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Combatting Drug-Resistant *Clostridium perfringens* Type B: *In vitro* effect of *Eucalyptus globulus* Essential Oil Fractions

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ABSTRACT

The development of antimicrobial resistance in *Clostridium perfringens* could pose a significant challenge to public health, necessitating the exploration of alternative treatment options such as essential oils. Essential oils have promising antimicrobial properties that need further exploration. Plant essential oils including *Eucalyptus globulus* essential oil has gained attention for its diverse therapeutic attributes. The present study aimed to evaluate the efficacy of cinnamon, clove, coriander, and Eucalyptus (*E. globulus*) essential oils. The activity of *E. globulus* essential oils fractions was evaluated in laboratory settings against 3 antibiotic-resistant *C. perfringens* type B isolates recovered from local sheep. The isolates were confirmed by polymerase chain reaction (PCR) targeting 16S rRNA gene. The Kirby-Bauer disc diffusion method was used to ascertain drug resistance. The *C. perfringens* isolates were resistant to ciprofloxacin and pefloxacin. Of the four tested essential oils, *E. globulus* exhibited the highest zone of inhibition (14 mm), followed by coriander oil (1 mm), while the Cinnamon and Clove essential oils did not show any zone of inhibition. The potential of nine distinct fractions of *E. globulus* essential oil to inhibit growth of drug-resistant *C. perfringens* was evaluated. The minimum inhibitory concentration (MIC) for the *E. globulus* oil fractions was 4-26 g/ml against antibiotic-resistant *C. perfringens*. The n-hexane+chloroform fraction was found to be the most effective with an MIC of 4.833±2.09 µg/ml suggesting *in vitro* antibacterial activity against drug-resistant *C. perfringens*.

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ZA, AAA and SA contributed to the study's conception and design. ZA conducted the experiments, collected data and wrote the first draft of the manuscript. SA supervised the study, analyzed data, critically reviewed the manuscript. AAA provided the chemical, reagents, and laboratory facility for the research work. SA and MMA critically reviewed and revised the manuscript. All authors approved the final manuscript.

Key words
Minimum inhibitory concentration, Polymerase chain reaction, Antimicrobial resistance, Gas chromatography, Mass spectrometry

INTRODUCTION

Clostridium perfringens is a Gram-positive, spore-forming, toxigenic, anaerobic, rod-shaped bacterium that is ubiquitous in nature (Johnston *et al*., 2022). It is a fast-growing bacterium found in soil, manure, rotting vegetation, and environments contaminated with feces (Bush and Vazquez-Pertejo, 2023). While it is commonly found in human and animal intestines as a part of normal gut microbiota, it has the potential to become pathogenic when the gut microbiome is perturbed due to stress caused by hunger, abrupt changes in diet, or the therapeutic use of antibiotics (Redondo *et al*., 2015; Mohiuddin *et al*., 2020).

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C. perfringens is most commonly associated with food-borne illnesses owing to its ability to produce enterotoxins. Based on toxins, *C. perfringens* can be categorized into seven toxinotypes: A through G. The bacterium produces specific toxins, such as alpha, beta, epsilon, iota, enterotoxin, beta-2, and NetB (Fernandez-Miyakawa *et al*., 2007).

C. perfringens Type B causes enteric diseases in various animals, including cattle, goats, and sheep (Uzal *et al*., 2018). In sheep, *C. perfringens* is the main cause of necro-hemorrhagic enteritis (Uzal *et al*., 2018). *C. perfringens* type B is the predominant isolate in sheep, followed by type A and type D (Nayel *et al*., 2013). *C. perfringens* type A and D are commonly isolated from clinically healthy sheep (Nayel *et al*., 2013). *C. perfringens* Type B can cause a range of diseases in sheep, including neonatal lamb dysentery, a significant cause of mortality in young lambs. Recently a case of fatal neonatal lamb gastroenteritis was reported in New Zealand (Munday *et al*., 2020). The lamb was found dead at day one of age, and a post-mortem examination revealed severe mucosal necrosis and ulceration in the small intestine, consistent

