Antibacterial activity of *Citrus limonum* fruit juice extract

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Abstract: The fruit juice extract of *Citrus limonum* was investigated for antibacterial activity. The antibacterial activity of the extract on ten strains of bacteria was determined by both agar well diffusion and macro-broth dilution methods. The extract was variously bacteriostatic and bactericidal against *Bacillussubtilis* ATCC 6051, *Staphylococcus aureus* ATCC 12600, *Escherichia coli* ATCC 11775, *Pseudomonas aeruginosa* ATCC 10145 as well as locally isolated clinical strains of the above bacteria and *Salmonella kintambo* (Human: 13, 23: mt:-), *Salmonella typhi* and *Proteus sp*. The MICs ranged from 0.78mg/ml to 50mg/ml; MBCs, 25.0mg/ml to >100mg/ml and MBC/MIC ratios 2.0 to >16.0. These results provide scientific justification for the medicinal use of *Citrus limonum* fruit juice by Nigerian herbalists in the treatment of diseases in which strains of the test organisms have been implicated as etiologic agents.

Keywords: Antibacterial, lemon juice, *Citrus limonum*, enthomedicine, Nigeria.

INTRODUCTION

Lemon juice is believed to have antimicrobial properties in many cultures of the world. In the Caribbean, South America and Africa, lemon juice is believed to be effective against diphtheria and upper respiratory tract infections (Pamplona-Roger, 1999). It is claimed that the cholera epidemic in Venezuela in 1885 was largely resolved by massive consumption of lemon fruit and lemon juice (Pamplona-Roger, 1999). In South Africa, lemon juice has been used in the treatment of oral thrush in HIV/AID patients (Wright *et al.,* 2009). In many parts of the world, lemon juice is also used as sanitizers to remove food borne pathogens from fresh fruits, vegetables and fish (Sengun and Karapinar, 2004; Tomotake *et al.,* 2006). Studies have shown that concentrated or freshly squeezed lemon juice has antibacterial activity against *Vibrio species*(Tomotake *et al.,* 2006), *Salmonella typhimurium* (Sengun and Karapinar, 2004), *Pseudomonas aeruginosa* and *Escherichia coli* (Kumar *et al.,* 2012).

In Nigeria, herbalists use lemon juice in the treatment of diarrhea, dysentery, typhoid fever, wound infections, urinary tract infection and arthritis (Kafaru, 1994). Freshly squeezed lemon juice diluted with water or mixed with honey was believed to be potent against diseases of bacterial etiology (Kafaru, 1994). At present there is paucity of scientific data on the rationale behind the medicinal use of lemon juice in Nigerian ethnomedicine. This study was setup to authenticate the folklore claims regarding the antibacterial activity of *C. limonum* fruit juice. In this regard, the antibacterial activity of lemon juice extract against bacteria strains isolated locally from clinical specimens and typed strains from the American Type Culture Collection (ATCC) was determined.

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MATERIALS AND METHODS

Collection, identification and extraction of plant material

Fresh fruits of *Citrus limonum*Burm were collected from the residence of Dr. AC Uwaegbute whose residence is located at the University of Nigeria of Nigeria Nsukka. The voucher specimens were confirmed by Mr. AO Ozioko and deposited at the Botany Department Herbarium, University of Nigeria Nsukka. Five lemon fruits were peeled and the juice was squeezed out using a plastic juice extractor. The extracted fruit juice was subjected to double filtration with Whatman Number One filter paper and 0.45µm membrane filter (Sigma) respectively. The filtrate was measured into a weighted sterile Petri Dish and evaporated to a paste at room temperature. Subsequently, weight of the residue was determined and recorded as yield (w/v) of the fruit juice extract. The fruit juice extract was sterilized by UV irradiation for 20 hours. An aliquot of the reconstituted extract was checked for sterility by plating on nutrient agar plates. The extract was stored in sterile containers at 4^{o}C and used for phytochemical analysis and anti-microbial susceptibility testing within 2-4 days of preparation.

