Short Research Article

In-Vitro Antibacterial potentials of Essential oil from *Citrus limon* against selected pathogenic bacteria isolated from cultured Nile Tilapia (*Oreochromis niloticus*)

ABSTRACT

Essential oils (EOs) from medicinal plants like lemon (*Citrus limon*) are considered promising to replace synthetic antibiotics in aquaculture production due to their ability to improve fish health. The aim of the research was to investigate the *in vitro* antibacterial potentials of varying levels of lemon EO against *Streptococcus agalactiae, Escherichia coli, Staphylococcus aureus* and *Aeromonas hydrophila* isolated from Nile Tilapia. This research was conducted at the Microbiology unit., Kenyan Marine and Fisheries Research Institute, Mombasa, Kenya and Environmental Microbiology and Biotechnology Laboratory, Department of Microbiology, University of Ibadan, Ibadan, Nigeria in December 2021. Extraction of EO from lemon peel was performed using steam hydro distillation. The quantitative and qualitative profiling of extracted oil was performed using Gas Chromatography-Mass Spectrometry. The antibacterial potential of lemon EO was tested against some pathogens using the agar well diffusion method while the Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC) were determined by the broth dilution method. The most abundant chemical was D-Limonene (37.56%), 3-Carene (15.88%) and Terpinen-4-ol (93.4%). The lemon EO at 100% and 50% concentration respectively showed the highest antibacterial activity against *S. agalactiae* (26.00mm and 28.33mm) and *E. coli* (33.00mm and 30.00 mm); while the least activity was observed against *A. hydrophila* (8.33mm and 6.67 mm) at $P = .05$. The MIC and MBC values revealed that 50% lemon EO inhibited bacterial growth for *S. agalactiae* whereas it was greater than 50% for *S. aureus*, *E. coli* and *A. hydrophila*. Lemon EO has potential antibacterial properties against tested pathogens that affect cultured fish and could be considered a good candidate for further research to develop new antibacterial drugs in aquaculture.

Keywords: Citrus limon, Essential oil, Antibacterial, Fish pathogens, Tilapia

1. INTRODUCTION

Food security and poverty alleviation are major focuses of the world's development agenda. The lack of regular access to nutritious and sufficient food put humans at greater risk of malnutrition and poor health. According to the UN Food and Agriculture Organization [1], at least 9.2 million people in Nigeria faced a crisis or worse levels of food insecurity between March and May of 2021 amid armed conflicts, COVID-19's effects and climate change. Fish contributes to food security and poverty reduction because it represents the principal animal protein food source for the human population. Fish contributes over 40% of the total dietary protein consumption of Nigerians and fish consumption at 13.3 kg/person/per year [2]. There is still a fish supply deficit resulting from unstable and declining captured fisheries. Foresight Model projections have revealed that the fish supply-demand gap in Nigeria will continue to widen over the coming decades. Fish imports are used to augment deficits and these currently cost the Nigerian government around 1 billion USD in valuable foreign exchange annually [3].

Aquaculture has an important role to play in this effort by providing fish products rich in protein, essential fatty acids, vitamins, and minerals. It also provides income and employment opportunities and is more likely to meet future fish demand increases. The Nile tilapia is an important commercially cultured fish species in Nigeria due to the high demand for fish and tolerance to extensive and intensive culture conditions as well as high yield potentials [4]. Despite the huge potential and strides made in the aquaculture sector, a negative growth trend has been observed in the industry. It has been predicted that the annual rate of growth of aquaculture is expected to decrease from 11 per cent (2010–2015) to 7% (2015–2025) to 5% (2025–2035) and then to 2% (2035–2050) (3). One of the major constraints limiting the expansion of the aquaculture industry is the invasion of infectious diseases (5). Diseases in farmed fish usually arise when stocking density is high and feed quality is poor. Infectious agents are mostly caused by pathogens of bacteria [6] and have been shown to be responsible for about 70 to 80 % of disease infections in farmed fish.

