons, alcohols, and aldehydes in the structure of *P. endlicherianum* essential oil, a natural product that can be used as an alternative to existing antibiotic agents, have high antimicrobial properties. Accordingly, in this study it was aimed to obtain the essential oil instead of extracts prepared with various solvents of the plant and to combine the essential oil with antibiotics to achieve a high synergistic effect, thereby reducing both the minimum effective dose and the side effects of the antibiotics.

2. Materials and methods

2.1. Pelargonium endlicherianum Fenzl. essential oil

In this study, plant samples collected from the Develi district of Kayseri in July 2015 were used. The herbarium sample of the plant is stored in the Herbarium of Erciyes University, Faculty of Sciences (Plant Collector No: GK-1003). As per the methods of the European Pharmacopoeia, the essential oil of *P. endlicherianum* was obtained by hydrodistillation method on a Clevenger apparatus.

2.2. GC-FID and GC/MS analysis

Gas chromatography-flame ionization detector (GC-FID) analysis was carried out using an Agilent 6890 N GC system. The FID temperature was 300 °C. To obtain the same elution order with GC/MS, simultaneous auto-injection was done on a duplicate of the same column, applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The analysis results are given in Table 1.

Gas chromatography-mass spectrometry (GC/MS) analysis was carried out with an Agilent 5975 GC/MSD system. An Innowax FSC column (60 $m \times 0.25$ mm, 0.25-μm film thickness) was used with helium as a carrier gas (0.8 mL/min). The GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, then kept constant at 220 °C for 10 min and then programmed to 240 °C at a rate of 1 °C/min. The split ratio was adjusted to 40:1. The injector temperature was set to 250 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 35 to 450.

Identification of the essential oil components was carried out by comparison of their relative retention times (RRTs) with those of authentic samples or by comparison of their relative retention index (RRI) to n-alkane series. Computer matching against a commercial database (Wiley GC/MS Library, MassFinder 4 Library) (McLafferty and Stauffer, 1999; Hochmuth, 2008) and the in-house Baser Library of Essential Oil Constituents built up of genuine compounds and components of known oils was used for the identification, together with MS literature data (Joulain and Koenig, 1998).

2.3. Antimicrobial activity

Antimicrobial activities of antibiotics, essential oil, and antibiotic + essential oil combinations were determined using the agar well diffusion method. Initial concentrations of antibiotics were obtained by diluting two upper concentrations according to the EUCAST clinical limit value table. MIC values were taken into consideration while antibiotics were combined with the essential oil. The antimicrobial experiments were performed in triplicates.

The antibiotics used in our study were prepared according to the following formula:

$$\textbf{Amount of antibiotics to be weighed (mg)} = [\textbf{Studied concentration } (\mu g/\textbf{mL}) \times \textbf{Solvent volume (mL)}]/[\textbf{Antibiotic potency } (\mu g/\textbf{mg})]$$

2.3.1. Bacterial culture

Klebsiella pneumoniae ATCC 33,495 was obtained from American Type Culture Collection (ATCC). In accordance with CLSI recommendations, the tested *K. pneumoniae* was incubated under aerobic conditions for 24 h in nutrient agar medium. Colonies from the cultures were adjusted to 0.5 McFarland (approximately 1×10^8 cfu/mL) in physiological saline (0.9% $NaCl_2$).

2.3.2. Minimum inhibitory concentration (MIC)

After the microorganism was developed under appropriate culture conditions, Cation-Adjusted Muller Hinton Broth (CAMHB) was transferred to U-bottomed, 96-well lidded sterile microplates with 100 μL from a micropipette respectively. Serial twofold dilutions of essential oil, antibiotics, and antibiotic + essential oil combinations were performed in culture medium (100 μL). After inoculating 5 μL of microorganism cultures into each well, the plates were covered to prevent drying and incubated without agitation at 35 ± 2 °C for 16 to

Table 1
Chemical composition of *Pelargonium endlicherianum* essential oil.

RRI	Compound	%
1032	α-Pinene	5.8
1118	β-Pinene	5.1
1203	Limonene	1.7
1218	β-Phellandrene	1.0
1244	2-Pentyl furan	0.3
1280	*p*-Simen	4.1
1400	Nonanal	1.3
1443	Dimethyl tetradecane*	1.3
1452	α,*p*-Dimetlystyrene	0.1
1471	2-Nonly acetate	0.4
1495	Bicycloelemen	0.1
1497	α-Copaene	0.6
1499	α-Kamfolen aldehyde	0.2
1500	Pentadecane	0.3
1506	Decanal	1.3
1528	α-Burbonen	0.6
1535	β-Burbonen	15.5
1568	*trans*-α-Bergamoten	0.2
1589	β-Yilangen	1.3
1600	β-Elemen	0.4
1597	β-Copaene	1.6
1612	β-Caryophyllen	0.5
1617	Undecanal	0.6
1617	6,9-Guayadien	0.5
1628	Aromadendren	1.1
1648	Mirtenal	0.5
1650	γ-Elemen	0.5
1655	(*E*)−2-Decenal	0.7
1659	γ-Gurjunen	0.7
1668	(*Z*)-β-Farnesene	0.6
1700	Heptadecane	0.1
1726	Germacrene D	5.3
1765	(*E*)−2-Undecanal	0.9
1773	δ-Cadenine	0.9
1776	γ-Cadinene	0.3
1786	*ar*-Kurkumen	0.7
1830	Tridecanal	0,6
1868	(*E*)-Geranyl acetone	1.5
1896	Phenyl ethyl isobutyrate	0.6
1900	Nonadecane	0.5
1941	α-Calacorene	0.6
1945	1,5-Epoxy-salvial(4)14-en	1.1
1988	2-Phenylethyl-2-methylbutyrate	10.5
1992	2-Phenylethyl-3-methylbutyrate	0.5
2008	Caryophyllen oxide	0.7
2041	Pentadecanal	1.5
2069	Germacrene D-4β-ol	0.5
2073	β- Caryophyllenx alcohol	0.3
2100	Heneicosane	0.9
2131	Hexahydrofarnesyl acetone	7.7
2144	Spatulenol	1,3
2179	1-Tetradecanol	0.3
2179	Nor-Kopaonon	0.6
2226	Methylhexadecanoate	0.4
2255	α-Cadinol	0.3
2256	Cadalene	0.2
2269	Guaya-6,10(14)-dien-4β-ol	0.3
2273	Celine-11-en-4α-ol	0.8
2300	Tricosenes	0.9
2384	Farnesil acetone	0.8
2500	Pentacosenes	0.1
2503	Dodecanoic acid	0.2
2622	Phytol	0.4
2670	Tetradecanoic acid	0.5
2822	Pentadecanoic acid	0.1
2900	Nonacosan	0.2
2931	Hexadecanoic acid	0.5
	Total	92.5
	% Yield	0.012

RRI: The relative binding time index was calculated according to the n-alkane sequence;%: Calculated based on FID data. * Correct isomer could not be identified.

