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Synergy between *Pelargonium endlicherianum* essential oil and conventional antibiotics against *Neisseria meningitidis* and *Haemophilus influenzae*

Berrak Dumlupinar[a,*], Damla Damar Celik[b], Gokce Şeker Karatoprak[c], Ümran Soyoğul Gürer[b]

[a] Faculty of Health Sciences, Nutrition and Dietetics, Istanbul Gelisim University, Istanbul, Turkey
[b] Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Marmara University, Istanbul, Turkey
[c] Department of Pharmacognosy, Faculty of Pharmacy, Erciyes University, Kayseri, Turkey

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ABSTRACT

This study investigated the bactericidal effects of the essential oil of *Pelargonium endlicherianum* in combination with four antibiotics commonly used in the treatment of bacterial meningitis (penicillin, ampicillin, ciprofloxacin, and gentamicin) against the meningitis causative pathogens *Neisseria meningitidis and Haemophilus influenzae.* The phagocytic effects of these combinations were also tested against human leukocyte cells. The bactericidal effect of *P. endlicherianum* essential oil (PEO) and antibiotic combinations was dynamically detected by time-kill assay. The function of PEO and antibiotic in permeating outer membrane barriers, when used singly or in combination, was analyzed by UV spectrophotometer.

The interactions between antibiotic and essential oil were calculated according to the fractional inhibitory concentration (FIC) index. While a synergistic effect of the ciprofloxacin + PEO combination was determined on *N. meningitidis* (FIC$\leq$ 0.5), an additive effect was observed on *H. influenzae* (FIC= 1). Combined use of PEO with gentamicin showed a synergistic effect against *N. meningitidis* and *H. influenzae* (FIC $\leq$ 0.5). The antimicrobial effect of the penicillin + PEO combination was higher than that of penicillin + PEO used alone. The ampicillin + PEO combination had a synergistic effect on *N. meningitidis* and an additive effect on *H. influenzae*. The results of our study showed that the essential oil increases membrane permeability activity and also has phagocytic activity in human leukocyte cells. Combining antibiotics with essential oils that target resistant bacteria may open up new options in combating microbial resistance.

1. Introduction

Among the infectious diseases, meningitis types are one of the disease groups in which modern treatment approaches and antibiotic treatment success are most prominent. Despite a significant reduction in mortality with antibacterial agents, morbidity remains a serious problem today. Therefore, it is very important to start effective and targeted treatment in the early period. Today, antibiotic combinations are used in the treatment of bacterial meningitis. However, combined antibiotic therapy not only increases the cost, but also when an inappropriate combination of antibiotics is used the expected therapeutic benefit cannot be achieved and undesirable effects may occur. Moreover, when a side effect occurs with combined antibiotic therapy, it is difficult to link it to a particular antibiotic; therefore, discontinuation of all antibiotics results in prolonged treatment and increased costs. This is time consuming and expensive and delays the patient's treatment. Our study found that combining of the essential oil obtained from *Pelargonium endlicherianum* with antibiotics commonly used in the treatment of bacterial meningitis resulted in increased antibacterial activity of existing antibiotic treatment options. This may well be an alternative to reduce the number of antibiotics used in combination with one another and to avoid associated side effect profiles and additional costs related to polypharmacy. In addition, the current debate on resistance to penicillin and third generation cephalosporins may encompass antibiotics such as vancomycin and meropenem in the near future (Hsu, 2009; Techasaensiri, 2010). The increase in antibiotic resistant bacteria leads to treatment failure in infections caused by drug resistant bacteria. Therefore, alternative strategies are needed to combat bacterial infections. In the present study, it was shown that the antibiotics used in the treatment of meningitis combined with *P. endlicherianum* essential oil increase the antibiotic activity and thus prevent the bacteria from developing resistance to antibiotics and providing effective treatment.

* Corresponding author.
E-mail addresses: baltinsoy@gelisim.edu.tr (B. Dumlupinar), umran.gurer@superonline.com (Ü. Soyoğul Gürer).

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The bactericidal effect on a certain bacterium with a single antibacterial agent against a single agent during treatment, reducing the likelihood of resistance development, and reducing the dose of a toxic antibacterial agent are reasons for the use of combined antibiotics. The combined use of antibiotics is justified, but the antagonist effect among antibacterial agents is an undesirable aspect of applications. Superinfections occurring during combination therapy are much more common than with a single agent. If fever does not decrease, especially in patients who are treated with broad-spectrum combined antibiotics, a fungal superinfection must be considered. Therefore, antibiotic treatment should be as narrow spectrum as possible (Çolak et al., 1997). When planning treatment with an antibiotic combination, drug–drug and drug–host interactions should be considered and the potential benefits and harmful effects of such treatment should be carefully evaluated. In the literature, it has been reported that the combined use of antibiotics with essential oils increases the antibacterial effect on microorganisms by showing a synergistic effect. This approach also promises to reduce the risk of toxicity in combined therapies (Aelenei, 2019; Arasu, 2019; Vitanza, 2019).

Nowadays, with the increasing interest in natural sourced products, scientific studies have accelerated. *Pelargonium* species have gained great importance with the discovery of traditional tribal remedies in South Africa (Kolodziej et al., 1998). *Pelargonium sidoides* DC is one of the geophyte species of the family Geraniaceae used as a traditional medicine in South Africa. The red tuber or rhizomes of this plant are widely used for gastrointestinal disorders, chest pain, respiratory infections, tuberculosis, and diarrhea by different cultural groups. Many *Pelargonium* species, hybrids, and cultures derived from them have fragrant leaves that produce *P. endlicherianum* essential oil (PEO) (Williams and Harborne, 2002). Geranium essential oil obtained from *Pelargonium* species is known to be significantly beneficial in the treatment of the skin for fungal and general infections, acne, burns, bruises shingles, eczema, and dermatitis (Lis-Balchin, 2006). The flowers of *P. endlicherianum* Fenzl. (Geraniaceae) are used as a traditional medicine for intestinal parasites among the people. In the literature, the properties of the essential oil obtained from *P. endlicherianum* for the inhibitory effect on pathogenic microorganisms have not been studied. Inspired by the immune system-enhancing effect of *Pelargonium sidoides*, a species that grows in this country, *P. endlicherianum* was expected to show the same synergistic effect on the immune system by increasing the phagocytic effect on leukocyte cells. This suggests that *P. endlicherianum* oil may have a place in modern medicine. Antimicrobial and antifungal activities of α-pinene and β-pinene hydrocarbons have been reported Alma et al. (2004). The correlation between antimicrobial activity and chemical composition showed that the antimicrobial activity of PEO may be associated with the presence of high concentrations of monoterpene hydrocarbons such as α-pinene and β-pinene. Sesquiterpenes and monoterpenes such as β-bourbonene and germakren D, which are responsible for the bacteriostatic activity, were expected to contribute to the antimicrobial activity of the oil (Brehm-Stecher and Johnson, 2003, Pepeljnjak et al., 2005, Dumlupinar et al., 2020). Likewise, it was aimed to determine whether it will have the same effect on pathogens that are frequently encountered in meningitis. Therefore, the purpose of the present study was to determine the synergistic effect by combining the essential oil obtained from the aerial part of *P. endlicherianum* with antibiotics in order to increase the antibacterial effect against meningitis pathogens and the phagocytic activity in human leukocyte cells. As a result of our study, it will be possible to reduce the use of antibiotics by combining the essential oil obtained from *P. endlicherianum* with antibiotics, which are frequently used in the treatment of bacterial meningitis, and inhibiting the resistance mechanisms of bacteria by acting together with the physicochemical interactions of the antibiotic and essential oil.

