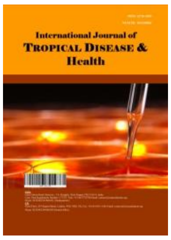

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In silico ATP Synthase Inhibition Activity and Antibacterial Activity of Selected Essential Oil against *Escherichia coli* and Resistant *Acinetobacter baumannii*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The rise in the resistant strains has ignited the investigation of the ability of these essential oils to have an effect against them. The aim of this study was to investigate the antibacterial activity of clove oil, oregano oil and tea tree oil against *Escherichia coli* and resistant patient isolate *Acinetobacter baumannii*. Ciprofloxacin was used as standard and DMSO at 1 % was the negative control. To understand the inhibitory action on ATP synthase, docking analysis was done by using Autodock ver. 4.2.6. The study found out that essential oils had potent antibacterial activity against both *E. coli* and *A. baumannii* as all the essential oils recorded zones above 15 mm against *E. coli* and above 20 mm against *A. baumannii*. Tea tree oil had the highest activity among the three essentials against *E. coli* with 21.000±0.00 mm and against *A. baumannii* 28.500±0.500 mm respectively. These results support the antibacterial activity of essential oils. Docking analysis confirmed the ability of active constituents of selected essential oils to inhibit ATP synthase enzyme which is a crucial drug target in antimicrobial activity. Natural products have been at the center stage in the research for alternative agents with antibacterial agents.

Keywords: Acinetobacter baumannii; Escherichia coli; antibacterial resistance; clove oil; oregano oil; tea tree oil.

1. INTRODUCTION

Antimicrobial resistance is a problem that requires urgent attention as it's threatening the human race. The ability to control these infectious diseases is becoming difficult across the entire globe [1]. Many factors such as inappropriate prescription of antibiotics and the use of antibiotics in agriculture have immensely contributed to the widespread resistance among many pathogenic bacteria. By 2050, 10 million deaths are expected due to antimicrobial resistance and are huge in comparison to 8.2 million estimated deaths due to cancer [2].

The continued loss of effectiveness of antibiotics and no new antibacterial agents has seen the rise in the use of herbal medication as the conventional alternative. The world today has embraced the use of naturally occurring products that are thought to have antimicrobial properties due to less resistance. In addition, the safety associated with these products as well as their easy availability has contributed greatly to their use. Such natural products include essential oils derived from the commonly occurring medicinal herbs and plants that are mostly used as spices in foods. Many studies have reported the biological activities of essential oils including antimicrobial activity against resistant bacteria [3].

A. baumannii which is common in hospital and community acquired infections, is a ubiquitous and gram negative bacterium. This is emerging as the reason for global outbreaks due to its increased resistance. Many countries reported the multi-drug resistant strains of the bacterium [4]. A report published in 2017, by World Health Organization indicated the list of 12 priority pathogenic threats to human health and *A. baumannii* is one among them [5]. Therefore, the present study investigated the antibacterial activity of selected essential oils (selection based on antibacterial reports in literature and wide availability) against *E. coli* and resistant strain of *A. baumannii*.

2. MATERIALS AND METHODS

2.1 Materials

All the chemicals and solvents that were used in this study were of analytical grade and will be obtained from well-identified suppliers. The chemicals and solvents will include methanol, dimethyl sulfoxide, tween 80, ciprofloxacin standard and normal saline.

2.2 FTIR Studies of the Essential Oils

FTIR studies of the selected essential oils were performed by using FTIR spectrometer (Shimatzu, Japan) by using KBr palette method. The scanning range of 400 to 4000cm^{-1} with the resolution of 4cm^{-1} was maintained during the study.

2.3 Antibacterial Activity of Essential Oils

The study will employ the use of nutrient agar as the media culture and it will be prepared per the instructions outlined on the packaging container by the manufacturers. This will involve suspending 28g of nutrient agar powder in 1000ml clean distilled water. The media will then be heated on a hot plate to boiling and then sterilized by autoclaving for 15 minutes at temperature conditions of 121$^{0`}$C and 15 bars pressure. Sterile media will then be cooled to around 45^{0}C and then exactly 20 ml of the media dispensed in disposable Petri dishes in lamina flow and allowed to solidify.