Phytochemical analysis

The presence of glycosides, tannins, flavonoids, alkaloids, saponins, carbohydrates, proteins and water soluble vitamins in the reconstituted fruit juice extract was examined by standard phytochemical methods (Harbone, 1973).

Bacterial strains

The Bioresources Development and Conservation Project (BDCP), Nsukka provided the typed strains. Typed strains obtained from BDCP were *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 12600, *Escherichia coli* ATCC 11775, and *Pseudomonas aeruginosa* 10145. *Salmonella kintambo* (Human: 1,13,23: mt:-) strain was obtained from the Department of Veterinary Microbiology and Pathology of the University of Nigeria, Nsukka. The rest (untyped clinical isolates), *Staphylocuccus aureus*, *Escherichia coli*, *Pseudomona aeruginosa*, *Salmonella typhi*, and *Proteus sp* were provided by the Department of Microbiology, University of Nigeria Nsukka. The purity and identity of the bacterial strains used in this study were confirmed by standard biological methods (Collins and Lyne, 1970).

Determination of antibacterial activity

The standardization of each bacterial inoculum was done following the National Committee for Clinical Laboratory Standards (NCCLS, 1993). Briefly, each bacterial strain inoculated into Mueller Hinton broth (MHB; Oxoid) was incubated to a turbidity equivalent to 0.5 McFarland units (measured with a Nephelometer). Bacterial strains were incubated for 12 hours at 35° C to achieve the turbidity of 0.5 McFarland units. A standardized bacterial inoculum containing $5x10^5$ colony forming units (cfu) per millimeter (ml) were used for antibacterial screening. Screening of bacterial strains for susceptibility to *C. limonum* fruit juice extract was by a modification of agar well diffusion technique (Okeke *et al.,* 2001). Standardized suspension of each bacteria strain in 1.0ml aliquot (5 x 10^5cfu/ml) was spread on MHA plate. The inoculated MHA plates were allowed to dry at room temperature and 6 mm diameter wells were bored in the agar. The fruit juice extract was re-constituted in distilled water to a concentration of 100mg/ml and 100µl of the reconstituted juice extract was added unto three wells for each bacteria. The plates were incubated at 37° C for 24 hours after allowing the fruit juice to diffuse into the agar at room temperature. Thereafter, the inhibition zone diameter (IZD) in the plates was recorded to the nearest millimeter (mm). The whole root of *Landolphia owerrience* (cold water extract) (Okeke *et al.,* 2001) and gentamycin (at 8µg/ml and 16µg/ml concentrations) were included as controls.

Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration

The minimal inhibitory concentration (MIC) for each test organism was investigated by both of agar well diffusion method (Okeke *et al.,* 2001) and the macro-broth dilution method (NCCLS, 1993). The MIC determination by the well diffusion method involved serially diluting reconstituted juice extract two fold with sterile distilled water as diluent in order to achieve a decreasing concentration ranging from 100mg/ml to 0.1mg/ml. Three wells in the MHA plates pre-seeded with the standardized inoculum of each bacterium were filled with 100 µl per well of each dilution. The plates were then incubated for 24 hours 37°C. The MIC is the lowest concentration or the highest dilution of the juice extract showing clear zone of inhibition.

In the macro-broth dilution method, the reconstituted *Citrus limonum* fruit juice extract was serially diluted two fold using MHB as the diluent. Each dilution was seeded in triplicate with a suspension of each strain of bacteria such that a final concentration of 5 x 10^5cfu/ml was obtained. The incubation for all the cultures was for 24 hours at 37°C. The MIC determined by macro-broth method is the highest dilution of juice extract showing no visible bacterial growth.

