Original Research Article

Invitro interaction of essential oil of *Cymbopogon citratus* (Lemongrass) and ciprofloxacin against fluoroquinolone-resistant *Staphylococcus aureus and Escherichia coli.*

Comment [t1]: Title to be modified.

Abstract

Background: Essential oil (EO) of *Cymbopogon citratus* has been demonstrated to have antibacterial activities against both Gram negative and Gram positive bacteria. The study was, therefore, conducted to evaluate the effects of combined use of *C. citratus* and ciprofloxacin against fluoroquinolone-resistant *Staphylococcus aureus* (FQRSA) *and Escherichia coli* (FQREC).

Methods: The antibacterial activity of the essential oil was evaluated by agar dilution method while the combination studies were done using Checker-board agar dilution technique.

Results: The mean MIC values of EO against the FQRSA and FQREC were 0.043 ± 0.01 mg/ml and 0.049 mg/ml respectively. When ciprofloxacin was combined with EO of lemongrass, 53.9, 28.3, 17.8 and 0.0 % of the FQRSA isolates showed indifference, additive, synergistic and antagonistic effects respectively. Similarly, when ciprofloxacin was combined with EO of lemongrass at different ratios, 44, 35.5, 20.6 and 0.0 % of the FQREC isolates showed combined effects of additivity, synergism, indifference and antagonism respectively.

Conclusions: The results revealed the *invitro* antibacterial potency of *Cymbopogon citratus* oil against fluoroquinolone resistant *S. aureus and E. coli*. More importantly, the study has proved that the combined use of ciprofloxacin and essential oil of lemongrass has increased the sensitivity of both FQRSA and FQREC bacteria.

Keywords: Essential oil; Lemongrass*; S. aureus; E. coli*;, Fluoroquinolone-resistant.

Comment [t2]: Please correct the spelling to be *coli*

Introduction

Lemongrass has been cultivated over eons for medicinal purposes in different parts of the world. It is a genus of Asian, African, Australian, and tropical island plants in the grass family, designates two different species, East Indian *C. flexuous* and West Indian, *C. citratus* [1]. The consumption of *C. citratus* leaves as well as its oil was found in folk remedy for respiratory tracts infections, elephantiasis, malaria,

ophthalmia and cardiovascular disorders [1]. Many scientists have documented the antibacterial and antifungal activities of lemongrass oil against bacteria, yeasts and fungi [2,3,4]. In our previous work, the sensitivity test on the essential oil of *Cymbopogon citratus* against fluoroquinolone-resistant *S. aureus and E. coli* isolates showed that the oil has a promising antibacterial activity against the bacteria tested [5]. We also conducted GC-MS analysis of the essential oil of *C. citratus* and investigated sixteen compounds with the most abundant constituents being citral (3,7-dimethyl-2,6-octadienol) and citral derivatives (2,6-dimethyl-3,7-octadie-2-ol, followed by 2,7-dimethyl-2,7-octanediol, and 2,6-octadienoic acid) which occurred at moderate level (5-30%)[5]. Since the middle ages, essential oil of *C. citratus* has been widely used for parasitical, insecticidal, medicinal, and cosmetic applications, especially nowadays in pharmaceutical, sanitary, cosmetic, agricultural, and food industries [6]. A study in patients with lower uncomplicated UTI showed that non-antibiotic herbal therapy was not inferior to antibiotic therapy in the treatment of the acute phase of UTI [7]. In addition, essential oils of *Pelargonium graveolens* and *Coriandrum sativum* were found to potentiate the effectivity of ciprofloxacin and gentamycin, respectively, against selected uropathogens [8].