The three essential oils; oregano, clove oil and tea tree oil were evaluated for their antibacterial activity at the concentration range of 1% - 0.03125%. These essential oil concentrations were prepared by diluting the 100% oil in 1% DMSO solution fortified with 1% tween 80. The tubes containing the essential oils were put in the sonicator to aid in mixing the oil. The diluted essential oils were then transferred into the clean and sterile Eppendorf tubes.

The bacteria strains used in this study were selected based on those associated with gastrointestinal infections. Three bacteria species will be used and this will be pure cultures of *E. coli*, and patient isolate culture *A. baumannii*, before the culturing, during the antibacterial activity screening, the two bacteria were sub-cultured on nutrients for 24 hours to check the viability and as well to obtain fresh colonies that were still in the growth phase [6].

2.4 Preparation of the Sensitivity Disks

Filter paper disks will be used in this study upon which the extracts will be impregnated. By use of a clean paper punch sterilized by rubbing with 70% ethanol solution, cylindrical disks of 6 mm diameter will be punched out of the filter papers. The disks will be filled in a clean glass bottle and sterilized by autoclaving at conditions of 121^{0}C and 15 Bars of pressure. The sterilized disks will then be stored at 4^{0}C in the fridge until the use day.

2.5 Preparation of the Study Bacteria Inoculums

The bacteria cultures of *E. coli*, and *A. baumannii* were suspended in sterile normal saline before inoculation. The freshly isolated colonies of 24 hours old were scoped using hot flame sterilized wire loop and suspended in normal saline. With the help of a wire loop, the colonies were rubbed onto the wall of the test tube to ensure they dissolve in the solution and the turbidity adjusted to 0.5 McFarland standard.

2.6 The Antibacterial Susceptibility Test

The potential antibacterial activity of selected three essential oils was evaluated by the disk diffusion method. Onto freshly prepared and sterilized nutrient agar which was plated in sterile Petri dishes, the respective bacteria inoculum was inoculated uniformly to cover the entire surface of the media using sterile cotton wool swabs. Using sterile forceps, the filter paper disks were laid onto the bacteria inoculated media and each plate was allowed a maximum of four disks. Exactly 15µl of the respective extract at 1different concentrations was dispensed onto disks and allowed 30 minutes to dry. Ciprofloxacin at 30µg/ml was used as the positive control and 1% DMSO was used as the negative control. All the plates were then incubated for 18-24 hours at 37^0 C. The antibacterial activity was indicated by the zones of inhibition around the disks and measured using a ruler in millimeters.

2.7 Docking Analysis

Docking studies using AutoDock Tools Ver 1.5.7 were performed with mycobacterial ATP synthase as the target receptor (PDB ID: 4V1F) and Carvacrol, Eugenol and Terpine -4- ol as the ligands. The protein crystal structure for 4V1F was downloaded from Protein Data Bank. The chemical structures of active constituents of selected essential oils were downloaded as SDF files from the library of PubChem database and this SDF files were converted into PDBQT format using free online software Open Babel. Docking was done by comparing the RMSD of internal ligand and extracted internal ligand (Bedaquiline) [7].

2.8 Data Analysis

The data obtained in this study consisted of zones of inhibition that were measured in millimeters. The values were recorded in the laboratory project book in triplicate and thereafter entered into the excel spreadsheet. Descriptive statistics of the zones of inhibition data was conducted to obtain the mean and respective standard error of the means. The analysis of variance to compare the antibacterial activity of the different essential oils was done and the level of significance determined at $p>0.05$. The analysis was conducted using the Prism graph pad statistical software and the results presented in tables and graphs.

