

Antibacterial Activity of Citrus Aurantifolia Leaves Extracts Against Some Enteric Bacteria of Public Health Importance


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Received:  December 08, 2018

Published:  December 20, 2018

Abstract

The study was conducted to determine the phytochemical composition and antibacterial activity of Citrus aurantifolia leaves extracts against clinical isolates of some enteric bacteria of public health importance. The result of phytochemical screening of the leaves extracts shows the presence of alkaloid, glycoside, saponin, tannin, flavonoid, steroids, terpenoid and phenol. The result of antibacterial efficacy of the extracts against the isolates indicated that the extracts were active against the isolates with higher activity in ethanol extract (with average zone of inhibition of 14.91mm) when compared to aqueous extract (12.67mm). The result of susceptibility of the isolates to the extracts showed Shigella was more sensitive to the extract with average zone of inhibition of 14.90mm, followed by Klebsiella (14.49mm), Escherichia coli (13.77mm) and Salmonella typhi with average zone of inhibition of 12.01mm. The minimum inhibitory concentration (MIC) of the extracts showed that dilutions of various concentrations of aqueous and ethanol of leaves extracts can inhibit the growth or kill the isolates at a concentration of between 2.125-20mg/ml. Statistical analysis of the results indicated that there is no significant different in the activity of the extracts against the isolates used at $p < 0.05$. Findings from this study support the use of Citrus aurantifolia leaves extracts for medicinal purposes.

Keywords: Antibacterial activity; Citrus aurantifolia; Phytochemical; Enteric bacteria extract

Introduction

Natural products such as plants have been integral part traditional medicine system in ancient such as Chinese, Ayurvedic and Egyptian [1]. Several published reports had described the antimicrobial activity of various crude extracts of plants either in single or in combination [2]. It has been estimated that about 2.5 million species of higher plants and their therapeutic values are yet to be determined. However, herbal extracts are becoming popular as natural medicine; preservatives and additives [3]. According to World Health Organization [4] medicinal plants contain substances in one of its organs such as stem, root, leaves, rhizomes, fruits, flower and seeds that can be used for therapeutic purposes or which are precursor for chemo-pharmaceutical semi-synthesis [4]. The medicinal plants are employed in the treatment or control of disease

condition due to the presence of biochemical components called phytochemical [5]. Phytochemicals are considered as bioactive substances of plant origin and are of medical importance. They are otherwise known as secondary metabolites as they are of little important to the plant who manufactured them. These secondary metabolites synthesized naturally in various organs of plants such as leaves, stem, root, flower and seed [6]. Most of the modern drugs used today were originally obtained from plants [7]. Many researches confirmed the herbal extract boost immune system by stimulating the production of white blood cell which fight diseases [8]. Citrus aurantifolia belongs to Rutaceae, it is a polyembryonic plant cultivated in several part of the world especially hot subtropical or tropical region such as India, USA, Nigeria, Mexico,

West Indies and Egypt [9]. The plant is a shrub in nature and height of about 2 meters tall, evergreen with dense and irregular branches which possess short and stiff spines. The *Citrus aurantifolia* fruits are ovoid berries of about 3-6 cm in diameter and sometimes possess apical papilla. When ripe, the fruits turn yellow from initial blue [10]. The plant is used in traditional medicine for treatment of several diseases such as cold and stomach ailment. It can also be used as an antiseptic, mosquito repellent, antifungal, antibacterial and antiviral agent. The health benefits of *Citrus aurantifolia* plant are highly associated with the high amount of bioactive constituents it contained such as phenols, flavonoids, carotenoid, vitamins and minerals [11]. Limes contain unique flavonoid compounds that have antioxidant and anti-cancer properties. The flavonoids help to inhibit cell division in many cancer cell lines in addition to its antimicrobial efficacy [12]. The plant also demonstrated bioactive activities for cold, fever, sinusitis, sore throats, asthma and bronchitis [13]. Antibacterial assessment of *Citrus aurantifolia* aqueous ethanol, acetone, chloroform, ethanol and petroleum ether leaves extract conducted by Pathan R et al. [14] against various pathogens showed significant activity against *Staphylococcus aureus*, *Pseudomonas* spp, *Klebsiella pneumoniae* along with antifungal activity against *Mucor* spp., *Aspergillus fumigatus* and *Aspergillus niger*. Kandpal et al. [15] isolated actinomycetes from *C. aurantifolia* and tested its antibacterial efficacy against different pathogens. In this study, five actinomycetes isolated from the plant exhibited antibacterial activity against various pathogens including *S. aureus*, *E. coli*, *K. pneumoniae* and *S. typhi*. The present study was aimed at determining the phytochemical constituent and antibacterial activity of *Citrus aurantifolia* leaves extracts against clinical isolates of some enteric bacteria.

Materials and Methods

Collection, identification and authentication of plant materials

The leaves of *C. aurantifolia* were used in this study. The leaves were collected from the botanical garden of School of Technology, Kano State Polytechnic. After collection, the leaves were identified and authenticated at the herbarium in the Department of Plant Science, Bayero University Kano with the following voucher number BUKHAN 0341, voucher specimen of the plant's leaves were deposited in the herbarium for future references. The leaves were then rinsed with distilled water and air dried at room temperature for 2 weeks. The dried leaves were made into fine powder under laboratory condition using sterile pestle and mortar. The powder was stored in a dark and airtight container.