Table 1: Phytochemical analysis of *Citrus limonum* fruit juice

Phytochemical compounds	Presence in juice[a]
Alkaloid	+
Cyanogenetic glycosides	++
Anthracene glycosides	-
Cardiac glycosides	+
Steroidal glycosides	+
Tannin	+++
Saponin	+
Flavonoid	+
Carbohydrate	++
Protein	+
Water soluble vitamins	++

[a]:-,not detectable; +, low concentration; ++, medium concentration; +++, high concentration

Minimum bactericidal activity and MIC index determination

The minimum bactericidal concentration (MBC) was determined as follows; a 0.1ml volume of broth from each (macro-broth MIC testing) tube showing no evidence of bacterial growth was taken and sub-cultured on sterile MHA. Cultures were incubated for 24 hours at 37° C and the highest dilution without bacterial growth following subculture was taken as the MBC. The well diffusion and macro-broth dilution MIC values were used to calculate the MIC index(Sanders *et al.,* 1993). MIC indices were adopted from Fractional Inhibitory Concentration Index (FICi) as modified by Sanders *et al.,* (1993).

RESULTS

Phytochemical analysis and antibacterial activity

The yield of the fruit juice extract was 0.33% (w/v). The phytochemical analysis showed presence of alkaloids, cyanogenetic, cardic and steroidal glycosides, tannins, saponins, flavonoids and water-soluble vitamins (table 1). The studied strains of bacteria were inhibited by *Citrus limonum* fruit juice extract. The fruit juice extract

Table 2: Inhibition Zone Diameter, IZD (mm) of *Citrus limonum* fruit juice extract, *Landolphia owerrience* whole-root extract and gentamycin (controls) against test bacterial strains

Test bacterial strain		IZD (mm)[b]	Gentamycin	
	Fruit juice extract (100mg/ml)	*Landolphia owerrience* whole-root extract	(8µg/ml)	(16µg/ml)
Staphylococcus aureus ATCC 12600	21.0	13.0	18.0	24.0
Staphylococcus aureus (clinical)	19.0	14.0	11.0	12.0
Bacillus subtlis ATCC 6051	19.0	0.0	31.0	33.0
Escherichia coli ATCC 11775	17.0	0.0	30.0	35.0
Escherichia coli (clinical)	14.0	13.0	10.0	13.0
Pseudomonas aeruginosa ATCC 10145	20.0	0.0	31.0	34.0
Pseudomonas aeruginosa (clinical)	20.0	0.0	18.0	21.0
Salmonella kintambo (Human:1,13,23:mt:-)	17.0	Nt	22.0	28.0
Salmonella typhi (clinical)	16.0	0.0	18.0	22.0
Proteus sp. (clinical)	15.0	0.0	19.0	22.0

[b]Each value is a mean of three replicates. Nt, not tested.

Table 3: Minimum Inhibitory Concentration (MIC) of *Citrus limonum* juice extract against test organisms as determined by Agar well diffusion and Macro-broth dilution methods

Test bacteria strains	Agar well MIC[c]		Macro-broth MIC[c]		MIC Index		Effect of test methods[d]	
	Fruit juice extract (mg/ml)	Gentamycin (µg/ml)	Fruit juice extract (mg/ml)	Gentamycin (µg/ml)	Fruit juice extract	Gentamycin	Fruit juice extract	Gentamycin
Staphylococcus aureus ATCC 12600	25.0	0.125	12.5	1.0	2.0	0.125	NS	S
Staphylococcus aureus (clinical)	25.0	4.0	6.25	64.0	4.0	0.063	NS	S
Bacillus subtilis ATCC 6051	50.0	0.5	6.25	0.125	8.0	4.0	S	NS
Escherichia coli ATCC 11775	50.0	0.5	6.25	4.0	8.0	0.125	S	S
Escherichia coli (clinical)	50.0	8.0	25.0	64.0	2.0	0.125	NS	S
Pseudomonas aeruginosa ATCC 10145	12.5	0.25	6.25	2.0	2.0	0.125	NS	S
Pseudomonas aeruginosa (clinical)	25.0	1.0	6.25	2.0	4.0	0.5	NS	NS
Salmonella kintambo (Human: 1, 13, 23:mt-)	0.78	0.25	12.5	2.0	0.063	0.125	S	S
Salmonela typhi (Clinical)	12.5	0.5	12.5	4.0	1.0	0.125	NS	S
Proteus sp (clinical)	6.25	0.5	6.25	8.0	1.0	0.163	NS	S

