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Original Article

Antibacterial Efficacy of Eucalyptus Essential Oil against Respiratory Infection Pathogens and Characterization of its Bioactive Compounds

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ABSTRACT

Antibacterial activity of eucalyptus essential oils (EEO) against some respiratory pathogens and its mechanism of action were studied. Clinical pathogens used in the study were *Enterobacter cloacae, Klebsiella pneumoniae, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Disk diffusion, macro and microbroth dilution methods were respectively used to assess antibacterial assay, minimum inhibitory concentration (MIC) and growth inhibition assay using standard antibiotics as references. Gas chromatography Mass spectrometry was used to characterize the bioactive compounds present in EEO. Scanning electron microscopy was used to determine the mode of action of EEO on test pathogens. A total of 12 compounds were found to have bioactive functions with p-Cymene having 34.07%, gamma-Terpinene (7.01%), Cyclohexasiloxane (5.30%), 2-Aminobenzoic acid (4.59%). The EEO exerts varying antibacterial effects on susceptible organisms. At 100 mg/ml concentration the EEO antibacterial activities were observed on *E. cloacae* with 23.0 mm mean zone of inhibition, *K. pneumoniae* (22.7 mm) and *S. aureus* (16.0 mm) while no activity was recorded on *P. aeruginosa*. The MIC and MBC of the EEO on susceptible organisms were *E. cloacae* (6.25mg/ml, 12.5mg/ml); *S. aureus* (25mg/ml. 50mg/ml) respectively, while both MIC and MBC of EEO on *K. pneumoniae* was 50mg/ml. Scanning electron microscopy displayed noticeable damages of cell morphology and ultrastructure on two most susceptible pathogens (*E. cloacae and K. pneumoniae*), thus increase cell permeability and subsequent cell death. This study showed EEO is a potent antibacterials against some respiratory pathogens and can be further explored in treating respiratory and other infections caused by these pathogens. The positive effect of eucalyptus essential oil on the selected respiratory infection pathogens could make it an important part of drug (ointment formation) for the treatment of such infections.

Keywords: Essential Oils; Bioactive Compounds; Respiratory Pathogens; Susceptibility; GCMS

INTRODUCTION

The rate of proliferation of resistant clinical isolates worldwide has led to the search for new antimicrobial agents[1,2]. Meanwhile, the use of health threatening chemicals as antimicrobial agents has been heavily criticized due to the negative impact on humans and animals which made researchers focus their search on natural antimicrobial agents[3]. Of the many novel areas being explored is the use of medicinal plant parts, herbs, and spices which have reportedly shown positive results. Hence, their proposal as a significant source of natural antimicrobials and as sustainable alternatives to synthetic drugs to treat bacterial infections[4]. Essential oils (EOs) extract from different medicinal plant parts (leaves, peels, bark, roots, seeds, and flowers) are complex mixtures of phytochemical comprising of phenols, terpenes, ketonic bodies, terpenoids, carotenoids, curcumins, coumarins and aldehydes. These are classified as plant secondary metabolite and are responsible for their antimicrobial properties[5–8].

Eucalyptus is a member of the *Myrtaceae*, and a well-known medicinal plant with more than 400 species. Members of this family are a rich source of polyphenols and terpenoids, with eucalyptol or cineol as its main composition. They are important source of EOs with vast proven biological activities including antibacterial, antifungal, anti-inflammatory, antioxidant and antiviral[9]. The EO extracted

from eucalyptus leaves is widely used as disinfectant, also to reduce the symptoms of cough, congestion, sore throat, wound healing, antibacterial, etc. [10,11].

Although, the effect of medicinal plant EOs on respiratory infections caused by *Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus* has all been previously reported [12–14]. However, there is dearth of information on the bioactive components of the oils and their mode of actions on the susceptible bacteria. Hence, this present study aims to investigate the antimicrobial efficacy of Eucalyptus EO on respiratory infection causing bacteria; determine its bioactive compounds and their collective mode of actions on the susceptible organisms.

MATERIALS AND METHODS

Collection of samples and test isolates

Samples of eucalyptus leaves were collected around the University of Ilorin campus. Clinical test isolates used were collected from the organisms' bank of the University of Ilorin Teaching Hospital, Ilorin, Nigeria and they include *Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus*, which were Standard biochemical tests were carried out to further confirm the identity of the test isolates according to the Bergey's manual of systematics of Archaea and Bacteria [15].