3. RESULTS AND DISCUSSION

3.1 FTIR Studies of the Essential Oils

The FTIR spectra of Oregano oil shows the peaks at 2950 cm^{-1}, 1377 cm^{-1} and 722cm^{-1} corresponding to the C-H stretching of alkane, 1,8- Cineol group due to CH_3(CO) group and characteristic aromatic group peak specific to the camphor compounds respectively. A band at 1460 cm^{-1} indicates a strong methyl band and 809 cm^{-1} indicate C-H vibration of benzene rings.

The FTIR spectra of Tea tree oil shows the peaks at 2987-2905 cm^{-1}, 1718cm^{-1} and 741, 688cm^{-1} corresponding to the H bonded benzene ring, -C=O group stretching vibration and C-H (Benzene ring) plane bending vibration respectively. Another peak at 1600cm^{-1} indicates C-C benzene ring stretching vibration and peaks between 1170-1020cm^{-1} indicate C=O (ester) stretching vibration and C=O (tertiary alcohol in terpenes) stretching vibrations.

The FTIR spectra of Clove oil shows the peak at 3359cm^{-1} indicates C-H stretching, at 1648 cm^{-1} peak indicates the C-H stretching vibration of benzene ring and at 1375cm^{-1} peak corresponding to the C-H deformation vibration of eugenol methyl group, 1082 cm^{-1} prominent peak indicates C-O-C stretching vibration of aromatic ether specific to eugenol derivatives.

The FTIR spectral studies shown in Figs. 1, 2 and 3 confirm the constituents of the essential oils with the presence of peaks indicating the presence of terpene, eugenol methyl group, camphor specific aromatic group and aromatic benzene.

