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Antibacterial Activity of Essential Oil from *Citrus aurantifolia* (Christm.) Swingle Peels against Multidrug-Resistant Bacterial Isolates

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Abstract

The study investigated the antibacterial activity of essential oil from the peel of *Citrus aurantifolia* against eleven multidrug-resistant (MDR) bacterial isolates of clinical origin. The Kirby-Bauer disc diffusion method was used to determine the antibiotic resistance profile of the isolates. Essential oil (EO) from the peels of lime purchased at a market in Ile-Ife was extracted by the hydro-distillation method, while the sensitivity of the isolates to EO was done via agar well diffusion method. The minimum inhibitory and minimum bactericidal concentrations (MIC and MBC) of the EO against the tested isolates were determined following standard methods. All the tested isolates exhibited multidrug resistance (MDR) characteristics. The multiple antibiotics resistant indexes (MARI%) for Gram-positive bacterial isolates ranged between 70% and 100% while that of Gram-negative was 100%. The yield of EO was 1% and the EO demonstrated activities at 25%, 50% and 100% v/v against the MDR bacterial isolates. The activity of EO was mostly not significantly different at the same concentration for all the isolates, and at different concentrations for each of the isolates. The MIC range for Gram-negative and Gram-positive isolates was between 0.195% to 3.125% v/v and 0.39% to 3.125% respectively while the range was between 1.563% to 3.125% and 0.781% to 6.250% v/v for MBC respectively. The study showed that EO from the peel of lime fruits demonstrated excellent *in vitro* antibacterial activity against MDR bacterial isolates. This potential can be further explored as an alternative for the treatment and management of infections caused by MDR bacterial isolates.

Keywords

Citrus aurantifolia, Essential Oil, Bacteria, Antibacterial, Resistance

1. Introduction

The antimicrobials obtained from plants have been given serious attention due to the development of resistance to conventional antibiotics by some microorganisms [1]. It has been reported that a significant number of the world's population depend on traditional medicine for primary healthcare [2]. Over the years, essential oils and other plant extracts have stirred up curiosity as sources of natural products and have thus, been screened for their potential uses as alternatives for the treatment of many infectious diseases [3]. It has been severally hypothesised and empirical data have shown that the antibacterial potential of agents from natural sources, such as essential oils from plants could serve as means of combating multidrug resistance challenges [4] [5] [6] [7]. One of the plants which serves as a source of essential oil is *C. aurantifolia* and it is used in various applications [8].

It has been reported that *C. aurantifolia* is utilized commonly in West Africa in its natural state, as part of the essential ingredients used in the preparation of most herbal mixtures in the treatment of some illnesses [9] [10]. The antibacterial, antifungal, antiflatoxigenic and anticancer activity of *C. aurantifolia* has been well documented [8] [10] [11] [12] [13]. It has also been reported that extracts from *C. aurantifolia* has significant antimycobacterial activity, chiefly against the isoniazid-resistant strain of mycobacteria [14]. The presence of high amounts of phytochemicals and bioactive compounds in the plant has been reported [15] and amongst these polyphenols, carotenoids and essential oils have been identified as biologically active compounds [16].

The infections caused by the MDR pathogens have been observed as a major threat in the middle and low-income countries [17] and are also a growing trend in high-income countries. According to the Center for Disease Control Antibiotics Resistance Report, it was pointed out that more than 2.8 million antibiotics-resistant infections occurred in the United States of America with 35,000 mortality cases [18]. Studies have shown the potential of essential oil for medicinal purposes from different components of *C. aurantifolia* [10] [15] [16], however, there is a paucity of information on the antibacterial potential of essential oil from the fruit peels against multidrug-resistant bacteria. Hence, this study investigated the *in vitro* antibacterial activity of essential oil from the fruit peels of *C. aurantifolia* against some multidrug-resistant bacterial isolates of clinical origin.