20 h in an ambient air incubator within 15 min of adding the inoculum. At the end of the incubation period, the microplates were measured on the spectrophotometer at a wavelength of 625 nm. However, since the spectrophotometer values of the wells including the essential oil were outside the measuring range, there was no antimicrobial effect in the MIC and MBC studies by microdilution method, and these steps were continued by using the agar well diffusion technique (EUCAST, 2018). The synergistic effect of the fractional inhibitor concentration index (FICI) was determined according to the following formulas (Yap et al., 2013):

FIC of essential oil = Essential oil's MIC value in the presence of antibiotic / Essential oil's MIC value

FIC of antibiotic = Antibiotic's MIC value in the presence of essential oil / Antibiotic's MIC value

FICI $\leq$ 0.5 synergistic, 0.5 $\langle$ FICI $<$ 1 partially synergistic, FICI = 1 additive, 1 $<$ FICI $\leq$ 4 ineffective, FICI $\rangle$ 4 antagonistic

2.3.3. Agar well diffusion

Antibiotics were dissolved in distilled water, the essential oil was dissolved in DMSO and 0.5% Tween 80, and the concentrations used in the study were prepared. Microorganism suspensions were prepared from 24-hour agar cultures. Bacterial suspensions washed twice with 0.9% physiological saline were adjusted to McFarland standard turbidity at a concentration of 10^8 cfu/mL. Wells of 6 mm in diameter were opened using a sterile punch at regular intervals on solidified agar medium, and antibiotics dissolved in distilled water, essential oil dissolved in DMSO, and antibiotic + essential oil combinations were added. Meropenem (10 μg/well) was used as a positive control and solvents (DMSO, Tween 80, and distilled water) were used as negative controls. All of the petri dishes were incubated for 24 h at 37 °C for the growth of bacteria and growth inhibition zones were measured in millimeters at the end of the period. The experiment was performed in triplicates. MIC values of the antibiotics and essential oil were determined by using the agar well diffusion method and antibiotic + essential oil combinations were prepared with these values starting above 2 MIC values (Perez et al., 1990).

2.3.4. Minimum bactericidal concentration (MBC)

The MBC is based on the counting of live bacteria after one night of incubation (18–24 h). Live bacterial counts were performed at concentrations of the MIC value and above the MIC value. The bactericidal effect was determined as the lowest concentration of the MBC value that decreased 99.9% of the initial live bacteria (Bryan and Godfrey, 1991).

2.4. Time-kill assay

Depending on time and antibiotic concentration, the time-kill assay is a method that dynamically demonstrates the bactericidal effect of antibiotics. In this method, the decrease in the amount of bacteria depending on time is given separately for the concentration of bacteria. Live bacteria counts were performed at different times for time-dependent reduction. Brain Heart Broth (BHB) medium (5 mL) containing antibiotics and essential oil separately and also antibiotic + essential oil combinations were prepared at 2 lower and 2 upper concentrations of MIC. Bacteria at 10^5 cfu/mL were initially used for the time-kill assay. Control tubes and tubes containing antibiotics, essential oil, or antibiotic + essential oil were sampled at 0, 3, 6, 12, and 24 h after inoculation with bacteria. The colonies were counted for each concentration during working hours (Yap et al., 2013). The experiment was conducted in replicates.

2.5. Determination of postantibiotic effect (PAE)

The 1 × MIC concentrations were determined for the antibiotics, essential oil, and antibiotic + essential oil combinations and the

prepared bacteria were inoculated in BHB medium for 1 hour. In order to remove the antibiotics, the bacterial pellet was washed three times with 0.9% physiological saline and centrifuged at 4000 × *g* for 5 min. The suspended bacteria were incubated at 37 °C in a shaking water bath. To evaluate bacterial development, serial dilutions were prepared from each tube at 0, 1, 2, 3, 4, 5, 6, and 24 h. They were then incubated on BHA medium with a swab and incubated at 37 °C for 24 h. Colonies formed at the end of incubation were counted. Before and after contact with antibiotics, samples were taken every hour after centrifugation, and after transfer to an antibiotic-free medium and colony counts of the bacteria, the logarithmic growth curves were drawn and the time required to increase the number of bacteria by 10 times was calculated (1 log10). In the same experimental conditions, the time required to increase the number of bacteria by 10 times (1 log10) was calculated. PAE was determined by the following formula and the experiment was conducted in replicates (Boswell et al., 1997; Craig and Gudmundsson, 1996; Giamarellos-Bourboulis et al., 2005; Li and Tang, 2004):

$$PAE = TA - TC$$

TA = Time required to increase the number of bacteria treated with antibiotics, essential oil, or essential oil + antibiotic combinations after the counts

TC = In the same experimental conditions, the time taken for a 1 log10 increase in the number of non-antibiotic bacteria

2.6. WBC 264–9C atcc HB-8902 cell line and culture

WBC 264–9C ATCC HB-8902 human leukocyte cells were used for an in vitro leukocyte cell model. WBC 264–9C cells were cultured in a sterile incubator containing 5% carbon dioxide at 37 °C. Cells were cultured in flasks with heat-inactivated 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in Eagle's Minimum Essential Medium for a collagen-1 coated flat cell culture at 55 °C for 30 min (Yin et al., 2005).

