SUSCEPTIBILITY OF MULTIDRUG RESISTANT BACTERIA TO LYOPHILIZED CITRUS LIMON JUICE EXTRACT

By

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ABSTRACT

This research was carried out to evaluate the antibacterial activity of lyophilized Citrus limon on multi-drug resistant Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi, Macrococcus luteus and Clostridium difficile. Phytochemical constituent of the lyophilized citrus limon was determined using standard laboratory methods, while the agar well diffusion technique was used to evaluate the antibacterial potential of the lyophilized extract. Phytochemistry of the lyophilized Citrus limon extract revealed the presence of saponins, tannin, flavonoid, glycoside, cardiac, terpenes, anthraquinone, resins phenol, alkaloid, volatile oils and steroid. Lyophilized Citrus limon juice extract had a significant activity ($p<0.05$) with zone of inhibition as follows 26.00 ± 1.4, 21.00 ± 1.41, 24.50 ± 0.70, 16.50 ± 0.70, 29.50 ± 2.12 and 20.50 ± 0.00 mm at 40 mg/mL against Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi, Macrococcus luteus and Clostridium difficile, respectively. The minimum inhibitory concentration ranges from 10-80 mg/mL and the minimum bacteriocidal concentration ranges from 80-160 mg/mL. This study provides insight on the antibacterial potential of Citrus limon juice extract against multidrug resistant bacterial pathogens. It is recommended that further studies should be conducted to explore the Citrus limon bioactive compound in the development of therapeutic drug.

Keywords: Citrus Limon, Therapeutic, Rutaceae, Lyophilized, Bacteriocidal.

INTRODUCTION

Antibacterial resistance is rising to perilously high levels in all parts of the world, and the emergency of antibiotic resistance is undoubtedly threatening our ability to treat common infectious diseases in the last few decades. These bacteria have abilities to develop ways of evading treatment and can transfer genetic material that permits other bacteria to become impervious to antibacterial. Due to this, the unwanted side effect that usually accompany antibiotic treatment (Adu et al., 2016; Zhang et al, 2019), there is a great challenge for scientific research in seeking most effective, efficient and less expensive antibiotics from natural sources.

To guide this effort, World Health Organizations (WHO) presents a report of priority pathogens list that require research and drug development. *Escherichia coli, Pseudomona aeruginosa, Salmonella typhi, and Klebsiella pneumoniae* have all been identified as one of the most important multidrug resistant bacteria. This is because of their ever-persistent ability to resist antibiotics as well as transfer the mechanism to other bacteria (Zhang et al, 2019).

Fruits are known to decrease the chances of several chronic diseases including cancer, this is partly due to the presence of bioactive constituents like poly phenolic compounds (Pandey et al., 2011; Cotelle et al.,1996). Many fruits like oranges, mandarins, limes. lemons and grapes categorized into Citrus group are reported for enormous number of biological activities such as anti-cancer, anti-diarrheal, antibacterial, antifungal, antiviral insecticidal and antioxidant (Adu et al., 2016; Pandey et al., 2011; Cotelle et al., 1996; Parekh et al., 2006). *Citrus limon* belongs to the family rutaceae (Adu et al., 2016). It is the most abundant and widespread of the genus occurring in the savannah regions. *Citrus limon* (lemon) is an elliptically shaped berry fruit. *Citrus limon* or lime juice exhibits a range of protective health benefits including antioxidative, anti-inflammatory, antitumor and antimicrobial (Adu et al., 2016; Pandey et al., 2011; Cotelle et al., 1996).

Undoubtedly, plants have been a good source of natural products for maintaining human health from time immemorial due to their important antibacterial principle, phyto-constituents and wider therapeutic potentials (Parekh et al., 2006; Tyagi & Malik, 2010). Plants are expected to be a future alternative source of new antibacterial to curtail the present menace of antibiotic resistance. However, there is a little or no information on the antibacterial potentials of many medicinal plants, particularly those mentioned in the traditional and folk medicine on multidrug resistant bacterial pathogens. Hence, this study was designed to determine the antibacterial potentials of *Citrus limon* extract on some multidrug resistance bacterial pathogens.

1. Materials and Methods

1.1 Collection and Identification of Plant Material

Fresh *Citrus limon* fruits (lemon) were purchased from Kure Market, Minna, Nigeria. The fruits were taken to the Department of Plant Biology, Federal University of Technology Minna, Nigeria, for identification by botanists. The Citrus limon fruits were moved to the Department of Microbiology for further analysis.

