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RESEARCH ARTICLE

In vitro antimicrobial, antioxidant and anti-inflammatory evaluation of *Eucalyptus globulus* essential oil

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Abstract

Eradication of *Propionibacterium acnes* and associated skin pathogenic species such as *Staphylococcus aureus* and *S. epidermidis* involve anti-oxidant as well as anti-inflammatory effects besides antimicrobial action. For this purpose, Pharmacopoeia Grade (PhEur) *Eucalyptus globulus* essential oil was evaluated against the human pathogenic species such as *P. acnes* ATCC 6919, *P. acnes* ATCC 11827, *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 using an *in vitro* microdilution method. The composition and quality of the essential oil was confirmed both by GC/FID and GC/MS techniques, respectively. The *in vitro* radical-scavenging activity was evaluated using the photometric 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay; the anti-inflammatory activity assay performed by using the *in vitro* lipoxygenase (5-LOX) enzyme inhibition assay. Essential oil analysis confirmed the presence of 1,8-cineole (80.2 %), *p*-cymene (6.6 %), and limonene (5 %) as main components. The antibacterial performance of the tested oil was more susceptible against *Staphylococcus* species (MIC=625 µg/mL) compared to *P. acnes* (MIC=1250 µg/mL). 5-LOX inhibitory activity was determined as IC_{50} = 58 ±1,4 µg/mL for the essential oil, compared to the inhibition of the standard nordihydroguaiaretic acid = NDGA. The preliminary experimental results suggest that the *Eucalyptus* essential oil and its major constituent 1,8-cineole acts against skin pathogenic bacteria as a mild natural antimicrobial with anti-inflammatory effects, for further potential topical applications.

Keywords: Antibacterial, anti-inflammatory, *Eucalyptus globulus*, *Propionibacterium, Staphylococcus*

Introduction

Inflammation is a physiological response, which occurs in the human system against tissue damage caused by various physical, biochemical effects, and microbial infections. Lipoxygenases (LOX) are well known as an important group of enzymes associated with inflammatory processes. The ethiology of acne is an inflamed and painful swollen unpleasant topical situation, which is associated with *Propionibacterium* sp. and *Staphylococcus* sp. etc. pathogenic microorganisms located on the skin pores (Athikomkulchai et al., 2008; Lertsatitthanakorn et al., 2006; Perry & Lambert, 2006; Serpi et al. 2012). Today, resistance of *P. acnes* to antibiotics (Abascal& Yarnell, 2002; Chomnawang et al., 2005; Ergin et al., 2007; Akolade et al., 2012), and the extensive use of external antimicrobials may cause dryness, irritation and peeling among others which urged the need for new treatment approaches (Lertsatitthanakorn et al., 2006).

For many years, essential oils (EOs) are used to treat and protect from diseases due to their pharmacological effects. EOs and their preparations can be used orally after dilution, by inhalation and by topical routes (Başer & Buchbauer, 2020; Babar et al., 2015). Herbal preparations or cosmetics containing *Eucalyptus* oils are also used in several countries for the treatment of infectious diseases and several conditions (Maccioni et al., 2002). Additionally, it is also documented that in some countries, *Eucalyptus* preparations are used for the

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treatment of acne (Dey et al., 2014). On the basis of traditional use, the present study aimed to evaluate the combined *in vitro* antibacterial, antioxidant, and anti-inflammatory activity of the Pharmacopoeia (PhEur) quality *Eucalyptus globulus* Labill. (Myrtaceae) essential oil (EGEO). To the best of our knowledge, there is only limited data on EGEO against *P. acnes* and skin pathogenic microorganisms, as well as on 5-LOX inhibitions. Antibacterial activity was studied against the skin pathogens *Propionibacterium acnes*, *Staphylococcus aureus*, and *S. epidermidis* by *in vitro* microdilution method. In addition, antioxidant activity of EGEO was examined using the *in vitro* 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and the anti-inflammatory effect was evaluated using the lipoxygenase (LOX) inhibition.