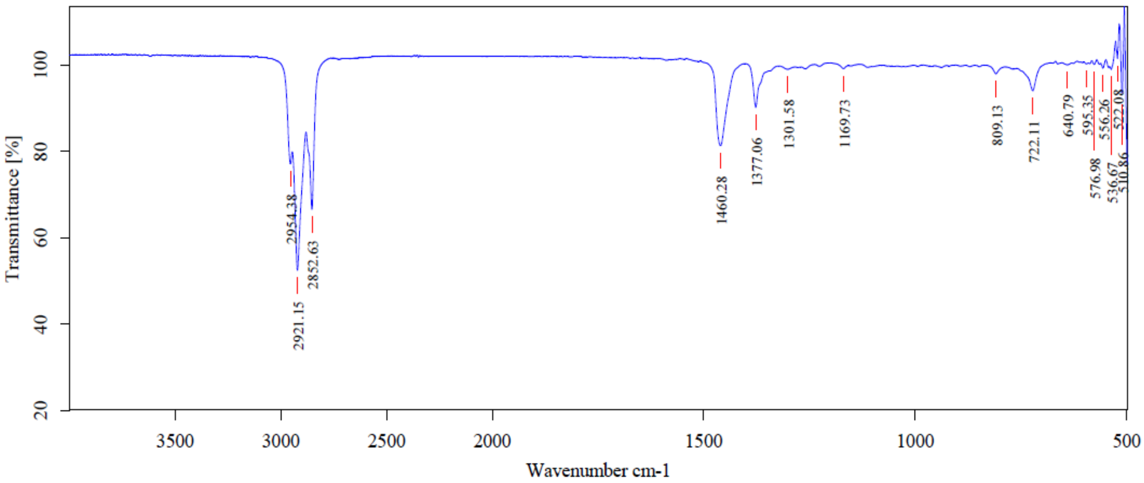

Fig. 1. FTIR spectra of Oregano oil

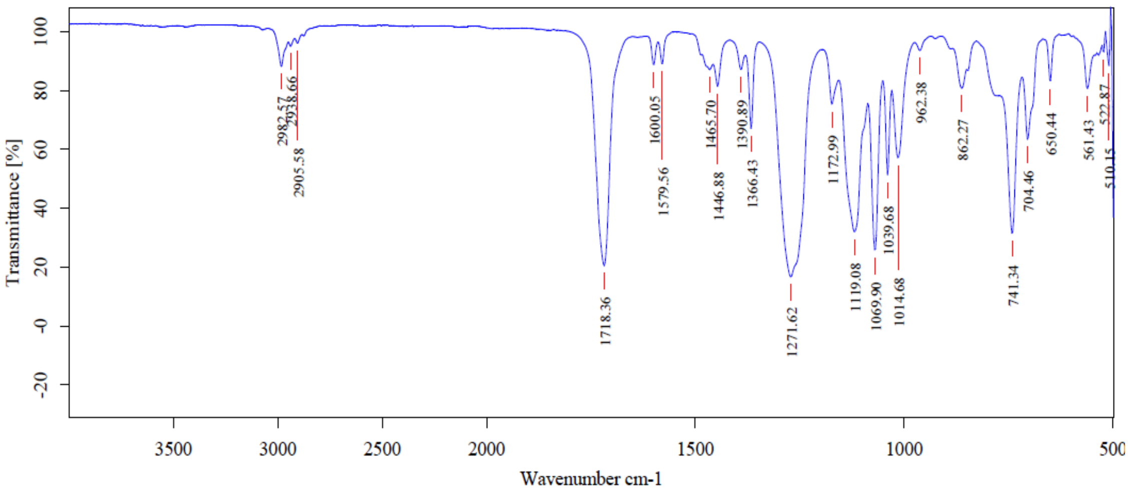

Fig. 2. FTIR spectra of Tea tree oil

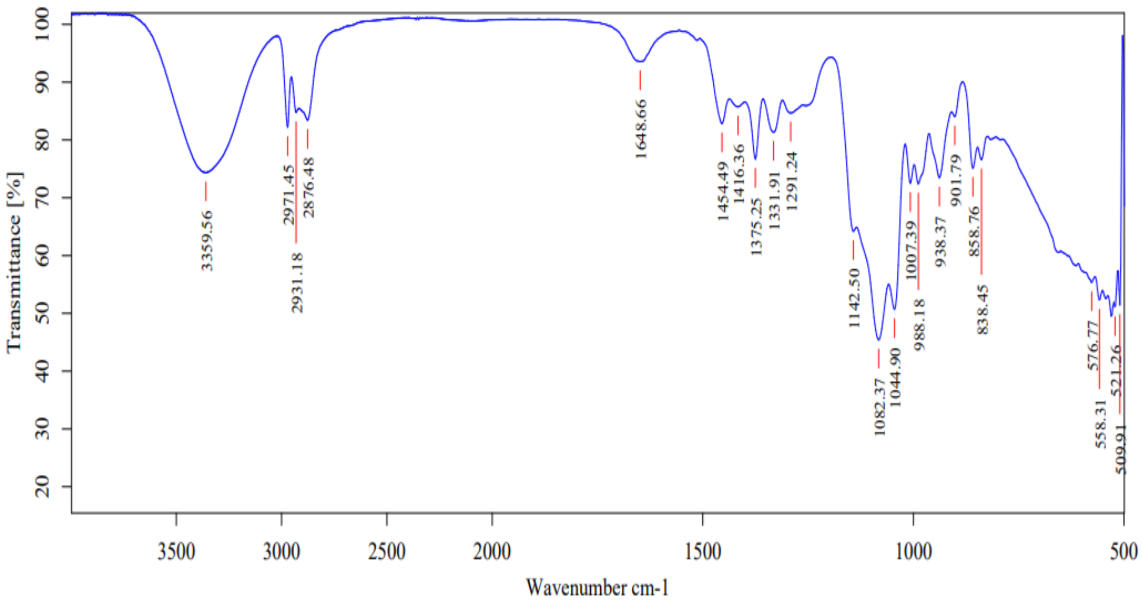

Fig. 3. FTIR spectra of Clove oil

3.2 Antibacterial Activity of Essential Oils against *E. coli*

The antibacterial activity of the three selected essential oils was investigated against *E. coli* pure culture bacteria. Disc diffusion method involving loading an exact volume of the oil on the filter paper disc was used. The ability of the essential to diffuse from the discs to the bacteria inoculated media was assessed and the zones of inhibition around the discs were recorded.