1.2 Preparation of Citrus Lemon Juice Extract

The fresh *Citrus lemon* fruits were rinsed thoroughly with distilled water and were cut into halves. The juice was extracted from the fruits using a juice extractor. The extracted juice was subjected to double filtration with Whatman No.1 (Germany) filter paper (pore size 25 μm). The filtrate was lyophilized using a Freeze Dryer (Germany, LGJ-18). Sterility of the lyophilized lemon juice was checked by plating an aliquot of the reconstituted extract on nutrient agar (Oxoid) plates. The plates were incubated at 37 ^{o}C for 24 hours aerobically. The extract was stored in sterile containers at 28 $^{o}C \pm 2$ for further analysis.

1.3 Qualitative Determination of Phytochemicals Resent in Lyophilized Citrus Limon Juice Extracts

Citrus limon juice extracts were screened using the standard methods (Sofowora, 1993) to detect the presence or absence of various secondary metabolites, such as saponins, flavonoids, alkaloids, steroids, anthraquinones, cardiac glycosides, tannins, phenols, terpenes and resins.

1.4 Source of Bacterial Cultures

Six multidrug resistant bacterial pathogens from diarrheic and wound swabs of patients attending General Hospital, Minna, Nigeria were selected to investigate the antibacterial effect of lyophilized *Citrus limon* juice. The bacteria isolates including *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella enterica typhi, Macrococcus luteus, Clostridium difficile* were obtained from stock culture in the Microbiology Laboratory, General Hospital, Minna, Nigeria. Biochemical test was carried out to confirm the identity of the isolates using the methods described in Chessbrough (2006).

1.5 Antibiotic Susceptibility Test

Kirby Bauer's agar disc diffusion method was used for antibiotic susceptibility test as described in Clinical and Laboratory Standards Institute (2017) guidelines. Discs of antibiotics (Oxoid) commonly used for the treatment of bacterial infection (Gram negative and positive) were tested. Individual colonies were suspended to 0.5 McFarland standards in normal saline and using sterile swabs, the suspensions were inoculated on Muller Hinton agar for 18 hours. Sterile 6 mm Whatman filter paper No.3 (Germany) was used as control.

According to the diameter of the zones of inhibition around the antibiotic discs, the organisms were classified as susceptible, intermediate or resistant to a specific antibiotic (Chessbrough, 2006). Multidrug Resistant (MDR) bacterial pathogen was defined by resistance to ≥ 1 agent in ≥ 3 antimicrobial classes (Magiorakos et al., 2012).

The various classes of antibiotics including streptomycin, nalidixic acid, ampicillin, erythromycin, ofloxacin, reflaxin, ciproflox, augmentin, ceporex and gentamycin used in this study for the antibiotic susceptibility test are shown in Table 1.

The CLSI chart was used to determine the susceptibility pattern of the organism to the different antibiotics used.

1.6 Determination of the Antibacterial Activity of Citrus Limon Juice Extract

The method was adopted in standardizing the bacterial culture (Chuku et al., 2016). The antibacterial assay of the crude extracts was performed using agar well diffusion techniques described by Collins et al. (2006). A 10 mm cork borer was used to puncture holes in the solidified medium, and two wells were punctured on each petri plate. The entire surface of the plates were inoculated with 0.5 McFarland standards of the test bacteria (Chuku et al., 2016).

Thereafter, 1.60 μL of different concentrations of *Citrus limon* juice extracts (10, 20, 30 and 40 mg\mL) were introduced into each well using microtiter-pipette. This was allowed to diffuse at room temperature for one hour. The plates were incubated at 37 °C for 24 hours aerobically. The activities of the extracts were compared with the control antibiotic discs (Ampiclox-Beecham (30 μg)). Briefly, standard antibiotic powder (Ampiclox-Beecham) was obtained commercially. 30 μg/mL solution was prepared and impregnated on 6 mm whatman filter paper No.3 (Germany). The impregnated discs were dried in clean incubator at 37 °C for 4 hours. The observed inhibition zones were measured and recorded after 24 hours of incubation (Collins et al., 2006).

1.7 Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC) of the *Citrus limon* juice extracts were determined (Chuku et al., 2016). Serial dilution of the *Citrus limon* juice extract was done using 2 mL of nutrient broth in six test tubes to obtain varying concentrations (160, 80, 40, 20, 10 and 5 mg/mL). The tubes were inoculated with 0.1 mL of standardized inocula of the test bacteria cultures. The tubes were incubated over night at 37 °C. The tubes containing serially diluted *Citrus limon* juice extracts without inocula and tubes containing broth and inoculum served as positive and negative controls, respectively.

Visual observation was used to determine the growth of the test bacterial cultures. The culture tube with the lowest concentration of extract which did not exhibit any growth was considered as the MIC value and the tubes lacking turbidity were re-inoculated in fresh medium to obtain the MBC value. The minimal concentration of extract that inhibited the growth of bacterial cells upon re-inoculation in Muller Hinton agar was considered as the MBC value (Collins et al., 2006).