K. pneumoniae was incubated overnight at 37 °C with BHA medium. After taking 4–5 colonies, bacteria were suspended in 10 mL of fresh BHB medium prepared at 37 °C before being allowed to come to turbidity of 0.5 (approximately 1×10^8 cfu/mL) in a shaking water bath at 37 °C. The bacterial suspension was incubated for 2 h at 37 °C in a shaker oven with each combination according to the MIC values of the essential oil and antibiotics by taking 0.1 mL of the combination. After incubation, the tubes were centrifuged at 1000 × *g* for 10 min and then washed with physiological saline 3 times to remove the antibiotics and essential oil combinations. The number of bacteria was first adjusted to 5×10^7 cfu/mL at 0.5 McFarland turbidity in BHB medium followed by 1/2 dilution with the same medium. For the same bacterium in the non-antibiotic BHB medium, all the above procedures were performed and bacterial suspension to be included in the control series was prepared.

2.6.1. Activation of leukocyte cells

As described above, WBC 264–9C ATCC HB-8902 human leukocyte cells (1×10^7 cells/mL) developed under suitable conditions were added to the suspension to contain approximately 2×10^7 bacteria. Finally, after adding inactive human serum to a final concentration of 10%, the tubes were incubated at 37 °C in a water bath. The control was incubated with leukocytes under the same conditions for bacteria not treated with antibiotics or essential oil. In order to find out the amount of leukocytes treated with antibiotics and to not kill the test and control bacteria, samples were taken from tubes at 0, 2, 4, 8, and 12 h and vortexed for leukocytosis explosion, and 0.1 mL of pre-prepared BHA medium was made by dilution in appropriate proportions. After incubation at 37 °C for 24 h, colonies were counted and the number of bacteria/mL was determined. The values were compared with the control values and the number of bacteria killed by leukocytes was determined (Novelli et al., 2000; Pruul and McDonald, 1979). The activation of leukocyte cells experiment was performed in triplicates.

2.7. Outer membrane permeability

Bacterial outer membrane permeabilities of the essential oil, antibiotics, and antibiotic + essential oil combinations were determined according to the methods of Hemaiswarya and Doble and Marri et al. (Davis and Hedge, 1967; Pereira et al., 2014). Microorganisms incubated overnight were washed with PBS (pH 7.4), suspended (OD = 0.3 at 625 nm), and treated with antibiotics, essential oil, and essential oil + antibiotic combinations, and then 0.1% sodium dodecyl sulfate (SDS) was added in a 1/2 ratio. For the treated sample, each 10 mL of culture was divided into two 5-mL portions. One of the portions was added with SDS and the other without SDS. SDS was used at a final concentration of 0.1%. SDS initiates cell lysis, leading to deterioration of the outer membrane. Sudden cellular death caused by SDS was determined at 625 nm with a UV–Vis spectrophotometer at regular intervals (0, 5, 10, 30, and 60 min) (Yap et al., 2013). The outer membrane experiment was performed in triplicates.

2.8. Scanning electron microscopy (SEM)

In order to determine the effect of the combined use of essential oils and antibiotics on bacteria, bacteria cells were observed by scanning electron microscopy (SEM) after 16 h of incubation at 37 °C in BHB medium. Bacterial cells were treated with essential oil and antibiotics alone or in combination for 5 min and then centrifuged at 13,000 × *g* for 7 min, and the cell pellet was washed with PBS (pH 7.4). Samples were dropped and dried on slides and then, in order to get the images via SEM, electrons were not deposited on the surface and the samples were spin-plated with gold (Au) to provide conductivity.

2.9. Statistical analyses

Samples for MIC determination were taken as a single group with increasing concentrations of essential oils tested for *K. pneumoniae*, with studied antibiotics at reduced concentrations; gentamicin and gentamicin + essential oil and cefepime and cefepime + essential oil were divided into groups according to essential oil. For the comparison of multiple groups, one-way ANOVA was performed and the Tukey test was applied with Bonferroni correction. Differences in concentrations between the gentamicin and gentamicin + essential oil groups were evaluated by the Wald-Wolfowitz test as a significant difference was found only for *K. pneumoniae* in the gentamicin group. There was no significant difference between the groups with $p = 0.333$ and $p > 0.05$. For the time-kill assay and leukocyte determination, the studied antibiotics used in decreasing concentrations and the increasing concentrations of essential oils were taken as a single group; gentamicin and gentamicin + essential oil and cefepime and cefepime + essential oil were divided into groups as essential oils. One-way ANOVA and post hoc tests were performed for comparison of multiple groups and determination of synergetic effects. Since the number of n was less than 5, Bonferroni correction was performed and the Tukey biweight test was applied. The Wilcoxon test was used to compare the binary groups (time-kill method). For leukocyte determination, groups were compared using the Freidman test.

3. Results and discussion

3.1. Chemical analysis of essential oil with gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS)

The chemical composition of *Pelargonium endlicherianum* essential oil is shown in Table 1. The main constituents of the oil were

β-bourbonene (15.5%), 2-phenylethyl-2-methylbutyrate (10.5%), hexahydrofarnesyl acetone (7.7%), α-pinene (58%), germinen D (5.3%), and β-pinene (5.1%). Plants produce numerous secondary metabolites that attract coloring, odor, or pollinators as natural protection against microbial and pesticide attacks. These metabolites, also known as essential oils, are the products of the secondary metabolism of aromatic plants. There are significant differences in purity and composition between essential oils and raw plant extracts. There are many studies showing the high bioactivity of the essential oil derived from *Pelargonium*, but no clinical studies have been found on *P. endlicherianum* essential oil. However, in vitro susceptibility studies of the efficacy of various organisms may suggest that *P. endlicherianum* oil may have a place in modern medicine. Many *Pelargonium* species have hybrids and fragrant leaves that yield essential oils from the cultures derived from them (Lis-Balchin, 1996). High-quality oil is found in the leaves, body, and stems of the plant (Lis-Balchin, 2002). Table 1 shows the 67 compounds found in the essential oil. The main constituents of the essential oil were β-bourbonene (15.5%), 2-phenylethyl-2-methylbutyrate (10.5%), hexahydrofarnesyl acetone (7.7%), α-pinene (58%), germacrene D (5.3%), and β-pinene (5.1%). There are studies conducted with the above-ground parts of *P. endlicherianum* grown in Turkey. The essential oil extracted from the above-ground parts of the plant was analyzed by GC/MS. In the essential oil germacrene-D, β-caryophyllene, T-cadinol, α-caryophyllene, α-pinene, and β-pinene were found as the main constituents (Bozan et al., 1999).