2. Materials and methods

2.1. P. endlicherianum Fenzl. essential oil (PEO)

The *P. endlicherianum* plant used in the study was collected from Develi district of Kayseri, Turkey, in 2015. A sample was deposited in the Erciyes University Faculty of Sciences Herbarium (Plant Collector No: GK-1003). According to the European Pharmacopoeia methods, the essential oil was obtained by hydrodistillation using a Clevenger apparatus.

2.1.1. Preparation of essential oil dilutions

To prepare, after 320 mg of pure essential oil was dissolved in 1280 μL of DMSO, 400 μL of the essential oil solution dissolved in DMSO was taken from it for a concentration of 80 mg/mL, and 1000 μL was completed with medium containing 0.5% Tween 80. Other concentrations were equally reduced (80, 40, 20, 10.5, 2.5, 1.25, 0.62, 0.31, 0.15, 0.07, 0.03).

2.2. Minimum inhibitory concentration (MIC)

Agar well diffusion was employed for determining the antibacterial activity of the antibiotic, PEO, and antibiotic + PEO combinations. The initial antibiotic concentrations were based on the EUCAST clinical limit value table (EUCAST, 2018) and the study progressed by diluting the two upper concentrations of this concentration. The antibacterial experiments were performed in triplicate.

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

Amount of antibiotic to be weighed (mg) = [Desired concentration (μg/mL) x Solvent volume (mL)] / [Antibiotic Potency (μg/mg)]

The synergistic effect was calculated according to the fractional inhibitory concentration (FIC) index (Yap et al., 2013).

FIC of PEO = PEO's MIC value in the presence of antibiotic / PEO's MIC value

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

FIC $\leq$ 0.5 synergistic, 0.5 $<$ FIC $<$ 1 partially synergistic, FIC = 1 additive, 1 $<$ FIC $\leq$ 4 ineffective, and FIC $>$ 4 antagonistic

2.2.1. Bacterial culture

Haemophilus influenzae ATCC 49766 and *Neisseria meningitidis* ATCC 13077 were obtained from the American Type Culture Collection (ATCC). Growth of the studied bacteria was achieved by incubating in brain heart infusion agar / broth medium for 24 h under anaerobic conditions (5% CO_2, 37 °C) and in accordance with CLSI recommendations. Colonies obtained from the cultures were adjusted to 0.5 McFarland standard ($\approx 1 \times 10^8$ cfu/mL) using physiological saline (0.9% NaCl).

2.2.2. Agar well diffusion

The concentrations to be used in the study were prepared using dissolved antibiotics and PEO. *H. influenzae* and *N. meningitidis* were grown in 24 h agar cultures. Bacteria suspensions were adjusted to McFarland standard turbidity at a concentration of 10^8 cfu/mL. MIC values of the prepared combinations were analyzed by agar well diffusion (Perez et al., 1990). The experiment was performed in triplicate.

2.3. Time-kill assay

According to Yap et al. (2013), the bactericidal effect of the antibiotic was dynamically demonstrated, depending on the time and the antibiotic density. In our experiment, a reduction in the number of viable bacteria was determined over time. The control tube and tubes containing antibiotic / PEO /antibiotic + PEO were sampled 0, 3, 6, 12,

and 24 h after inoculation with bacteria. The plates were incubated at 37 °C for 24 h in triplicate experiments. During the working hours, 6 petri dishes for each concentration were cultivated with dilutions and the colonies were counted.

2.4. Determination of postantibiotic effect (PAE)

The 1xMIC concentrations were determined by antibiotic / PEO / antibiotic + PEO combination and the bacteria were cultured in BHB medium for 1 h. Bacteria removed from the antibiotic / / PEO / antibiotic + PEO combination were incubated at 37 °C in a shaking water bath. Serial dilutions were prepared from each tube at 0–6, and 24 h to assess bacterial growth, and all tubes were incubated at 37 °C for 24 h. Colonies were determined by counting at the end of incubation. PAE duration was obtained by plotting log10 cfu/mL versus time. PAE was determined by the following formula and the experiment was performed in triplicate (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 antibiotic / PEO / PEO + 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.

2.5. WBC 264-9C ATCC HB-8902 cell line and culture

In this experimental system *in vitro*, WBC 264-9C ATCC HB-8902 human leukocyte cells were used. The WBC 264-9C cells were cultured with Eagle's Minimum Essential Medium containing 10% inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/ mL streptomycin in 5% CO_2 at 37 °C in an incubator (Yin et al., 2005).

H. influenzae and *N. meningitidis*, incubated overnight at 37°C with BHA medium, were suspended in BHB medium. The bacterial suspension was incubated for 2 h at 37 °C in a shaker oven according to the MIC values of the antibiotic / PEO / antibiotic + PEO combination. The antibiotics / PEO / PEO + antibiotic combinations were removed from the tubes after the incubation. The bacterial count was first adjusted to 5×10^7 cfu/mL with McFarland 0.5 turbidity in BHB medium and then diluted 1/2 with the same medium. For the bacteria in non-antibiotic BHB medium, the above procedures were performed in the same way and this prepared bacterial suspension was included in the control series (Yin et al., 2005).

2.5.1. Activation of leukocyte cells

WBC 264-9C ATCC HB-8902 human leukocyte cells were suspended (1×10^7 cells/mL) to contain approximately 2 times the bacteria. Finally, after 10% inactive human serum was added to the tubes, they were incubated at 37 °C. The bacteria not treated with antibiotics and PEO were grown under the same conditions as the control group. At 0, 2, 4, 8 and 12 h, samples were taken from the tubes and vortexed to leukocytosis explosion, and by diluting in the appropriate ratio the previously prepared BHA medium was inoculated on the surface and after 24 h incubation at 37 °C the colonies were counted. The numbers of bacteria killed by leukocytes were determined by comparing the values found with the control values (Novelli et al., 2000; Pruul and McDonald, 1979). This experiment was carried out in triplicate.

2.6. Outer membrane permeability

Microorganism cultures were treated with the antibiotics, PEO, and PEO + antibiotic combinations, and then 0.1% sodium dodecyl sulfate (SDS) was added to the culture at a ratio of 1/2. Then differences in absorbance were measured at regular intervals (0, 5, 10, 30, and 60 min) to detect sudden cellular death caused by SDS with a UV-vis spectrophotometer at 625 nm (Yap et al., 2013). The outer membrane experiment was performed in triplicate. Sudden cellular death caused by SDS in the bacterial outer membrane was detected as described by Hemaiswarya and Doble (2009) and Marri et al. (2021), Davis and Hedge (1967), Pereira et al. (2014).

2.7. Statistical analyze

Samples for MIC determination: antibiotics tested separately for *N. meningitidis* and *H. influenzae* and studied at decreasing concentrations and essential oils at increasing concentrations were taken as a single group; ciprofloxacin and ciprofloxacin + essential oil; gentamicin and gentamicin + essential oil; penicillin and penicillin + essential oil; ampicillin and ampicillin + essential oil were divided into 8 groups. For the comparison of multiple groups, one-way ANOVA was performed. Since the number of n was less than 5, Tukey's test was applied with Bonferroni correction. Samples for time-kill and leukocyte determination tested separately for *N. meningitidis* and *H. influenzae* and studied antibiotics at decreasing concentrations and essential oils at increasing concentrations were taken as a single group; ciprofloxacin and ciprofloxacin + essential oil; gentamicin and gentamicin + essential oil; penicillin and penicillin + essential oil; ampicillin and ampicillin + essential oil were divided into 8 groups. In order to compare multiple groups and to determine the synergetic effect, one-way ANOVA and post hoc tests were performed, respectively, and since the number of n was less than 5, Tukey's biweight test was applied with Bonferroni correction. Wilcoxon's test was used for comparison of binary groups (time-kill method). For leukocyte determination, groups according to diseases were compared using Freidman's test.