The three selected essential oils: clove oil, oregano oil, and tea tree inhibited the growth of the *E. coli* at different degrees. The antibacterial activity decreased with a decrease in the concentration of the essential oil (Table 1). The zones of inhibition in the range of 21.000± 0.00-10.000± 0.00 mm, 16.500± 0.500 -9.500± 0.500 mm, and 20.500± 0.500 - 9.000± 0.000 mm were recorded against *E. coli* by tea tree oil, clove oil and oregano oil respectively. The standard antibiotic ciprofloxacin recorded zone of inhibition of 31.500± 0.500 mm (Table 1).

The comparison analysis (Fig. 4) showed that ciprofloxacin was the best inhibitor of *E. coli* with a zone of 31.500± 0.500 mm. all the essential oils had antibacterial activity that was lower than that of ciprofloxacin (Table 1). At the highest concentration of 1%, tea tree and oregano oil recorded zones of inhibition that were significantly not different from each other ($p>0.05$). However, the two essential oils zones of inhibition were significantly larger than the one recorded by clove oil ($p<0.05$). At concentration levels, 0.5%, 0.0625%, and 0.03125% no significant difference was noted in the zones of inhibition recorded by clove oil and oregano oil ($p<0.05$).

Table 1. Antibacterial activity of selected Essential oils against *E. coli*

Concentration (%)	Zone of inhibition (MEAN±SEM)		
	Tea tree oil	**Clove oil**	**Oregano oil**
1	21.000± 0.00	16.500± 0.500	20.500± 0.500
0.5	18.500± 0.50	15.000± 0.00	15.500± 0.500
0.25	15.500± 0.500	14.000± 0.000	12.500± 0.500
0.125	14.000± 0.00	13.000± 0.00	10.00± 0.00
0.0625	12.000± 0.00	10.000± 0.500	9.500± 0.00
0.03125	10.000± 0.00	9.500± 0.500	9.000± 0.000
Correlation coefficient (R)	0.935463353	0.865869513	0.99428476
Ciprofloxacin 30 mcg	31.500± 0.500	31.500± 0.500	31.500±0.500

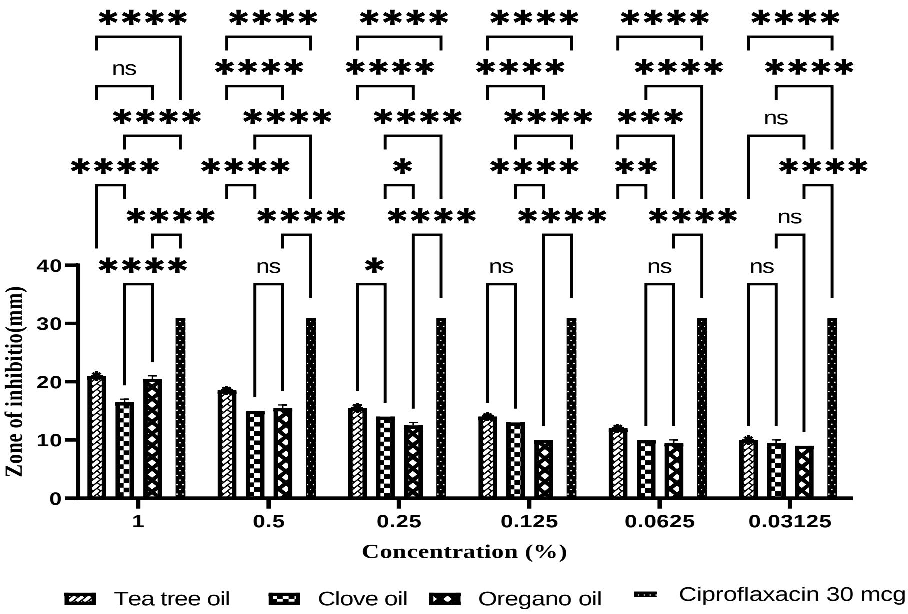

Fig. 4. Comparisons of zones of inhibition of selected essential oils against *E. coli*