with Clostridial enteritis. The isolation and characterization confirmed the presence of *C. perfringens* type B (Munday *et al*., 2020). Another study described a case of suspected neurotoxicity in a tiger (*Panthera tigris*) caused by *C. perfringens* type B. The tiger, a 5-years old male, was presented to a veterinary clinic with neurological signs including ataxia, tremors, and seizures. A diagnosis of suspected neurotoxicity was made based on the clinical signs and the presence of *C. perfringens* type B in the tiger's feces. The tiger was treated with antibiotics and supportive care but ultimately died due to the severity of neurological signs. This case highlights the potential of *C. perfringens* type B to cause neurotoxicity in tigers, and the importance of considering this in the differential diagnosis in cases of neurological signs in captive tigers (Zeira *et al*., 2012). *C. perfringens* type B has also been found associated with multiple sclerosis in humans (Rumah *et al*., 2013).

Several antibiotics are used to treat *C. perfringens* infections in humans and animals. However, antimicrobial resistance to many of the commonly used antibiotics has been documented which renders treatment ineffective (Khanna, 2008; Beres *et al*., 2023). Plant-derived essential oils possess antibacterial properties and can be used as an alternative to antibiotics (Tariq *et al*., 2019; Liu *et al*., 2020). These essential oils are composed of volatile/phenolic chemicals and aromatic compounds (Dhifi *et al*., 2016). The phenolic compounds disrupt the proton motive force, cytoplasmic membrane, active transport, and electron flux, causing the coagulation of bacterial cells (Divya *et al*., 2014). Additionally, phenolic compounds lead to the breakdown of the lipid connections linking the protein layers, and the coagulation of cellular components in the cytoplasm (Trombetta *et al*., 2005). Essential oils function in a manner that is comparable to antibiotics target sites suggesting essential oils may substitute antibiotics (Yap *et al*., 2014). Essential oils from *Eucalyptus globulus* have antimicrobial and anticoccidial properties (Javed *et al*., 2023). The essential oil of various Eucalyptus species have antioxidant and antimicrobial properties (Almas *et al*., 2021). Eucalyptus oil's antimicrobial activity could be attributed to the presence of 1,8-cineole, α-pinene, β-pinene, and limonene. Additionally, isoborneol, a bicyclic monoterpene alcohol, present in *E. globulus* essential oil, disrupts the bacterial cell membrane and inhibits its essential enzymes hindering bacterial growth (Merghni *et al*., 2023). The present study was designed to evaluate the ability of cinnamon clove, coriander, and Eucalyptus (*E. globulus*) essential oils against antibiotic-resistant *C. perfringens* type B in laboratory settings.

MATERIALS AND METHODS

Characterization of Clostridium perfringens *isolates*

Three isolates of *Clostridium perfringens* type B were obtained from the Quality Operations Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan. The samples originated from sheep in the Lahore and Kasur districts of the Punjab Province of Pakistan. Perfringens agar base medium was used to revive the bacterium. The agar plates were incubated for 24 h at a temperature between 37 to 40ºC in anaerobic conditions. Any black and round colonies observed on the Perfringens agar base medium plates were suspected to be *C. perfringens.* The suspected colonies were subjected to Gram staining, microscopy, and biochemical testing. The colonies presumptively positive for *C. perfringens* were subjected to PCR. The bacterial DNA was extracted using a commercial DNA extraction kit (GeneAll, Seoul, Korea). Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) was used to estimate the concentration of the extracted DNA. A multiplex PCR targeting alpha, beta, and epsilon toxins genes of *C. perfringens* was set up using published primer information (van Asten *et al*., 2009). The PCR reaction mixture was composed of 12.5 µL of 2X PCR master mix (ThermoFisher Scientific, Waltham, MA, USA), 1.5 µL each of forward and reverse primers (10 pmol) for each of the three toxin genes, 2 µL of DNA template, and 7.5 µL of nuclease-free water. PCR cycling conditions were as follows: polymerase activation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were analyzed using 1.5% agarose gel containing 0.5 µg/mL ethidium bromide. The gel was visualized using a gel documentation system (BioRad, Hercules, CA, USA).