3. Results and discussion

3.1. Antibacterial activity

In our previous study (Dumlupinar et al., 2020), it was shown that a total of 67 components were present in the chemical composition of PEO. These include β-bourbonene, 2-phenylethyl-2-methylbutyrate, hexahydrofarnesyl acetone, α-pinene, germinen D, and β-pinene, which have antimicrobial activity according to the literature (Alma et al., 2004; Brehm-Stecher and Johnson, 2003, Pepeljnjak et al., 2005; Al-Macqtari et al., 2011; Dumlupinar et al., 2020). These main components in PEO have contributed to its antibacterial effectiveness.

Polyphenols have the potential to penetrate the cell membrane of bacterial cells. They can easily pass through the cell membrane and enter the cell (Hemaiswarya and Doble, 2009; Wang et al., 2012). PEO showed high antibacterial activity against *N. meningitidis* and *H. influenzae* due to these various phenols. The antibacterial activity of PEO may be related to the presence of monoterpene hydrocarbons such as α-pinene and β-pinene in high concentrations in its chemical composition (Dumlupinar et al., 2020). In addition, terpenic compounds such as β-bourbonene and germacrene *D* also contributed to the antibacterial activity (Al-Macqtari et al., 2011; Pluchtová et al., 2018).

3.2. Minimum inhibitor concentration (MIC)

It was shown that *H. influenzae* and *N. meningitidis* are sensitive to penicillin, ampicillin, gentamicin, and ciprofloxacin. The MIC values were determined as 2 mg/L, 1 mg/L, 2 mg/L, and 0.25 mg/L, respectively, for *H. influenzae*; and 2 mg/L, 4 mg/L, 2 mg/L, and 0.5 mg/L, respectively, for *N. meningitidis.* PEO was found to have a MIC of 5 g/L against *H. influenzae* and of 20 g/L against *N. meningitidis* (Tables 1 and 2).

Table 1
MIC zone values (mm) of antibiotic + PEO combination against *H. influenzae*.

Concentrations (μg/mL)	Penicillin+PEO	Ampicillin+PEO	Gentamicin+PEO	Cyprofloxacin+PEO
8	-	14.5 ± 0.5	15 ± 0	-
4	16 ± 0	10 ± 0	12 ± 0	-
2	12 ± 1	8.5 ± 0.5	9 ± 0	-
1	9.5 ± 0.5	-	6.5 ± 0.5	18.5 ± 0.5
0.5	7 ± 0	-	3.5 ± 0.5	14.5 ± 0.5
0.25	3 ± 0	-	-	7.5 ± 0.5
0.125	-	-	-	4 ± 0
0.062	-	-	-	-

Table 2
MIC zone values (mm) of antibiotic + PEO combination against *N. meningitidis*.

Concentrations (μg/mL)	Penicillin+PEO	Ampicillin+PEO	Gentamicin+PEO	Cyprofloxacin+PEO
8	-	13.5 ± 0.5	13 ± 0	-
4	11 ± 0	12 ± 0	10.5 ± 0.5	-
2	9 ± 0	8.5 ± 0.5	8 ± 0	-
1	6.5 ± 0.5	5 ± 0	6.5 ± 0.5	19 ± 0
0.5	3 ± 0	4 ± 1	4 ± 0	15 ± 0
0.25	-	-	3 ± 0	12 ± 2
0.125	-	-	-	7 ± 0
0.062	-	-	-	5 ± 0

The MIC value of PEO against *H. influenzae* was 5 mg/mL and the zone diameter was 8 ± 0 mm. For penicillin 2 μg/mL MIC value and 3 ± 0 mm zone diameter were determined. In the combination of penicillin and PEO, the MIC value for the antibiotic was 0.25 μg/mL, while it was 0.625 mg/mL for PEO. The MIC zone diameter of the penicillin + PEO combination was 3 ± 0 mm. For ampicillin 1 μg/mL MIC value and 4 ± 0 mm zone diameter were determined. In the combination of ampicillin and PEO, the MIC value for the antibiotic was 0.5 μg/mL, while it was 2.5 mg/mL for PEO. The MIC zone diameter of the ampicillin + PEO combination was 8.5 ± 0.5 mm. The MIC value of gentamicin was 2 μg/mL and the zone diameter was 3 ± 0 mm. When gentamicin was combined with PEO the MIC value was 0.5 μg/mL for the antibiotic contained in the combination and it was 0.625 mg/mL for the PEO. The MIC zone diameter of the gentamicin + PEO combination was 3.5 ± 0.5 mm. The MIC value of ciprofloxacin was 0.25 μg/mL and the zone diameter was 3 ± 0 mm. When ciprofloxacin was combined with PEO, the MIC for the antibiotic in this combination was 0.125 μg/mL and for PEO it was 2.5 mg/mL. The MIC zone diameter of the ciprofloxacin + PEO combination was 4 ± 0 mm.

The MIC value of PEO against *N. meningitidis* was 20 mg/mL and the zone diameter was 10 ± 0 mm. For penicillin 2 μg/mL MIC value and 5 ± 1 mm zone diameter were measured. In the penicillin + PEO combination the MIC value for the antibiotic was 0.5 μg/mL and it was 5 mg/mL for the PEO. The MIC zone diameter of the penicillin + PEO combination was 3 ± 0 mm. The MIC value of ampicillin was 4 μg/mL and the zone diameter was 3 ± 0 mm. The MIC value for the antibiotic in combination with ampicillin and PEO was 0.5 μg/mL, while it was 2.5 mg/mL for PEO. The MIC zone diameter of the ampicillin + PEO combination was 4 ± 1 mm. The MIC value of gentamicin was 2 μg/mL and the zone diameter was 3 ± 0 mm. In the gentamicin + PEO combination the MIC value was 0.25 μg/mL for the antibiotic and 2.5 mg/mL for the PEO. The MIC zone diameter of the gentamicin + PEO combination was 3 ± 0 mm. The MIC value of ciprofloxacin was 0.5 μg/mL and the zone diameter was 5.5 ± 1.5 mm. In the combination of ciprofloxacin and PEO, the MIC values were 0.062 μg/mL for the antibiotic and 2.5 mg/mL for PEO, while the MIC zone diameter of the combination was 5 ± 0 mm.

It was found that the combination of ciprofloxacin + PEO had a synergistic effect on *N. meningitidis* and an additive effect on *H. influenzae*. The combined use of PEO with gentamicin had a synergistic effect against *N. meningitidis* and *H. influenzae*. The antibacterial effect of the penicillin + PEO combination was higher than that of penicillin and PEO alone. A synergistic effect was observed on *N. meningitidis* and *H. influenzae*. The ampicillin + PEO combination showed a synergistic effect on *N. meningitidis* and an additive effect on *H. influenzae*.

The MBC effect of benzylpenicillin was determined at a concentration of 8 mg/L on *H. influenzae* and against *N. meningitidis* at a concentration of 16 mg/L. The MBC effect of ampicillin was 8 mg/L and 16 mg/L for *H. influenzae* and *N. meningitidis*, respectively. The bactericidal effect of ciprofloxacin was 4 mg/L for *N. meningitidis* and *H. influenzae*. The bactericidal effect of gentamicin was determined at a concentration of 16 mg/L in all microorganisms studied. The MBC value of PEO was 80 mg/L for *N. meningitidis* and 20 mg/L for *H. influenzae*.