Escherichia coli and *Staphylococcus aureus* are bacteria frequently isolated from human specimens in both community-acquired and nosocomial infections. Currently, the treatment of such infections poses a serious health challenge due the increasing rates of resistance of the common pathogens to commonly used antibiotics such as fluoroquinolones and others [9, 10]. The emergence of resistance to fluoroquinolones in virtually all species of bacteria was recognized soon after the introduction of these compounds for clinical use [11]. Undoubtdly, there are several reports showing increased resistance to fluoroquinolones among bacteria causing community-acquired infections, such as *E. coli* and *S. aureus.* [1, 12]. In addition, most of the fluoroquinolone-resistant *Staphylococcus aureus and Escherichia coli* isolates are multidrug-resistant**.** To fight against these resistant organisms, alternative treatment options, such as combinations of two antimicrobials, need to be investigated. Moreover, both lemongrass essential oil (EO) and citral are generally regarded as safe for use as flavouring agents and the oil has also been approved for use as a food additive and for human consumption [13]. As a result, it is important that the combined effects of the oil and the commonly used antibiotics such as fluoroquinolones be investigated to avoid undesirable combined effects emanating

Comment [t3]: Pleas re-write the sentence as, "GC-MS analysis of the essential oil of *C. citratus* showed the presence of sixteen compounds with the most abundant constituents being citral (3,7-dimethyl-2,6-octadienol) and citral derivatives (2,6-dimethyl-3,7-octadie-2-ol, followed by 2,7-dimethyl-2,7-octanediol, and 2,6-octadienoic acid) which occurred at moderate level (5-30%).

Comment [t4]: Add the latest reference to support this statement : (Malik T. Eat Healthy to Keep UTI's at Bay. J Infect Dis Microbiol. 2023; 1(2):1-3. DOI: https://doi.org/10.37191/Mapsci-JIDM-1(2)-01

Comment [t5]: The reference cited here, does'nt mentions *Pelargonium graveolens* essential oil , another reference has to be mentioned for it , Phytother. Res. 25: 1225 - 1228 (2011)

from drug-food or drug-drug interactions. The study was ,therefore, conducted to investigate the antibacterial combined effects of essential oil of *C. citratus* with ciprofloxacin against fluoroquinolone resistant *S. aureus* (FQRSA) *and E. coli* (FQREC).

Materials and methods

Materials

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin, France) while the fresh leaves of *C. citratus* were collected in the morning in December 2018 from Enugu, Eastern Nigeria. The identity of the plant was authenticated by a botanist - Mr. A .O .Ozioko- of the Bioresource Development and Conservation programme (BDCP) Nsukka, Enugu State and voucher specimen was deposited at the Herbarium of the same institution.

Comment [t6]: Please include the voucher no. & identification no. in this regard.

Test microorganisms

Fluoroquinolone-resistant *Staphylococcus aureus and Escherichia coli* were obtained from the Pharmaceutical Microbiology Unit of Department of Pharmaceutical Microbiology, Enugu State University of Science and Technology, Enugu State, Nigeria. The two bacteria were maintained by forthnightly subculturing on nutrient agar slants stored at 4 ^{0}C after previous 24 h incubation at 37 ^{0}C. Before each experiment, the organism was activated by successive subculturing and incubation.

Standardization of test microorganisms

A 12 ml volume of sterile water was added to the agar slant containing a 24 h old culture of the purified test microorganism and shaken carefully to harvest the organism. Subsequently, dilutions were carried out to get microbial population of 10^5 cfu/ml by comparing with Mcfarland 0.5 opacity standard.

Comment [t7]: Include a reference here

Extraction of essential oil of Lemongrass

A 2.0 kg of the freshly harvested leaves of *C. citratus* plant material were chopped, loaded into a 4 litre round bottom flask with sufficient quantity of water and then hydro-distilled in a Clevenger type apparatus for 3 h to get essential oil as previously described [14]. On heating the flask, essential oil glands present in the plant material got ruptured. The steam essential oil vapour generated in the flask passed through a condenser to remove the energy which finally converts the vapour into liquid. The temperature of the condenser was kept low by connecting it to a water circulators loaded with ice blocks. The condensate (mixture of essential oil and water) was collected in an extraction burette. Since the water and essential oil have different densities, essential oil floated on the surface of the water in the extraction burette. The essential oil (EO) was measured directly in the extraction burette. The oil yield was calculated in percentage of volume per weight (v/w) of plant samples. The oil sample was stored in an air-tight container at 0°C for further use.