3.3 Antibacterial Activity of Essential Oils against *A. baumannii*

The ability of the three essential oils to have an antibacterial effect against the patient isolate *A. baumannii* was investigated. The essentials oils had shown different inhibitory effects with the zone of inhibition of tea tree ranging in 28.500±0.500-6.000± 0.00 mm, clove oil 22.500± 0.500-9.000± 0.000 mm, and oregano oil 20.000±0 0.000 - 9.000± 0.000 mm. *A. baumannii* was not sensitive against ciprofloxacin the standard antibiotic and recorded zone of 6.000± 0.00 mm.

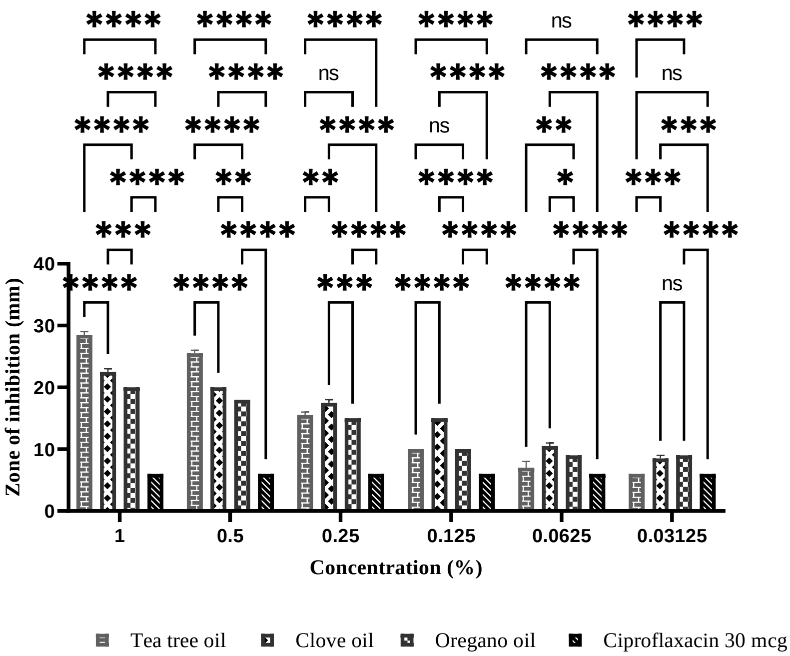

Fig. 5. Comparisons of zones of inhibition of selected essential oils against *A. baumannii*

Fig. 5 and Table 2 show the comparison of the antibacterial activity of the three essential oils against *A. baumannii*. From the graph it's evident that all the three essential oils were more potent against *A. baumannii* as larger zones of inhibition were recorded as compared to those recorded by ciprofloxacin. At concentration level 1% and 0.5% antibacterial efficacy was in the order tea tree>clove oil > oregano oil. At concentration level 0.25%, 0.125% and 0.0625% clove oil recorded significantly larger zones of inhibition than both tea tree and oregano essential oil (P>0.05). Regression coefficient values between the concentration and zone of inhibition indicate a good concentration dependent activity of tea tree oil and oregano oil in comparison with clove oil against the selected resistant strain.

3.4 Docking Analysis

ATP synthase protein is gaining important role in antibacterial activity against drug resistant bacteria. Drug resistant mutations of ATP synthase and drug target interactions studies reveal ATP synthase as an important target for the antibacterial compounds [8].

Present investigation aimed in docking analysis of major components of selected essential oils against ATP synthase enzyme (PDB: 4V1F). Details of analysis and the respective parameters were presented in Table 3 and Fig. 6. Docking analysis clearly indicated the capability of all the selected essential oil main constituents to inhibit the target protein. Carvacrol of oregano oil indicated the maximum docking score with least inhibitory concentration of 969.33µM in comparison to the other essential oils and also the standard complexing compound bedaquiline.

