JOPAT Vol 22(2), 1121 - 1149, July - December. 2023 Edition
ISSN2636 – 5448 https://dx.doi.org/10.4314/jopat.v22i2.4

IN SILICO STUDY AND BIOPROSPECTION OF THE ANTIBACTERIAL AND ANTIOXIDANT EFFECT OF THE ESSENTIAL OILS OF *Cymbopogon citratus* AND *Zingiber officinale* AGAINST BACTERIA ASSOCIATED WITH OTITIS MEDIA IN CHILDREN.

***A.O. KOLAWOLE[1], T. M. OBUOTOR[1], F. O. ADEYANJU[1], E.O. ONI[1] AND Y.M. FERUK – BELLO[2]**

[1]Department of Microbiology, College of Biosciences, Federal University of Agriculture Abeokuta, Nigeria.

[2] Department of Microbiology, Faculty of Natural and Applied Science, Hallmark University Ijebu – Itele, Ogun State.

ABSTRACT

This present study was conducted to investigate the efficacy of the essential oils (EO) of *Cymbopogon citratus* (DC.) Stapf. (Lemon grass) and *Zingiber officinale* Roscoe (ginger) against bacteria associated with otitis media (OM) in children. Ear swab samples were collected from 12 children diagnosed with OM between the ages of 0 – 5 years. Essential oils were extracted from the plants using the hydro-distillation method. The antibacterial activity of the essential oils was determined using the agar well diffusion method against isolated bacteria including *Proteus mirabilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Moraxella catarrhalis*, *Klebsiella pneumoniae* and *Staphylococccus epidermidis*. The antibiotic susceptibility patterns of the bacteria isolated was determined using 10 standard antibiotics as control. Greater zones of inhibition were observed in essential oil of Lemon grass as compared to Ginger. Minimum inhibitory concentrations (MIC) and Minimum Bactericidal concentrations (MBC) were carried out for each essential oil where higher values (12.5 -100%v/v) were recorded for ginger essential oil than lemon grass (0.78 – 1.56%v/v). Phytochemical analysis of the EOs of ginger and lemon grass carried out using GC-MS showed the presence of zingiberene (26.85%) and generaldehyde (21.13%) respectively as the most abundant phytocompounds. *In silico* study was carried out through the molecular docking method using the AutoDock version 2.1 by taking two proteins- osmoporin and mevalonate synthetase as targets and different phytocompounds from the essential oils as ligands. Molecular docking highlighted the potential of several phytocompounds in the essential oils to inhibit protein targets required by bacteria for survival.

Keywords: Otitis media, Ginger, Lemon grass, *In silico*, Essential oil, Antibacterial activity

Email: *Corresponding author: kolawoleamos2014@gmail.com +2348063570820
obuotortm@funaab.edu.ng; +2348037156800
mofolaoluwasadeyanju@gmail.com; +2348136190697
onieo@funaab.edu.ng; +2348032237108
yetunde.ferukebello@hallmarkuniversity.edu.ng**;**

INTRODUCTION

Otitis media (Om) refers to a group of complex infectious and inflammatory diseases affecting the middle ear [1]. Aroll [2] opined that it is the inflammation of the middle ear drum and the inner ear, including a duct known as the Eustachian tube and is a common infection among children of school age worldwide [3]. It has been reported that children less than 5 years are more prone to otitis media due to shorter and more horizontal Eustachian tube, lower immunity compared to adults and the fact that bacteria adhere to the epithelial cells of children than in adults [4]. Om may be presented in different clinical forms including acute otitis media (AOM), Otitis media with effusion (OME) and chronic suppurative otitis media (CSOM). The most common cause of Om is the bacterial infection of the middle ear. AOM has been reported to be predominantly caused by *Streptococcus pneumoniae*, *Heamophilus influenza* and *Moraxella catarrhalis* [5] [6]. However, *Pseudomonas aeruginosa* and *Staphyloccocus aureus* are known as the most common aerobic microbial isolates in patients with CSOM, followed by *Proteus vulgaris* and *Klebsiella pneumoniae* [7] [8] [9].

The increase in the emergence of antibiotic-resistant pathogens [10] has called for the need to carry out research on safer phytomedicines and biologically active compounds with acceptable therapeutic index for the development of novel drugs. Traditional uses of plants have led to investigating their bioactive compounds, which have resulted in the detection of a significant number of therapeutic properties [11].

Ginger has been cultivated for thousands of years as a spice and for medicinal purposes [12]. Bellick *et al* [13] considered it a safe herbal medicine with only few and insignificant adverse side effects. It has been reported that the main ingredients of ginger like volatile oil, gingerol, shogaol and diarylheptanoids work as an antioxidant, anti-inflammatory, anti-lipid, anti-diabetic, analgesic, antipyretic and anti-tumor [14] [15].

Lemongrass is a perennial herb widely cultivated in the tropics and sub-tropics, which designates two different species, East Indian *Cymbopogn flexuosus* and West Indian, *Cymbopogon citratus* [16]. The antimicrobial effect of whole lemon grass EO has been shown in previous studies with a wide range of in vitro activity including effects against antimicrobial resistant pathogens [17] [18] [19]. The strong antimicrobial activity of lemon grass has been attributed to a high citral content [20] [19] [21] [22]. Both lemongrass EO and citral are generally regarded as safe for use as flavouring substances and is also an approved compound for use as a food additive and for human consumption [23] [24].

Obuotor *et al.*, [25] stated that *in silico* investigations have assisted in explaining the mechanism of action of the potential antimicrobial compounds. It has also allowed drug – ligand interaction studies to be performed

in shorter periods and aids the designing of better therapeutic compounds [26]. Hence, the aim of this study was to determine the antibacterial effects of the essential oils of *Zingiber officinale* and *Cymbopogon citratus* on both Gram positive and Gram negative multi-drug resistant bacteria isolated from children with otitis media using *in silico* and *in vitro* approaches.

MATERIALS AND METHODS

Collection of samples

Ear swab samples were obtained from 12 children between the age range of 0-5 attending Ogun State Hospital, Ijaiye, Abeokuta, Nigeria and having suspected otitis media. The ear swab samples were immediately transported between 1 – 4 hours after collection using buffered peptone water as transport medium to the Postgraduate Laboratory, Department of Microbiology, Federal University of Agriculture, Abeokuta for further analysis. Fresh leaves of *Cymbopogon citratus* were obtained from the botanical garden of the College of Plant Sciences, Federal University of Agriculture Abeokuta, Ogun state, Nigeria while dry rhizomes of ginger were purchased from Lafenwa market and then milled into fine powder.

Extraction of oils from the plants

Essential oils of Ginger and Lemon grass were obtained by hydro distillation using a vertical hydro distillation unit [27]. This was done using the Clavenger's apparatus which comprises of the heating mantle, round bottom flask (3000mls), condenser and the separating funnel. During hydro distillation, the samples were exposed to temperature of 50°C for Ginger and lemon grass. The essential oils were trapped in the condenser, pipette and dried over anhydrous sodium sulphate and stored in an amber bottle in the refrigerator at 4°C until needed.