Antibiotic resistance patterns

The PCR-confirmed *C. perfringens* isolates (n=3) were tested for their susceptibility to various antibiotics. The Kirby-Bauer disc diffusion method was used following Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines. The bacterial broth suspension was adjusted to 0.5 McFarland units by measuring the optical density at 630 nm using a spectrophotometer (Neogen, Lansing, MI, USA). The inoculum was cultured on nutrient agar plates using the spread plate method. Antibiotic discs containing nalidixic acid, meropenem, cefepime, penicillin, nitrofurantoin, norfloxacin, pefloxacin, cefotaxime, linezolid, ciprofloxacin, vancomycin, amoxicillin, ceftazidime, levofloxacin, and ceftriaxone were placed on the agar plates and gently pressed using sterile forceps.

After 24 h of incubation at 37°C, the plates were examined for bacterial growth and a clear zone of inhibition (ZOI) indicating antibiotic sensitivity was noted. The diameter of the ZOI was measured to the nearest millimetre using a ruler and compared to the CLSI (2022) guidelines to determine whether the bacteria were sensitive to antibiotics (CLSI, 2022).

Determination of the activity of Eucalyptus globulus *essential oil fractions*

The study employed the broth microdilution method to evaluate the minimum inhibitory concentration (MIC) of *E. globulus* essential oil. A commercial product containing *E. globulus* essential oil (Marhaba Laboratories Private Limited, Lahore, Pakistan) was procured from the local market. Column chromatography was used to fractionate the essential oils according to increasing solvent polarity, using silica gel as the stationary phase, as previously described (Moein *et al*., 2015; Kaur *et al*., 2019). Different solvents, including n-hexane (14.15 µg/ml), chloroform (6.96 µg/ml), ethyl acetate (18.64 µg/ml), methanol (19.16 µg/ml), and acetonitrile (26.87 µg/ml), along with their combinations, were used to elute the essential oil.

The MIC determinations were made by using 96-well microtiter plates. Serial dilution of essential oil was prepared in liquid Perfringens agar base (PAB) medium. Test bacterial strains were suspended in the medium. McFarland standard 0.5 units was used to adjust the cell densities through a spectrophotometric method. A 96-well microtiter plate was prepared by adding 0.1 mL PAB broth to each well. One hundred microliters of test extracts were added in the first well and a two-fold dilution of oil extract was made by using a micropipette up to the 10th well. The microtiter plate was inoculated with approximately 0.1 mL of the bacterial inoculum up to the 11th well and incubated anaerobically at 37°C for 24 to 48 h. Optical density was measured at 0 and 24 h. The 11th well served as the positive control, while the 12th well was the negative control.

Gas chromatography and mass spectrometry

To analyse the samples using gas chromatography and mass spectrometry (GCMS), a CARBOWAX capillary column was utilized along with helium as the carrier gas. An injector was heated to 260 ºC, and *E. globulus* essential oil's n-hexane and n-hexane + chloroform solvent fractions were injected at a rate of 1 µL/min (Kaur *et al*., 2019). The identification of active compounds in the test samples was accomplished by comparing their retention time to that of standard compounds.

Statistical analysis

Data based on the zones of inhibition and MIC values of oil fractions were analyzed using one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test. Statistical Package for Social Sciences (SPSS) version 20.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The level of significance was set at 0.05.

RESULTS

The *Clostridium perfringens* type B isolates, formed characteristic black, round colonies due to sulphite reduction, when cultured on Perfringens agar base medium under anaerobic conditions at 37–40°C for 24 h. Gram staining revealed Gram-positive, rod-shaped bacilli with blunt ends. Microscopic examination showed non-motile, short chains or single cells with a thick peptidoglycan layer.

Characterization of isolates

The PCR targeting alpha (324 bp), beta 158 (192 bp), and epsilon (376 bp) toxin genes of *C. perfringens* indicated the presence of suspect bacterium (Fig. 1).