2. Materials and Methods

2.1. Antibiotic Susceptibility Testing of the Bacterial Isolates

The eleven multi-drug resistant bacterial isolates used for this work were of clinical origin and were collected from the culture collections of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. The bacterial isolates were tested against some selected classes of antibiotics using the Kirby-Bauer disc diffusion method as described by CLSI (2016) [19]. Ste-

rile Mueller-Hinton agar (MHA) plates were seeded with standardized inoculum (0.5 McFarland standard) and the following antibiotic discs such as COT—Cotrimoxazole 25 µg, CPX—Ciprofloxacin 5 µg, ERY—Erythromycin 15 µg, AX—Amoxicillin 25 µg, OFL—Ofloxacin 5 µg, STR—Streptomycin 10 µg, CHL—Chloramphenicol 30 µg, CRO—Ceftriaxone 30 µg, GEN—Gentamycin 10 µg, PFX—Pefloxacin 5 µg, AUG—Augmentin 30 µg, NIT—Nitrofurantoin 300 µg, TET—Tetracycline 30 µg were applied on the plates. The plates were incubated at 37˚C for 24 h after which zones of inhibition were observed, measured and interpreted. The experiment was carried out in duplicates. The multiple antibiotics resistant indexes (MARI %) for the isolates were determined following standard method [20].

2.2. Extraction of the Essential Oil

The essential oil was extracted from the peels of fresh lime purchased at the market in Ile-Ife by hydro-distillation using a Clevenger extractor. The fresh lime peels were chopped into small pieces; 900 g of the peels were transferred into the distillation flask (5 L) and 3 litres of water was added to the sample. The mixture was heated on a heating mantle at 85˚C for 3 h [21] and the essential oil was subsequently collected.

2.3. Sensitivity Testing of Extract

The sensitivity testing of the essential oil was determined using agar well diffusion method as described by Irobi *et al.* (1994) [22]. The bacterial isolates were first grown in nutrient broth for 18 h and standardized (0.5 McFarland standard) before use. About 0.1 ml of standardized test isolates was later spread on solidified sterile Mueller-Hinton agar (MHA) plates. Wells were bored into the MHA plates using a sterile 6 mm cork-borer. The wells were filled up with different concentrations of the solution of the essential oil at two-fold serial dilution. The solution was prevented from spilling onto the surface of the medium. The plates were allowed to stand on the laboratory bench for between 1 - 2 h to allow proper inflow of the solution into the medium before incubating the plates at 37˚C for 24 h.

2.4. Determination of Minimum Inhibitory Concentrations (MICs)

The minimum inhibitory concentration (MICs) of the essential oil was determined using the method as described [23]. Two-fold serial dilution of the essential oil was prepared and 2 ml of different concentrations of the solution were added to 9 ml of pre-sterilized molten nutrient agar to give a final concentration of the essential oil. The medium was poured into sterile Petri dishes and allowed to set. Dry surface of the media was streaked with 18 h old test bacterial cultures. The plates were incubated at 37˚C for 72 h after which they were examined for the presence or absence of growth. The MIC was taken as the lowest concentration that prevented the growth of the bacterial isolates.

2.5. Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration (MBC) was determined by using the modified method of Spencer and Ragout de Spencer (2004) [24]. Samples from plates with no visible growth in the MIC assay were taken and sub-cultured on fresh nutrient agar plates incubated at 37˚C for 24 h. The MBC was taken as the concentration of the essential oil that did not show any growth on a new set of sterile agar plates.

2.6. Statistical Analysis

A between groups and within groups ANOVA was performed to test the hypothesis that concentration has effect on the degree of susceptibility of the tested isolates to essential oil. Assumptions of normality were determined and satisfied prior to conducting the ANOVA. Also, the homogeneity of variance test was conducted before conducting Post Hoc multiple comparisons. Three different concentrations (25% v/v, 50% v/v and 100% v/v) of the essential oil were employed and the mean zone of inhibition (ZI) results obtained for the isolates at each of the different concentrations served as the group's data. All statistical analysis was computed using the statistical packages SPSS version 16 and the p-value was set at 0.05.

3. Results

The antibiotic susceptibility profile of the bacterial isolates is as shown (**Table 1**). All the tested Gram-positive bacterial isolates exhibited 100% resistance against cotrimoxazole, ciprofloxacin, amoxicillin, ofloxacin, streptomycin, chloramphenicol and pefloxacin while 80% exhibited resistance against erythromycin and gentamycin. Only 60% of the Gram-positive isolates exhibited resistance against ceftriaxone. Furthermore, all the Gram-negative isolates exhibited resistance against all the antibiotics tested. The MARI% for Gram-positive bacteria ranged between 70% and 100% while that for Gram-negative was 100%.