Extraction and preparation of Essential oil concentrations

Eucalyptus essential oil (EEO) was extracted from fresh leaves by hydro-distillation method as described by Umereweneza et al. [16]. A 2-l flask containing 500 g of chopped and homogenized leaves material was heated for 3 hours and the vapor was condensed and separated throughout an oil/water separator. Crude essential oil extract was collected and used for further experiments. Concentration preparation was done using 0.5% (v/v) tween 80. The stock essential oil concentration prepared was 100 mg/ml.

Characterization of bioactive compounds

Gas Chromatography-Mass Spectrometry using SHIMADZU QP2014 ULTRA apparatus operated in EI mode at 70ev. A Restek-5MS column (30 m x 0.25 mm x 0.25 μm) capillary column coated with non-polar cross-linked fused silica was used. The oven temperature was maintained at 40 °C for 1min and then increased progressively to 70 °C at the rate of 3 °C /min. After 1 min, the temperature was again increased at a rate of 15 °C /min from 70 °C for 1 min, and then increased to 220 °C for 10 min before sample injection. Helium was used as a carrier gas at the flowrate of 1.2 mL/min. To enhance the sensitivity for minor constituents, 10 % (v/v) solution of each essential oil in hexane were prepared. While the major constituents were determined using a 1 % (v/v) solution of essential oil in hexane. To conduct chemical analysis, 1μl of the solution was injected at 220 °C and the effluent obtained from GC column was directly introduced into mass spectrometer with m/z 5-500 mass range. Scanning was done at an interval of 0.5 sec with a scanning speed of 1000 amu/s and ionization voltage of 70eV. Identification of components was based on computer library software and by comparing the obtained retention time indices (RI) with those from the literature [17]. Quantification of different constituents, expressed in percentage, was done by peak area normalization measurements.

Antibacterial activity of Essential oil and standard antibiotics disk on selected pathogens

The antimicrobial activity of EEO was determined using the agar disc diffusion method as described by Wimonrut and Chahomchuen [18]. Inoculum standardization was done by adjusting bacterial cell suspension to 0.5 McFarland to obtain approximately 1.5 × 108 CFU/ml cell number. The bacterial suspension was spread uniformly onto the surface of Mueller Hinton agar in a sterile Petri dish using a sterile cotton swab. The surface of the medium was allowed to dry. Sterile filter paper discs (6 mm in diameter) impregnated with 100 mg/ml of EEO were then placed aseptically on the surface of these agar plates and were incubated at 37°C for 24 hours. Diameter of zone of clearance observed was measured and recorded accordingly.

Antibiotics susceptibility test was carried out on all test pathogens using disk diffusion method. Standard antibiotics disks used for this study were Gram positive and Gram negative disks from Abtek Biologicals Ltd, UK.

Determination of minimum inhibitory concentration and minimum bactericidal concentration (MIC and MBC)

The MIC was determined using the method reported by Asiaei et al. [19] against the test organisms. One milliliter of different EEO concentrations (100, 50, 25, 12.5, 6.25 and 3.13mg/ml) was added each to 1 ml of sterile nutrient broth in different test tubes respectively. Fifty microliter (50 μl) of eighteen hours old culture (adjusted to 0.5 MacFarland standard) of each organism was respectively inoculated into each EEO concentration and incubated at 37°C for 24 hours. Control tubes included growth medium and test isolates while a blank was set containing only sterile broth. The tube with the lowest concentration of the EEO which had no detectable bacterial growth or turbidity when visually compared with the control tube was considered the MIC. The MBC was the lowest concentration of EEO which showed no growth on the growth medium after 24 hrs of incubation.

Growth inhibition curve

Growth inhibition curve was determined using the microdilution method in 96-well microplates[20] . A 100 µl of Mueller Hinton (MH) broth was aseptically dispensed into individual wells. Thereafter, a 100 µl of EEO at 100 mg/ml was introduced into the first well and two-fold serial dilutions of the oil was made using concentrations ranging from 100 to 3.13 mg/ml in consecutive wells to yield final cell plate volumes of 100 µl. Ten (10) µl standardized inoculum of the different test pathogens were introduced separately into the wells. As a negative control, 100 µl of MH broth/well were used while the positive control was a 100 µl of the bacterial inoculum without the oil. The microplates were then incubated at 37°C for 24 hours. After incubation, the OD was measured using a microplate reader at 600 nm.