Isolation and Identification of Bacteria

The characterization and identification of isolates was based on macroscopic morphology, microscopic morphology and several biochemical tests including but not limited to Catalase, Coagulase, Indole, Oxidase, Urease, Citrate tests.

ANTIBACTERIAL ASSAY

Antibiotic Susceptibility testing using disc diffusion method

Susceptibility to antibiotics was assessed using the Kirby-Bauer disc diffusion technique. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) [28] and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [29]. The panel of antibiotics used include: (Tetracycline, Amikacin, Gentamycin, Nalidixic acid, Amoxicillin/Clavulanic acid, Trimethoprime/Sulfomethoxazole, Ciprofloxacin, Chloramphenicol, Ampicillin, Cefoxitin). The degree of susceptibility of the bacterial isolates to each antibiotic was determined.

Sensitivity testing of the essential oils on the bacterial isolates

The test organisms used were standardized with sterile saline (NaCl 0.9%), and the turbidity was adjusted to the standard inoculums of a McFarland scale of 0.5 (1.0×10^8 colony forming units/ml). Briefly, agar plates containing 20 ml of Mueller Hinton Agar (Oxoid Ltd., Hampshire UK) were inoculated with the bacterial strains under aseptic conditions and wells (diameter=8 mm) were filled with 100µ L of the oils. The experiment was repeated in triplicates and the mean zone of inhibition was recorded after incubating the test organisms at 37 °C (24 h)

Determination of Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of the Essential oils on bacterial isolates

The method used was similar to that as described by Adukwu *et al* [19] and Obuotor *et al.,* [25] with a few modifications. This assay was done to determine the lowest concentration of the oil that will inhibit microbial growth. A two - fold dilution of each essential oil was prepared by adding 2ml of the essential oil into sterile test tube containing 2ml of absolute methanol as reconstituting solvent to give 50%v/v concentration of the essential oil and 2ml from this was taken and dispensed into another test tube containing 2ml of methanol (this gives 25%v/v). This process was continued to obtain 2ml of different concentration (100% v/v – 0.003%v/v) of the essential oil. These different concentrations of the essential oil were each poured into 18ml pre-sterilized molten nutrient agar and mixed properly (methanol only was used as control). The molten nutrient agar was poured into sterile Petri dishes and allowed to set. The surface of the media was allowed to dry before streaking with the 18 h old standardized bacterial culture. The plates were later incubated at 37°C for 72 h after which they were examined for the presence or absence of growth. The MIC value was taken as the lowest concentration that does not show bacterial growth.

Based on the MIC results obtained, the concentrations of the essential oils that showed no growth were sub-cultured onto sterile nutrient agar plates and incubated for 48 hours for bactericidal activity. The MBC was taken as the least concentration that did not show any growth on the incubated nutrient agar plates.

PHYTOCHEMICAL SCREENING OF THE ESSENTIAL OILS

The various essential oils were subjected to phytochemical screening using the method as described by Kalaivani and Vidhya, [30]. This method was used to test for saponins, tannins, terpenoids, glycosides, alkaloids, flavonoids and reducing sugars.

ANTIOXIDANT ACTIVITY OF THE ESSENTIAL OILS

Radical Scavenging ability

The radical scavenging ability of the oil was determined using the stable radical DPPH (2,2-

diphenyl-1-picrylhydrazyl hydrate) as described by Brand-Williams *et al.,* [31]. The reaction of DPPH with an antioxidant compound which can donate hydrogen, leads to its reduction [32]. The change in colour from deep violet to light yellow was measured using a spectrophotometer at 517nm.To 1ml of different concentrations (10, 5, 2.5, 1.25, 0.625, 0.3125mg/ml) of the essential oils or standard (vitamin C) in a test tube was added 1ml of 0.3mM DPPH in methanol. The mixture was mixed and incubated in the dark for 30mins after which the absorbance was read at 517nm against a DPPH control containing only 1ml methanol in place of the extract.

The percent of inhibition was calculated in following way:

I% = [(Ablank-Asample)/Ablank] x 100

Where A blank is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound. Sample concentration providing 50% inhibition (IC50) was calculated from the graph plotting inhibition percentage against extract concentration.

Determination of Total Antioxidant Capacity

This method is based on the reduction of Molybdenum (VI) to Molybdenum (V) by the extract and the subsequent formation of a green phosphate/Molybdenum (V) complex at an acidic pH [33]. To 0.1ml of the essential oils or standard solutions of ascorbic acid (20, 40, 60, 80, 100µg/ml) was added 1ml of the reagent solution which consisted of 0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. The tubes containing the reacting mixture were incubated in a water bath at 95^0C for 90mins. The mixture was then allowed to stand and cool to room temperature and the absorbance measured at 695 nm against a blank which consisted of the reacting mixture containing distilled water in place of the extract. The antioxidant activities of the extracts were expressed as an ascorbic acid equivalent.

Determination of Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method with absorbance measured with a spectrophotometer [34]. The principle of this method is based on the reduction of a colourless ferric-tripyridyltriazine complex to its blue ferrous coloured form owing to the action of electron donating in the presence of antioxidants. A 300mmol/L acetate buffer of pH 3.6, 10 mmol/L 2, 4, 6-tri-(2-pyridyl)-1, 3, 5-triazine and 20mmol/L $FeCl_3$.6H2O were mixed together in the ratio of 10:1:1 respectively, to give the working FRAP reagent. A 50µl aliquot of the essential oils at 0.1mg/ml and50µl of standard solutions of ascorbic acid (20, 40, 60, 80, 100µg/ml) was added to 1ml of FRAP reagent. Absorbance measurement was taken at 593nm exactly 10 minutes after mixing against reagent blank containing 50µl of distilled water. All measurements were taken at room temperature with samples protected from direct sunlight. The reducing power was expressed as equivalent

concentration (EC) which is defined as the concentration of antioxidant that gave a ferric reducing ability equivalent to that of the ascorbic acid standard.

GAS CHROMATOGRAPHY MASS SPECTROSCOPIC (GC - MS) ANALYSIS OF ESSENTIAL OILS

The essential oil samples were submitted for GC-MS analysis to the Laboratory of Chromatography, Federal Institute of Research Oshodi, Lagos state, Nigeria. The Physicochemical properties of the essential oils of *Zingiber offiicnale* and *Cymbopogon citratus* were identified using Gas Chromatography Mass Spectrometry (GC-MS) analyzer (Shimadzu GC-MS-QP 2010 Ultra). SLB-5ms Column fused with silica capillary 0.20mm X 30.0m with film thickness 0.20µm was used for this purpose. The initial temperature was maintained at 40°C for 3 minutes and then heated at a rate of 15°C per minute up to 290°C with a director voltage relative to the tuning result. Carrier gas Helium was used at a rate of 1ml per minute.

IN SILICO STUDIES

Generation of three-dimension (3D) structures for components of the Essential oils.

Three-Dimensional (3D) structures of the component of the essential oils of *Zingiber officinale* and *Cymbopogon citratus* were drawn using Chem3D Pro 12.0 after which the generated structures were saved as pdf formats. The .pdb format is for the actual docking process as the software requires ligands to be docked to be in such formats.