On the other hand, the yield of essential oil from the *Citrus aurantifolia* peel was 0.5 mL/50g. The antibacterial activities of the various concentrations of the essential oil against bacterial isolates that were earlier subjected to the antibacterial effect of some conventional antibiotics are shown in **Figure 1**.

The highest numerical mean ZI value for each of the tested isolates was observed at 100% v/v of EO while the numerical mean ZI values for each of the tested isolates at 50% v/v of EO was greater than that observed at 25% v/v of EO. Furthermore, the mean ZI values for the isolates were numerically different at each of the EO concentrations tested except in few occasions when two or three isolates had the same mean ZI at the same concentration of EO as shown in **Figure 1**.

The Welch test was used for statistical comparison of mean ZI at different concentrations for each of the isolates and at the same concentration for all the isolates and Games-Howell test was computed for Post Hoc analysis. The Welch

test result (Table 2) showed significant differences in mean ZI between concentration groups (25% v/v, 50% v/v and 100% v/v EO) for only isolates B2 and B3. Games-Howell Post Hoc test (Table 2) showed that the significant difference in mean ZI for the two isolates was only at concentration 25% v/v EO versus 100% v/v EO at $p < 0.05$. Also, the Welch test result (Table 3) showed significant difference in the mean ZI between isolates at concentration 25% v/v EO only and the Games-Howell Post Hoc test (Table 3) showed that the significant difference was only for the mean ZI for isolate B2 versus mean ZI for isolate B6 at 25% v/v EO at $p < 0.05$.

The result of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the EO against the tested isolates are shown in Table 4.

Table 1. Antibiotics sensitivity pattern of the test bacterial isolates.

Test organisms	**ZI (mm)**									
Gram-positive organisms	COT	CPX	ERY	AX	OFL	STR	CHL	CRO	GEN	PFX
Staphylococcus aureus (B3)	0 (R)	0 (R)	0 (R)	0 (R)	10 (R)	0 (R)	0 (R)	5 (R)	8 (R)	5 (R)
Staphylococcus aureus (N1)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	25 (S)	0 (R)	0 (R)
MRSA (N18)	0 (R)	0 (R)	16 (I)	0 (R)	0 (R)	0 (R)	0 (R)	32 (S)	15 (S)	0 (R)
MRSA (N11)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	4 (R)	0 (R)
Streptococcus pneumoniae (B6)	4 (R)	10 (R)	0 (R)	5 (R)	10 (R)	5 (R)	0 (R)	7 (R)	5 (R)	5 (R)
Gram-negative organisms	COT	OFL	AX	CPX	TET	PFX	AUG	CRO	NIT	GEN
Escherichia coli (B1)	0 (R)	2 (R)	0 (R)	5 (R)	0 (R)	8 (R)	0 (R)	0 (R)	0 (R)	6 (R)
Klebsiella sp. (B2)	0 (R)	8 (R)	0 (R)	8 (R)	0 (R)	5 (R)	0 (R)	0 (R)	2 (R)	6 (R)
Klebsiella pneumoniae (B4)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
Pseudomonas aeruginosa (*B5*)	0 (R)	3 (R)	0 (R)	3 (R)	0 (R)	8 (R)	0 (R)	5 (R)	0 (R)	0 (R)
Shigella sp. (S15)	0 (R)	8 (R)	0 (R)	5 (R)	5 (R)	4 (R)	0 (R)	0 (R)	5 (R)	10 (R)
Escherichia coli (EK58)	0 (R)	4 (R)	0 (R)	5 (R)	0 (R)	8 (R)	0 (R)	0 (R)	0 (R)	10 (R)

Key; COT—Cotrimoxazole 25 µg, CPX—Ciprofloxacin 5 µg, ERY—Erythromycin 15 µg, AX—Amoxicillin 25 µg, OFL—Ofloxacin 5 µg, STR—Streptomycin 10 µg, CHL—Chloramphenicol 30 µg, CRO—Ceftriaxone 30 µg, GEN—Gentamycin 10 µg, PFX—Pefloxacin 5 µg, AUG—Augmentin 30 µg, NIT—Nitrofurantoin 300 µg, TET—Tetracycline 30 µg, R—resistant, S—susceptible, I—intermediate, MRSA—Methicillin-resistant *Staphylococcus aureus*.