Materials and Methods

Materials

Pharmacopoeia Grade (PhEur) EGEO was kindly provided by Enafarma Ltd., Istanbul, Turkey. All bacteria were obtained from Microbiologics, USA. Antibacterial activities of the EGEO against *P. acnes* ATCC 6919 and ATCC 11827 (American Type Culture Collection), *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 strains were screened. The antibacterial compounds [tetracycline, ampicillin and cefuroxime] were provided by Deva Pharmaceutical Company, Istanbul, Turkey.

Lipoxygenase (1.13.11.12, type I-B, soybean), linoleic acid Nordihydroguaiaretic acid (NDGA), resazurin sodium salt and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were acquired from Sigma-Aldrich. All chemical substances were used analytical grade if not otherwise stated.

GC-FID analysis

The GC analysis was carried out using an Agilent 6890N GC system. FID detector 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.

GC/MS analysis

The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column [60 m x 0.25 mm, 0.25 mm film thickness] was used with helium as carrier gas [0.8 ml/min]. GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 450.

Identification of components

Identification of the EGEO components was carried out by comparison of their relative retention times (RRT) with those of authentic samples or by comparison of their relative retention index [RRI] to a series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, MassFinder 3 Library) (McLafferty & Stauffer, 1989; Koenig et al., 2004) and *in-house* "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils. Additionally, MS literature data (Joulain & Koenig, 1998; ESO, 2000) was also used for the identification.

Antibacterial activity (MIC, µg/mL)

Antibacterial activity of EGEO against *P. acnes* ATCC 6919, *P. acnes* ATCC 11827, *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 were screened *in vitro* by the modified CLSI microdilution method (CLSI, 2006). The

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tests were carried out in 96-well micro plates. The sample (100 µL per well) was diluted two fold, with a final concentration range of 5000 to 9.76 µg/mL, respectively. Standard antibacterial agents ampicillin, cefuroxime (64 to 0.125 µg/mL) and tetracycline (16 to 0.03 µg/mL) were used under the same conditions as positive controls. *P. acnes* ATCC 6919 and *P. acnes* ATCC 11827 were inoculated in Reinforced *Clostridium* Medium (RCM), and cultured % 5 CO_2 at 37°C for 72 h under anaerobic conditions. *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 were incubated in Mueller Hinton Broth (MHB) overnight at 37°C for 24h or 48h. Cultures, with a final inoculum size of 1 x 10^6 colonies forming units (CFU/mL) were used. Microbial growth was observed by adding 20 µL of resazurin of 0.01% with minor modifications of CLSI standards (Pfaller et al., 2008). A change from blue to pink indicated the reduction of resazurin and, therefore, microbial growth. The minimum inhibitory concentration (MIC) was determined as the lowest drug concentration that prevented the color change. All experiments were repeated in triplicate, and average results were reported.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant activity was measured by using the microplate spectrophotometric method for EGEO and its main compound 1,8-cineole (Sigma Aldrich). Stock solutions of test samples (10 mg/mL) were dissolved initially in methanol. Serial dilutions (100 µL) were prepared using the stock solutions of the EGEO and 1,8-cineole. A stock solution of the DPPH (2 mg /25 mL) in methanol was prepared freshly. 100 µL DPPH solution was then added each well and mixed, and after 30 min storage in a dark environment at 25°C the UV absorbance was recorded at 517 nm. Blank samples containing the same amounts of methanol and DPPH solution were also prepared. The experiment was performed in duplicate for the EGEO, and 1,8-cineole with positive standard control, vitamin C. The average of the absorptions was noted for each concentration. The percentage inhibition (I%) was calculated using the Equation. The IC_{50} value, which is the concentration of the test material that inhibits 50% of the free radical concentration, was calculated as mg/mL using Sigma Plot statistical program (Kumarasamy et al., 2007).