Preparation of drug stock solutions and discs

Stock solution containing 100 mg/ml of ciprofloxacin was prepared by weighing out accurately 0.5 g each of the drugs and dissolving in 5 ml sterile water. Two fold serial dilutions were carried out to obtain 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 mg/ml of the drug solutions. These solutions were used to prepare the antibiotic discs using Whatmann No 1 filter paper in accordance with the NCCL standards [15].

Comment [t8]: Potency & manufacturer of the antibiotic has to be mentioned.

Evaluation of minimum inhibitory concentration (MIC) of the essential oil against FQRSA and FQREC

The MIC of the EO was determined using agar dilution method [16]. Eight different dilutions of each of the oil in DMSO were prepared by two-fold dilution. The range of the concentrations of the oil against the test isolates was 0.0013 – 0.16 mg/ml. With an automatic micropipette, 0.08 ml each of these different dilutions (one dilution per plate) of the Lemongrass essential oil was introduced into individual agar plates respectively. The molten agar and the diluted agent were mixed carefully and thoroughly and allowed to set. With the aid of a sterile wire loop the standardized test microorganisms were delivered on the agar surface of the plates containing different concentrations of the agent. This was done by streaking (four different strains of the isolates per plate) on the surface of the set agar. These inoculated agar plates were incubated at 37 °C for 24 h. At the end of the incubations, the MICs were determined as previously reported [17, 18]

Comment [t9]: The observance of MIC should be properly reported... i.e how to report which conc. is the MIC?

***In-Vitro* interactions of ciprofloxacin and the EO**

The combined effects of the ciprofloxacin and the lemongrass essential oil against the test microorganisms were evaluated using the agar dilution checker board method as described previously reported [19]. The two agents used for the study were combined and incorporated into molten agar at concentration 3x MIC as previously described [19]. For each test organism, different concentrations of the two combined agents were prepared and evaluated by combining them at different ratios starting from 0:10 (that is, zero part of the ciprofloxacin to 10 parts of the oil), then moving through 1:9, 2:8, 3:7,… and 10:0 The same procedure was repeated for all the test isolates. For each isolate, the fractional inhibitory concentrations (FIC) of all the ratios of the

combined agents were determined and then combined. Their sum gives the combined effect. The FIC value for each agent was calculated using the formula.

$$FIC(A) = \frac{\text{MIC of the extract A in Combination}}{\text{MIC of A alone}}$$

The addition of FIC_A and FIC_B gives the FIC index from where an inference can be drawn.

$$FIC\ (index) = \Sigma FIC = FIC\ (A) + FIC\ (B)$$

The effects of the combinations were classified as synergistic, additive, indifference and antagonistic if the FIC index is ≤ 0.5, >0.5 to 1, > 1 to 2 and >2 respectively [20].

Result

Extaction Yield

The yield (%) of the essential oil was 1.20 % of the mass of the leaves.

Combined effect of EO of Lemon grass and ciprofloxacin against FQREC and FQRSA

The interactions of EO of *C. citratus* and ciprofloxacin against FQRSA and FQREC were studied using Checkerboard techniques and the results are shown in Tables 2 and 3 respectively. At different ratios of combination of ciprofloxacin and EO of lemongrass, 53.9, 28.3 and 17.8% of the FQRSA isolates showed indifference, additive and synergistic effects respectively. The synergistic and additive effects were more at ratios when the ciprofloxacin concentrations were more than the oil (ie cpx: oil ratio of 9:1 to 6:4). With the FQREC isolates, the proportion of the bacteria that showed antagonistic, additive, synergistic and indifferent combined effects were 0, 44.4, 35.5 and 20.6% respectively. Zero (0%) antagonism was recorded with all the FQREC and FQRSA isolates.

Table 1 MIC values (MIC ± SEM)mm of Lemongrass essential oil against the test microorganisms.

Comment [t10]: MIC values to be mentioned in mg/ml not in mm, it has to be checked.