[c]Mean of triplicate experiments. MIC index=Agar well MIC/Macro-broth MIC; S=significant (MIC index>4.0 or ≤0.125); NS=Non-significant (MIC index of >0.125 to 4.0). [d]The interpretation of MIC indices for significance or non-significance was as defined in Materials and Methods section. By definition, MIC indices which lie outside the inherent two-fold error of MIC (MICi>4.0 or ≤0.125) is considered significant, that is, the test method has an effect on the MIC value determined.

produced inhibition zone diameters (IZD) varying from 14.0mm to 21.0mm (table 2). The controls, cold water extract of whole-root of *L.owerrience* (Okeke *et al.*, 2001) and Gentamycin (used at 8.0µg/ml concentration) gave IZD ranges of 0.0-14.0mm and 10.0-31.0mm respectively (table 2).

Minimum inhibitory concentration (MIC) and MIC Index

The well-dilution MIC values of the juice extract varied from 0.78mg/ml for *Salmonella kintambo* (Human: 1, 13, 23: mt:-) to 50mg/ml for *Bacillus subtilis* ATCC 6051, *E. coli* ATCC 11775 and *E. coli* (clinical) (table 3). The

Table 4: Minimum Bactericidal Concentration (MBC) of *Citrus limonum* fruit juice extract

Test bacterial strains	MBC[e]		MBC/MIC Ratio[f]	
	Fruit juice (mg/ml)	Gentamycin (mg/ml)	Fruit juice	Gentamycin
Staphylococcus aureus ATCC 126000	100.0	4.0	8.0	4.0
Staphylococcus aureus (clinical)	25.0	512.0	4.0	8.0
Bacillus subtilis ATCC 6051	>100.0	1.0	>16.0	8.0
Escherichi coli ATCC 11775	25.0	8.0	4.0	2.0
Escherichia coli (clinical)	25.0	512	1.0	8.0
Pseudomonas aeruginosa ATCC 10145	>100.0	16.0	>16.0	8.0
Pseudomonas aeruginosa (clinical)	25.0	12.0	4.0	6.0
Salmonella kintambo (Human: 1, 13, 23: mt:-)	25.0	4.0	2.0	2.0
Salmonella typhi (clinical)	25.0	8.0	2.0	2.0
Proteus sp (clinical)	>100.0	8.0	>16.0	1.0

[e]Each value is the mean of triplicate experiments. [f]Macrobroth MIC is used in calculating MBC/MIC ratio.

macro-broth determined MIC values varied from 6.25mg/ml for *S. aureus* (clinical), *B. subtilis* ATCC 6051, *E. coli* ATCC 11775, *P. aeruginosa* ATCC 10145, *P. aeruginosa* (clinical) and *Proteus sp* (clinical) to 25.0mg/ml for *E. coli* (clinical) (table 3). The macro-broth dilution MIC values of the fruit juice extract (range, 6.25-25.0mg/ml) were generally lower than corresponding agar well diffusion MIC values (range, 6.25-50mg/ml) with the exception of the MIC value for *S. kintambo* (Human: 1, 13, 23: mt:-). However, the differences in the MIC values obtained by the two techniques were not significant except in the cases of *B. subtilis* ATCC 6051, *E. coli* ATCC 11775 and *S. kintambo* (Human: 1, 13, 23: mt:-) (table 3). MIC indices, which lie within the two-fold error (MICi>0.125 to 4.0) imply that the test methods have no significant on the MIC value (Sanders *et al.,* 1993). The Gentamycin (control) MICs recorded for each strain of bacteria were also significantly different for the two techniques with the exception of *B. subtilis* ATCC 6051, and *P. aeruginosa* (clinical). MIC indices which lie outside the inherent two-fold error of MIC (MICi>4.0 or ≤0.125) is considered significant, that is, the test method has an effect on the MIC value determined. We did not determine the MIC values of the cold-water extract of *L. owerrience* whole root.