An emerging bacterial pathogen that infects armed fish and leads to substantial mortalities in developing countries is the *Streptococcus* species*. Streptococcus* species causes Streptococcosis in freshwater aquaculture and the most common pathogenic *Streptococcus* species affecting fish, especially tilapia is *Streptococcus agalactiae* [7]. Streptococcosis can cause mortality of up to 30–90% in farmed tilapia [8], leading to severe economic losses from outbreaks. The symptoms of Streptococcosis infection in cultured fish include weakness, loss of appetite, abnormal swimming behavior, exophthalmia, and intra-ocular, muscle, anus, and fin base hemorrhage [9]. Another virulent bacterium affecting aquaculture is *Aeromonas hydrophila*, a Gram-negative bacterium, with a cosmopolitan distribution, that thrives in freshwater as well as marine environments with the ability to inflict diseases in catfish and tilapia [10]. The pathogen causes skin ulcerations and hemorrhagic septicemia [11]. *Escherichia coli* is a bacterium that commonly lives in the intestine of farmed fishes. Most strains of *E. coli* are normal inhabitants of the small intestine and colon are non-pathogenic. Nevertheless, this non-pathogenic *E. coli* can cause disease if they spread outside the intestine to other organs. The pathogenic strains of *E. coli* may cause diarrhea by producing and releasing toxins that are enterotoxigenic [12]. *Staphylococcus aureu*s is another important opportunistic bacterium that affects cultured fish, and their population gives an indication of the general quality of fish. It is not a normal part of the microbiota assemblage of aquatic animals and its presence may be associated with diseases [13].

The application of chemotherapeutic drugs is practical and easy to implement in aquaculture. But the overuse or continuous use of antibiotics in aquaculture health management has resulted in the emergence of drug-resistant genes and multiple antibiotic resistance bacteria [14]. The efficacy offered by chemical-based drugs, and their application not only causes destruction to the environment but also poses a health risk to humans when consumed. These have made it essential to find alternative ways of disease prevention in aquaculture [15]. Recently, phytogenic extracts such as essential oils (EOs) have shown promise as preventive and environmentally friendly alternatives to antibiotics in aquaculture [10]. Diverse medicinal plants have been reported worldwide and among the valuable sources of plants used in the pharmaceutical and nutritional industry are the lemon fruits.

The EO from lemon fruits mainly exist in the fruit peels which are usually considered as agro-industrial wastes, it has active chemical compounds known as D-limonene [16]. Lemon EO has been reported to have beneficial biological effects such as antimicrobial, antioxidant, radical scavenging, anti-cholinesterase, anthelmintic, and anticancer properties [17] on aquatic animals. Despite available information on the beneficial use of plant extracts to improve sustainable aquaculture production, there is a scarcity of information on the potential of EO from lemon fruits to enhance disease resistance against pathogenic bacteria of commercially important cultured fish in Nigeria. Therefore, this research investigated the *in-vitro* antibacterial potentials of varying levels of lemon EO as an alternative to commercial antibiotics against emerging pathogenic bacteria isolated from cultured *Oreochromis niloticus*.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

2.1 Extraction of Lemon essential oil

Lemon (*Citrus limon*) fruit was procured from the National Horticulture Research Institute, Ibadan, Nigeria. The fruit was washed with de-ionized water, peeled and afterwards, the peel was cut into small pieces to obtain 500g. The extraction of the essential lemon oils was done by steam distillation [18] with modifications using a Clevenger device during the period of 4 h. The Clevenger set-up consists of a 1000-mL round–bottomed flask (Isolab, Wertheim Germany), a volatile oil determination tube and a reflux condenser (Norm Cam, Ankara, Turkey). The pre-treated Lemon peel sample was placed in a 1000-mL round–bottomed distillation flask (Isolab, Wertheim Germany) and 1,000ml of water was added. The extraction process was done at a temperature of 100^0C for 180 minutes. The distillate was collected into a conical flask and a separating funnel was used to separate the citrus oil from the water.