Tea tree oil in literature was reported for its ability to inhibit wide varieties of bacteria [9]. Oregano oil with its constituents other than carvacrol is reported for antibacterial activity and also a promising adjuvant and a replacement of exiting anti-bacterial agents. Investigation of oregano oil on multi drug resistant microorganisms revealed it's potential as an alternative to antibiotics especially for wound associated infections [10]. The same investigation also reported about its skin safety at 10mg/mL up to three days. The mechanism of antibacterial activity of oregano oil can be by its irreversible damage to cell membrane causing leakage of macromolecules, inhibition of metabolites and inhibition of pvl gene [11]. Tea tree oil effect on organisms expressing drug resistance indicated a rapid killing time with mode of action unclear [12]. Many reports confirm the ability of antibacterial activity of tea tree oil against resistant bacteria [13,3]. Controlled trial in 2001 involving 224 patients infected with Methicillin- resistant *Staphylococcus aureus* indicated aerosolized tea tree oil resulted in 41% relief from infection [14].

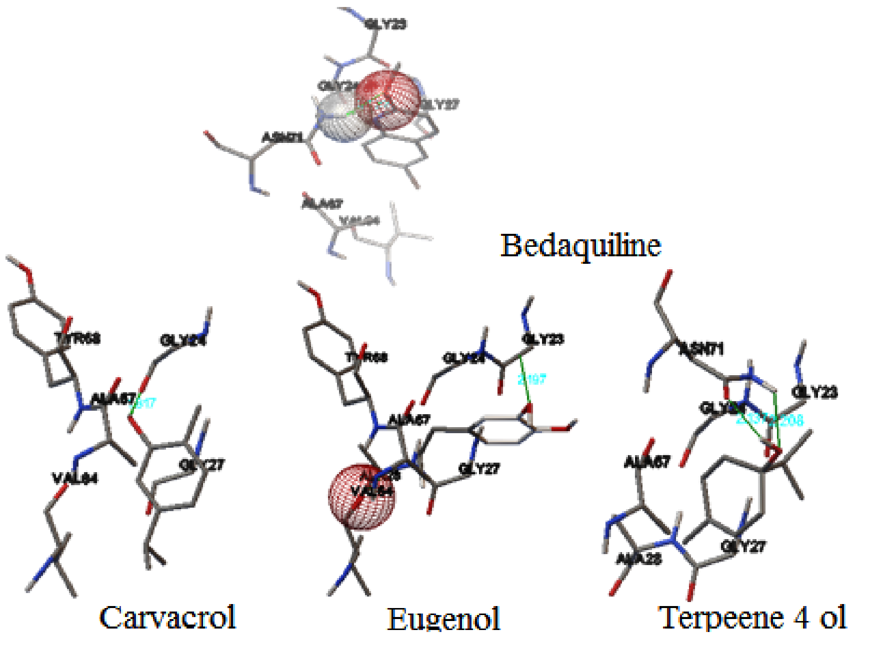

Fig. 6. Binding interaction analysis of active constituents of selected essential oils with mycobacterial ATP synthase (PDB 4V1F)

Table 2. Antibacterial activity of selected Essential oils against *A. baumannii*

Concentration (%)	Zone of inhibition (MEAN±SEM)		
	Tea tree oil	Clove oil	Oregano oil
1	28.500±0.500	22.500± 0.500	20.000±0 0.000
0.5	25.500±0 0.50	20.000± 0.000	18.000± 0.000
0.25	15.500± 0.500	17.500± 0.500	15.000± 0.000
0.125	10.000± 0.00	15.00± 0.000	10.000± 0.000
0.0625	7.000± 1.00	10.500± 0.500	9.000± 0.000
0.03125	6.000± 0.00	8.500± 0.500	9.000± 0.000
Correlation Coefficient (R)	0.934775994	0.877477307	0.919972175
Ciprofloxacin 30 mcg	6.000± 0.000	6.000± 0.000	6.000± 0.000