In the present study, it was shown that PEO alone against *N. meningitides* and *H. influenzae* is less effective than the use of antibiotics alone, while combinations of PEO with antibiotics are much more effective against the same standard microorganisms. Many studies have reported the antimicrobial and antifungal activity of essential oils of *Pelargonium* species. Rosato et al. (2007) found that the use of norfloxacin together with *Pelargonium graveolens* essential oil in the treatment of infections caused by some bacterial species reduces the minimum effective dose of norfloxacin, and thus the side effects caused by antibiotics can be minimized. In another study, some *Pelargonium* species and cultivar essential oils showed strong antimicrobial activity against *Salmonella enteritidis* and *Listeria innocua* (Balchin, 1998). Previous studies have reported that *P. graveolens* essential oil showed antimicrobial activity against various pathogens, namely *S. aureus, Bacillus cereus, K. pneumoniae*, and *Candida albicans*. Andrade et al. (2011) stated that essential oil from *Pelargonium odoratissimum* showed antifungal potential by inhibiting various fungi.

Today, the incidence of multiple resistant organisms is increasing and this is becoming a global problem. As the resistance to antibiotics has increased, the importance of understanding the resistance mechanisms of infectious bacteria and controlling these bacteria has also increased. Aminoglycoside + cephalosporin combinations are used in current meningitis treatments; aminoglycosides and cephalosporins can chemically interact and inactivate each other (Ayaz, 2001). Invasive meningococcal disease is a serious disease caused by the Gram-negative diplococcus *N. meningitidis*, and can quickly become fatal if left untreated (Pathan, 2003; Rosenstein et al., 2001; Virji, 2009).

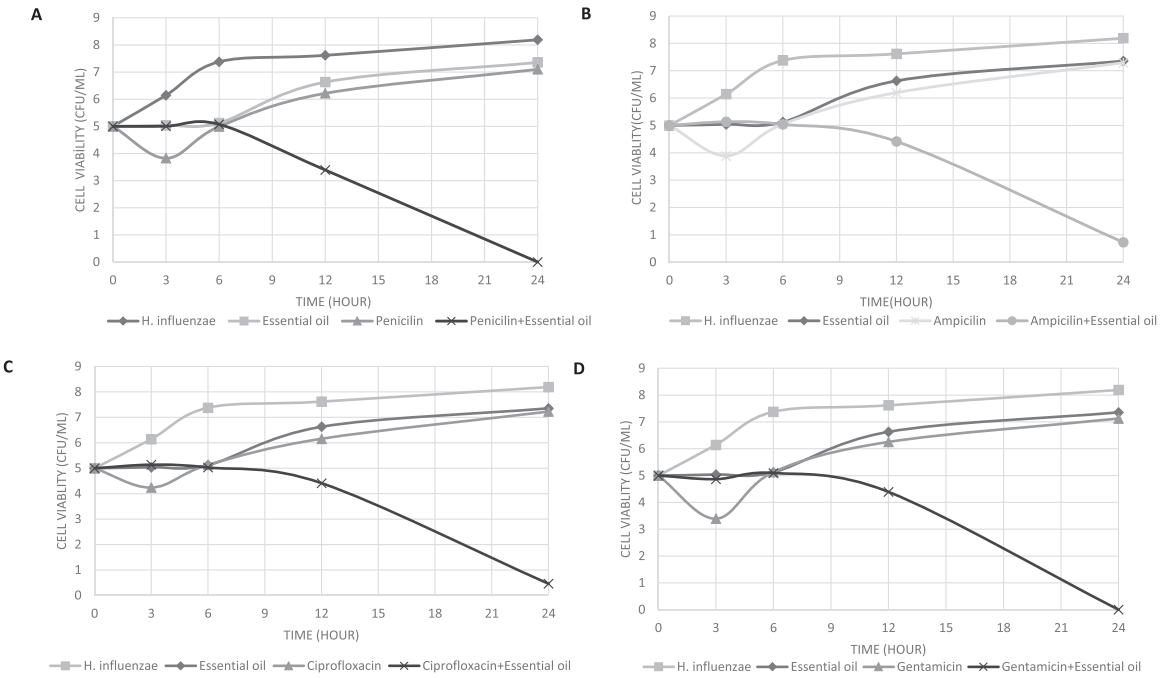

Fig. 1. Time-kill analysis of essential oil, antibiotics and combination of both of them against *H. influenzae*. Penicillin (A), Ampicilin (B), Ciprofloxacin (C), Gentamicin (D).

Most *N. meningitidis* isolates are sensitive to penicillin and ampicillin. Although antimicrobial resistance in *N. meningitidis* strains is rare, reduced susceptibility to third-generation cephalosporins and penicillin *G* has recently been reported (Vacca et al., 2018). *H. influenzae*, an important community-acquired pathogen, causes significant morbidity and mortality worldwide. It has been reported that there is an increase in resistance to ampicillin and penicillin, which are the main antibiotics used in the treatment of infections caused by *H. influenzae* (Barry et al., 2001; Cerquetti, 2004; Yamada, 2019). The high prevalence of resistance to beta-lactam antibiotics, which are the primary treatment choice, indicates the importance of selecting an alternative antimicrobial agent for the empirical treatment of infections caused by this pathogen worldwide. Another problem is that by giving the bactericidal and bacteriostatic antibiotics together the lethal effect of the antibiotic can be lost. The best clinical example of this is that in patients with pneumococcal meningitis treated with a combination of penicillin and chlortetracycline the mortality is higher than that of penicillin alone (Ceyhan et al., 2008; Techasaensiri, 2010).

However, resistance to these antibiotics is increasing, leading to the search for new antibiotics. The results we obtained in our *in vitro* study show promise in the control of pathogens by the combination of the beta-lactam derivative penicillin G, aminoglycoside antibiotic gentamicin, ciprofloxacin, quinolone group antibiotic, and PEO on *N. meningitidis* and *H. influenzae*, which are known to be common meningitis agents.

3.3. Time-kill assay

A synergistic interaction between gentamicin and penicillin and PEO combinations against *H. influenzae* was observed in the time-kill study to determine the decrease in the number of live bacteria over time. A statistically significant difference was found between the penicillin and penicillin + PEO groups in the time-kill study ($p < 0.05$). The difference between the ciprofloxacin and ciprofloxacin + PEO groups was significantly limited ($p = 0.050$). A synergistic interaction was observed between gentamicin and penicillin and PEO combinations against *H. influenza* ($p = 0.05$ and $p < 0.001$, respectively). There was a decrease in the number of viable cells in the detection of synergy compared to the antibiotic + PEO with combination PEO treatment only 24 h after treatment. Using the drug in combination with PEO did not cause any significant inhibition of cell proliferation. Except for combinations of PEO with gentamicin and penicillin, PEO alone did not show cell death compared to the PEO + drug combination (Fig. 1A–D).

A significant difference was observed between the ciprofloxacin and ciprofloxacin + PEO groups, which were studied against *N. meningitidis* in determining the reduction in the number of live bacteria over time ($p = 0.037$). In addition, a synergistic interaction was observed between the antibiotics and PEO against *N. meningitidis*. In the detection of synergy, a decrease in the number of viable cells was observed when the antibiotic combination was compared only with PEO application 24 h after treatment. Conversely, the antibiotic alone did not dramatically decrease the cell count (Fig. 2A–D).