Ligand preparation

The SDF designs of the ligands were recovered from the "PubChem information base (www.pubchem.ncbi.nlm.nih.gov)" [35]. The ligands were converted to .pdb format using the "PYMOL atomic illustrations framework (1.7.4.5 Edu)" [36]. The ligand atoms were thereafter completely converted to the dockable. pdbqt format using the "Autodock vina program"

Enzyme preparation

The crystal structure of the enzymes of the test organisms Osmoporin (OMPK36) of *Klebsiella pneumoniae* and 3-hydroxyl-3-methylglutaryl CoA (HMG-CoA) reductase (Figure 6 and 7), (a mevalonate synthetase which is responsible for the synthesis of peptidoglycan in *Staphylococcus aureus*) [25].

Molecular docking of components of the essential oils with selected proteins from the test microorganisms.

The molecular docking analysis was done to ascertain the binding conformation of the protein–ligand complex using AutoDock vina [37]. The binding conformation would aid to reveal the binding energy of the HMG-CoA and OMPK36 with the enzymes. The ligands side chain and the torsional bonds kept flexible

while the HMG-CoA and OMPK36 fixed rigid. All the ligands were docked to the residue involved in catalytic activity with x, y, and z coordinates of 7.000, −7.250 and 68.750 respectively. The grid box was set at 74 Å ×78 Å ×56 Å and with an exhaustiveness of 8. The free binding energy (ΔG bind) was calculated using the sum of van der Waals energy (ΔG vdw), the sum of electrostatic energy (ΔG elect), the sum of hydrogen bond and desolvation energy (ΔG h bond), the sum of final total internal energy (ΔG conform), the sum of torsional free energy (ΔG tor) and the sum unbound system energy (ΔG solv) [38]. The compounds were then ranked by their binding affinity scores. Molecular interactions between the receptors and compounds with most remarkable binding affinities were first viewed with PYMOL after which further graphical analysis was obtained using Discovery Studio Visualizer, BIOVIA, 2016.

RESULTS

Prevalence of organisms implicated in Otitis Media

The prevalence of the organisms isolated from Otitis media ear swab samples showed that *Proteus mirabilis* had the highest occurrence of 31.25% while *Proteus vulgaris* and *Klebsiella pneumonia* had the lowest occurrence of 3.13% as depicted in Figure 1.

Characteristics of the Essential oils obtained from *Zingiber officinale* and *Cymbopogon citratus*.

The essential oil obtained from *Zingiber officinale* had a bright yellow colour with a strong pungent odour and a percentage yield of 1.5% on the other hand, the essential oil obtained from *Cymbopogon citratus* had a light lemon colour with a very sweet odour and a percentage yield of 0.8%.

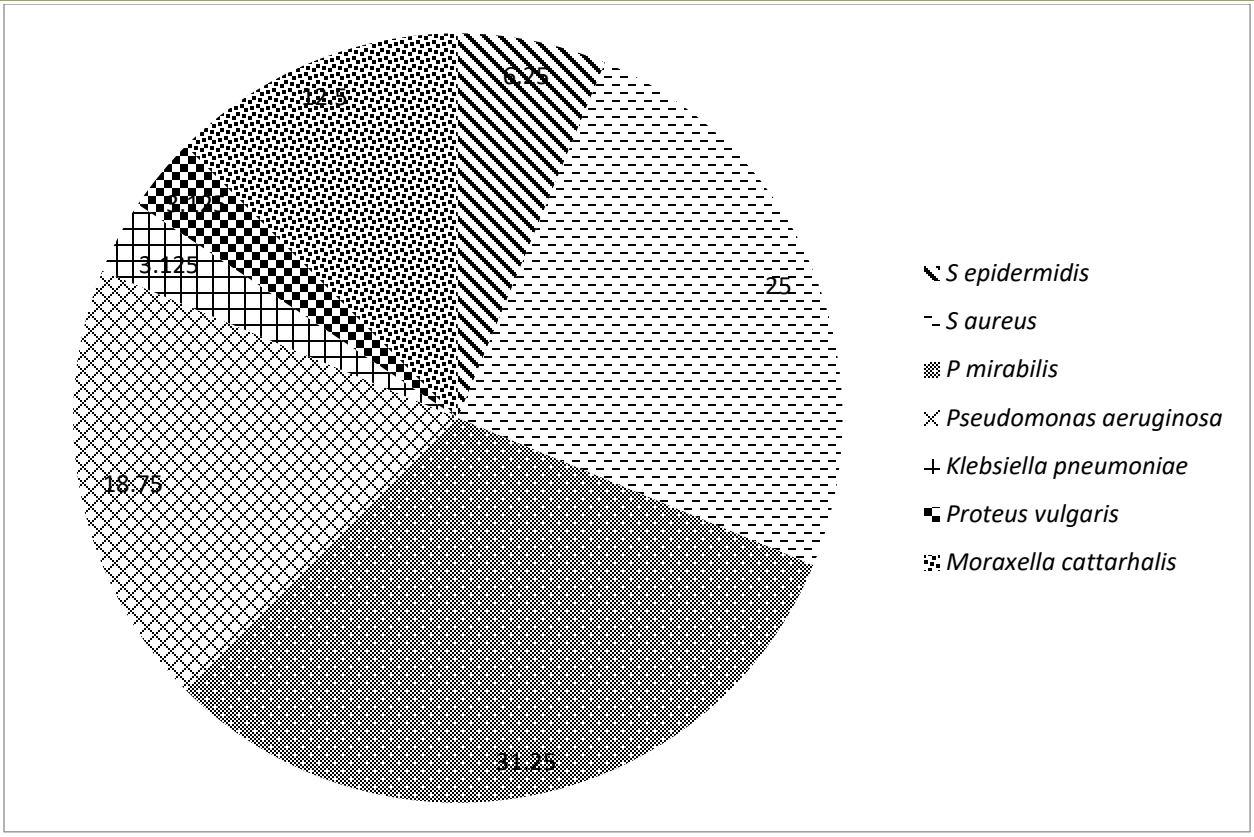


Figure 1: Frequency of Isolated organisms from ear swab samples

Sensitivity testing of antibiotic disc on bacterial isolates

The antibiotic sensitivity testing on bacterial isolates results are shown in Table 1. It was observed that *Pseudomonas aeruginosa* and *Moraxella cattarhalis* were both resistant to 7 out of the 10 antibiotics used, followed by *Klebsiella pneumoniae* and *Proteus vulgaris* which were resistant to 6 antibiotics. *Staphylococcus aureus* and *Proteus mirabilis* were susceptible to 6 antibiotics in contrast with other bacterial isolates.