3.2. Antimicrobial activity

3.2.1. Minimum inhibitory concentration (MIC)

It was shown that *K. pneumoniae* is sensitive to gentamicin and cefepime. MIC values were determined as 2 mg/L and 1 mg/L, respectively. The essential oil was found to have a MIC of 5 g/L against *K. pneumoniae*. The MIC value of the essential oil against *K. pneumoniae* was measured as 5 mg/mL and the zone diameter was 7.5 ± 0.5 mm. The MIC value of cefepime was determined as 0.5 μg/mL and the zone diameter was 6 ± 0 mm. For cefepime combined with essential oil, the MIC value was 0.06 μg/mL for the antibiotic contained in the combination and 0.625 mg/mL for the essential oil. The MIC zone diameter of cefepime + essential oil was determined as 5 ± 0 mm (Table 2). The MIC value of gentamicin was determined as 2 μg/mL in a zone of 3 ± 0 mm. With the combined use of gentamicin with essential oil, the MIC was 2 μg/mL for the antibiotic included in the combination and 5 mg/mL for the essential oil. The MIC zone diameter of the gentamicin + essential oil combination was 3.5 ± 0.5 mm (Table 2).

Combined use of cefepime + essential oil has been found to inhibit the development of *K. pneumoniae* by showing a synergistic effect. The use of essential oil with gentamicin was found to be ineffective against *K. pneumoniae*.

It was determined that *K. pneumoniae* is sensitive to gentamicin and cefepime. MBC values were determined as 16 mg/L and 8 mg/L, respectively. The essential oil was found to have a MBC of 20 g/L against *K. pneumoniae*.

In Gram-negative bacteria, β-lactamases are secreted into the periplasmic space and act together with increased membrane permeability (Georgopapadakou, 1993). β-Lactam antibiotics are known to target the bacterial cell membrane by affecting different targets such as transpeptidases, transglycosylases, and carboxypeptidase (Walsh, 2000). In order to increase the effectiveness of antibiotics, especially against Gram-negative bacteria, it is necessary to investigate and examine methods that increase the diffusion of antibiotics and bypass the bacterial membrane barrier. In this study, the mechanism of action of gentamicin and cefepime antibiotics used in the treatment of meningitis against *K. pneumoniae* was further extended in combination with the essential oil obtained from *P. endlicherianum*, resulting in increased efficacy of said antibiotics.

3.3. Time-kill assay

The time-kill assay used to determine the decrease in the number of time-dependent live bacteria showed the synergistic interaction between cefepime and essential oil against *K. pneumoniae*. A significant difference was found between the cefepime and cefepime + essential oil groups against *K. pneumoniae* at the $p = 0.05$ level. At the 24th hour after treatment, the drug combination was observed to have reduced the number of viable cells compared to treatment with the essential oil. In contrast, the drug alone did not effectively reduce cell numbers. Only the essential oil showed no complete cell destruction compared to the combination of gentamicin and essential oil (Figs. 1-2). The results of time-kill studies show that there is a synergistic effect between the essential oil and antibiotics and therefore their combination is more effective than treatment with essential oil or antibiotics alone. Gentamicin, essential oil, and gentamicin + essential oil had no effect on *K. pneumoniae*. The essential oil + antibiotic combinations for cefepime resulted in a significantly faster reduction in the number of living cells from the 6th hour.

3.4. Postantibiotic effect

The mean PAE duration determined by the 1 × MIC solution of *K. pneumoniae* was determined as 1.70 h for gentamicin, 0.80 h for cefepime, and 0.10 h for essential oil. For the combination of gentamicin with essential oil the PAE duration was 1.30 h, while for cefepime with essential oil it was 0.40 h (Figs. 3-4).

PAE represents the continuous suppression of bacterial growth after brief exposure to an antimicrobial agent. It has been reported that DNA, RNA, and protein syntheses in microorganisms exposed to antibiotics are inhibited by the PAE (D'Arrigo et al., 2010; Gottfredsson et al., 1995). In our study, gentamicin and cefepime stimulated a PAE at MIC levels. When bacterial growth is constantly

Table 2
MIC zone values of antibiotics and antibiotic + essential oil combinations against *K. pneumoniae*.

Cefepime and Gentamicin (μg/mL)	Cefepime zone diameter (mm)	Gentamicin zone diameter (mm)	Essential oil (mg/mL)	Essential oil zone diameter (mm)	*Cefepime+ Essential oil (μg/mL)	Cefepime+ Essential oil zone diameter (mm)	*Gentamicin + Essential oil (μg/mL)	Gentamicin+ Essential oil zone diameter (mm)
16	21 ± 0	9 ± 0	80	19 ± 1	2	19.5 ± 0.5	8	6.5 ± 0.5
8	19 ± 0	7 ± 0	40	18.5 ± 0.5	1	15.5 ± 0.5	4	5 ± 0
4	17 ± 0	4 ± 0	20	17 ± 1	0.5	13.5 ± 0.5	2	3.5 ± 0.5
2	14 ± 0	3 ± 0	10	9.5 ± 0.5	0.25	13 ± 0	1	–
1	9.5 ± 0.5	–	5	7.5 ± 0.5	0.125	10 ± 0	0.5	–
0.5	6 ± 0	–	2.5	–	0.06	5 ± 0	0.25	–
0.25	–	–	1.25	–	0.03	–	0.125	–
0.125	–	–	0.62	–	0.01	–	0.06	–

* The concentration of antibiotic in the combination of antibiotic + essential oil is given. +/- SD, $n = 3$.