There are similar studies using different plant species, strains, and antibiotics. The antimicrobial properties of *Thymus vulgaris* essential oils were investigated against multidrug resistant strains of Enterobacteriaceae. It has been reported that the combined use of the essential oil obtained from this plant with cefotaxime has significant antimicrobial activity and shows a synergistic effect (Benameur et al., 2019). In a study investigating the antimicrobial effect of the use of *Cladanthus arabicus* and *Bubonium imbricatum* essential oils alone or in combination with amoxicillin and neomycin antibiotics, it was reported that essential oils had a high antimicrobial effect on Enterobacteriaceae isolates by showing a synergistic effect with antibiotics (Aghraz et al., 2018).

Cell damage caused by combinations of PEO, antibiotic, and antibiotic + PEO were determined in time-kill analysis. It is suggested that irreversible membrane damage results from acidification of the cell membrane and protein denaturation due to the accumulation of PEO components (Borges et al., 2013). It causes membrane damage by creating essential oil and antibiotic synergism. In this way, it helps antibiotics to enter the cell through the cytoplasmic membrane by allowing them to bind to the antibiotic binding protein. This hypothesis proposed by Bolla et al. (2011) is in agreement with the outer membrane permeability test results in our study.

According to the results of the time-kill analysis, the inhibitory effect of antibiotics in the presence of PEO was greater than when they were used alone. The combined use of PEO and antibiotics

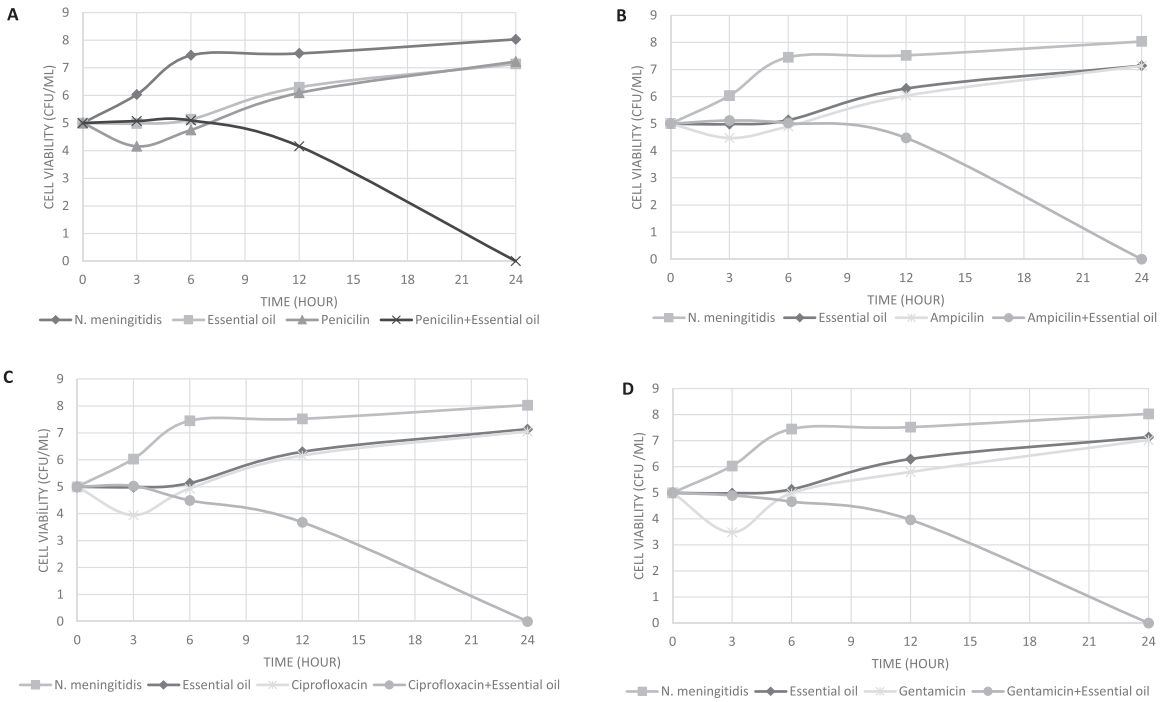

Fig. 2. Time-kill analysis of essential oil, antibiotics and combination of both of them against *N. meningitidis*. Penicillin (A), Ampicilin (B), Ciprofloxacin (C), Gentamicin (D).

showed a synergistic effect on bacteria. The activity of PEO + antibiotic combinations showed different effects against different bacterial species. *N. meningitidis* and *H. influenzae* were completely killed by combinations of PEO with penicillin at the end of 24 h. *N. meningitidis* was completely destroyed at the end of 24 h, showing high sensitivity to all antibiotic + essential combinations studied. However, the slower dying *H. influenzae* was more susceptible to combinations with ciprofloxacin + PEO and ampicillin + PEO than antibiotics and PEO. The combination with gentamicin, PEO, and gentamicin + PEO produced a significantly rapid decrease in the number of living bacteria from 6 h.

3.4. Postantibiotic effect (PAE)

Herein, all antibiotics studied stimulated PAE at MIC levels. When bacterial growth is constantly suppressed, the antibiotic concentration is below MIC. For *H. influenzae*, the average PAE time determined with 1xMIC solution of the antibiotic was ampicillin 0.30, gentamicin 0.95, ciprofloxacin 1.25, penicillin 0.90, and PEO 0.65 h. The duration of PAE of the combination of ampicillin with PEO was 0.50, of the combination of gentamicin with PEO was 1.35, of the combination of penicillin with PEO was 0.45, and of the combination of ciprofloxacin with PEO was 1.55 h. The average duration of PAE determined with 1xMIC solution of antibiotic for *N. meningitidis* was determined as ampicillin 0.00, gentamicin 1.00, ciprofloxacin 1.30, penicillin 0.30, and PEO 1.10 h. The PAE time of the combination of ampicillin with PEO was 1.20, of the combination of gentamicin with PEO was 2.70, of the PEO combination of penicillin was 2.00, and of the combination of ciprofloxacin with PEO was 2.25 h (Figs. 3 and 4).

In particular, the combination of gentamicin and ciprofloxacin with PEO showed synergistic effects, causing significant structural changes in *H. influenzae* and *N. meningitidis*. Antibiotic + PEO combinations reacted with cell membranes and showed synergistic activity by interacting between bacterial cells and antibacterial compounds. In our study all antibiotics stimulated PAE at MIC levels. The antibiotic concentration is below MIC when bacteria growth is constantly suppressed. Especially the combination of gentamicin and ciprofloxacin with PEO showed a synergistic effect and caused significant structural changes in *H. influenzae* and *N. meningitidis*. Antibiotic + PEO combinations showed synergistic activity by interacting between bacterial cells and antibacterial compounds by reacting with cell membranes. Combined use of gentamicin with PEO had a synergistic effect on the studied microorganisms and prolonged the PAE. Alternatively, it may reduce the inhibitory activity by causing interactions between different compounds in multicomponent systems (Lis-Balchim and Deans, 1997). Combined use of penicillin with PEO reduced the inhibitory effect on *H. influenzae* and shortened the duration of PAE. Aeschlimann et al. (1999) found significant increases in the PAEs of ciprofloxacin and norfloxacin against *Staphylococcus aureus*. They suggested that this effect is due to membrane-associated multidrug proteins that reduce sensitivity to fluoroquinolones (Aeschlimann et al., 1999). Although the mechanisms that cause PAE are not known precisely, it is thought to occur via slowdown in the repair of cellular damage in intracellular active regions and delay in protein synthesis (Craig and Gudmundsson, 1996).