Percentage Inhibition (I%) = $[(Abs_{control} - Abs_{sample})/ Abs_{control}] \times 100$ Equation

$Abs_{control}$: Control absorbance
Abs_{sample}: Sample absorbance

In vitro lipoxygenase [LOX] enzyme inhibition assay

The anti-inflammatory activity was evaluated by modifying the spectrophotometric method developed by Baylac and Racine (Baylac & Racine, 2003). Potassium phosphate buffer (1,94 mL; 100 mM; pH 9.0), 40 µL of test compound solution and 20 µL of lipoxygenase solution were mixed and incubated for 10 min at 25°C. The reaction was then initiated by the addition of 50 µL linoleic acid solution, the change of absorbance at 234 nm was followed for 20 min. Test compounds and the positive control Nordihydroguaiaretic acid (NDGA) were dissolved in methanol. All the kinetic experiments were performed in quartz cuvette. The concentration that gave 50% inhibition (IC_{50}) for test samples was calculated. All experiments were repeated in triplicate and average results were reported.

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Results and Discussion

Chemical analysis of the essential oil

The compounds of *Eucalyptus globulus* essential oil were determined by GC/FID and GC/MS, the results are listed in Table 1. Twelve main compounds were identified in the oil representing 100%. The major compounds were characterized as 1,8-cineole (80.2%), *p*-cymene (6.6%), and limonene (5%) as shown in Figure 1. The analytical results met the required Pharmacopoeia limits (Anonymous, 2008).

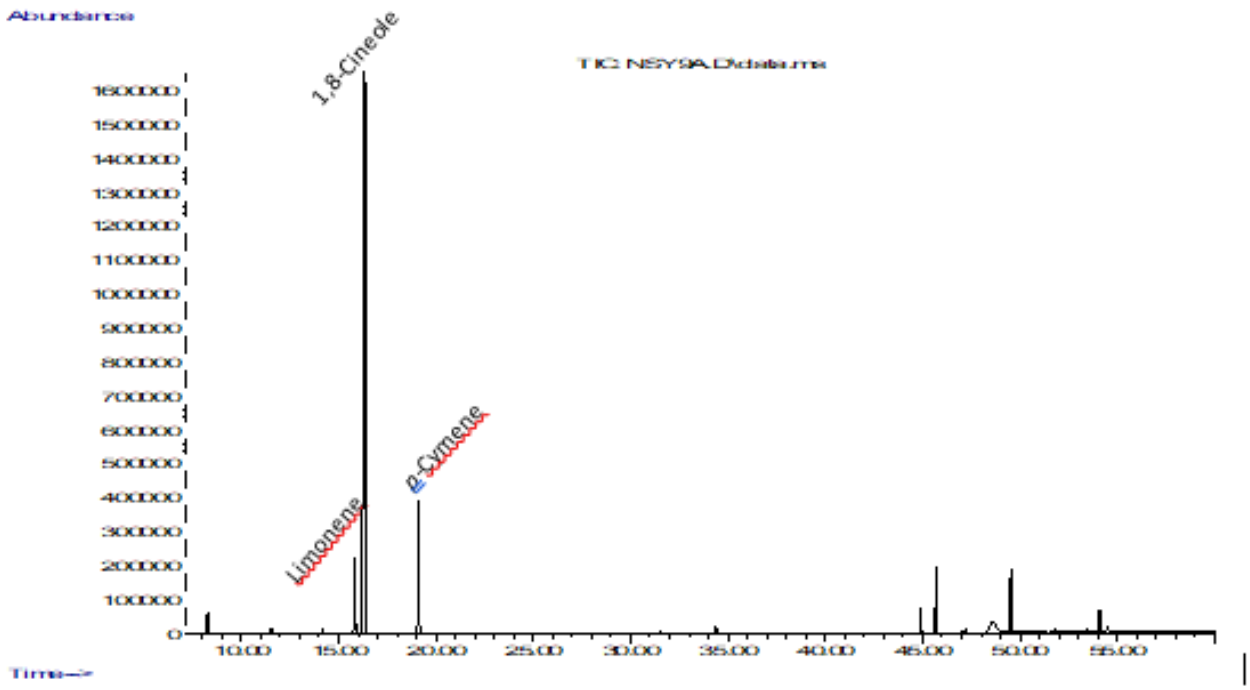
Numerous studies exist on the *Eucalyptus globulus* oil composition, where varying amouts of 1,8-cineole were reported (Damjanovic-Vratnica et al., 2001; Harkat-Madouri et al., 2015; Luis et al., 2016). 1,8-Cineole (85.8%), α-pinene (7.2%), and β-myrcene (1.5%) were the main components (Damjanovic-Vratnica et al., 2001). In another report, 1,8-cineole (55.3 %), spathulenol (7.4 %) and α-terpineol (5.5 %) were found as main components (Harkat-Madouri et al., 2015). In another study, major oil components were reported as 1,8-cineole (63.8 %), α-pinene (16.1 %), aromadendrene (3.7 %), and *o*-cymene (2.4 %) (Luis et al., 2016).