Table 1: Antibiotic disc sensitivity testing on bacterial isolates (in mm)

Isolates	FOX (mm)	CN (mm)	CIP (mm)	AMC (mm)	SXT (mm)	NA (mm)	AMP (mm)	C (mm)	AMK (mm)	TET (mm)
Klebsiella pneumoniae	12±1.4	23.5±0.7	26±0	15.5±0.7	22±1.4	21.5±0.7	11.5±2.1	6±0	21.5±0.7	6±0
Staphylococcus aureus	26±0	30±0	12.5±0.7	33±2.8	7.5±2.1	6±0	32.5±0.7	31±1.4	23±0	13±0
Pseudomonas aeruginosa	6±0	23.5±0.7	28±1.4	6±0	6±0	11.5±0.7	6±0	14±0	21±1.4	11.5±0.7
Proteus vulgaris	24±0	24±0	18.5±2.1	8±0	6±0	22.5±0.7	6±0	7±0	23±0	6±0
Proteus mirabilis	25±0	6±0	18±0	27.5±0.7	17±1.4	20.5±3.5	19.5±0.7	18.5±0.7	6±0	7±0
Moraxella catarrhalis	7±0	8±1.4	31±1.4	35±1.4	6±0	6±0	25±0.7	13.5±2.1	11.5±0.7	19.5±0.7

Key: FOX- Cefoxitin (30µg); CN- Gentamycin (120µg); CIP- Ciprofloxacin (5µg); AMC- Amocicillin/clavulanic acid (30µg); NA- Nalidixic acid (30µg); AMP- Ampicillin (10µg); C- Chloramphenicol (30µg); AMK- Amikacin (30µg); TET- Tetracyclin (30µg); SXT- Sulfomethoxazole/Trimethoprin (25µg).

Antibacterial activities of the essential oils of *Zingiber officinale* and *Cymbopogon citratus* against bacterial isolates.

The antibacterial activities of the essential oils of *Zingiber officinale* and *Cymbopogon citratus* against bacteria isolated in this study is as shown in Table 2, 90% of the bacterial isolates were susceptible to the essential oil of *Zingiber officinale* with zones of inhibition ranging from 11.5 ± 0.7 – 20.0 ± 1.4. *Klebsiella pneumoniae* had the lowest zone of inhibition and *Staphylococcus aureus* had the highest zone of inhibition, only *Proteus mirabilis* was resistant.

All the bacterial isolates except *Pseudomonas aeruginosa* were susceptible to the essential oil of *Cymbopogon citratus. Staphylococcus aureus* had the highest zone of inhibition of 62.0 ± 2.8 whereas *Klebsiella pneumonia* had the lowest zone of inhibition of 21.0 ± 1.4. In addition, all bacterial isolates were also susceptible to the combinations of the essential oils of *Cymbopogon citratus* and *Zingiber officinale*, with *Staphylococcus aureus* having the highest zone of inhibition *of* 47.5mm±0.7mm and *Pseudomonas aeruginosa* having the lowest zone of inhibition of 14.0mm±1.4mm.

Table 2: Antibacterial activities exhibited by the essential oils of Ginger and Lemon grass against test isolates

	GL	G	L
Klebsiella pneumoniae	33.0 ± 1.4	11.5 ± 0.7	21.0 ± 1.4
Pseudomonas aeruginosa	14.0 ± 1.4	12.5 ± 0.7	8.5 ± 0.7
Proteus vulgaris	15.0 ± 1.4	17.0 ± 0.0	32.5 ± 0.7
Proteus mirabilis	16.0 ± 0.0	8.0 ± 0.0	29.5 ± 2.1
Moraxella catarrhalis	36.0 ± 1.4	11.5 ± 0.7	50.5 ± 0.7
Staphylococcus aureus	47.5 ± 0.7	20.0 ± 0.7	62.0 ± 1.4

KEY: G = Ginger, L = Lemon grass, GL = Ginger and Lemon grass.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) exhibited oils of *Zingiber officinale* and *Cymbopogon citratus* against test isolates.

The Minimum Inhibitory Concentration and Minimum Bactericidal Concentration exhibited by the essential oils against the bacterial isolates are as shown in Table 3. The MIC exhibited by

the essential oil of *Zingiber officinale* ranged between 12.5%v/v and 100%v/v whereas the MIC exhibited by the essential oil of *Cymbopogon citratus* ranged between 0.78%v/v and 1.56%v/v. The MBC exhibited by the essential oils of *Zingiber officinale* and *Cymbopogon citratus* ranged between 12.5%v/v and 100%v/v, 0.78%v/v and 1.56%v/v respectively.

Table 3: The Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations exhibited by the essential oils of Ginger and Lemon grass against test isolates.

S/N	Isolates	Ginger		Lemon grass	
		MIC (%v/v)	MBC (%v/v)	MIC (%v/v)	MBC (%v/v)
1	*Klebsiella pneumoniae*	25.00	25.00	0.78	0.78
2	*Staphylococcus aureus*	12.50	12.50	0.78	0.78
3	*Pseudomonas aeruginosa*	25.00	25.00	1.56	1.56
4	*Proteus vulgaris*	50.00	50.00	1.56	1.56
5	*Proteus mirabilis*	100.00	100.00	1.56	1.56
6	*Moraxella catarrhalis*	12.50	12.50	0.78	0.78

Phytochemical screening of the essential oils

The phytochemical screening of the essential oils of *Zingiber officinale* and *Cymbopogon citratus* indicated the presence of several phytochemical compounds in the oils including; Tannins, Glycosides, Resins, Saponins, Phlobatannins, Flavonoids, Sterols, Phenols, Carbohydrates, Alkaloids and Terpenoids.

Table 5 shows the phytochemical screening results for the essential oils.

Table 5: Phytochemical screening of the essential oils

Phytochemical	Ginger oil	Lemon grass oil
Tannin	-	+
Saponin	-	+
Terpenoids	-	-
Flavonoids	+	+
Glycosides	+	-
Sterols	+++	-
Phlobatannins	-	-
Phenols	-	++
Carbohydrates	+	+
Alkaloids	-	_
Resins	-	_

KEY: (-): Negative test; (+): Weak positive test; , (++): Positive test; (+++): Test strongly positive

Antioxidant activity of the essential oils of *Zingiber officinale* and *Cymbopogon citratus*.

Table 4 shows the antioxidant capacity of the essential oils. The essential oil of *Zingiber officinale* had a higher Total Antioxidant Capacity (TAC) value of 0.14mg/ml. Similarly, for Ferric Reducing Antioxidant Power, the essential oil of *Cymbopogon citratus* had a lower value of 0.55mg/ml than the 1.63mg/ml value of the essential oil of *Zingiber officinale*. However, reverse is the case in Di-phenyl Picryl Hydrazyl Hydrate (DPPH) assay where a higher value of 1.21mg/ml was recorded for the essential oil of lemon grass than ginger essential oil.

Table 4: Antioxidant assay of essential oils

S/N	ANTIOXIDANT ASSAY	Zingiber *officinale* oil (mg/ml)	*Cymbopogon citratus* oil (mg/ml)
1.	Di-phenyl Picryl Hydrazyl Hydrate (DPPH) (mg/ml)	0.53 ± 0.3	1.21± 0.11
2.	Ferric Reducing Antioxidant Power (FRAP) (mgAAE/ml)	1.63±0.94	0.55±0.014
3.	Total Antioxidant Capacity (TAC) (mg/ml)	0.14±0.18	0.18 ± 0.05

GC - MS analysis of the Essential oils

The results from Gas Chromatography-Mass Spectroscopy (GC-MS) analysis of the essential oils of *Zingiber officinale* and *Cymbopogon citratus* are as shown in Figure 2 and 3. A total of 57 phytocompounds was present in ginger essential oil which are represented as peaks in Figure 2 and listed in table 5 while lemon grass essential oil had a total of 16 phytocompounds represented as peaks in Figure 3 and phytocompounds listed in table 6.