3.5. Activation of leukocyte cells

The *in vitro* phagocytic activity of *P. endlicherianum* PEO in human leukocyte cells was tested. In PEO + antibiotic combinations, PEO and antibiotics showed synergistic effects, and antibiotic and antibiotic + PEO treatment dramatically improved the phagocytic activity of WBC 264-9C cells compared to control values. ($p < 0.022$). The data on enhancing the bactericidal activity of WBC 264-9C cells are given in Tables 3–6. The phagocytic effect of PEO was found to be greater than when it was combined with gentamicin in *N. meningitidis* ($p = 0.009$). PEO was more effective than ampicillin and ciprofloxacin combinations in *H. influenzae* ($p = 0.004$ and 0.003, respectively). Penicillin treatment from 2 h decreased the logarithmic growth of *H. influenzae* cells by about 3 logs, while penicillin + PEO treatment showed a synergistic effect, reducing the number of viable cells by about 4 logs. From 4 h, the number of viable cells decreased approximately 3 logs with the synergistic effect of the ampicillin + PEO combination compared to the ampicillin treatment group. It showed a high phagocytic effect on *N. meningitidis* in leukocyte cells. In simultaneous measurements, when compared with the control group, an

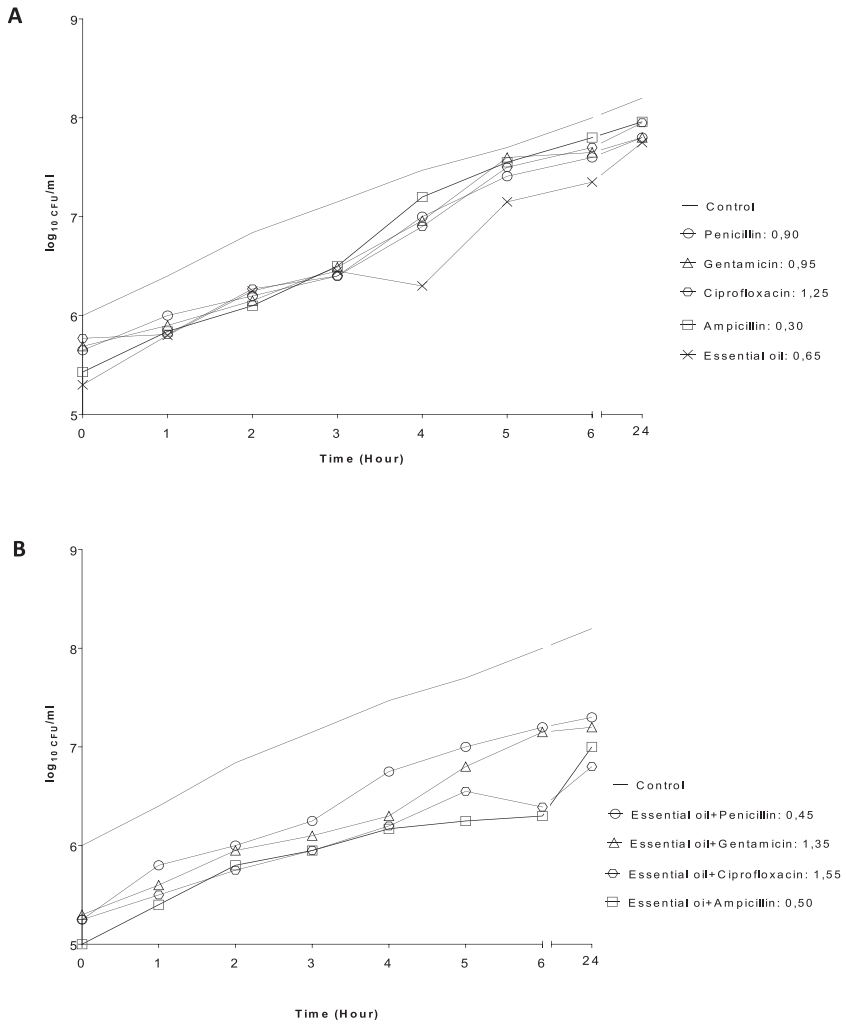

Fig. 3. PAE values as a result of treatment of *H. influenzae* strain with antibiotics and essential oil (A). PAE values as a result of treatment of *H. influenzae* strain with antibiotics + essential oil combination (B).

approximately 3 log decrease in the number of viable bacteria was observed in PEO combined treatments of penicillin, ciprofloxacin, and gentamicin. A similar effect was observed after 2 h in the ampicillin + PEO group. All the antibiotics studied showed a synergistic effect with PEO. The interaction of the antibiotic with the host defense or changes in the bacterial cell surface during pre-exposure may support the phagocytic activity of leukocyte cells by making microorganisms more susceptible to phagocytosis and intracellular killing (Bruddger et al., 1986; Craig and Gudmundsson, 1991).

3.5. Outer membrane permeability

In order to determine the sudden cellular death caused by SDS, the differences in absorbance at certain intervals (0, 5, 10, 30, and 60 min) are shown in Tables 7 and 8. The least deaths were found in the control groups using only SDS in the analyses ($p < 0.001$ for all groups). The group in which PEO and penicillin were used together for *H. influenza* and the groups in which PEO and penicillin and ciprofloxacin were used for *N. meningitidis* showed significantly higher bactericidal membrane damage and the lower survival values compared to the other groups ($p < 0.001$).

In the present study, the effects of penicillin, ampicillin, gentamicin, and ciprofloxacin on pathogens causing meningitis were further expanded in combination with PEO from *P. endlicherianum* and resulted in increased efficacy of these drugs. These antibiotics alone did not increase outer membrane permeability despite the SDS influx in cells, and therefore OD readings were not significantly reduced during this experiment due to the hydrophilic nature of beta-lactam antibiotics ($p > 0.05$). Fatal injury to bacterial cell membranes can impair cell permeability and therefore affect the osmosis ability of the membrane (Gilbert, 1991). SDS easily dissolves the cytoplasmic membrane of bacteria due to its chemical structure, but in our study the treatment with 0.1% SDS caused no significant lytic effects compared to the controls of antibiotics and/or PEO without 0.1% SDS. However, the duration of this experiment was limited to 60 min to prevent possible cell lytic reaction as a result of prolonged exposure to SDS. The PEO in inhibitor concentration sensitized the bacteria to SDS. These findings are largely in agreement with studies with sesquiterpenes such as bourbonene and germacrene *D*, which are the main components of PEO. Since sesquiterpenoids have the ability to increase bacterial membrane permeability, they cause significant damage to the bacterial cell membrane, leading to cell death. Other cellular structures also cause bacterial cell wall lysis due to cell membrane disruption, followed by intracellular loss of intracellular substance (Carson et al., 2002).