Table 1. Chemical composition of *E. globulus* Labill. essential oil

RRI	Compounds	%	IM
1032	α-Pinene	1.0	RRI, MS
1118	β-Pinene	0.4	RRI, MS
1174	Myrcene	0.2	RRI, MS
1203	Limonene	5.0	RRI, MS
1213	1,8-Cineole	80.2	RRI, MS
1280	*p*-Cymene	6.6	RRI, MS
1611	Terpinen-4-ol	0.1	RRI, MS
1706	α-Terpineol	0.4	RRI, MS
2100	Heneicosane	0.8	RRI, MS
2131	Hexahydrofarnesyl acetone	2.6	MS
2300	Tricosane	1.9	RRI, MS
2492	Ethyl oleate	0.8	MS
	Total	100	

RRI: Relative retention indices calculated against *n*-alkanes, %: calculated from FID data, IM: Identification method based on the relative retention indices (RRI) of authentic compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data

Figure 1. GC chromatogram of *E. globulus* essential oil



Antibacterial activity (MIC, µg/mL)

In the present study*; P. acnes* ATCC 6919, *P. acnes* ATCC 11827, *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228, were selected as test microorganisms based on their pathological potential. Minimum inhibitory concentrations (MIC) of test samples are listed in Table 2. The EGEO showed more antibacterial effect against *Staphylococcus* (MIC=625 µg/mL) species compared to *Propionibacterium* species (MIC=1250 µg/mL). In addition, the major component 1,8-cineole was also tested and was relatively more effective against *S. epidermidis* compared to other strains.

Table 2. *Eucalyptus globulus* essential oil Minimum Inhibitory Concentration (MIC, µg /mL) results

Samples	***S. aureus*** **ATCC 6538**	***S. epidermidis*** **ATCC 12228**	***P. acnes*** **ATCC 6919**	***P. acnes*** **ATCC 11827**
EGEO	625	625	1250	1250
1,8-Cineole	1250	312.5	1250	2500
Ampicillin	>64	2	0.125 >	0.125 >
Cefuroxime	>64	4	-	-
Tetracycline	4	4	2	4

EGEO: *Eucalyptus globulus* essential oil

Acne vulgaris is a skin disorder, and pathogenesis of acne include many factors such as follicular hyperkeratosis, increased sebum secretion, inflammation and *P. acnes,* which is one of the main causative microorganisms. In addition, *S. epidermidis* and *S. aureus* may be present in acne lesions. *P. acnes* secretes several proinflammatory products, which play an important role in the development of inflammation. These include lipases, proteases, hyaluronidases, and chemotactic factors (Kanlayavattanakul & Lourith, 2011; Das & Raynolds, 2014). Azelaic acid, salicylic acid, retinoic acid and derivatives, benzoyl peroxide, macrolides, tetracylines are extensively used in acne treatment (Kanlayavattanakul & Lourith, 2011). However, widespread use of antibiotics in the treatment of acne has led to the development of resistance against

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strains. To overcome the problem of antibiotic resistance, EOs have been extensively studied for the treatment of acne and other skin diseases. (Orafidiya et al., 2002; Orchard et al., 2018; Hammer, 2015; Taleb et al., 2018).