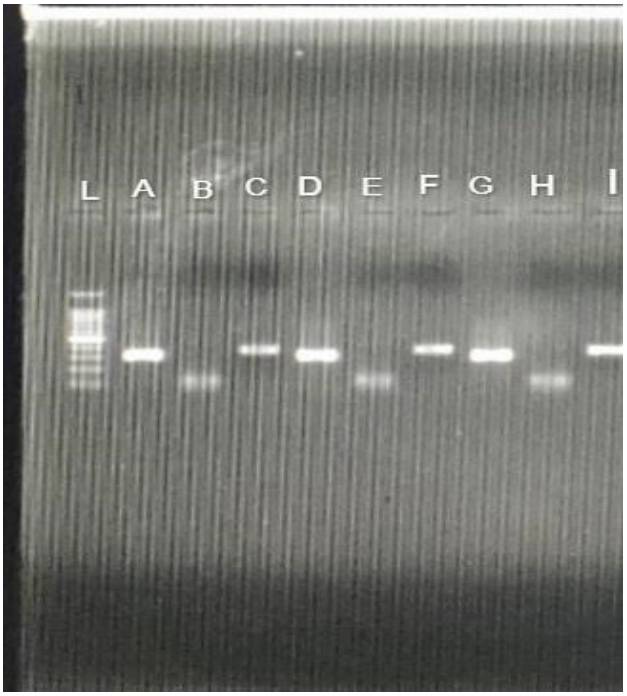

Fig. 1. Gel image of *Eucalyptus globulus* 16S rRNA PCR. Column L shows the 100 bp ladder. Column A is the Alpha gene of 324 bp. Column B is the Beta gene of 192 bp. Column C shows the bands of the Epsilon gene at 376 bp. Columns A-C, D-F, and G-I correspond to Alpha, Beta, and Epsilon genes, respectively of *Clostridium perfringens* Type B isolates CZ-01, CZ-02, and CZ-03, respectively.

Table I. Evaluation of antibiotic activity against *Clostridium perfringens* type B.

Antibiotics	Concentration	CZ-01 (mm)	CZ-02 (mm)	CZ-03 (mm)	Mean ±S.D	Sensitive/ Resistant
Nalidixic acid	30µg	18	20	23	20±2.51^{ab}	Sensitive
Meropenum	10 µg	50	58	60	56±5.29^{f}	Sensitive
Cefepime	30 µg	37	35	46	39±5.85^{c}	Sensitive
Penicillin	10 µg	78	69	78	75±5.1^{g}	Sensitive
Nitrofurentoin	300 µg	39	42	44	41.66±2.51^{cd}	Sensitive
Norfloxacin	10 µg	21	20	20	20±0.57^{ab}	Sensitive
Pefloxacin	5mcg	12	13	20	15±4.35^{a}	Resistant
Cefotaxime	30 µg	52	50	57	53±3.60^{ef}	Sensitive
Linezolid	30 µg	35	43	50	42.66±7.50^{cd}	Sensitive
Ciprofloxacin	5 µg	23	25	28	25±2.51^{b}	Resistant
Vancomycin	30 µg	45	48	50	47.66±2.51^{de}	Sensitive
Amoxicillin	30 µg	54	59	60	57.66±3.21^{f}	Sensitive
Ceftazidime	30 µg	43	46	48	45.66±2.51^{abc}	Sensitive
Levofloxacin	5 µg	24	22	30	25±4.16^{b}	Sensitive
Ceftriaxone	30 µg	48	59	60	55.66±6.65^{f}	Sensitive

*Means with different superscripts differ significantly.

Antibiotic resistance profile and well diffusion assay

The highest zone of inhibition (ZOI) was demonstrated by penicillin (75±5.1 mm) followed by amoxicillin (57.66±3.21 mm). The least ZOI was noted for pefloxacin (15±4.35 mm) followed by ciprofloxacin (25±2.51 mm). The resistance patterns against various antibiotics are presented in Table I.

The well diffusion assay demonstrated a distinct zone of inhibition (14 mm) around the *Eucalyptus globulus* in comparison to the cinnamon oil, clove oil, and coriander oil (Fig. 2).

Activity of E. globulus *oil fractions*

Nine different fractions of the *E. globulus* oil were evaluated in this study. The n-hexane+chloroform fraction demonstrated the least MIC (4.833±2.09 µg/ml) followed by the chloroform fraction (6.96±0.00 µg/ml). These fractions were further processed for GCMS analysis. The highest MIC was noted for the acetonitrile fraction (26.87±0.00 µg/ml) followed by the chloroform + ethyl acetate (23.22±8.04 µg/ml) fraction. The MICs of various fractions are presented in Table II.