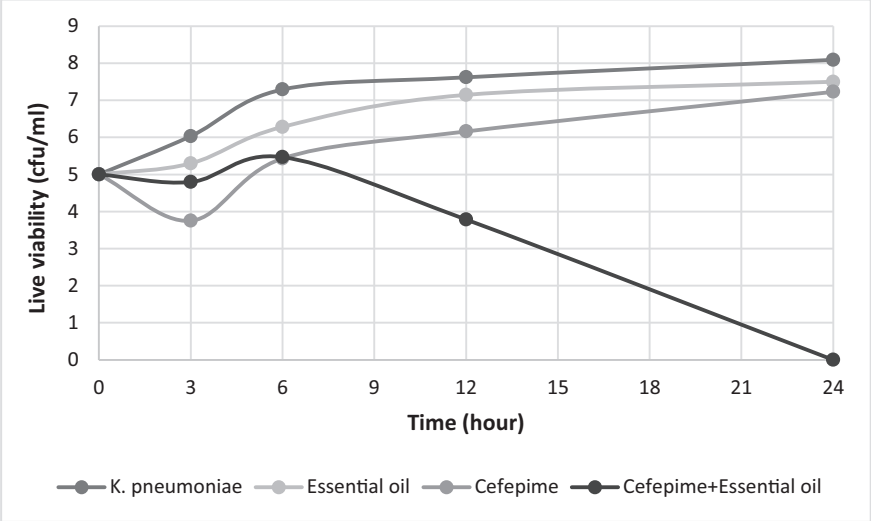

Fig. 1. Time-kill analysis of essential oil, cefepime and combination of both of them against *K. pneumoniae*.

suppressed, the antibiotic concentration is below the MIC value. Combinations of cefepime + essential oils reacted with cell membranes to interact with bacterial cells and antimicrobial compounds to show synergistic activity. In order to investigate the direct effect of *P. endlicherianum* essential oil on the phagocytic activity of human leukocyte cells, the detected MIC values of this essential oil were tested in vitro in combination with cefepime and gentamicin to test for their phagocytic activity. Synergism between essential oil and antibiotics was observed in all essential oil + antibiotic combinations tested, and the phagocytic activity of the leukocyte cells increased and a significant decrease in the number of live bacteria was determined ($p < 0.05$). This effect can also be observed in essential oils isolated from other plants, but no studies in the literature have shown that essential oils have increased phagocytic activity in leukocyte cells against microorganisms. Pruul and McDonald (1990) showed that *E. coli* strains became more sensitive to the killing activity of phagocytes after being exposed to high doses of quinolone and chloramphenicol antibiotics for a short period of 10 to 20 min. The combination of conventional antimicrobial agents and essential oils is a new formulation. When essential oils are used in combination with standard drugs, the combined effect increases their performance and shows improved antimicrobial activity (Gibbons et al., 2003). Rosato et al. (2007) found that the use of norfloxacin with *P. graveolens* essential oil in the treatment of infections caused by some bacterial strains reduced the minimum effective dose of norfloxacin and thus minimized the side effects caused by the antibiotic. Rosato et al. (2010) investigated combinations of Australian tea tree (*M. alternifolia*) oil with aminoglycoside antibiotics and synergism of gentamicin with the essential oil was found against *E. coli, Y. enterocolitica, S. marcescens*, and *S. aureus.* Aminoglycosides inhibit protein synthesis and tea tree oil has been reported to exhibit a highly targeted synergy by damaging the cytoplasmic bacterial membrane. These promising studies show that the combination of essential oils with conventional antibiotics provides significant potential for the development of new drug therapies and the treatment of infectious diseases caused by multidrug-resistant microorganisms.

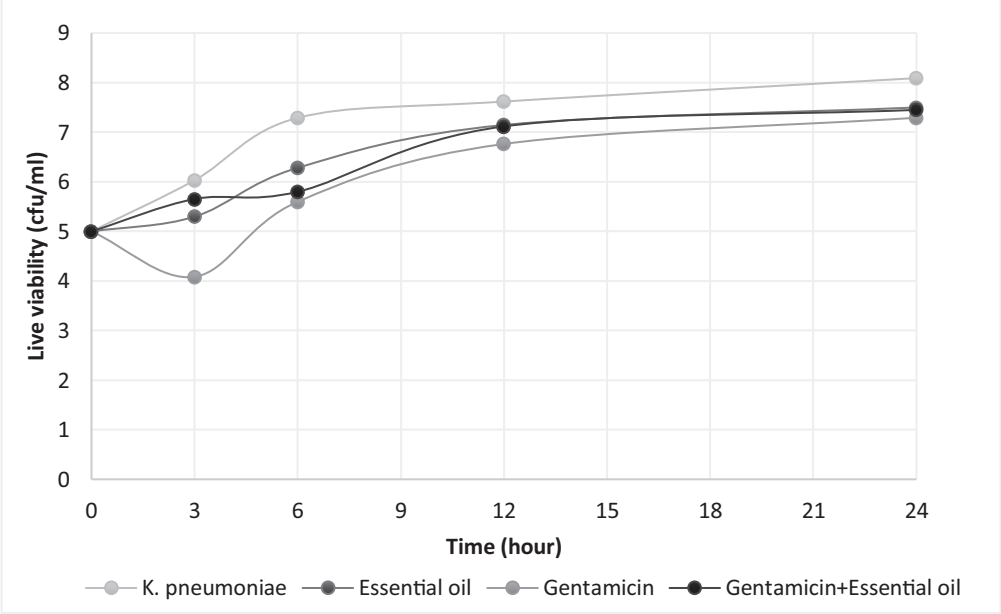

Fig. 2. Time-kill analysis of essential oil, gentamicin and combination of both of them against *K. pneumoniae*.