4. Conclusion

Essential oils, which have been widely used as medicinal products since medieval times, are thought to be promising in preventing bacterial resistance because these oils are important components of plant chemistry and are naturally occurring and multicomponent

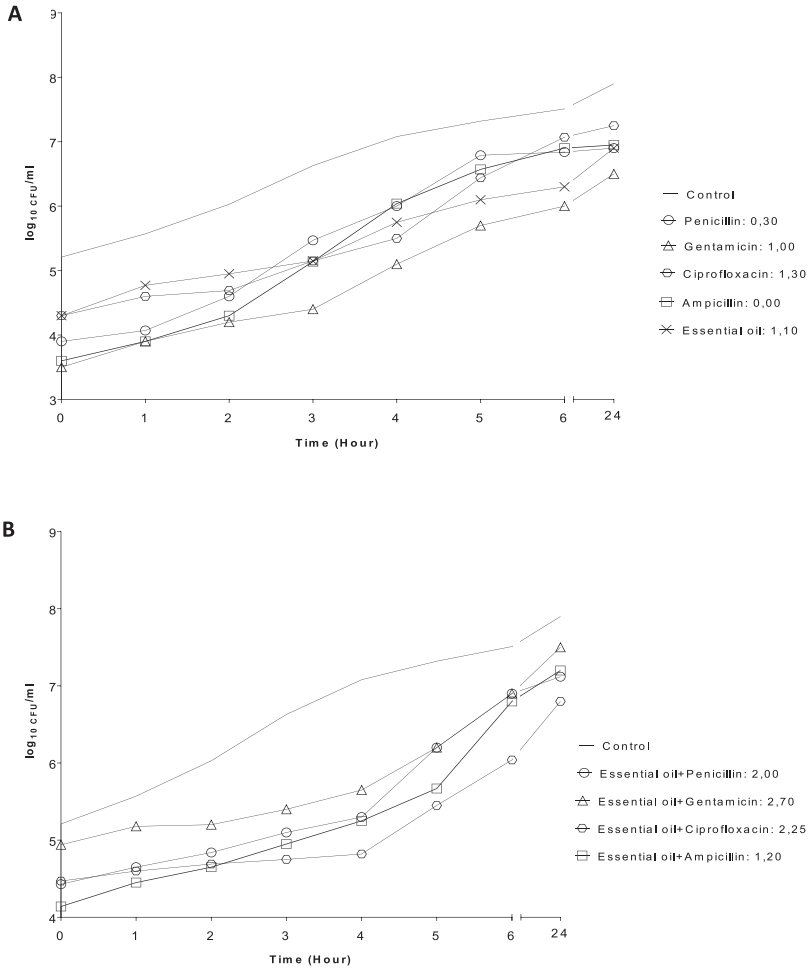

Fig. 4. PAE values as a result of treatment of *N. meningitidis* strain with antibiotics and essential oil (A). PAE values as a result of treatment of N. meningitidis strain with antibiotics + essential oil combination (B).

Table 3
Phagocytic effect of leukocytes on *H.influenzae* exposed to antibiotics.

H. influenzae incubated with leukocytes and time (hours)	Control (log cfu/mL)	Penicillin (log cfu/mL)	Ampicilin (log cfu/mL)	Cyprofloxacin (log cfu/mL)	Gentamicin (log cfu/mL)
0	6.88 ± 0.10	6.78 ± 0.07	6.78 ± 0.07	6.34 ± 1.00	6.38 ± 0.53
2	6.91 ± 0.09	3.25 ± 0.00	6.45 ± 0.70	5.27 ± 1.44	4.79 ± 1.13
4	6.78 ± 0.24	2.88 ± 0.19	4.99 ± 1.50	2.69 ± 0.18	2.70 ± 0.47
8	2.83 ± 0.22	2.48 ± 0.06	2.08 ± 0.14	2.07 ± 0.34	1.44 ± 0.48
12	2.15 ± 0.02	0.60 ± 0.00	0.50 ± 0.00	0.53 ± 0.00	0.30 ± 0.00

Table 4
Phagocytic effect of leukocytes on *H. influenzae* exposed to PEO and PEO + antibiotic combinations.

H. influenzae incubated with leukocytes and time (hours)	PEO (log cfu/mL)	Penicillin + PEO (log cfu/mL)	Ampicilin + PEO (log cfu/mL)	Cyprofloxacin + PEO (log cfu/mL)	Gentamicin + PEO (log cfu/mL)
0	6.34 ± 0.73	6.44 ± 0.82	6.44 ± 0.82	6.88 ± 0.01	4.24 ± 2.14
2	2.68 ± 0.16	2.62 ± 0.01	4.54 ± 1.65	5.22 ± 1.50	4.05 ± 2.14
4	2.24 ± 0.57	1.75 ± 0.25	1.98 ± 0.68	2.31 ± 0.34	2.19 ± 0.78
8	2.04 ± 0.46	0.95 ± 0.00	1.73 ± 0.38	1.90 ± 0.07	0.30 ± 0.21
12	0.46 ± 0.17	0.00 ± 0.00	0.47 ± 0.02	0.85 ± 0.03	0.00 ± 0.00

when compared to many antibacterials with only one target site. In the present study, PEO was tested with antibiotics as a new treatment method against bacterial infections of meningitis. The recommended main action of PEO is to break down the bacterial membrane at both lethal and lethal concentrations, and then to increase the nonspecific cell activity of the antibiotic. In our study, it was shown that PEO not only inhibits bacterial growth, but also causes damage to the bacterial membrane and postantibiotic effects and increases the phagocytic activity of leukocyte cells, resulting in a decrease in the number of bacteria. Given the heterogeneous composition of PEO, the mode of

Table 5
Phagocytic effect of leukocytes on *N. meningitidis* exposed to antibiotics.

N. meningitidis incubated with leukocytes and time (hours)	Control (log cfu/mL)	Penicillin (log cfu/mL)	Ampicilin (log cfu/mL)	Cyprofloxacin (log cfu/mL)	Gentamicin (log cfu/mL)
0	6.84 ± 0.15	6.28 ± 0.94	5.03 ± 1.75	3.92 ± 1.11	6.14 ± 0.82
2	6.58 ± 0.10	4.38 ± 2.01	2.83 ± 0.33	2.87 ± 0.14	2.65 ± 0.00
4	5.84 ± 0.59	2.95 ± 0.27	2.09 ± 0.60	2.57 ± 0.00	1.86 ± 0.07
8	3.88 ± 1.39	2.52 ± 0.00	1.53 ± 0.39	1.37 ± 0.05	1.40 ± 0.20
12	2.13 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 6
Phagocytic effect of leukocytes on *N. meningitidis* exposed to PEO and PEO + antibiotic combinations.

N. meningitidis incubated with leukocytes and time (hours)	PEO (log cfu/mL)	Penicillin + PEO (log cfu/mL)	Ampicilin + PEO (log cfu/mL)	Cyprofloxacin + PEO (log cfu/mL)	Gentamicin + PEO (log cfu/mL)
0	3.51 ± 1.81	3.04 ± 0.13	5.94 ± 0.77	3.22 ± 0.12	2.97 ± 0.21
2	2.66 ± 0.20	2.62 ± 0.01	2.15 ± 0.42	2.50 ± 0.34	2.05 ± 0.32
4	1.79 ± 0.33	1.82 ± 0.22	1.17 ± 0.15	1.86 ± 0.07	1.57 ± 0.03
8	0.00 ± 0.00	0.84 ± 0.50	0.69 ± 0.09	1.62 ± 0.46	0.41 ± 0.12
12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 7
Reduction of membrane permeability of *H. influenzae* by the combination of PEO. Antibiotics and PEO + antibiotic.