The lemon yield was calculated as Yield = $\frac{Amount\ of\ citrus\ oil\ extracted}{Mass\ of\ geated\ lemon\ peels} \times 100$

The extracted essential oil was kept in a refrigerator at 4 °C prior to the analysis of chemical constituents using a gas chromatographer coupled with a mass spectrometer [19].

2.2 Evaluation of the chemical composition of lemon essential oil

Different molecules were identification in the essential oil sample following the method of Ligor *et al.* [20] with some modification, using an Agilent Technologies 7890B gas chromatograph coupled with a mass spectrometer system 5975 Agilent Technologies. The principle behind the analysis was the separation technique. The mobile phase was helium gas, and the stationary phase was the column of Agilent technologies HP5MS with a length of 30m, an internal diameter of 0.320mm and a thickness of 0.25µm. The initial temperature was 80^0C held for 2 minutes at 12^0C/min to the final temperature of 240^0C held for 6 minutes, the scan range was 50 – 550, the interface temperature between the gas chromatograph and mass spectrometer was 250^0C. One µL volume of diluted oil in hexane was injected and the component of lemon oil was identified and calculated with regard to the retention time of a series of alkanes (C4-C28) used as the reference and the similarity of their mass spectra with those gathered in the NIST-MS and WILEY-MS libraries were used.

2.3 Test organisms

The cultured stock of *Streptococcus agalactiae* (ATCC 12386), *Escherichia coli* (ATCC 25922) *Staphylococcus aureus* (ATCC 923) and *Aeromonas hydrophila* (ATCC 1307) isolated from diseased *Oreochromis niloticus* were obtained from the Microbiology Unit of the Kenya Marine and Fisheries Research Institute, Mombasa Kenya. All cultures of bacteria were maintained on appropriate agar slants at 4°C throughout the study.

2.4 Preparation of culture disc or antibacterial assay

Determination of the antibacterial activity of lemon EO was done using the agar well diffusion method [21] with slight modifications. It was performed using an 18-24 h culture at 37°C. One or two colonies of each test isolate was inoculated into sterile normal saline and adjusted to 0.5 McFarland standard. Sterile swab stick was used to introduce the suspension onto already prepared Mueller-Hinton agar (Oxoid, UK). A-6mm cork borer was used to make wells in the plates and aliquot (50 µl) of lemon EO was filled into each well. The extracted lemon EO was dissolved in 10% aqueous Dimethyl sulfoxide with Tween 80 (0.5% v/v for easy diffusion) and sterilized by filtration through a 0.45 µm membrane filter for the preparation of the other concentrations under aseptic conditions. Each well was filled with 50 µL of each concentration (1, 1:1, 1:5, 1:10, 1:20) of lemon EO. A standard disc containing Streptomycin (25µg/disc) was used as the positive control.

2.4.1. Evaluation of zone of inhibition and Minimum Inhibitory Concentrations (MIC)

At the end of the incubation period, the zone of inhibition was measured with a Vernier caliper. The microdilution method recommended by the National Committee for Clinical Laboratory Standards [21] was used with some modifications to determine the MIC. A series of dilutions (1, 1:1, 1:5, 1:10, 1:20) of lemon essential oils were incorporated into Mueller Hinton broth containing approximately 20 µL (0.5 McFarland standard) of each test bacteria. The MIC value of the extract was determined as the lowest concentration that completely inhibited bacterial growth after 48h of incubation at 35±2^{o}C. All procedures were performed in triplicate and mean values were calculated.

2.4.2. Evaluation of Minimum Bactericidal Concentration (MBC)

The bactericidal action of the lemon EO was evaluated by adding 20µL of the microbial culture of test pathogens removed from the MIC wells, inoculated on Mueller Hinton agar plates incorporated with the lemon essential oil. The concentration equal to the MIC was incubated at 35±2^{o}C for 24 h. The lowest concentration that revealed no visible bacterial growth after sub-culturing was calculated as the MBC. Procedures were performed in triplicate and the mean values were calculated.