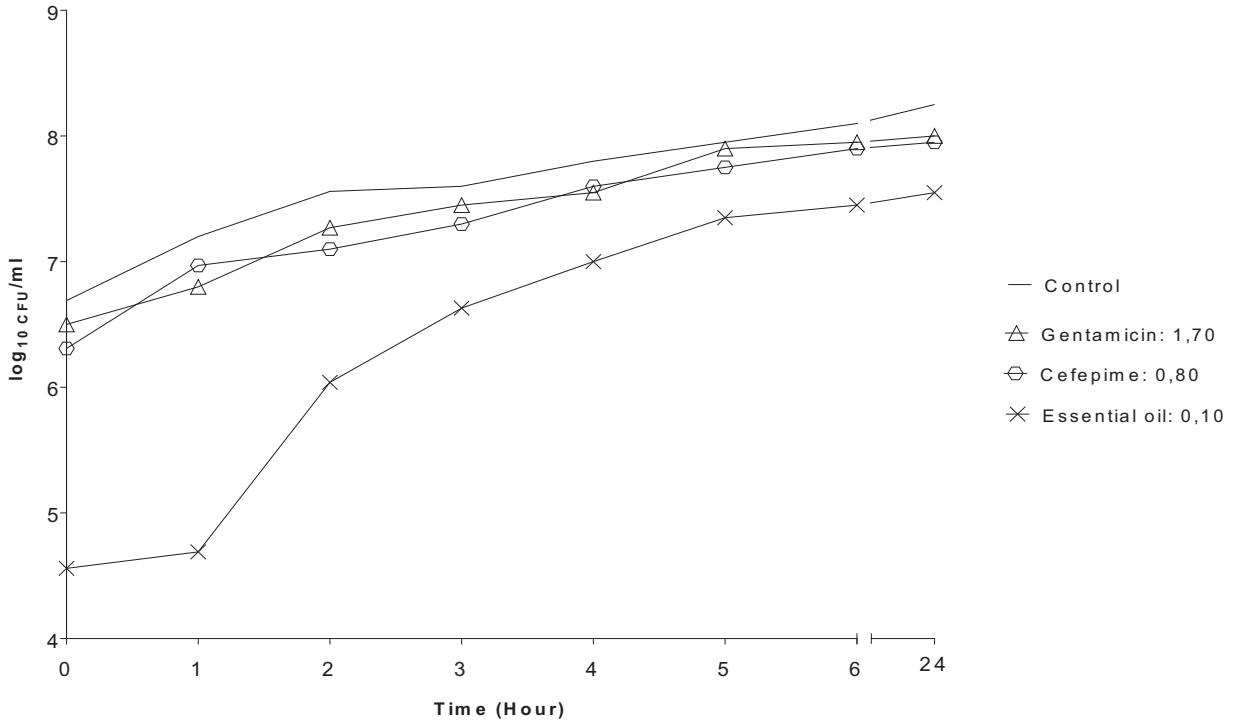

Fig. 3. PAE values as a result of treatment of *K. pneumoniae* strain with antibiotics and essential oil.

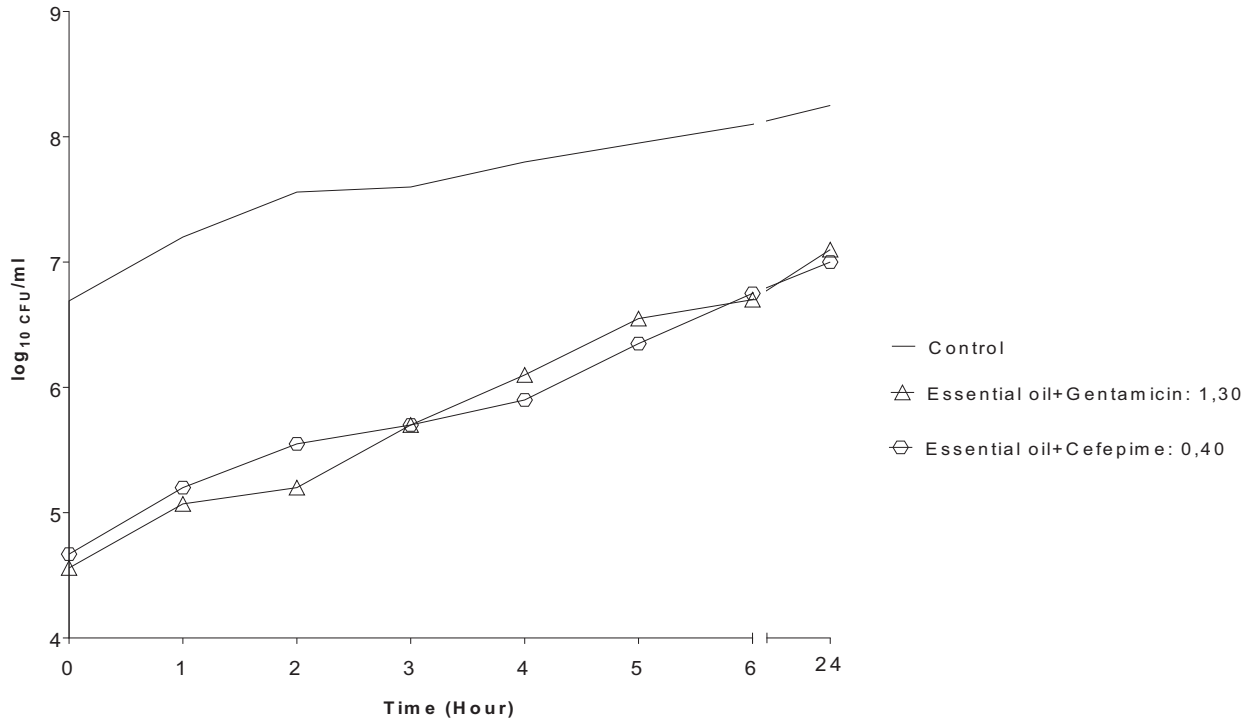

Fig. 4. PAE values as a result of treatment of *K. pneumoniae* strain with antibiotics + essential oil combination.

3.5. Activation of leukocyte cells

Data on strengthening the bactericidal activity of leukocyte cells determined by pre-exposure to the studied antibiotics are given in Table 3. Antibiotics and antibiotic + essential oil treatments significantly increased the phagocytic activity of human leukocyte cells compared to control values ($p < 0.022$). Essential oil was more effective than the gentamicin combination ($p = 0.022$). The logarithmic growth of *K. pneumoniae* showed a synergistic effect with the combination of cefepime + essential oil at the second hour, and the number of bacteria cells decreased by about 2 log. A similar effect was observed with the combination of gentamicin and gentamicin + essential oil at 4 h, but no synergistic effect was found between the antibiotic and essential oil. The effect of gentamicin was not different from the effect of the gentamicin + essential oil combination.