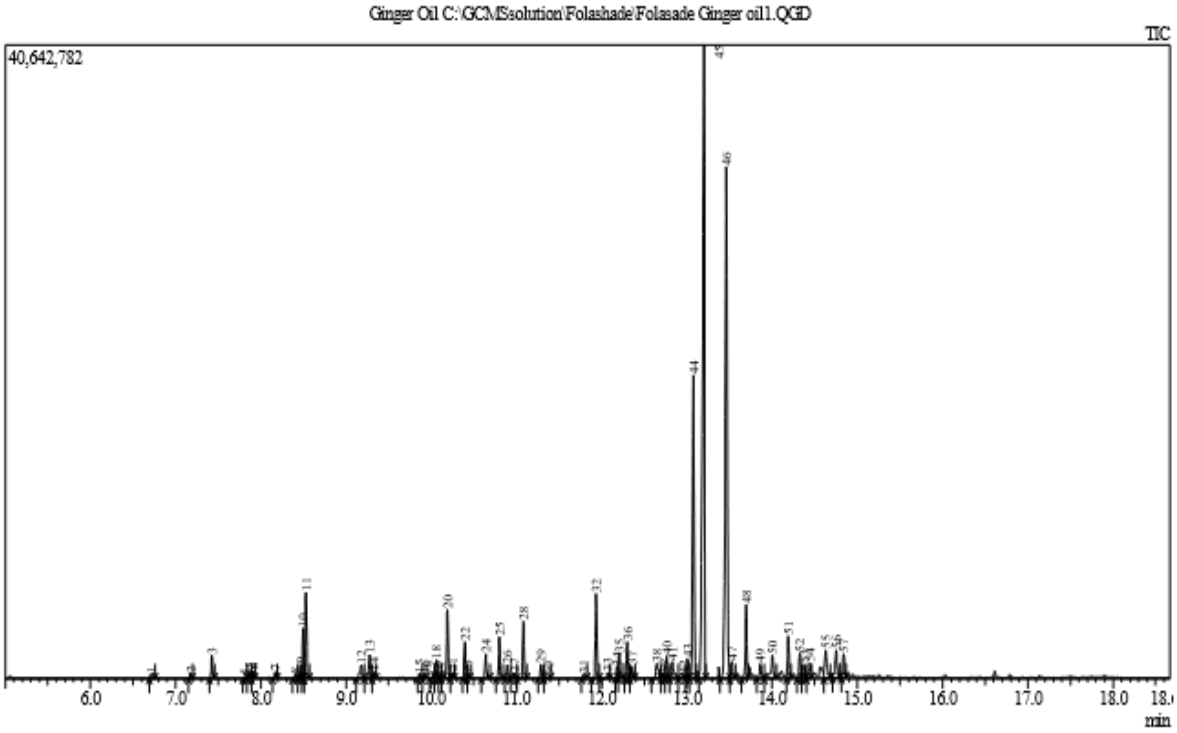


Figure 2: Peaks of the components of *Zingiber officinale* essential oil

Table 5: GC-MS analysis of the components of *Zingiber officinale* essential oil

PEAK REPORT TIC							
Peak#	R.Time	Area	Area %	Height	Height%	A/H	Name
1	6.710	388532	0.16	286521	0.17	1.36	2-Heptanol
2	7.176	407868	0.17	340819	0.21	1.20	alpha.-Pinene
3	7.426	1830743	0.77	1496483	0.91	1.22	Camphene

4	7.812	107901	0.05	95349	0.06	1.13	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylen
5	7.864	626092	0.26	533051	0.32	1.17	5-Hepten-2-one, 6-methyl-
6	7.909	563231	0.24	503672	0.31	1.12	.beta.-Myrcene
7	8.170	470682	0.20	414009	0.25	1.14	.alpha.-Phellandrene
8	8.402	222872	0.09	196235	0.12	1.14	1,3,8-p-Menthatriene
9	8.467	1134386	0.48	878844	0.53	1.29	D-Limonene
10	8.498	3924880	1.65	3228078	1.96	1.22	.beta.-Phellandrene
11	8.529	6674672	2.80	5526953	3.35	1.21	Eucalyptol
12	9.178	1484072	0.62	870779	0.53	1.70	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-
13	9.276	1961700	0.82	1524538	0.93	1.29	1,6-Octadien-3-ol, 3,7-dimethyl-
14	9.325	482369	0.20	428876	0.26	1.12	3,Oxatricyclo[4.1.1.0(2,4)]octane, 2,7,7-trimet
15	9.860	358816	0.15	282689	0.17	1.27	6-Octeal, 3,7-dimethyl-, (R)-
16	9.925	300386	0.13	220464	0.13	1.36	Camphor
17	10.017	202576	0.09	166967	0.10	1.21	Bicyclo[2.2.1]heptan-2-ol, 2,3,3-trimethyl-
18	10.063	1368011	0.57	117647 1	0.71	1.16	2,6-Dimethyl-1-nonen-3-yn-5-ol
19	10.100	148434	0.06	137307	0.08	1.08	Isobornyl acetate
20	10.188	5733941	2.41	4396516	2.67	1.30	endo-Borneol
21	10.250	432362	0.18	372158	0.23	1.16	Terpinen-4-ol
22	10.395	2796904	1.17	2344205	1.42	1.19	.alpha.-Terpineol
23	10.430	264992	0.11	234634	0.14	1.13	5-Tetradecen-3-yne, (E)-
24	10.640	1800069	0.76	1540261	0.93	1.17	Citronellol
25	10.798	3121232	1.31	2654387	1.61	1.18	2,6-Octadienal, 3,7-dimethyl-, (Z)-
26	10.890	955014	0.40	839701	0.51	1.14	Geraniol
27	10.969	382203	0.16	329138	0.20	1.16	trans-p-mentha-1(7),8-dien-2-ol
28	11.082	4271008	1.79	3652489	2.22	1.17	2,6-Octadienal, 3,7-dimethyl-, (E)-
29	11.288	1192424	0.50	883665	0.54	1.35	2-Undecanone
30	11.374	143288	0.06	111068	0.07	1.29	4-Methyl-2-hexanol
31	11.785	470110	0.20	238287	0.14	1.97	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl
32	11.935	6421686	2.70	5424097	3.29	1.18	Eugenol