Gas chromatography and mass spectrometry analysis

For component analysis and identification of the concentration of an active chemical in the n-hexane+ chloroform fraction, gas chromatography and mass spectrometry (GCMS) were performed. The identification and quantification of components by GCMS were based on comparing the mass spectrum of the *E. globulus* essential oil with the spectra reported previously. The concentrations of the active components found in the essential oil fractions after GCMS are presented in Table III.

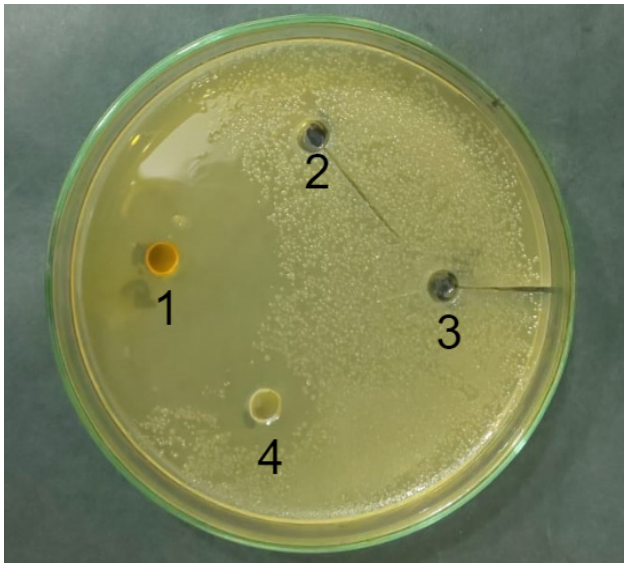

Fig. 2. The zone of inhibitions of *Eucalyptus globulus* essential oil in a well diffusion assay. A distinct zone of inhibition (14 mm) of *E. globulus* (Well. # 1); No zones of inhibition (0 mm) of cinnamon essential oil and clove essential oil (Well # 2 and 3, respectively); Very limited zone of inhibition (1 mm) of coriander oil (Well # 4).

Table II. Mean MIC Values of *Eucalyptus globulus* oil fractions.

Mean MIC values of *Eucalyptus globulus* oil fractions				
Solvent fraction	**MIC values**			**Mean ±S.D**
	Iso-01	**Iso-02**	**Iso-03**	
n-hexane	14.15	14.15	14.15	14.10 ± 0.00^{ab}
n-hexane+chloroform	3.625	7.25	3.625	4.833 ± 2.09^{a}
Chloroform	6.96	6.96	6.96	$6.96\pm0.0^{0}a$
Chloroform+ ethyl acetate	27.87	27.87	13.93	$23.22\pm8.0^{4b}c$
Ethyl acetate	27.96	13.98	13.98	18.64 ± 8.07^{bc}
Ethyl acetate+methanol	14	28	14	18.66 ± 8.08^{bc}
Methanol	28.75	14.37	14.37	19.16 ± 8.30^{bc}
Methanol+acetonitrile	7.1	14.37	14.37	19.16 ± 8.30^{bc}
Acetonitrile	26.87	26.87	26.87	$26.87\pm0.0^{0}c$

*Means with different superscripts differ significantly. The inhibitory effect observed in the study is primarily attributed to the active compounds present in the essential oil of Eucalyptus globulus, not the solvents used for fractionation. The main components, such as Bicyclo(2,2,1)heptan-2-ol,1,7,7-trimethyl, Benzene, Isoborneol, and Bicyclo(2,2,1)heptan-2-ol, have been identified through GCMS analysis and are known for their antimicrobial properties. The effectiveness of the Eucalyptus globulus essential oil against antibiotic-resistant Clostridium perfringens is mainly due to these bioactive components present in the oil, indicating that the inhibitory effect is a result of the essential oil itself.

The bicyclo(2.2.1)heptan-2-ol, 1,7,7-trimethyl fraction had the highest concentration (14.4%), followed by cyclohexane (13%), benzene (9.5%), and o-cymene (6.4%). The lowest concentration was noted for camphor (0.39%) followed by exo-2,7,7-trimethylbicyclo (2.2.1) heptan-2-ol (0.62%), and Terpinene (0.62%) fractions.