2.5. Statistical analyses

The zone of inhibition, MIC and MBC were evaluated using one-way ANOVA according to treatments and the Turkey Multiple Range Test (P = .05) was used to compare the potentials of the lemon EO against each bacterium. All statistical analysis was conducted with Graphpad Prism version 8.

3. RESULTS AND DISCUSSION

3.1 Constituents of extracted lemon essential oil

The average yield of the extracted lemon essential oil using the steam hydro-distillation method was 1.40 % of lemon peels. The average yield of the extracted lemon essential oils is close to the percentage yield (1.56%) reported by Zeleke [14] (22). The main chemical classification detected in the lemon essential oil were monoterpene, oxygenated monoterpene, sesquiterpene, oxygenated sesquiterpene, hydrocarbons and oxygenated hydrocarbons with percentages of 55.95%, 25.18%, 9.71%, 4.86%, 1.16% and 3.14% as shown in figure 1. The chemical constitutes with the most abundance was D-Limonene (37.56c%), 3-Carene (15.88%) and Terpinene-4-of (9.34%) while p-Xylene (0.07%), Methyl palmitate (0.07%) α-Costol (0.05%) are detected as traces in the essential oil (Table 1). The composition of the lemon essential oil revealed that the most abundant component detected in the oil was D-limonene (37.56%). This corroborates with the findings of Bourgou *et al.* [23] that Limonene (37.63%) was the main constituent of lemon essential oil. Similarly, Yazgan *et al.* [24] reported that the main compound of lemon peel essential oil was D-limonene (52.85%).