33	12.070	394244	0.17	351811	0.21	1.12	Geranyl acetate
34	12.179	875975	0.37	707563	0.43	1.24	1,2,4-Metheno-1H-indene, octahydro-1,7a-dimethyl
35	12.206	1909707	0.80	1524281	0.93	1.25	Copaene
36	12.299	2671973	1.12	2308971	1.40	1.16	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-me
37	12.357	876290	0.37	745005	0.45	1.18	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexeny
38	12.647	1967037	0.83	941777	0.57	2.09	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8
39	12.721	344839	0.14	234526	0.14	1.47	beta.-copaene
40	12.763	1726122	0.72	1421827	0.86	1.21	cis-.beta.-Farnesene
41	12.831	1798697	0.75	1071246	0.65	1.68	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-m
42	12.960	443226	0.19	224554	0.14	1.97	Humulene
43	13.007	1705855	0.72	1292799	0.78	1.32	Alloaromadendrene
44	13.076	25481861	10.69	19473901	11.82	1.31	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-
45	13.200	63981967	26.85	40641483	24.67	1.57	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl
46	13.464	52701028	22.12	32841829	19.93	1.60	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-me
47	13.529	1685183	0.71	1106973	0.67	1.52	(-)-.alpha.-Panasinsen
48	13.695	6150660	2.58	4769500	2.89	1.29	Nerolidyl acetate
49	13.861	1664976	0.70	949392	0.58	1.75	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene
50	14.003	3084714	1.29	1467257	0.89	2.10	7-epi-cis-sesquisabinene hydrate
51	14.189	3630688	1.52	2718941	1.65	1.34	.alpha.-Bisabolol
52	14.325	2266767	0.95	1607814	0.98	1.41	7-epi-cis-sesquisabinene hydrate
53	14.375	1588513	0.67	908184	0.55	1.75	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,7-octahy
54	14.444	1804073	0.76	975070	0.59	1.85	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl

55	14.627	3475035	1.46	1800071	1.09	1.93	2-Naphthalenemethanol, decahydro-.alpha.,.alpha
56	14.750	3279494	1.38	1784603	1.08	1.84	7-epi-cis-sesquisabinene hydrate
57	14.839	2114108	0.89	1554950	0.94	1.36	6,10-Dodecadien-1-yn-3-ol, 3,7,11-trimethyl-
		238266	488	100.00	1647530 39	100.00	

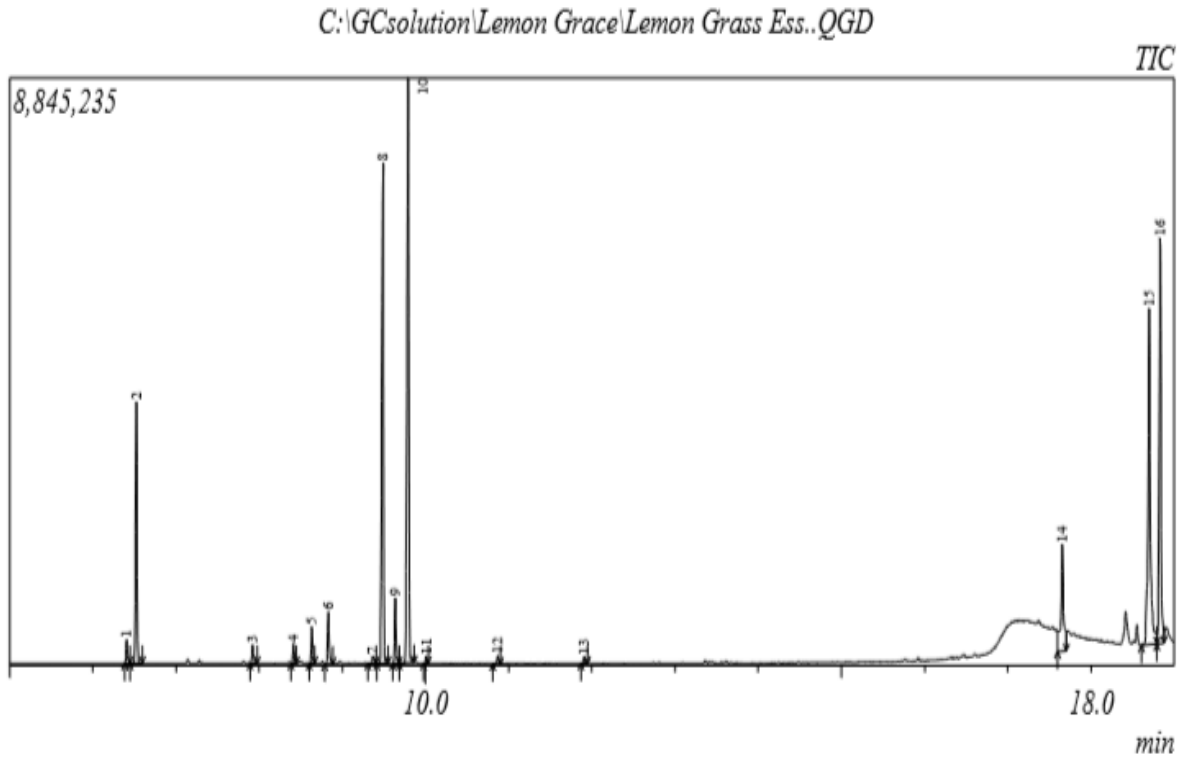


Figure 3: Peaks of the components of *Cymbopogon citratus* essential oil

Table 6: GC-MS analysis of *Cymbopogon citratus* essential oil

PEAK REPORT TIC							
Peak#	Retention Time	Area	Area%	Height	Height%	A/H	Name
1	6.407	462648	0.83	355926	0.97	1.30	5-Hepten-2-one, 6-methyl-
2	6.525	4587326	8.23	3929887	10.70	1.17	beta.-Myrcene
3	7.921	401717	0.72	279903	0.76	1.44	1,6-Octadien-3-ol, 3,7-dimethyl-

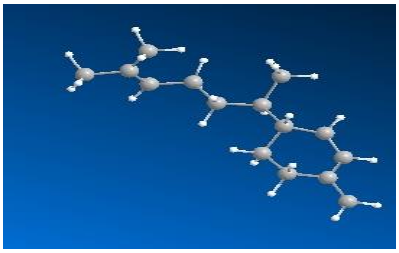
4	8.413	349420	0.63	295066	0.80	1.18	1,4-Hexadiene, 3,3,5-trimethyl-
5	8.634	623442	1.12	550952	1.50	1.13	Verbenol
6	8.832	1010170	1.81	784250	2.14	1.29	Carane, 4,5-epoxy-, trans
7	9.367	174531	0.31	107799	0.29	1.62	Citronellol
8	9.488	10406614	18.68	7539840	20.53	1.38	2,6-Octadienal, 3,7-dimethyl-, (Z)-
9	9.637	1273389	2.29	983643	2.68	1.29	Geraniol
10	9.793	13446658	24.13	8794239	23.95	1.53	2,6-Octadienal, 3,7-dimethyl-, (E)-
11	10.009	116909	0.21	102529	0.28	1.14	Butanimidamide
12	10.869	156037	0.28	120149	0.33	1.30	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z
13	11.906	120779	0.22	97495	0.27	1.24	Butanal, 3-methyl-
14	17.656	3503503	6.29	1608648	4.38	2.18	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-
15	18.700	9911132	17.79	5059647	13.78	1.96	13-Octadecenal, (Z)-
16	18.832	9176133	16.47	6110656	16.64	1.50	Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester
		55720408	100.00	36720629	100.00		

***IN SILICO* ANALYSIS**

Three Dimension (3D) structures for 5 phytocompounds with the highest area of peak for the essential oils of *Zingiber officinale* and *Cymbopogon citratus* were generated. For ginger essential oil (Figure 4), 3D structures of Zingiberene (26.85%), Cyclohexene (22.12%), Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl (10.69%), Eucalyptol (2.8%) and Endo-Borneol (2.70%) were generated. Also, 3D structures of 2,6-Octadienal, 3,7-dimethyl (24.73%), cis-3,7-Dimethyl-2,6-octadienal (18.68%), cis-13-Octadecenal (17.79%), Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester (16.47%) and 1,6-Octadiene, 7-methyl-3-methylene (8.23%) were generated for lemon grass essential oil (Figure 5).