Scanning electron microscopy (SEM)

To assess the mode of action of EEO on bacterial cells, SEM analysis was carried out according to the method Moghayedi et al.[21] with slight modifications. The morphology of bacterial pathogens before and after treatment with EEO was analyzed and checked using SEM images. In this assay, susceptible test pathogens were cultured and treated with EEO at MIC in broth medium and were incubated using shaker incubator at 37 °C while control culture without EEO were also carried out at same condition. After incubation, cell suspension was centrifuged at 5000 g for 10 minutes. The cell pellet was collected and washed twice with 0.1 M phosphate-buffered solution. The suspension was filtered and fixed in a 2.5% glutaraldehyde solution and kept at 4 °C for 2 hours. After several washing with double-distilled water the sample was dehydrated successively with different ethanol solutions (30%, 50%, 70%, 80%, 90% and 100%) for 10 min each. Finally, the samples were dried, coated with gold and examined under JEOL JSM–7600F field-emission SEM, USA.

Statistical analysis

All experiments were performed in three replicates. Results were analysed by one-way ANOVA using statistical package for social science (SPSS) software (version 20.0) and Tukey range test was used to measure the differences between data means at 95% confident level ($P < 0.05$).

RESULTS

Eucalyptus essential oil chemical composition

GC-MS analysis of EEO revealed the presence of compounds with antibacterial properties and the respective percentage peak area (Table 1). The major compound, p-Cymene was most abundant (34.07%); while other main compounds such as gamma-Terpinene (7.01%), Cyclohexasiloxane (5.30%), 2-Aminobenzoic acid (4.59%), and Benzoic acid (1.42%) were also identified. The spectrum of the GC-MS analysis is presented in Figure 1.

Table 1: Bioactive compounds found in Eucalyptus Essential Oil

S/N	Compound name	Retention time	% area
1	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)	4.620	0.20
2	p-Cymene	6.522	34.07
3	gamma-Terpinene	7.016	7.01
4	2-Carene	7.454	0.32
5	Benzoic acid	9.005	1.42
6	Carbamic acid	9.099	0.37
7	Acetic acid	9.130	0.70
8	Cyclohexasiloxane	9.393	5.30
9	Acetamide	10.500	0.22
10	p-Anisic acid	10.713	0.91
11	2-Aminobenzoic acid	11.051	4.59
12	Imidazole	12.320	0.53

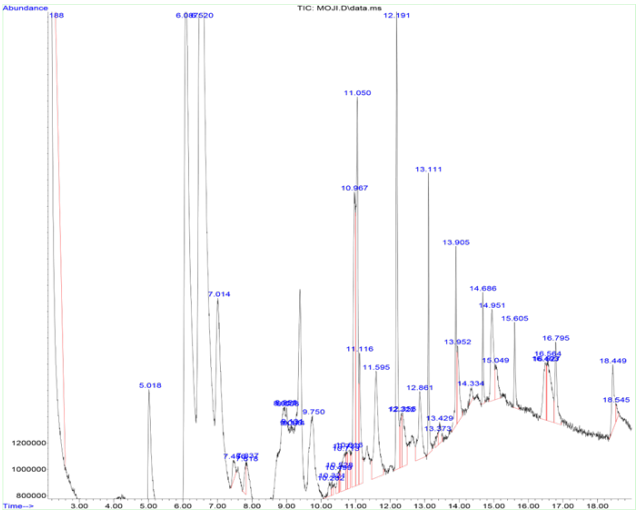

Fig. 1: Spectrum of GC-MS analysis of eucalyptus essential oil

Antibacterial assay of EEO and standard antibiotics on test pathogens

Antibacterial activity of EEO on test pathogens is presented in Table 2. It was observed that EEO at concentration 100 mg/ml had inhibitory effect against three of the four pathogens assessed in which case, the degree of inhibition varied depending on pathogens. The highest mean zone of inhibition was obtained in *E. cloacae* (23.0mm); K. pneumoniae had 22.7(mm) while *S. aureus* was 16.0 (mm). When compared with standard antibiotics, NIT, GEN and OFL all showed a better activity against *E. cloacae*

with 31.7mm, 25.3mm and 23.3mm zone of inhibitions respectively. NIT and OFL also had better activity than EEO against *K. pneumoniae* while only NIT was better than EEO when tested against *S. aureus. P. aeruginosa* was resistant to all antibiotics and EEO.

Determination of minimum inhibitory concentration and minimum bactericidal concentration (MIC and MBC)

The results of MIC and MBC of EEO was presented in Table 3. For *E. cloacae*, the MIC was observed at concentration 6.25 mg/ml; while MBC was at 12.5 mg/ml. For *K. pneumoniae*, MIC and MBC were at 50 mg/ml of the oil. The MIC of EEO against *S. aureus* was observed at 25 mg/ml; while MBC was at 50 mg/ml.