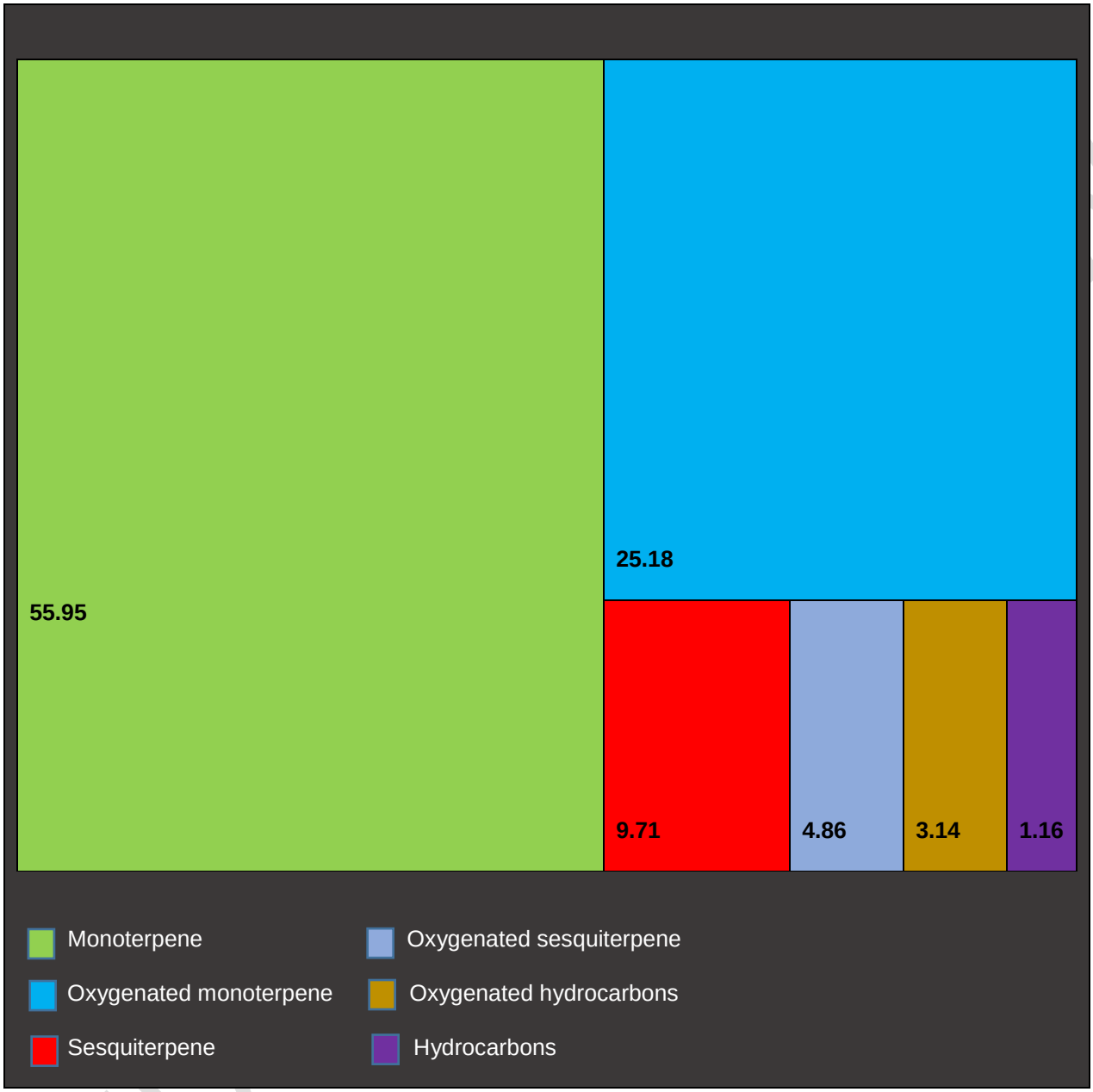

Figure 1: Major classification of lemon essential oil components identified

Table 1: Chemical constituents of extracted lemon essential oil

S/N	Retention time	% Composition	Constituent	Chemical formula
1	5.221	0.07	p-Xylene	C_8H_{10}
2	6.042	4.39	α-Pinene	$C_{10}H_{16}$
3	7.160	15.88	3-Carene	$C_{10}H_{16}$
4	9.629	37.56	D-Limonene	$C_{15}H_{16}$
5	9.640	0.42	γ-Terpinene	$C_{10}H_{16}$
6	9.671	0.14	4-ethyl oxylene	$C_{10}H_{14}$