Table 2. Comparison of Means Zone of Inhibition at constant concentration of EO across all tested isolates.

Robust Tests of Equality of Means ZI*					
Conc.	Test	Statistic	df1	df2	Sig.
25% v/v	Welch	11.86	10	4.37	0.01

Multiple Comparisons (Games-Howell Post Hoc)*							
Conc.	(I) Org	(J) Org	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
25% v/v	B2	(B6)	9	0.71	0.03	1.80	16.19

Key: * = Robust Test of equality of means ZI at one concentration for all the isolates, Conc. = concentration, df = degree of freedom, Sig. = significant level at $p < 0.05$, Std. = standard deviation, Org = organism, B2 = *Klebsiella* sp., B6 = *Streptococcus pneumoniae*.

Table 3. Comparison of means zone of inhibition at the three concentrations of EO per organism.

Robust Tests of Equality of Means ZI**							
Org	Test	Statistic	df1	df2	Sig.		
B2	Welch	26.7	2	1.90	0.04		
B3	Welch	21.00	2	2	0.04		
Multiple Comparisons (Games-Howell Post Hoc)**							
Org	(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
B2	25% v/v	50% v/v	−3.50	1.12	0.21	−13.345	6.35
		100% v/v	−6.00	0.71	0.02	−10.17	−1.83
	50% v/v	25% v/v	3.50	1.12	0.21	−6.34	13.35
		100% v/v	−2.50	1.12	0.31	−12.35	7.35
	100% v/v	25% v/v	6.00	0.71	0.02	1.83	10.17
		50% v/v	2.50	1.12	0.31	−7.35	12.34
B3	25% v/v	50% v/v	−8.00	1.41	0.05	−16.33	0.33
		100% v/v	−10.00	1.41	0.03	−18.33	−1.67
	50% v/v	25% v/v	8.00	1.41	0.05	−0.33	16.33
		100% v/v	−2.00	1.41	0.47	−10.33	6.33
	100% v/v	25% v/v	10.00	1.41	0.03	1.67	18.33
		50% v/v	2.00	1.41	0.47	−6.33	10.33

Note: The table is an excerpt of SPSS result and only significant results are presented. **Key:** * = Robust Test of equality of mean ZI at one concentration for all the isolates, Conc. = concentration, df = degree of freedom, Sig. = significant level at $p < 0.05$, Std. = standard deviation, Org = organism, B2 = *Klebsiella* sp., B3 = *Staphylococcus aureus*.

Table 4. The MIC and MBC determination of the essential oil from *Citrus aurantifolia* peels.

Bacterial Isolates	Essential oil (%)		
	MIC	MBC	MBC/MIC
Escherichia coli (B1)	0.390	3.125	8
Klebsiella sp. (B2)	0.195	1.563	8
Staphylococcus aureus (B3)	3.125	6.250	2
Klebsiella pneumoniae (B4)	3.125	6.250	2
Pseudomonas aeruginosa (B5)	0.781	3.125	4
Shigella sp. (S15)	0.391	1.563	4
Staphylococcus aureus (N1)	0.391	0.781	2
MRSA (N18)	0.391	3.125	8
MRSA (N11)	0.391	3.125	8
Streptococcus pneumoniae (B6)	0.391	1.563	4
Escherichia coli (EK58)	0.391	3.125	8

Key: MIC = Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration; MRSA = Methicillin Resistant *Staphylococcus aureus*.