Minimum bactericidal concentration (MBC) and MBC to MIC ratio

The *C. limonum* fruit juice extract was bactericidal against all test organisms. With the exception of *B. subtilis* ATCC 6051, *P. aeruginosa* ATCC 10145 and *Proteus sp* (clinical) the fruit juice extract was bactericidal at MBCs ranging from 25mg/ml to 100mg/ml (table 4) The control, Gentamycin was bactericidal to all the test organisms at MBC range of 1.0-16.0µg/ml except for*S.uareus* (clinical) and *E. coli* (clinical) (MBC= 512.0µg/ml). The lemon fruit juice extract MBC to MIC (determined by the macro-broth dilution method) ratio ranged from 1.0 to >16.0 (table 4).

DISCUSSION

The *C. limonum* fruit juice extract showed broad-spectrum activity similar to Gentamycin against the investigated strains of bacteria. The MIC values of *C. limonum* fruit juice extract as well as its broad spectrum antibacterial activity compare favorably with those of ethanolic extract of whole-root of *L. owerrience* (Okeke *et al.,* 2001). Previously, we have shown that the ethanolic extract of *L. owerrience* has strong antibacterial activity (Okeke *et al*., 2001) and thus could serve as a positive control for antibacterial activity of medicinal plant extracts. Of particular interest is the activity of the lemon juice extract against both typed and clinical strains of *Pseudomonas aeruginaosa*, an organism notorious for its resistance to many antibiotics (Van Eldere, 2003). Taking into cognizance that many nosocomial infections are caused by *Pseudomonas aeruginosa*, hand washing with lemon fruit juice in hospital settings may reduce the spread of nosocomial infection and provide reasonable alternative to alcohol based hand washers.

With the exception of *B. subtilis* ATCC 6051, *P. aeruginosa* ATCC 10145 and *Proteus sp* (clinical), the lemon fruit juice extract has low MBC/MIC ratio, indicating that the concentration at which the juice extract is bacteriostatic is similar to that at which it is bactericidal. Low MBC/MIC ratio is indicative of strong antimicrobial properties (Sader *et al*., 2006).

The MIC values obtained by agar diffusion technique were higher than those achieved for the same bacterial strains using the macro-broth dilution method. The exceptions were the test with *S. kintambo* (Human 1, 13, 23: mt:-), *S. typhi* (clinical) and *Proteus sp* (clinical). Conversely, with the control drug (Gentamycin), the MIC values obtained with the well dilution method were significantly lower than corresponding values obtained with macro-broth method. The reason(s) for the disparities in the two MIC determination methods in this

work and several others (Baltch *et al.,* 1998; Masuda and Tomioka, 1978; Okeke *et al.,* 2001; Okoli *et al.,* 2002; Sader *et al.,* 2006) remain unexplained. Although differences in the diffusion rate of the antimicrobial agent in the agar have been tendered as an explanation for such disparities, it is a phenomenon that needs further investigation in order to achieve acceptable standards for both methods. The antibacterial activity of lemon fruit juice may be attributed to the presence of organic acids, constituent vitamins, secondary metabolites and their interactions with each other. Citric acid, but not malic acid has been shown to contribute to antibacterial activity of *Citrus sudachi* juice. Further investigation is required to identify the active ingredient(s) that either singly or in combination account for the antibacterial properties of *C. limonum* fruit juice.

CONCLUSION

Overall, the antimicrobial activity of the lemon fruit juice extract against know etiologic agents of typhoid fever, gastroenteritis and wound sepsis validates the folkloric claims of its efficacy in the treatment of these and other bacterial infections (Kafaru, 1994). Thus, there is some scientific justification for its use in Nigerian ethnomedicine.

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