7	9.704	0.79	Isoterpinolene	$C_{10}H_{16}$
8	9.731	1.16	Linalool	$C_{10}H_{18}O$
9	9.796	0.19	Trans-Carveol	$C_{10}H_{16}O$
10	9.942	1.20	Cis-Limonene oxide	$C_{10}H_{16}O$
11	9.964	0.80	Cis-p-mentha-2,8-dien-1-ol	$C_{10}H_{16}O$
12	10.115	0.22	Dihydro Carveol	$C_{10}H_{18}O$
13	10.380	9.34	Terpinen-4-ol	$C_{10}H_{18}O$
14	10.634	3.13	Fenchol	$C_{10}H_{18}O$
15	10.812	1.79	Cis-Carveol	$C_{10}H_{16}O$
16	10.860	1.02	Citronellol	$C_{10}H_{20}O$
17	11.093	1.76	Carvotanacetone	$C_{10}H_{16}O$
18	11.131	0.49	Nerol	$C_{10}H_{16}O$
19	11.476	0.58	Perilla alcohol	$C_{10}HO$
20	11.530	0.26	p-menth-1-en-9-ol	$C_{10}H_{18}O$
21	11.714	2.01	Trans-p-menth-2,8-dien-1-ol	$C_{10}H_{16}O$
22	11.968	1.13	δ-Elemene	$C_{15}H_{24}$
23	12.103	0.58	Citronellyl acetate	$C_{12}H_{22}O_2$
24	12.265	1.12	Geranyl isovalerate	$C_{15}H_{26}O_2$
25	12.778	0.15	Isocaryophyllene	$C_{15}H_{24}$
26	12.838	0.45	2-epi-α-Funebrene	$C_{15}H_{24}$
27	12.951	0.82	Caryophyllene	$C_{15}H_{24}$
28	13.135	2.41	α-trans-Bergamotene	$C_{15}H_{24}$
29	13.270	0.19	(E)-β-Farnesene	$C_{15}H_{24}$
30	13.329	0.17	α-Humulene	$C_{15}H_{24}$
31	13.362	0.18	β-Santalene	$C_{15}H_{24}$
32	13.594	0.10	α-Curcumene	$C_{15}H_{24}$
33	13.626	0.31	Germacrene D	$C_{15}H_{24}$
34	13.799	0.23	α-Bisabolene	$C_{15}H_{24}$
35	13.983	3.02	β-Bisabolene	$C_{15}H_{24}$
36	14.496	0.34	Germacrene B	$C_{15}H_{24}$
37	14.788	1.11	Caryophyllene oxide	$C_{15}H_{24}O$
38	15.263	0.15	Iso Spatulenol	$C_{15}H_{24}O$
39	15.387	0.05	α-Costol	$C_{15}H_{24}O$
40	15.857	0.38	α-Bisabolol	$C_{15}H_{26}O$
41	16.149	0.05	Oplopenone	$C_{15}H_{24}$
42	18.240	0.07	Methyl palmitate	$C_{17}H_{34}O_2$
43	18.747	2.11	Palmitic acid	$C_{16}H_{32}O_2$
44	18.974	0.26	Ethyl palmitate	$C_{18}H_{34}O_2$
45	19.909	0.12	Methyl linoleate	$C_{19}H_{34}O_2$
46	21.454	0.21	β-Humulene	$C_{15}H_{24}$
47	22.502	0.07	Tetracosane	$C_{24}H_{50}$
48	26.197	1.02	α-Selinene	$C_{15}H_{24}$

3.2 Determination of the antimicrobial activity of extracted lemon essential oil

The antimicrobial activity of different concentrations of lemon EO against *Streptococcus agalactiae, Staphylococcus aureus*, *Escherichia coli* and *Aeromonas hydrophila* is presented in Table 2. The pathogens (*Streptococcus agalactiae, Staphylococcus aureus* and *Escherichia coli*) were significantly (*P* = .05) sensitive to varying concentrations of the lemon EO. On the other hand, the oil exhibited lesser activities against *Aeromonas hydrophila* at 100% and 50% concentrations. *Streptococcus agalactiae* and *Escherichia coli* showed, statistically high sensitivity at high concentrations of 100% and 50% essential oil with the zone of inhibition ranging from 26.00mm to 33.00mm. However, the lowest inhibition zone was obtained at 1:20 lemon oil against all the bacteria. The results from the zones of inhibition, minimum inhibitory concentration and minimum bactericidal concentration tests support the general characterization of the test pathogens (*S. agalactiae,* and *S. aureus*) which are Gram-positive bacteria. The test bacteria were sensitive to the lemon essential oils because they do not possess the outer membrane surrounding the cell wall which restricts the diffusion of hydrophobic compounds through its lipopolysaccharide covering. Seow *et al.* [25] stated that the phenolic compounds in essential oil from plants interact with the lipids of the cell membrane which disturbs the structures of cells, thus, resulting in leakage of ions and cytoplasmic content. The low activity of the oil against *Aeromonas hydrophila* and *E. coli*, which are Gram-negative bacteria could be due to the outer phospholipidic membrane which provides the permeability barrier in the cell wall [26].