Replicates of each of FQRSA and FQREC strains	**Mean MIC of lemongrass against FQRSA strains**	**Mean MIC of lemongrass against FQREC strains**
A	0.041 ± 0.01	0.049 ± 0.02
B	0.047 ± 0.01	0.051 ± 0.01
C	0.038± 0.02	0.050 ± 0.01
D	0.042 ± 0.02	0.049 ± 0.01
E	0.047 ±0.02	0.046 ±0.01

Key; Letters A to E, each represents four strains each of the FQRSA and FQREC
FQRSA, fluoroquinolone-resistant *Staphylococcus aureus*
FQREC, fluoroquinolone-resistant *Escherichia coli.*

Table 2: Combined effects of Ciprofloxacin and lemongrass oil as determined by Checkerboard agar dilution against FQRSA (Fluoroquinolone-resistant *S. aureus*).

		Number of isolates (%)			
Ratio		Synergism	Additive	Indifference	Antagonism
CPX	Oil	(≤0.5)^x	(>0.5-1)^x	(>1 – 2)^x	(>2)^x
10	0	-	-	-	-
9	1	0(0)	9(45.0)	11(55)	0(0)

8	2	1(5)	12(60)	7(35)	0(0)
7	3	4(20)	9(45.0)	7(35)	0(0)
6	4	2(10)	13(65)	5(25)	0(0)
5	5	4(20)	12(60)	4(20)	0(0)
4	6	5(25)	10(50)	5(25)	0(0)
3	7	6(60)	10(50)	4(20)	0(0)
2	8	4(20)	12(60)	4(20)	0(0)
1	9	6(30)	10(50)	4(20)	0(0)
0	10	-	-	-	-
Mean		17.8	53.9	28.3	0
Percentage (%)					

Key CPX = Ciprofloxacin
Oil = Essential oil of lemongrass
X fractional inhibitory Conc (FIC) Index

Table 3: Combined effects of ciprofloxacin and oil of lemongrass as determined by Checkerboard agar dilution against FQREC.

Combination ratio		**Synergism**	**Additive**	**Indifference**	**Antagonism**
		(≤ 0.5)^{x}	**(> 0.5-1)^{x}**	**(>1 – 2)^{x}**	**(>2)^{x}**
CPX	Oil				
10	0				
9	1	3(15.0)	10 (50.0)	7(35.0)	0(0)

8	2	2(10.0)	11(55.0)	7(35.0)	0(0)
7	3	2(10.0)	10(50.0)	8(40.0)	0(0)
6	4	3(15.0)	9(45.0)	8(40.0)	0(0)
5	5	11(55.0)	7(35.0)	2(10.0)	0(0)
4	6	8(40.0)	10(50.0)	2(10.0)	0(0)
3	7	11(55)	9(45)	0(0)	0(0)
2	8	10(50)	8(40)	2(10)	0(0)
1	9	13(65)	6930)	1(5)	0(0)
0	10	-	-	-	-
Mean & Percentage		35%	44.4%	20.6%	0%

Key X = fractional inhibitory Concentration (FIC)

Discussion

Many fluoroquinolone antibiotics have been shown, in several reports, to be active against some strains of *Staphylococcus aureus* and *Escherichia coli* [22, 23, 24] However, recent investigations have shown that there is increased emergence of fluoroquinolone-resistant strains of bacteria including *Escherichia. coli* and *Staphylococcus aureus* [22, 23, 25]. The sensitivity of the strains used in the study were ascertained by determining the MIC of the test antibiotic (ciprofloxacin) (Table 1).