Table 3. Binding interaction parameters for docking studies

S. No.	Compound	Docking score (Kcal/mol)	Reference RMSD	Minimum inhibitory concentration
1	Bedaquiline	-4.06	13.24	1.06mM
2	Carvacrol	-4.13	170.40	969.33µM
3	Eugenol	-3.57	170.60	2.41mM
4	Terpene - 4-ol	-3.81	171.01	1.61mM

All the three selected essential oils show antibacterial action by similar mechanisms including cell morphology change, alteration of integrity and cell wall damage [15]. A recent work about the effectiveness of essential oils against resistant bacteria indicated the oils are active at different extents and among the selected tea tree oil was found to be the most effective [16].

A. baumannii is a pleomorphic, non-motile, aerobic gram-negative bacillus bacteria. This pathogenic bacterium is most sorting for in the medical sector due to the number of infections it causes that are on the rise. Similarly, the resistance to antibiotics by this bacteria is as well on the rise [17]. The occurrence of this bacterium is mostly in the hospital setting where patients hospitalized for the long term are at a greater risk. The infections as result of *A. baumannii* have as well great impact among individuals with compromised immune system such as people living with HIV and AIDS, transplant patients and cancer patients.

A. baumannii resistance mechanisms are because of spontaneous mutations and over expression of efflux pump. ATP synthase is a membrane protein prone for drug resistant mutations and a target for anti-microbial agents. It is a drug target beyond mycobacterium. It is essential enzyme in gram negative pathogens like *A. baumannii* but the role is scarcely investigated [18]. Selected essential oils are reported to have anti-microbial activity by disrupting membrane as one of their mechanism. Terpenes are the major constituents in the selected essential oils. Terpene derivatives are proved to possess ATP synthase inhibitory action [19]. In specific, carvacrol and eugenol possess ATP synthase inhibiting activity [20]. An attempt to understand the ATP synthase inhibitory capacity of the active constituents successfully revealed their potential. Carvacrol was found to be more efficient than the other two essential oils.

Efflux pumps are the transmembrane proteins that expel drugs from the cell and thus limit their action. A recent review explained the role of essential oils in inhibiting the efflux pump in order to reverse the drug resistance [21]. The selected essential oils oregano oil [10], clove oil [22] and tea tree oil [23] are reported for their inhibitory action on efflux pump of the microorganism under investigation. A combination of ATP synthase and efflux mechanisms might be the reason for the more inhibiting capability of essential oils than the drug Ciprofloxacin on drug resistant *A. baumannii.* A combination therapy of antibiotic along with essential oils may effectively reduce the antibacterial resistance and may help in reducing the dose of antibiotic.

The ever-increasing resistance of the bacteria to many antibiotics is a global health concern [24]. This has been followed by an increase in mortality rate as a result of the infectious diseases caused by bacteria. The reduction in the efficacy of the available antibacterial agents has been a contributor to antibacterial resistance being witnessed. Additionally, with increase in many pathogenic bacteria acquiring resistance has seen a re-emergence of the older pathogenic bacterial infections [25]. The control and treatment of the bacterial infection requires use of sophisticated equipment and close range health care which is very expensive. This has resulted into a huge financial burden hence a limiting factor to the many people with remote resources. Therefore, the need for new and alternative antibacterial agents to fight these pathogenic bacteria is urgently needed [26]. These alternatives can be found in natural products such as plants and herbs.

4. CONCLUSION

The results of the present study showed that essential oils are potential antibacterial agents. The studied extracts showed the ability to inhibit both gram-positive and gram-negative bacteria as well as the resistant strains. This was shown by the inhibition of the patient isolate; *A. baumannii* which was resistant against many antibiotics. Therefore the studied essential oils can be used in the management of the *A. baumannii* infections as well those resulting from *E. coli* like diarrhea. With the further investigation on this property, the essential oils can be recommended for application in various antibacterial drugs for both topical and oral administration.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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