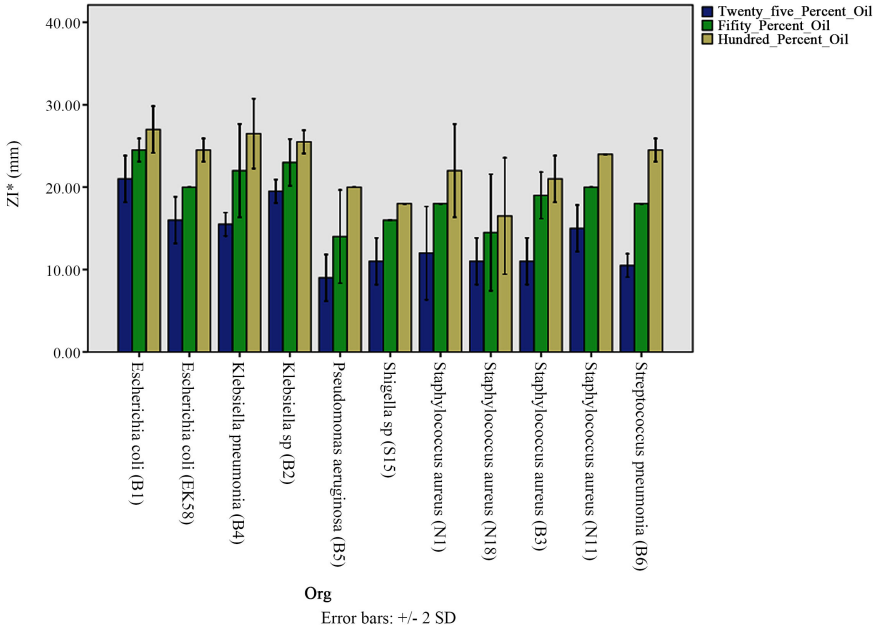

Figure 1. Antimicrobial activity of the essential oil against the test bacterial isolates. **Key:** ZI* = Mean Zone of Inhibition, Org = bacterial isolates. *Note*: *The diluent* (*methanol*) *which was used as control did not show any observable bacterial activity against any of the tested isolates*.

4. Discussion

The antibiotic susceptibility profile of the bacterial isolates showed that all the isolates tested were multidrug-resistant, an indication that infections caused by these isolates may be difficult to treat. The World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) have come out with reactive and preventive approaches to solving challenges of infections caused by MDR pathogens [25] [26]. The major preventive approach is to stop the spread of antibiotics resistance via Antibiotics Stewardship Programs (ASP) and the discovery of new antimicrobial drugs and/or new ways to treat MDR infection cases [25].

In this study, the yield of the essential oil extracted from the lime peel was 1%. This result agrees with the report of Intorasoot *et al.* (2017) [12], but at variance with other studies which reported lower or higher yield [27] [28]. Interestingly, the sensitivity of the MDR isolates to all the three concentrations of EO used in this study falls under "Moderately Sensitive", "Sensitive" and "Very Sensitive" categories based on the classification of the levels of sensitivity of EO [29]. This shows that our EO exhibited a very good *in vitro* antibacterial property against the MDR isolates that were tested for the various concentrations of the EO used.

Studies have elucidated antibacterial properties of essential oil from citrus plants [12] [13] [27] [28] [30]. Our work corroborates the result of earlier studies on the antibacterial potential of EO of lime peel although, most of the studies failed to report the antibiotic resistance profile of their test isolates. The reports on *Citrus aurantiifolia* especially EO from the peel seems to be on the average

based on the relevant studies considered in the present study. However, of the various studies on EO from citrus that we compared with our study, only Intorasoot *et al.* (2017) [12] and Pathirana *et al.* (2018) [13] conducted the antibiotics susceptibility profile of the bacterial isolates and the antibacterial activity of EO extracted from the lime peel.

The antibacterial activity of EO either singly or in combination with other agents against multidrug-resistant bacteria of public health importance has been reported [12] [13] [30]. The EO from the peel of lime has been observed to exhibit broad-spectrum activity [13]. In this study, it was observed that the EO exhibited a broad-spectrum antibacterial activity against the MDR isolates and there is no significant difference between the ZI against bacterial isolates tested. There was no significant difference in the activity of the EO in the MIC and MBC range in the MDR bacteria. The result of the present study corroborates the reports of Intorasoot *et al.* (2017) [12] and Pathirana *et al.* (2018) [13] and this may suggest that EO from the peel of lime fruit could be used in the treatment of infections caused by MDR bacteria.

5. Conclusion

In conclusion, the 1% yield of EO obtained in this study was sufficient for the antibacterial sensitivity testing carried out. The EO demonstrated "Moderately Sensitive", "Sensitive" and "Very Sensitive" categories of activities at 25%, 50% and 100% v/v against the MDR bacterial isolates. This study has shown that EO from the peel of lime fruit exhibits an excellent broad-spectrum *in vitro* antibacterial activity against the MDR bacteria tested. This antibacterial potential of EO can be explored as an alternative in the treatment and management of infections caused by MDR bacterial isolates.

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Conflicts of Interest

The authors declare there is no competing interest.

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