Currently, a systematic review demonstrated clinical use of EOs for the treatment of topical infections (Deyno et al., 2019). Clinical studies showed the efficacy of tea tree oil, against many diseases including acne, oral candidiasis, tinea, Onychomycosis, and *Molluscum contagiosum* (Hammer, 2015). Additionaly, it was reported that a topical preparation containing 2% *Ocimum* oil in cetomacrogol blend base is more effective in the treatment of *Acne vulgaris* than a 10 % benzoyl peroxide lotion (Orafidiya et al., 2002). The *in vitro* antibacterial activity of different EOs against acne associated bacteria like *P. acnes, S. epidermidis* and Multi drug resistant of *S. aureus* (MRSA) was reported (Orchard et al., 2017).

Antibacterial activity of *E. globulus* leaf EO against *Staphylococcus, Streptococcus, Escherichia, Haemophilus, Bacillus, Klebsiella* species, as well as its anti-candidal and antifungal activities were the subject of several reports (Bachir&Benali, 2012; Ghalem&Mohamed, 2008; Salari et al., 2007; Tohidpour et al., 2010; Noumi et al., 2011; Bansod&Rai, 2008; Vilela et al., 2009). MIC =15.75 mg/mL was found against *S. aureus* and *S. epidermidis* for EGEO, where the main component was 1,8-cineole (63 %) by microdilution (Mekonnen et al., 2016).

In this present study, MIC=625 µg/mL was obtained against *S. aureus* and *S. epidermidis.* According to the literature survey, a few studies were reported against *P. acnes, S. aureus,* and *S. epidermidis.* Antibacterial activity of EGEO by disc diffusion and agar dilution method were performed against *P. acnes* strains. Inhibition zones were not observed, and 4% concentration was reported as active concentration (Luangnarumitchai et al., 2007). Furthermore, EGEO containing *γ*-terpinene and *p*-cymene as the major components with a MIC=9375 µg/mL against *P. acnes* was reported (Athikomkulchai et al., 2008). In another study, antimicrobial activity of EGEO was evaluated against acne pathogenic microorganisms such as, *S. aureus* 2079 and *S. aureus* 5021 (Bhatt, 2011).

The possible reason for differences may be due to the characteristic compositions of EO or the microorganism strains used. Most of the antimicrobial activity of EOs were attributed to the oxygenated monoterpenes, while some hydrocarbons may also exhibit antimicrobial effects (Bassole & Juliani, 2012; Chouhan, 2017). The antimicrobial activity of *Eucalyptus* EO could be associated with the presence of 1,8-cineole, and linalool which are well-known constituents with pronounced antimicrobial properties (Damjanovic-Vratnica et al., 2001). 1,8-cineole, α-pinene, β-pinene, limonene, and linalool which have been known to exhibit antimicrobial activity against *Bacillus subtilis, Enterococcus faecalis, S. aureus, S. epidermidis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Candida albicans, Saccharomyces cerevisiae* and *Aspergillus niger* (Sonboli et al., 2006).

In recent years, there is an increased interest in the use of EOs and antimicrobial combination studies to control resistant microorganisms. Combinations of EOs and antimicrobial drugs may lead to synergistic, additive or antagonistic effects. Therefore, EGEO were combined with different EOs such as; *Citrus limon, Cedrus atlantica, Lavandula angustifolia, Melaleuca alternifolia, Pinus sylvestris, Rosmarinus officinalis, Thymus vulgaris, Mentha piperita, Mentha spicata* against *P. acnes* ATCC 11827 and *S. epidermidis* ATCC 2223 (Orchard et al., 2018). The combinations *E. globulus* and *Thymus mastichina* EOs were found to be synergistic against *S. aureus* (Vieira et al., 2017).