The 3D structures generated were docked with 3-hydroxyl-3-methylglutaryl CoA (HMG-CoA) reductase (Figure 6), (a mevalonate synthetase which is responsible for the synthesis of peptidoglycan in *Staphylococcus aurues*) and OmpK36- the osmoporin (channel-forming membrane proteins that confer solute permeability to the outer membrane of *Klebsiella pneumoniae* (Figure 7). beta-sequiphellandrene (Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene), alpha-curcumene (Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl) and alpha-zingiberene had the lowest binding energies against the enzymes tested for ginger EO (Fig 8) whereas Cis-3,7-dimethyl-2,6-octadienal and cis-13-octadecenal had the lowest binging energies for Lemongrass EO (Figure 9). This suggests their potential in inhibiting the growth of the bacteria.

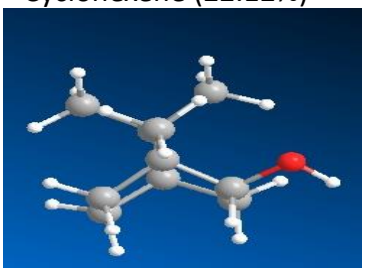
Zingiberene (26.85%)



Cyclohexene (22.12%)

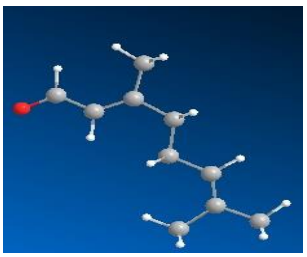
Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl (10.69%)

Eucalyptol (2.8%)

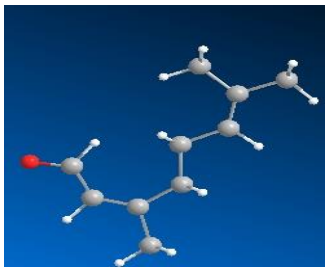


Endo-Borneol (2.70%)

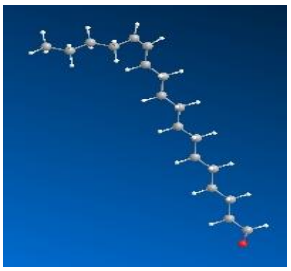
Figure 4: 3D structures of some phytocompounds in *Zingiber officinale* essential oil



2,6-Octadienal, 3,7-dimethyl (Generaldehyde) (24.73%)

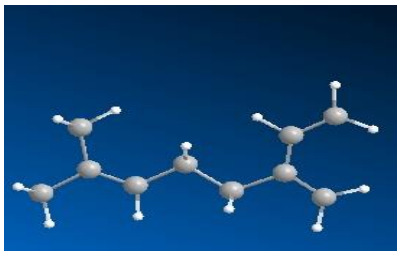


cis-3,7-Dimethyl-2,6-octadienal (18.68%)



cis-13-Octadecenal (17.79%)

Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester (16.47%)



1,6-Octadiene, 7-methyl-3-methylene (8.23%)

Figure 5: 3D structures of some phytocompounds in *Cymbopogon citratus* essential oil

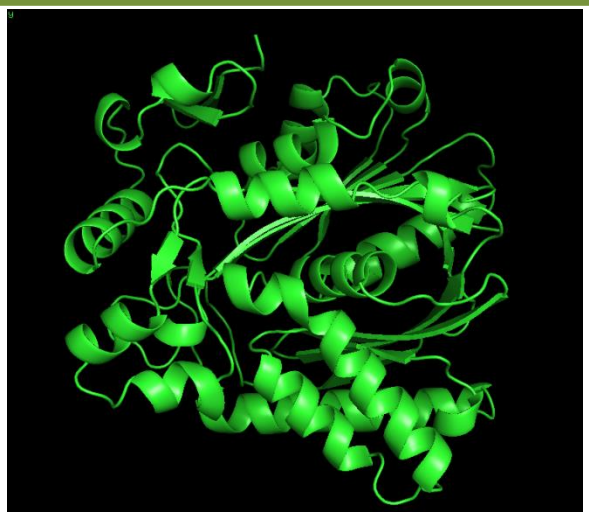


Figure 6: Crystal structure of mevalonate synthetase from *Staphylococcus aureus*.

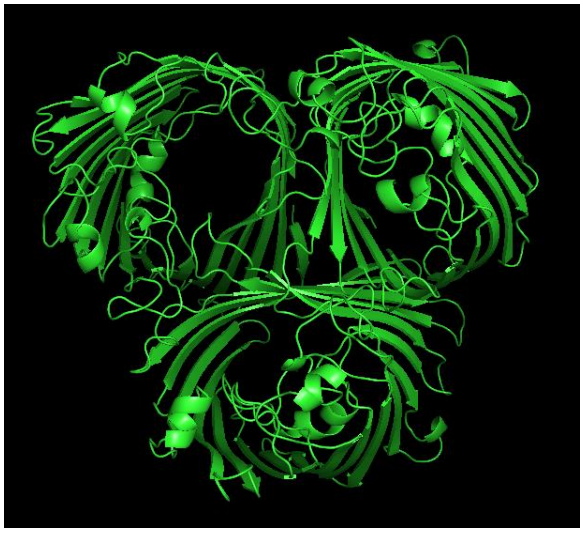


Figure 7: Crystal structure of Osmoporin (OMPK36) from *Klebsiella pneumoniae*

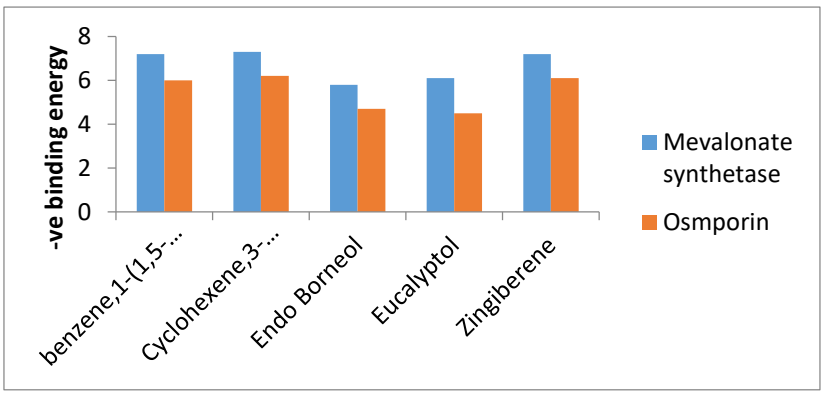


Figure 8: Binding energies of components of *Zingiber officicnale* against proteins from *Staphylooccus aureus* and *Klebsiella pneumoniae*