Growth inhibition curve

The growth inhibition curve of EEO against different test pathogens are presented in Figure 2. It was observed that EEO inhibited the growth of *E. cloacae* at all concentrations recording >50% inhibition rate. For *K. pneumoniae*, only concentrations 50 and 100 (mg/ml) recorded >50% inhibition rate while for *S. aureus,* 25, 50 and 100 (mg/ml) all recorded >50% inhibition rate.

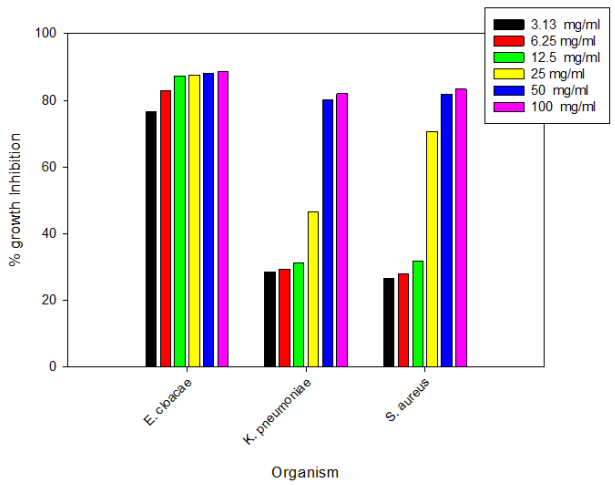

Fig. 2: Growth inhibition curve

Scanning electron microscopy

The electron micrographs of both untreated (control) and EEO treated bacterial cells for the two most susceptible pathogens (*E. cloacae and K. pneumoniae*) are presented in Figure 3 . In control group, the untreated bacterial cells showed their typical structures. In contrast, detrimental effects on the morphology of cell membranes were observed when cells were treated with EEO after 24 hours at the MIC values for both pathogens. Microstructural observations demonstrated that EEO caused an increase in the permeability of the cells and disrupted the membrane integrity. An incomplete and deformed shapes were observed in treated cells.

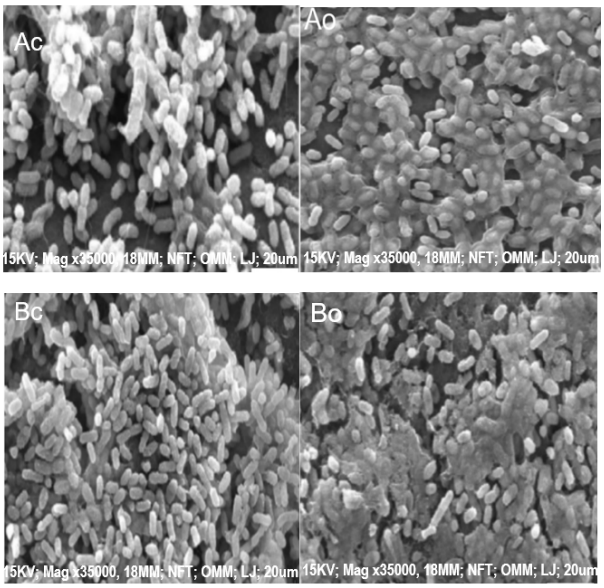

Fig. 3: Ac is the SEM image of *E. cloacae* without EEO; Ao is *E. cloacae* treated with essential oil; Bc is *K. pnuemoniae* without EEO, Bo is *K. pnuemoniae* treated with essential oil

DISCUSSION

The compounds revealed from the GCSM analysis of EEO was similar to those reported by Puvača et al.[22] who opined that gamma-Terpinene and p-Cymene were the main compounds found in Eucalyptus essential oil. The difference in chemical compositions of EO could be as a result of variation in environmental conditions, harvest period, variety type, growth stage of the medicinal herb. These bioactive compounds are responsible for the superb antibacterial effects shown against test pathogens. Özen et al.[23] and Jabbari et al.[24] had respectively reported that 2-Aminobenzoic acid and cyclohexasiloxane showed great antibacterial properties.

In this present study, *P. aeruginosa* showed no zone of inhibition when challenged with various concentrations of EEO. This was also reported by Behbahani et al.[25], where *P. aeruginosa* used showed resistance to EEO at all concentrations. However, Limam et al.[26] observed that *P. aeruginosa* was susceptible to higher concentrations of EEO. The differences in the reports could be due to the type of strains used and/or the obvious concentration difference as reported by Limam et al.[26] . The result of EEO when compared with standard drugs revealed