1.8 Data Analysis

Data were analyzed using Statistical Analysis Software (SAS) version 9.4 and presented as means ± standard error. Comparison between different groups was done using Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT). Values of $P<0.05$ were considered as statistically significant as described by Mahajan (1997).

2. Results

2.1 Biochemical Characteristics of the Test Isolates

The bacterial isolates were identified biochemically as presented in Table 2.

2.2 Phytochemical Constituent of Citrus Limon Juice Extracts

Phytochemical analysis showed the presence of saponins, resins, anthraquinone, volatile oil, glycosides, cardiac

Classes of Antibiotics	Antibiotics
Penicillin	ampicillin (PN) and augmentin (AU)
Quinolones	ofloxacin (OFX), ciproflox (CPX), nalidixic acid (NAL) and reflaxin (PEF)
Aminoglycoside	streptomycin (S) and gentamycin (CN)
Macrolides	erythromycin (E)
Cephalosporins	ceporex (CEP)

Table 1. Classes of Antibiotics Used

G - R	Sh	Cat	Cit	Pig	Glu	Man	Indole	Urease	MR	VP	Lactose	H_2S	Slant	Butt	Inference
_	Rod	+	+	green	+	+	_	+	_	_	_	_	Acid	Acid	P. aeruginosa
-	Rod	+	+	NA	+	+	_	+	_	+	+	_	NC	NC	K. pneumoniae
-	Rod	+	+	NA	+	+	_	_	+	_	_	_	Acid	Acid	S. typhi
+	Cocci	+	+	Yellow	_	_	_	+	NA	+	_	_	NA	NA	M. leteus
+	Rod	_		NA	+	_	_	_	NA	_	_	NA	NA	NA	C. difficle
_	Rod	+	_	NA	+	+	+	_	+	_	+	_	Acid	Acid	E. coli

Table 2. Biochemical Tests Results Obtained for the Test Isolates

steroids, steroids, tannins, alkaloids, phenols, flavonoids and terpenes.

2.3 Antibiotic Susceptibility Profile of Test Bacterial Pathogens

All test bacterial pathogens were resistant to ciproflox and ofloxacin except *S. typhi* and *E. coli* that showed intermediate resistance. Similarly, *P. aeruginosa, K. pneumoniae, M. luteus* and *Clostridium difficile* were resistant to ampicillin and erythromycin. *P. aeruginosa* and *Clostridium* species were both resistant to nalidixic acid and ceporex. In addition, *Clostridium* species were resistant to augumentin, gentamycin and streptomycin (Table 3).

2.4 Antibacterial Susceptibility Pattern of MDR Pathogen to Citrus limon Juice Extract

The *Citrus limon* juice extract showed the lowest mean zones of inhibition against *Macrococcus luteus* at 10 mg/mL and the highest average zones of inhibition against *Escherichia coli* at 40 mg/mL (Table 4). The mean zones of inhibition produced by *Citrus limon* juice extracts at 10, 20, 30 and 40 mg/mL ranged from 10.5±0.70- 24.50±0.70, 13.50±2.12 - 26.00±1.41, 7.50±2.12 - 29.50±2.12, 13.50±2.12 - 16.00±0.70,08±1.41 - 21.00±1.41 and 10.00±1.41-20.50±0.00 mm against *K. Pneumoniae, E. coli, M. luteus, S. typhi, P. aeruginosa,* and *Clostridium difficile,* respectively (Table 4).

2.5 Citrus Limon Juice Extract MIC and MBC Values

The MIC and MBC values produced by the Citrus limon juice extract against the MDR bacterial pathogens used in this study are presented in Table 5. The lowest MIC value was 10 mg/mL against *Clostridium* specie and *P. aeruginosa* while the highest MIC was 40 mg/mL against S. typhi. The lowest MBC value was 40 mg/mL against *K. Pneumoniae, S. typhi* and *P. aeruginosa.* Furthermore, 80 mg/mL was recorded against *E. coli, M. luteus* and *Clostridium difficile.*

3. Discussion

The menace of multidrug bacteria has no doubt necessitated the search for a new antibacterial agent for

Bacterial Pathogens	Concentration (mg/mL) MIC	MBC
K. Pneumoniae	20	40
E. coli	20	80
M. luteus	20	80
S. typhi	40	40
P. aeruginosa	10	40
Clostridium difficile	10	80