The comparative effects of lemon essential oil and the standard antibiotic disc on the various test pathogens indicate that the oil has antibacterial potential. *Streptococcus agalactiae* showed the highest sensitivity at a 1:1 concentration of the lemon essential oil which was not different when compared with the positive control. There is a dearth of information on the antimicrobial activity of lemon essential oils against *S. agalactiae*. However, Pathirana *et al*. [27] reported that essential oil from lemon grass had activities against *Streptococcus iniae* (FP5228, S186, S530 and S131), and this is in addition to the study of Hindi and Chabuckm, [28], who reported that extract from lemon juice showed antimicrobial activity against *S. agalactiae.* Lemon essential oil at 100% indicated higher antimicrobial activity (*P* =.05) against *S. aureus* and at 100%, 1:1 and 1:5 against *E.coli* when compared to streptomycin. A similar observation of higher inhibition zone diameter with *Citrus limon* essential oil has been reported for *S. aureus* [16, 23] and *E.coli* (28). The reason for antimicrobial activities may be associated with the chemical contents of lemon essential oils. The major component proved to be D-limonene which could be responsible for the antibacterial activity, particularly in Gram-positive bacteria (*S. agalactiae* and *S. aureus*) [29]. These oils are rich in limonene and other compounds belonging to the cyclic monoterpene hydrocarbon family. The cyclic monoterpene hydrocarbon family is considered to accumulate in the microbial plasma membrane and thus causes a loss of membrane integrity and dissipation of the proton motive force.

Table 2: Zone of inhibition of lemon essential oil against selected fish pathogens (mm)

Fish pathogen	Inhibition zone (mm)						
	Lemon essential oil						
	100%	1:1 (50%)	1:5 (16.6%)	1:10 (9.09%)	1:20 (4.76%)	Streptomycin	DMSO
Streptococcus agalactiae	26.00 ± 1.00^{b}	28.33 ± 1.16^{a}	19.67 ± 1.16^{c}	15.67 ± 2.52^{d}	14.00 ± 1.00^{d}	29.67 ± 0.58^{a}	0.00 ± 0.00^{e}
Staphylococcus aureus	11.33 ± 1.16^{a}	8.667 ± 1.53^{b}	7.33 ± 0.58^{b}	6.67 ± 0.58^{b}	6.00 ± 0.00^{b}	7.667 ± 1.53^{b}	0.00 ± 0.00^{c}
Escherichia coli	33.00 ± 4.36^{a}	30.00 ± 2.00^{a}	24.67 ± 2.52^{b}	15.00 ± 2.65^{d}	10.67 ± 1.53^{c}	19.33 ± 0.58^{c}	0.00 ± 0.00^{d}
Aeromonas hydrophila	8.33 ± 1.53^{a}	6.67 ± 1.16^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	19.00 0 ± 1.00^{c}	0.00 ± 0.00^{b}

*Values represents mean ± SD. The same superscript (a–e) in the same row were not significantly different (*P* =.05).

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *Streptococcus agalactiae* were recorded at 50% of lemon EO while that for *Staphylococcus aureus*, *Escherichia coli* and *Aeromonas hydrophila* were recorded at greater than 50% (Table 3). The growth of *S. agalactiae* was inhibited at 50% (MIC value). Similarly, the 50% dilution exhibited a strong bactericidal effect on the same bacteria (MBC value). The outcome of this current study indicated that 1:1 dilution strongly inhibited bacterial growth of *S. agalactiae* in the MIC and MBC method, but the dilution has less bacterial inhibition and bactericidal effect on the other three pathogenic bacteria, with MIC and MBC values greater than 50%. The results obtained may be due to many factors between assays including differences in microbial growth, exposure of microorganisms to the oil, and the solubility of oil or oil components [30].

Table 3: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration determination of Lemon essential oil against *Streptococcus agalactiae, Escherichia coli and Staphylococcus aureus*

Fish pathogen	**Lemon essential oil**		
	MIC %	MBC %	Tween 80
Streptococcus agalactiae	50	50	>50
Staphylococcus aureus	>50	>50	>50
Escherichia coli	>50	>50	>50
Aeromonas hydrophila	>50	>50	>50

4. CONCLUSION

The present study established that lemon essential oil has potential antibacterial properties against bacterial pathogens that affect aquaculture. It could be considered a good candidate for further research, to develop novel antibacterial drug against pathogens affecting cultured fish.

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