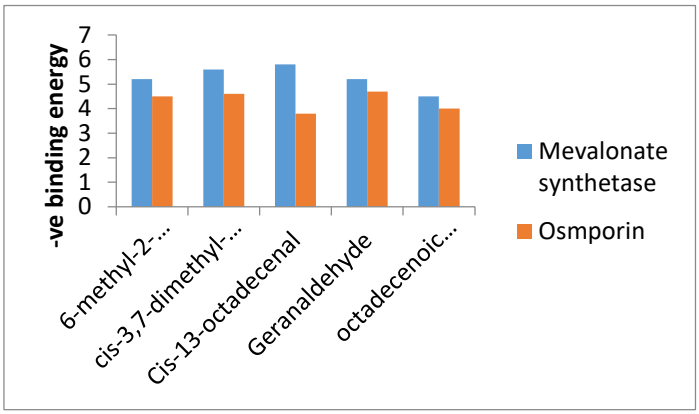


Figure 9: Binding energies of components of *Cymbopogon citratus* against proteins from *Staphylococcus aureus* and *Klebsiella pneumoniae*

DISCUSSION

In this study, a total of 7 bacterial species were isolated and it was observed that *Proteus mirabilis* had the highest percentage of occurrence followed by *Staphylococcus aureus* and *Pseudomonas aeruginosa* which is similar to the findings of Ilechukwu *et al*., [39], Ako-Nai *et al*., [40] and Oni *et al*., [41]. However, these findings are in contrast with the trend in the developed world where non-typeable *Heamophilus influenza*, *Streptococcus pyogenes* and *Moraxella catarrhalis* assume important predominant roles in Otitis media [42].

Ginger EO (GEO) showed inhibitory activity to all bacterial isolates except *Proteus mirabilis*. Moderate antibacterial activity of ginger EO has been reported by some researchers, with the EO showing stronger activities against Gram +ve than Gram –ve bacteria [43] [44] [45]. Stronger inhibitory patterns of GEO were observed in this study as compared to research done by Talla *et al*., [46] in Cameroon who reported much lower zones of inhibition of GEO against bacteria, this might be as a result of difference in method of extraction, components of the essential oil or species of the rhizome used in this study.

Lemon grass EO showed very high inhibitory activity against all bacterial isolates except for *Pseudomonas aeruginosa* with wide zones of inhibition ranging from 21.0mm to 62.0mm. Maximum activity of Lemon grass EO was observed against *Staphylococcus aureus* as compared to other isolates, this is agreement with researches conducted by Choubey *et al*., [47] and Naik *et al*., [16] whom also reported stronger activity of lemon grass oil against *S. aureus* whereas *P. aeruginosa* was resistant to the EO. Some other researchers who also studied the antimicrobial activity of essential oil of lemon

grass plant against pathogenic bacterial strains also reported that *Pseudomonas aeuriginosa* was most resistant to the EO [48] [49] [50] [51].

The combination of ginger and lemon grass EOs (GL) showed strong inhibitory effect compared to when GEO only was used. Conversely, LEO had a stronger inhibitory effect than in combination which might be as a result of the antagonsistic, synergistic or additive effects of the phytoconstituents in each essential oil, as essential oils are complex mixtures of volatile constituents. These suggest that the efficacy of combination therapy may depend very much on the organism in question, its mechanism of resistance and the mode of action of the essential oil.

In all cases of the LEO against the bacteria isolates, the value of MIC was the same as the value of MBC. This result is in agreement with the study by Nzeako *et al*., [52] who reported similar MIC values of 1.56%v/v for *S. aureus* and *P. aeuriginosa*. It was observed that GEO could not inhibit the growth of the bacteria isolates at low concentrations for all isolates tested. This is in agreement with the study by Hassan *et al*., [53] who also reported high MIC values between 12.5-100mg/ml for several bacteria isolates including *S. aureus*, *P. aeuriginosa* and *K. pneumoniae*.

GEO consisted of alpha-curcurmene, beta-sequiphellandrene and alpha-zingiberene, with the alpha-zingiberene as its largest component in that order similar to the findings of Ishiguro *et al*., [54]. Norajit *et al*., [55], Moon *et al*., [56], Sasidharan *et al*., [57], Yammato-Ribeiro *et al*., [58] and Khrimian *et al*., [59] also reported alpha-zingiberene as the largest component of the oil. However, this is in contrast to some researches which reported alpha-curcumene as the largest component [60] [61] [62] [63].

This study reported the predominant components of LEO as 2,6-Octadienal,3,7-dimethyl (Generaldehyde) (24.73%) and cis-3,7-Dimethyl-2,6-octadienal(neral) (18.68%) which is in accordance with the results of Mahanta *et al*., [64] and Negrelle and Gomes [65]. Adukwu *et al*., [12] and Andrade *et al*., [66] informed that citral was the main component in *C. flexuous* and *C. nardus*, respectively. It has been observed that citral has an inhibitory effect on bacteria and fungi [67] [68]. The variations in the chemical composition of essential oils might have been due to the existence of different species and also the differences in agro-climatic conditions [69[70] [71] [26].

Different studies have indicated that the electron donation capacity, reflecting the reducing power of bioactive compounds is associated with antioxidant activity [72] [73]. Plant phenolics have been reported to have great antioxidant potential because of high redox activity [74] [75]. The result of the antioxidant analysis of GEO indicates its ability to remove free radicals, prevent lipid peroxidation and also to reduce ferric ions. This is in line with the findings of Gulcin *et al*., [76] and Ghasemzadeh *et al*., [77]. Radical scavengers may directly react with and

quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products [78]. On the other hand, LEO in this study had a lower ferric reducing and lipid peroxidation inhibition ability as compared with GEO, this is in agreement with the reports of Adeola *et al*., [79] who reported that the antioxidant activity of lemon grass was not as strong as butylated hydroxyltoluene (BHT) which was used as positive control in that study.

In silico analysis conducted in this study revealed that the components of the essential oils are potential lead molecules in the inhibition of bacterial growth and thus justify its use in traditional folklore medicine. However, beta-sesquiphellandrene a phytocompound of ginger essential oil had the lowest binding energy as compared to the other phytocompounds of ginger and lemon grass used, hence its very high potential for the inhibition of the synthesis of osmoporin in *Klebsiella pneumoniae* and mevalonate synthetase in *Staphylococcus aureus.*

CONCLUSION

This study demonstrates the efficacy of both lemon grass and ginger essential oils in combating pathogens associated with otitis media. *In vitro* study shows that lemon grass essential oil inhibits the growth of bacteria at lower concentrations as compared to ginger while the *in silico* results point out that the phytocompounds of ginger have a stronger potential to inhibit the synthesis of proteins required for survival by bacteria. These suggest that the complexity of essential oils might have a lot to do with its antibacterial activity as a result of the synergistic and antagonistic interactions between several phytocompounds in the essential oil. Hence it is important that the activity of these phytocompounds (in combination or singly) as an alternative therapy to antibiotics be further investigated.

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