DISCUSSION

The emergence of antimicrobial resistance in *Clostridium perfringens* has highlighted the need for alternative treatment strategies. The present study was conducted to evaluate the effectiveness of cinnamon, clove, coriander, and eucalyptus (*E. globulus*) essential oils against antibiotic-resistant *C. perfringens* type B in laboratory settings. The study results indicated that *E. globulus* is effective against *C. perfringens* with an MIC of 4.833±2.09 µg/ml. The study highlights the potential antimicrobial of *E. globulus* essential oil against drug-resistant *C. perfringens* type B. By focusing on the most effective fraction, n-hexane+chloroform, we present *in vitro* evidence that *E. globulus* essential oil components can be effective against antimicrobial-resistant *C. perfringens* type B.

Table III. Concentrations and retention time of the active compounds of *Eucalyptus globulus* essential oil fractions.

Name	Retention time	Percentage concentration
Cyclohexane	6.1	13
Bicyclo(2.2.1)heptane, 2,2-dimethyl-3-methylene	13.6	4.5
7-Oxabicyclo(2.2.1)heptane,	15	0.87
1,3,5-cycloheptatriene	15.1	0.70
o-cymene	15.3	6.4
Cyclohexanol	15.4	1.48
Eucalyptol	15.54	5.71
Terpinene	16	0.62
Benzene	16.68	3.6
Bicyclo(2.2.1)heptane	16.76	1.7
Bicyclo(2.2.1)heptan-2-one	17.0	4.9
Bicyclo (2.2.1)heptan-2-ol	17.3	8.7
exo-2,7,7- trimethylbicyclo (2.2.1) heptan-2-ol	17.8	0.62
Camphor	18	0.39
Isoborneol	18.28	5.35
Bicyclo(2.2.1)heptan-2-ol, 1,7,7-trimethyl	18.42	14.4
Benzene	18.59	9.5
L-α-terpineol	18.7	1.5
Acetaldehyde	19	0.72
Bicyclo (3.1.0) hexane	19.15	3.9
Cinnamaldehyde	20.1	1
Octanal	21.2	0.9
(1,1'-Bicyclopropyl)-2-octanoic acid	21.4	1.7

The antimicrobial resistance findings of the present study are consistent with Slavić *et al*. (2011) who investigated the minimal inhibitory concentrations (MICs) of 12 antimicrobials against *C. perfringens* isolates of cattle, swine, turkeys, and chickens -origin and reported reduced susceptibility to bacitracin, clindamycin, erythromycin, florfenicol, and tetracycline (Slavić *et al*., 2011). The present study's results confirm the development of antibiotic resistance in *C. perfringens*. The development of antimicrobial resistance in *C. perfringens*, consistent across both studies, have implications for both animal and human health. In livestock, reduced susceptibility to key antibiotics jeopardizes the effectiveness of treatments, potentially leading to more severe infections and prolonged

recovery periods. Moreover, the zoonotic potential of *C. perfringens* raises alarms, as resistant strains can be transmitted from animals to humans highlighting the risk of antibiotic-resistant infections in both populations (Bendary *et al*., 2022).

The present study results of antimicrobial activity of *E. globulus* essential oil corroborate Ávila *et al*. (2023) findings who have reported antibacterial activity of aromatic plant ethanolic extracts against *Clostridium* spp. (Ávila *et al*., 2023). The present study also confirms the results of a Chinese study suggesting the antimicrobial activity of globulol, a compound found in *E. globulus* Labill plant. The study evaluated the antimicrobial activity of globulol against a range of microorganisms, including bacteria and fungi. The results indicated that globulol could be a main antimicrobial compound in the ethanol extract of *E. globulus* (Tan *et al*., 2008). The present study's findings are also consistent with those of Ullah *et al*. (2021) who reported the antimicrobial activity of *Eucalyptus* essential oil against *C. perfringens* (Ullah *et al*., 2021).