Table 3
Phagocytic effect of leukocytes on *K. pneumoniae* exposed to antibiotics, essential oil and essential oil + antibiotic combinations.

K.pneumoniae incubated with leukocytes and time (hours)	Control (log cfu/mL)	Cefepime (log cfu/mL)	Gentamicin (log cfu/mL)	Essential oil (log kob/mL)	Cefepime + Essential oil (log kob/mL)	Gentamicin + Essential oil (log kob/mL)
0	6.94 ± 0.10	6.45 ± 0.65	6.62 ± 0.17	6.74 ± 0.07	5.70 ± 0.00	6.64 ± 0.01
2	6.88 ± 0.10	5.91 ± 0.83	6.50 ± 0.40	3.95 ± 1.51	4.80 ± 0.69	6.48 ± 0.37
4	6.94 ± 0.10	3.29 ± 0.36	3.11 ± 0.19	2.45 ± 0.06	2.47 ± 0.47	3.07 ± 0.18
8	2.89 ± 0.19	1.29 ± 0.09	1.86 ± 0.07	0.75 ± 0.15	0.43 ± 0.23	1.67 ± 0.13
12	2.17 ± 0.00	0.54 ± 0.24	0.00 ± 0.00	0.68 ± 0.15	0.00 ± 0.00	0.00 ± 0.00

+/- SD, $n = 3$.

3.6. Outer membrane permeability

In this study, SDS was used as a permeabilizer probe. Microorganism cultures prepared under suitable conditions were treated with antibiotics, essential oil, and essential oil + antibiotic combinations and then periodically inspected to detect sudden cellular death caused by SDS (0, 5, 10, 30, and 60 min). The differences between the absorbances are shown in Table 4. In all studies, the least death was observed in the control groups using SDS ($p < 0.001$ for all groups). Among the groups examined, the highest bactericidal effect against *K. pneumoniae* was observed in the group where the essential oil was administered together with cefepime ($p < 0.001$). Concomitant administration with gentamicin showed less effective membrane damage in *K. pneumoniae* than cefepime.

The results of the outer membrane permeability test showed that the outer membrane barrier is impaired by the presence of essential oil and that the antibiotics significantly increase the binding effects of penicillin-binding proteins (PBPs) localized on the outer surface of the cytoplasmic membrane (Macheboeuf et al., 2006). *P. endlicherianum* essential oil contained in essential components such as bourbonene and germacrene D as well as studies with sesquiterpene are in agreement; sesquiterpenoids play a role in the deterioration of the bacterial cell membrane by increasing the sensitivity of the bacterial membrane to the exogenous antimicrobial compounds and bacterial membrane permeability (Byron et al., 2003). The SEM studies showed that the overall bacterial cell surface treated with the essential oil differs significantly from the essential oil-free bacterial cells. This lethal effect may be due to the deterioration of the membrane structure, and previous studies support this hypothesis (Bajpai et al., 2013; Paul et al., 2011; Polly et al., 2015; Sharma et al., 2013). Cells treated with the combination of essential oil and antibiotics showed gross cell damage with size and shape changes. In time-kill analysis, cell damage caused by the essential oil, antibiotics, and combination of antibiotic + essential oil was determined. Membrane damage allows antibiotics to have access to antibiotic-binding proteins, and this hypothesis is supported by the results of outer membrane permeability testing in this study (Bolla et al., 2011). Clinical resistance to β-lactams in Gram-negative bacteria is explained by low outer membrane permeability. Essential oils are known to contain a wide variety of polyphenols and terpenoids. These phenols have a strong binding affinity to different molecular structures such as protein or glycoproteins due to their high lipophilic properties. Therefore, they have great tendencies towards cell membranes and have the potential to penetrate through cell walls, leading to the leakage of cell contents (Hemaiswarya and Doble, 2009; Wang et al., 2012). Cox et al. (2000) reported that tea tree oil (*Melaleuca alternifolia*) showed membrane permeability by causing loss of bone loss for both Gram-positive and Gram-negative bacteria.

3.7. Scanning electron microscopy (SEM)

The SEM results showed detailed cell damage of the microorganisms after treatment with a combination of essential oil and antibiotics (Fig. 5). The size of the bacteria for gaining resistance to essential oil components has not yet been investigated systematically and comprehensively. Although tea tree oil has been approved for medicinal use in Australia since the 1920s, clinical resistance to essential oil has not yet been reported (Burt and Reinders, 2003). In general, these studies provide limited evidence of the spontaneous occurrence of essential oil resistance. The multicomponent structure of the essential oils can reduce the potential for the formation of essential oil resistance because many targets must be adapted to prevent the effects of essential oils.

Table 4
Reduction of membrane permeability of *K. pneumoniae* by the combination of essential oil, antibiotics and essential oil + antibiotic combinations.

OD_{625}= SD ($n = 3$) Time (minute)	0	5	10	30	60
K. pneumoniae (Control)					
% 0.1 SDS	0.31 ± 0.006	0.30 ± 0.006	0.31 ± 0.008	0.31 ± 0.007	0.31 ± 0.008
% 0.1 SDS (without)	0.30 ± 0.005	0.30 ± 0.008	0.30 ± 0.010	0,31 ± 0.002	0.31 ± 0.008
Essential oil (5 mg/L)					
% 0.1 SDS	0.30 ± 0.002	0.30 ± 0.003	0.29 ± 0.001	0.29 ± 0.006	0.29 ± 0.005
% 0.1 SDS (without)	0.29 ± 0.009	0.29 ± 0.005	0.29 ± 0.016	0.29 ± 0.006	0.28 ± 0.002
Cefepime (0.5 mg/L)					
% 0.1 SDS	0.29 ± 0.002	0.28 ± 0.005	0.28 ± 0.012	0.27 ± 0.008	0.26 ± 0.007
% 0.1 SDS (without)	0.29 ± 0.009	0.27 ± 0.007	0.27 ± 0.007	0.27 ± 0.009	0.26 ± 0.015
Essential oil+Cefepime					
% 0.1 SDS	0.28 ± 0.001	0.28 ± 0.008	0.26 ± 0.005	0.25 ± 0.002	0.24 ± 0.004
% 0.1 SDS (without)	0.28 ± 0.007	0.28 ± 0.009	0.26 ± 0.003	0.24 ± 0.009	0.23 ± 0.002
Gentamicin (2 mg/L)					
% 0.1 SDS	0.30 ± 0.014	0.30 ± 0.013	0.30 ± 0.009	0.29 ± 0.010	0.28 ± 0.004
% 0.1 SDS (without)	0.29 ± 0.004	0.29 ± 0.006	0.30 ± 0.007	0.29 ± 0.005	0.28 ± 0.003
Essential oil+Gentamicin					
% 0.1 SDS	0.30 ± 0.010	0.29 ± 0.008	0.29 ± 0.002	0.28 ± 0.002	0.27 ± 0.003
% 0.1 SDS (without)	0.29 ± 0.008	0.29 ± 0.002	0.28 ± 0.005	0.28 ± 0.006	0.27 ± 0.006