	OD_{625}= SD (n = 3)				
Time (minute)	0	5	10	30	60
H. influenzae (Control). % 0.1 SDS	0.31 ± 0.016	0.31 ± 0.006	0.31 ± 0.005	0.31 ± 0.003	0.31 ± 0.017
% 0.1 SDS (without)	0.30 ± 0.002	0.30 ± 0.014	0.30 ± 0.003	0.30 ± 0.012	0.31 ± 0.018
PEO (5 mg/L). % 0.1 SDS	0.30 ± 0.002	0.29 ± 0.008	0.28 ± 0.001	0.27 ± 0.003	0.26 ± 0.009
% 0.1 SDS (without)	0.30 ± 0.004	0.29 ± 0.009	0.28 ± 0.002	0.27 ± 0.003	0.26 ± 0.004
Penicillin (0.5 mg/L). % 0.1 SDS	0.29 ± 0.006	0.26 ± 0.007	0.24 ± 0.006	0.24 ± 0.005	0.23 ± 0.007
% 0.1 SDS (without)	0.29 ± 0.003	0.25 ± 0.004	0.24 ± 0.003	0.24 ± 0.010	0.22 ± 0.006
Penicillin + PEO. % 0.1 SDS	0.28 ± 0.001	0.28 ± 0.003	0.26 ± 0.002	0.22 ± 0.011	0.22 ± 0.007
% 0.1 SDS (without)	0.28 ± 0.002	0.27 ± 0.002	0.26 ± 0.0011	0.21 ± 0.007	0.21 ± 0.031
Ampicilin(0.5 mg/L). % 0.1 SDS	0.29 ± 0.002	0.28 ± 0.009	0.27 ± 0.005	0.26 ± 0.009	0.25 ± 0.003
% 0.1 SDS (without)	0.29 ± 0.008	0.27 ± 0.005	0.26 ± 0.003	0.25 ± 0.009	0.25 ± 0.008
Ampicilin + PEO. % 0.1 SDS	0.29 ± 0.012	0.28 ± 0.027	0.27 ± 0.013	0.26 ± 0.014	0.24 ± 0.004
% 0.1 SDS (without)	0.29 ± 0.011	0.28 ± 0.019	0.27 ± 0.015	0.26 ± 0.011	0.24 ± 0.003
Ciprofloxacin (0.5 mg/L). % 0.1 SDS	0.29 ± 0.00	0.28 ± 0.005	0.27 ± 0.006	0.26 ± 0.005	0.26 ± 0.008
% 0.1 SDS (without)	0.29 ± 0.012	0.27 ± 0.006	0.27 ± 0.012	0.25 ± 0.010	0.25 ± 0.010
Ciprofloxacin + PEO. % 0.1 SDS	0.29 ± 0.015	0.29 ± 0.025	0.28 ± 0.013	0.26 ± 0.015	0.25 ± 0.010
% 0.1 SDS (without)	0.29 ± 0.010	0.28 ± 0.015	0.28 ± 0.015	0.26 ± 0.012	0.24 ± 0.013
Gentamicin(0.5 mg/L). % 0.1 SDS	0.30 ± 0.002	0.27 ± 0.007	0.25 ± 0.006	0.25 ± 0.005	0.24 ± 0.007
% 0.1 SDS (without)	0.30 ± 0.003	0.26 ± 0.004	0.25 ± 0.003	0.25 ± 0.010	0.23 ± 0.006
Gentamicin + PEO. % 0.1 SDS	0.28 ± 0.005	0.27 ± 0.011	0.25 ± 0.017	0.23 ± 0.027	0.23 ± 0.023
% 0.1 SDS (without)	0.28 ± 0.007	0.27 ± 0.004	0.26 ± 0.015	0.23 ± 0.022	0.22 ± 0.016

Table 8
Reduction of membrane permeability of *N. meningitidis* by the combination of PEO. Antibiotics and PEO + antibiotic.

OD_{625}= SD (n = 3) Time (minute)	0	5	10	30	60
N. meningitidis (Control). % 0.1 SDS	0.30 ± 0.006	0.30 ± 0.002	0.30 ± 0.002	0.30 ± 0.005	0.30 ± 0.008
% 0.1 SDS (without)	0.30 ± 0.007	0.30 ± 0.006	0.29 ± 0.010	0.30 ± 0.002	0.30 ± 0.009
PEO (20 mg/L). % 0.1 SDS	0.30 ± 0.010	0.29 ± 0.003	0.29 ± 0.006	0.29 ± 0.002	0.27 ± 0.002
% 0.1 SDS (without)	0.30 ± 0.006	0.29 ± 0.000	0.28 ± 0.002	0.28 ± 0.003	0.27 ± 0.004
Penicillin (0.5 mg/L). % 0.1 SDS	0.30 ± 0.005	0.29 ± 0.006	0.29 ± 0.002	0.28 ± 0.005	0.27 ± 0.002
% 0.1 SDS (without)	0.29 ± 0.010	0.29 ± 0.002	0.29 ± 0.003	0.27 ± 0.005	0.27 ± 0.005
Penicillin + PEO. % 0.1 SDS	0.28 ± 0.005	0.27 ± 0.003	0.26 ± 0.003	0.25 ± 0.009	0.23 ± 0.002
% 0.1 SDS (without)	0.27 ± 0.002	0.26 ± 0.002	0.26 ± 0.006	0.25 ± 0.006	0.22 ± 0.008
Ampicilin(0.5 mg/L). % 0.1 SDS	0.29 ± 0.005	0.28 ± 0.006	0.26 ± 0.009	0.25 ± 0.005	0.24 ± 0.004
% 0.1 SDS (without)	0.29 ± 0.010	0.27 ± 0.002	0.26 ± 0.009	0.24 ± 0.005	0.24 ± 0.015
Ampicilin + PEO. % 0.1 SDS	0.28 ± 0.005	0.28 ± 0.009	0.27 ± 0.005	0.23 ± 0.011	0.21 ± 0.013
% 0.1 SDS (without)	0.27 ± 0.002	0.26 ± 0.002	0.26 ± 0.010	0.23 ± 0.015	0.21 ± 0.011
Ciprofloxacin (0.5 mg/L). % 0.1 SDS	0.29 ± 0.005	0.29 ± 0.002	0.27 ± 0.006	0.25 ± 0.020	0.24 ± 0.013
% 0.1 SDS (without)	0.29 ± 0.003	0.28 ± 0.005	0.27 ± 0.015	0.24 ± 0.007	0.24 ± 0.015
Ciprofloxacin + PEO. % 0.1 SDS	0.28 ± 0.005	0.28 ± 0.009	0.25 ± 0.005	0.24 ± 0.005	0.23 ± 0.009
% 0.1 SDS (without)	0.28 ± 0.006	0.28 ± 0.002	0.25 ± 0.006	0.23 ± 0.010	0.23 ± 0.007
Gentamicin(0.5 mg/L). % 0.1 SDS	0.30 ± 0.005	0.28 ± 0.013	0.26 ± 0.014	0.24 ± 0.003	0.24 ± 0.004
% 0.1 SDS (without)	0.29 ± 0.004	0.27 ± 0.006	0.26 ± 0.007	0.24 ± 0.007	0.24 ± 0.005
Gentamicin + PEO. % 0.1 SDS	0.28 ± 0.012	0.27 ± 0.005	0.25 ± 0.002	0.23 ± 0.009	0.23 ± 0.013
% 0.1 SDS (without)	0.27 ± 0.005	0.27 ± 0.008	0.24 ± 0.006	0.23 ± 0.004	0.22 ± 0.009

action is likely to be more complex than that shown here. From this point of view, although information on the mechanisms of action is limited, further research should be conducted to study and understand the usefulness of these compounds in the elimination of antibiotic-resistant microorganisms. It is very important to confirm the practical applications of PEO to be used as a therapeutic option in combination with current antibiotics applied in meningitis treatments. The *in vitro* susceptibility of a clinical isolate does not guarantee the success of clinical use of the therapeutic agent. The clinical results depend on various factors such as the site of infection, the pharmacological properties of the antibiotic, and the effectiveness of the specific and nonspecific defense mechanism. Thus, *in vitro* susceptibility testing is necessary but not sufficient for a positive clinical decision. The combination of essential oils with antibiotics has shown a decrease in effective doses of antibiotics in the treatment of infections. In this way, it is obvious that the negative effects of the antibiotic can be overcome. In the fight against microbial resistance, combining essential oils and antibiotics that target resistant bacteria is emerging as a new option.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2021.10.006.

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