Table 2: Antibacterial assay of EEO and standard antibiotics on test pathogens

Antibiotics/ Essential oil	Zone of inhibition (mm) Mean ± SD			
	E. cloacae	*K. pneumoniae*	*S. aureus*	*P. aeruginosa*
EO (100 mg/ml)	23.0 ± 3.61	22.7 ± 2.83	16.0 ± 2.65	0.0 ± 0.00
CAZ	0.0 ± 0.00	0.0 ± 0.00	NA	0.0 ± 0.00
CRX	0.0 ± 0.00	0.0 ± 0.00	NA	0.0 ± 0.00
GEN	25.3 ± 1.15	0.0 ± 0.00	10.3 ± 1.52	0.0 ± 0.00
CPR	22.7 ± 1.15	19.7 ± 2.52	0.0 ± 0.00	0.0 ± 0.00
OFL	23.3 ± 1.15	24.0 ± 2.00	NA	0.0 ± 0.00
AUG	0.0 ± 0.00	0.0 ± 0.00	NA	0.0 ± 0.00
NIT	31.7 ± 5.69	24.3 ± 2.08	34.0 ± 4.00	0.0 ± 0.00
AMP	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0 .0± 0.00
E	NA	NA	0.0 ± 0.00	NA
TET	NA	NA	12.7 ± 0.58	NA
PEN	NA	NA	0.0 ± 0.00	NA

Keys: EO= Essential oil; NA= Not applicable; CAZ= ceftazidime; CRX= cefuroxime; GEN= gentamycin; CPR= ciprofloxacin; OFL= ofloxacin; AUG= augmentin; NIT= Nitrofurantoin; AMP= ampicillin; E= erythromycin; TET= Tetracycline; PEN= Peniclin

Table 3: Minimum inhibitory and bactericidal concentrations

Isolate	Concentration (mg/ml)						MBC (mg/ml)
	100	50	25	12.5	6.25	3.13	
Enterobacter cloacae	**NG**	**NG**	**NG**	**NG**	**NG**	**G**	**12.5**
Klebsiella pneumoniae	**NG**	**NG**	**G**	**G**	**G**	**G**	**50**
Staphylococcus aureus	**NG**	**NG**	**NG**	**G**	**G**	**G**	**50**

Keys: NG = No growth; G = Growth

that only Nitrofurantoin (NIT) and Oflaxacin (OFL) had better inhibition than EEO against both *E. cloacae* and *K. pneumoniae*. It was also observed that none of the antibiotics including EEO was effective against *P. aeruginosa*. Observations in this study about the comparative advantage of EEO over some standard antibiotics have been earlier noted. Mumu and Hossain[27] concluded that EOs compared favorably with standard drug used except in few cases where highest activities were obtained on some antibiotics. As reported in our study, they observed that the zones of inhibition obtained for EEO around susceptible organisms were better than many of the antibiotics. The higher antibacterial potential displayed by essential oil could be directly linked to their main chemical constituents or with the interaction among the minor and major components of the oil[28].

Earlier reports had made similar observations regarding the Minimum inhibitory and bactericidal concentrations of EEO against tested organisms; albeit with little variations. Merghni et al.[29] reported a MIC of 10 mg/ml for eucalyptus oil against *S. aureus*; Bogavac et al.[30] also reported MIC against *S. aureus* at concentration 6.25 μl/ml. More interestingly, Puvaca et al.[2] reported the MIC and MBC of eucalyptus against *E. coli* were obtained at a lower concentration 2.9 mg/ml and 5.8 mg/ml respectively. This difference in the MIC and MBC is probably due to the type of pathogen, as well as the bioactive compounds composition of the EEO employed in the different studies.

Similar growth inhibition result was reported by Clerck et al.[31] where obtained >50% growth inhibition using several essential oils including eucalyptus oil.

The electron micrographs of damaged cells and the considerable increase of the cell constituents' release showed that EEO affected the cell membrane entirety. Also, the distorted cell shapes and membranes leads to cytoplasm secretion and cell death. The results of this study were consistent with those of Behbahani et al.[25] who assessed the antibacterial efficacies of eucalyptus oil on selected pathogens and Moghayedi et al.[21] who assessed the antibacterial activities of ethylene glycol as a common solvent against selected pathogens.

CONCLUSION

This study showed that eucalyptus essential oil had antimicrobial activity, which varied according to test pathogens. From the result obtained against *E. cloacae*, the percentage inhibition rate of the EEO even at a very low concentration of 3.13 mg/ml was above 50%. The data suggest that the EEO are potentially a good source of antibacterial agents as it compared favourably with many of the antibiotics tested against the test pathogens. The inability of EEO and all the antibiotics tested against *P. aeruginosa* to show any activity is as a result of the resistant nature of the pathogens. The

electron micrographs obtained revealed that EEO caused an increase in the permeability of the cells and disrupted the membrane integrity.

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