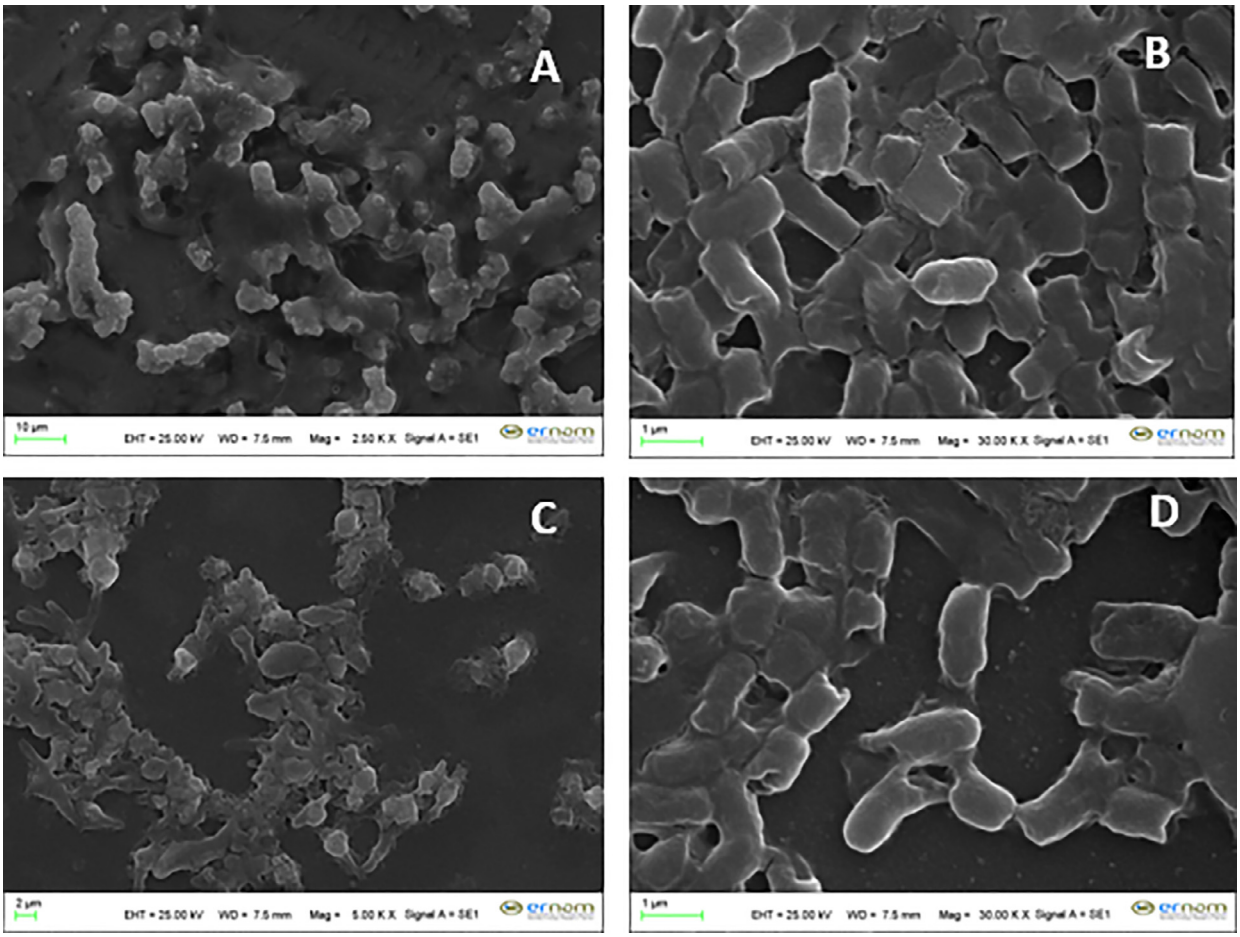

Fig. 5. *K. pneumoniae* under scanning electron microscope (A) Untreated cells (control), (B) P. endlicherianum essential oil (0.012% (v / v)), (C) cefepime (0.5 μg / mL) and (D) essential oil (0.012% (v / v) + cefepime (500 μg / mL).

4. Conclusion

The use of essential oils in combination with antibiotics has reduced the minimum effective antibiotic dose in the treatment of infections. In this way, the negative effects of antibiotics can be eliminated. Combining antibiotics with essential oils targeting resistant bacteria can lead to new options in combating microbial resistance. In this study, the potential of *P. endlicherianum* essential oil with antibiotics to be used as a new treatment method against infections caused by *K. pneumoniae* has been determined. The results of our study showed that the essential oil not only had membrane permeability activity but also phagocytic activity in human leukocyte cells. In addition, the use of essential oils in combination with antibiotics might be a potential source of antimicrobial agents in meningitis therapy. Although in vitro studies with essential oils are promising to show that they are synergistic, it is necessary to investigate the stability, selectivity, and bioavailability of these natural products and potential negative plant-drug interactions in the human body.

Ethical issue

Not applicable.

Declaration of Competing Interest

The authors declare no conflict of interest.

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