Table 5. MIC and MBC Produced by *Citrus limon* Juice Extract

Antibiotic(ug)	P. aeruginosa	E. coli	K. pneumoniae	M. luteus	S. typhi	Clostridium difficile
Ciproflox (10)	++	++	++	++	+	++
Gentamycin (10)	+	+	++	-	-	++
Streptomycin (30)	+	-	+	-	-	++
Nalidixic acid (30)	++	+	-	-	+	++
Ampicillin (30)	++	-	++	++	+	++
Erythromycin (30)	++	-	++	++	+	++
Ceporex (10)	++	-	-	-	++	++
Ofloxacin (5μg)	++	+	++	++	++	++
Augmentin (30)	-	-	-	-	++	++
Reflacine (10)	-	-	++	-	++	+

++: Resistance, +: Intermediate, -: Sensitive

Table 3. Antibiotic Susceptibility Testof the Test Isolate

Bacterial Pathogens	Concentration (mg/mL) 10	20	30	40	Ampiclox (30 μg)
K. Pneumoniae	10.5±0.70[d]	15.00±1.41[c]	19.00±1.41[b]	24.50±0.70[a]	17.00±1.41[bc]
E. coli	13·50±2·12[c]	15·00±1·41[c]	19·00±1·41[b]	26·00±1·41[a]	20·00±0·00[b]
M. luteus	7·50±2·12[c]	12·00±2·83[c]	18·50±2·12[b]	29·50±2·12[a]	20·00±2·12[a]
S. typhi	13·50±2·12[b]	14·00±2·12[b]	14·50±0·70[b]	16·50±0·70[ab]	19·50±2·12[a]
P. aeruginosa	8·00±1·41[c]	16·00±1·41[b]	19·00±1·41[ab]	21·00±1·41[a]	18·00±1·41[ab]
Clostridium difficile	10·00±1·41[c]	11·50±0·70[c]	17·00±0·00[b]	20·50±0·00[a]	20·50±0·70[a]

Values are mean ± SEM of 3 determinations. Means with dissimilar letter (s) differ significantly according to the Duncan's Multiple Range Test (DMRT).

Table 4. Zones of Inhibition Produced by *Citrus limon* Juice Extract

the successful control of infectious diseases. The findings of this study revealed that there are ten bioactive compounds with known therapeutic potentials in *Citrus limon* juice extract (Table 3). The presence of these metabolites in *Citrus limon* juice extracts may have contributed substantially to the observed antibacterial activities. This report corroborates the finding of Mahajan (1997), who investigated the presence of flavonoids, alkaloids, steroids, terpenoids, saponins, cardiac glycosides, and reducing sugars in *Citrus limon* juice.

Similarly, the findings of this study agree with previous reports (Rauf et al, 2014), and the present study found alkaloids and steroid in lyophilized *Citrus limon* juice. The differences could be accounted for by the variation in the geographical location of *Citrus limon* used for in present study.

The antibacterial activity of lyophilized *Citrus limon* was concentration dependent (Table 4). The highest antibacterial activities of *Citrus limon* juice extract observed at 40 mg/mL could be a function of increased concentration of individual bioactive ingredient in the extract at higher concentration. It is noteworthy to point out that the inhibitory effect of the crude extract compared favourably to the action of the standard drug used as control. This makes the fruit a potential source of drug for the treatment of infections associated with multidrug resistant *Clostridium difficile, S. typhi, P. aeruginosa, K. Pneumoniae, E. coli* and *M. luteus.* Besides, statistical analysis showed that the inhibitory actions of *Citrus limon* juice extract against *K. Pneumoniae* and *E. coli* are significantly higher ($p=0.05$) than zones of inhibition produced by the standard antibiotics. Previous reports (Adu et al., 2016; Oikeh et al., 2016; Hindi & Chabuck, 2013; Aburowais et al., 2017; Manconi et al., 2018; Hashemi et al., 2018) on antibacterial potential of various lemon fruit parts have established the antibacterial effect of *Citrus limon* juice on clinical bacterial isolates. Thus, the outcome of this study is in agreement with the previous reports on *Citrus limon.*

Conclusion

The minimum inhibitory concentrations and the minimum bactericidal concentrations of *Citrus limon* juice extracts were higher compared to the standard drug (control). Although the MIC and MBC were higher compared to that of the standard antibiotic, this does not invalidate the antimicrobial activity of the crude extracts. The lyophilized *Citrus limon* juice showed antibacterial action in the crude fashion validates its use locally as an antimicrobial agent and may be considered for the development of candidate antibacterial therapeutic drug for the control of multidrug resistance bacterial pathogens.

This study provides insight on the antibacterial potential of *Citrus limon* juice extract against multidrug resistant bacterial pathogens. A comprehensive study incorporating diverse bacteria should be carried out to further understand the broad-spectrum potential of the extract. In addition, studies on the toxicity of the extract should be carried out to ascertain its safety.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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