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1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH assay was used for antioxidant activity of EGEO, 1,8-cineole and vitamin C. EGEO and 1,8-cineole did not inhibit the DPPH free radicals at the tested concentrations (10 mg /mL), while significant DPPH radical inhibition was obtained for vitamin C. IC_{50} values are shown in Table 3.

Table 3. Antioxidant evaluation of *E. globulus* essential oil and 1,8-cineole

Samples	IC_{50} (mg/mL)
EGEO	>10
1,8-Cineole	>10
Vitamin C	0.017

EGEO: *Eucalyptus globulus* essential oil

Antioxidant activities of EGEOs were reported previously extensively (Akolade et al., 2012; Noumi et al., 2011; Sacchetti et al., 2005). Previous work on the radical scavenging activity of EGEO was not significantly active (Sacchetti, et al., 2005; Wei & Shibamoto, 2012). These results are in agreement with our results. Noumi and colleagues tested *in vitro* DPPH• antioxidant activity of EGEO and determined IC_{50}=57 µg/mL (Noumi et al., 2011). In another study, EGEO exhibited a weak antioxidant capacity. The activity of the tested oils is lower (IC_{50} = 33.33 ± 055 mg/mL) than that of the standard BHA (IC_{50} = 0.033 ± 0.002 mg/mL) (Harkat-Madouri et al., 2015). Fruit essential oil of *E. globulus* exhibited a weak antioxidant capacity (Said et al., 2016).

Essential oils with relatively higher monoterpenic compound amounts showed that they are relatively ineffective in many assays (Wannes et al., 2010; Dzamic et al., 2013). EGEO were rich in monohydroxylated compounds such as 1,8-cineole, which is not capable to chelate ferrous ions. α-Pinene, β-pinene, limonene, β-myrcene, terpinolene and sabinene are known to have good antioxidant properties, however, individually they may exhibit low antioxidant activity depending on the mechanism involved (do Rosario Martins et al., 2014). So, the low activity of the tested EO can be explained by the abundance of the ineffective compounds (Wannes et al., 2010).

In vitro lipoxygenase (LOX) enzyme inhibition

The oil was evaluated for its potential *in vitro* anti-inflammatory activity compared to the standard NDGA. The essential oil inhibited the enzyme with IC_{50}= 58 ± 1,41 µg/mL, while IC_{50}= 1,65 ± 0,05 µg/mL was observed for NDGA under the same conditions. The results showed that the oil is a moderate anti-inflammatory agent according to the *in vitro* evaluation.

As it is well known, inflammation plays an important role in different pathophysiological conditions. 5-LOX is a lipid peroxidase enzyme, which is found in plants and animals, it plays a role in the synthesis of strong pro-inflammatory mediators at the second major arachidonic acid pathway (Kuhn 2000; Leone et al., 2007). EGEO was previously evaluated for its 15-LOX inhibitory activity where 53% inhibition was reported at 0.5 µg/mL (Wei & Shibamoto, 2012). Also, three *Eucalyptus* oils were evaluated for their *in vivo* anti-inflammatory effects, on the rat paw edema induced assay, where neutrophil migration into rat peritoneal cavities was induced by carrageenan (Silva et al., 2003).

Conclusion

In the present study, pharmaceutical grade *E. globulus* essential oil was evaluated for its *in vitro* antibacterial, antioxidant and 5-LOX inhibitory activities. The evaluated essential oil showed *in vitro* antibacterial activity,

especially against the human pathogen *S. epidermidis*. Further detailed *in vitro* studies are required to confirm the effect of essential oil with different formulations against other pathogens as well.

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