In the present study, the n-hexane+chloroform fraction of *E. globulus* oil was found to have the least MIC (highest antibacterial activity) value of 4.833±2.09 µg/ml. The GCMS of the n-hexane+chloroform fraction revealed that bicyclo (2.2.1) heptan-2-ol, 1,7,7-trimethyl had the highest concentration (14.4%), followed by cyclohexane (13%), benzene (9.5%), and o-cymene (6.4%). A similar study using GCMS analysis revealed that the main chemical compounds in *E. globulus* were oxygenated monoterpenes (78.58%), 1,8-cineole (55.29%), spathulenol (7.44%), and a-terpineol (5.46%) (Harkat-Madouri *et al*., 2015). Findings of another GCMS analysis of *E. globulus* essential oil fraction revealed following key compounds: 1.8-cineole (22.35%), limonene, (7.01%), solanol (6.05%), p-pinene (5.20%), transverbenol (4.02%), terpinen-4-ol (3.10%), aristolene (2.35%), terpinyl acetate (2.10%), isosativene (1.85%), sabinene (1.49%), (alpha)-myrcene (1.15%), and a-terpineol (1.10%) (Jerbi *et al*., 2017). The active compounds suggested by GCMS in the present study were different from those reported by (Harkat-Madouri *et al*., 2015; Čmiková *et al*., 2023). The observed differences in the active compounds between studies can be attributed to variations in the plant's chemical composition owing to geographical differences and the use of different solvents for GCMS analysis (Almas *et al*., 2019). Environmental factors in different geographical regions influence the plant's natural chemical makeup, while varying solvents selectively extract different compounds from the essential oil, thus leading to variations in the identified components (Harkat-Madouri *et al*., 2015; Jerbi *et al*., 2017).

The fraction n-hexane+chloroform demonstrated the lowest MIC against the tested isolate: 4.833±2.09µg/ml. The MIC results suggest that the *E. globulus* oil can be used as an alternative to antibacterial agents. The major components of *E. globulus*, bicyclo (2,2,1) heptan-2-ol,1,7,7-trimethyl, benzene, isoborneol, bicyclo (2,2,1) heptan-2-ol, contribute to the antimicrobial activity corroborating Ali *et al*. (2023), who have also reported the antimicrobial activity of this component (Ali *et al*., 2023).

The mechanism of action of bicyclo (2,2,1) heptan-2-ol,1,7,7-trimethyl, benzene, isoborneol, and bicyclo(2,2,1) heptan-2-ol, as an antibacterial agent in *E. globulus* essential oil, is likely multifaceted. Bicyclo (2,2,1) heptan-2-ol, also known as α-terpineol, disrupts bacterial cell membrane, increases its permeability, and inhibits its protein synthesis, impeding bacterial growth. Benzene, as an aromatic hydrocarbon, may contribute to overall antimicrobial activity, although its exact mechanism of action remains unclear. Isoborneol present in *E. globulus* essential oil, disrupts the bacterial cell membrane and inhibits essential enzymes, hindering bacterial growth (Rajput *et al*., 2023).

Some of the limitations of the present study include, no assessment of the cytotoxicity of *E. globulus* essential oil fractions. Moreover, the study did not determine the activity of *E. globulus* essential oil against extensive drug-resistant strain of *C. perfringens*. These aspects require further investigation. Besides, therapeutic implications of the current study could be clarified by additional *in vivo* investigations on the effectiveness of *E. globulus* essential oil in treating *C. perfringens* type B infections in humans and animals (Assaggaf *et al*., 2022).

Future studies could evaluate the active ingredient of *Eucalyptus globulus* essential oil, bicyclo (2,2,1) heptan-2-ol,1,7,7-trimethyl, in the n-hexane+chloform fraction, for resistance modulation studies (Zielińska-Błajet and Feder-Kubis, 2020). Moreover, *Eucalyptus globulus* essential oils should be evaluated for oral administration in humans and poultry (Ahlem *et al*., 2009; Ullah *et al*., 2021) to prevent or control bacterial infections.

CONCLUSIONS

The present study indicates that *Eucalyptus globulus* is a promising antibacterial agent, and its n hexane+chloform fraction have *in vitro* antibacterial activity against *C. perfringens* type B. *E. globulus* oil can be used as an alternative to antibiotics.

DECLARATIONS

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Statement of conflict of interest

The authors have declared that there is no conflict of interest.

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