Ciprofloxacin, one of the first fluoroquinolones to gain extensive clinical use, is frequently prescribed in both the hospital and in the community. The antibiotic is frequently consumed with food and this could lead to possible drug–food interaction. Again, when the ciprofloxacin is consumed concomittantly with the drug source of lemongrass essential oil, this could also lead to possible drug–drug interaction. The

results of the combined antimicrobial activities indicate that lemongrass essential oil exhibited varied antimicrobial interactions with the tested antibiotic. In the study of antimicrobial interaction using agar dilution checker-board method, more than half of all the test isolates (FQREC and FQRSA) showed additivity while appreciable number exhibited synergism. There was no antagonistic effect recorded when the two agents were combined together against the test isolates. This is in agreement with the result of a research carried out in South Korea where lemongrass oil-amoxicillin and lemongrass oil-norfloxacin combinations showed synergistic interactions against *S. aureus* [26].

Comment [t11]: If the results could be interpreted as reduction in the dose of ciprofloxacin due to the combination of essential oil & antibiotic as there is synergism between these two agents. So it could be discussed in this manner also.

The mechanisms of action of EO of lemongrass on bacterial pathogens have been shown to include disruption of cellular architecture, leading to breakdown of membrane integrity, altering many cellular activities including energy production and membrane transport [27]. Membrane rupture induced by essential oils can lead to leakage of cellular components and loss of ions. The Citral component of lemongrass oil reduce the intracellular ATP of some bacterial pathogen[28, 29]. Intracellular ATP is necessary for storing and supplying metabolic energy, as well as for enzymatic reactions and signaling functions [28, 29]. In a similar study, another component, chlorogenic acid, was found to lower the level of intracellular ATP in *Staphylococcus aureus* isolates [30]. The reduction of intracellular ATP of these bacteria may be attributable to an increased rate of ATP hydrolysis inside the cells [31], or to increased membrane permeability which can cause intracellular ATP leakage through defective cell membranes [32]. Moreover, many biological processes within the cell are dependent on intracellular pH ($pH_{in)}$ including DNA transcription, gene expression and enzyme activities [33]. It has been shown that essential oil brings about changes in pH_{in} of some pathogenic strains of bacteria. It has been shown, that the addition of

essential oil significantly lowers pH_{in} from 6.23 to 5.20 in *Escherichia coli* O157:H7 [34]. The lowered pH_{in} is reported be indicative of membrane damage [31].

In *E. coli*, ciprofloxacin resistance is conferred by point mutations in the *gyrA* gene at the quinolone resistance determining region (QRDR) of the A-subunit of DNA gyrase (topoisomerase II), corresponding to amino acids 67-12 [35,36,37,38]. In *Staphylococcus aureus*, topoisomerase IV, of which ParC and ParE are homologous to GyrA and GyrB, respectively, is the primary target for fluoroquinolones. Mutations in the genes *parC* and *parE* at positions equivalent to those identified in *gyrA* and *gyrB* participate in the high level resistance to fluoroquinolones[39,40].

The exact mechanisms of the combined interactions observed between ciprofloxacin and essential oil of lemongrass are not yet clear. However, these effects may be induced by one or more of the following reasons. Firstly, it is possible that the disruption of the membrane integrity of the test bacteria by the essential oil, which leads to lyses of the cells, might facilitate the influx of ciprofloxacin into the bacterial cells. Such higher concentration in the cells will potentiate the damage on DNA caused by ciprofloxacin. Secondly, the action of ciprofloxacin in altering DNA synthesis may be potentiated by the oil, which also reduces intracellular concentration of ATP that functions in bacterial growth and replication. Moreover, the synergistic or additive effects may be as a result of the inhibition of the ciprofloxacin modifying enzyme by the oil which will potentiate the activity of ciprofloxacin. The reports described in this work provide a strong framework for future research on the molecular basis of interaction between essential oil of lemongrass and ciprofloxacin.

Conclusion:

Our results show that concomitant intake of ciprofloxacin and essential oil of lemongrass will lead to the potentiation of the antibacterial effect of ciprofloxacin or/and increased sensitivity of FQRSA and FQREC bacteria. The majority of the effects produced is a clear beneficial interaction and may imply combining the two agents in infections caused by fluoroquinole-resistant *Staphylococcus aureus* and *Escherichia coli*. It also implies that intake of the oil as a constituent of food may affect the effectiveness of a co–administered ciproflocaxin.

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