Test organisms

Five (4) enteric bacterial isolates recovered from stool of infected patients attending Murtala Muhammad General Hospital, Kano

namely; *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Shigella* were obtained from Microbiology Laboratory of Kano University of Science and Technology Wudil, Kano. The bacterial isolates were characterized to species level using various laboratory procedures which include Gram's stain, cultural characterization and Biochemical tests include (Indole, Methyl red, Voges Proskauer, Catalase, Citrate utilization and coagulase tests) as described by Holt et al. [16] and Cheesbrough [17]. The isolates were maintained on Nutrient agar slants at 4 °C.

Indole test

Indole test was conducted according to Cheesbrough [17] using Tryptophan broth. The broth was inoculated with the isolate of the test organism, and then incubated at 37 °C for 24 hours. About 0.5 ml of Kovack's reagents was added to the broth culture.

Methyl red test

Indole test was conducted using MR-VP broth as described by Holt et al. [16] and Cheesbrough [17]. The MR-VP broth was inoculated with an isolate of the test organism with the aid of sterile inoculating wire loop. The inoculated broth was incubated at 37 °C for 24 hours. About 5 drops of Methyl-red reagent was added to the broth culture.

Voges proskauer

MR-VP broth was inoculated with an isolate of the test organism using sterile inoculating loop and incubated at 37 °C for 24 hours. Six milliliters (6 ml) of 5% alpha naphthol was added followed by 0.2 ml of KOH. The tube was shaken gently and remained undisturbed for 5 minutes.

Citrate utilization test

Simmon's citrate agar was streaked back and forth with inoculum of the test organism and incubated aerobically at 37 °C for 24 hours.

Preparation of the leaves extracts

Water and ethanol were used as solvent for extraction. The aqueous and ethanol extracts of the plant leaves were prepared separately. Fifty grams (50 g) of leaves powder was prepared by mixing with 500 ml each of distilled water and ethanol respectively in 1 liter flask. The flask were kept for 3 days with intermittent shaking at room temperature. Filtration of the mixture was conducted using Whatman filter paper. The ethanol filtrate was evaporated using rotary evaporator at 50 °C while the aqueous filtrate was evaporated at 40 °C in water bath until dried extract samples were obtained. All the dried extract samples were dissolved in 10% DMSO separately to the final concentration of 200 mg/ml as a stock concentration. The prepared extracts were refrigerated for further use [18].

Qualitative phytochemical screening

The preliminary phytochemical screening of *C. aurantifolia* leaves extracts was conducted using standard laboratory methods as described by Sofowora [19] and Trease and Evans [20]. The presence of bioactive constituents such as alkaloids, reducing sugars, steroid, glycoside, phenol, Anthraquinones, terpenoids, flavonoids, saponin and tannin were determined.

Antibacterial assay of the extracts

Agar well diffusion method was used to determine the activity of the extracts against the test isolates as adopted by Nas et al. [21] with little modification. A bacterial suspension equivalent to 0.5 McFarland Standard (1.5×10^6 CFU) was introduced onto the surface of freshly prepared Mueller- Hinton agar medium in a sterile Petri-dish. Using 6 mm diameter sterile cork borer, 5 wells were constructed in the agar medium at equidistance. The agar well were filled with 0.1ml of the extracts at concentration of 100, 75, 50 and 25mg/ml. the fifth well was filled with 0.1ml of 50mg/ml Amoxicillin (Micro Lab Limited) as positive control for the experiment. The plates stand for about 1 hour until proper diffusion of the extracts into the medium was done. The plates were incubated at 37 °C for 24 hours, and thereafter the plates were observed for zones of inhibition and measured. Each experiment was conducted in triplicate and the average values of zone of inhibition were recorded.

Determination of minimum inhibitory concentration (MIC)

Broth dilution method was used for MIC determination. During the process, double fold serial dilution of the extracts was prepared in Nutrient broth by adding 2ml of 100mg/ml of the extract into a test tube, thus producing solution containing 50mg/ml of the extract. The process of serial dilution continues until a concentration of 25, 12.5, 6.25, 3.125mg/ml were obtained. The test tube Number 6 does not contain extract and served as negative control for the experiment. About 0.5ml of the standard organism (0.5 McFarland equivalents) was inoculated into each test tube containing the extracts and incubated at 37 °C for 24 hours. The test tubes were observed for bacterial growth by checking the presence of turbidity [21].

Antibacterial activity of aqueous extract

Table 2: Antibacterial activity of *C. aurantifolia* leaves aqueous extract.

Isolates	25	50	75	100	Control
Salmonella typhi	08.67±0.00 ^a	09.84±0.12 ^a	10.34±0.31 ^a	13.18±0.22 ^a	19.34
Escherichia coli	08.44±0.25 ^a	12.40±0.34 ^a	13.70±0.29 ^a	16.41±0.19 ^b	18.67
Klebsiella pneumoneae	10.40±0.27 ^a	11.28±0.29 ^a	13.62±0.19 ^b	15.21±0.45 ^b	19.67
Shigella sp	11.83±0.21 ^a	12.43±0.18 ^a	16.78±0.22 ^b	18.23±0.33 ^b	20.34

Key: Values having different superscript on the same row are considered significantly different at $p < 0.05$.

Determination of minimum bactericidal concentration

Minimum bactericidal concentration of the extracts was determined using method adopted by Nas et al. [21]. The tubes showing no visible growth from MIC were used. Briefly, 0.1ml bacterial culture was pipette from the mixture obtained in the determination of MIC tubes which did not show any growth and sub cultured onto the surface of Mueller Hilton agar plates and incubated at 37 °C for 24h. the plates showing no visible bacterial growth were considered as MBC.

Statistical analysis

One-way Analysis of variance (ANOVA) was used to analyze the data obtained from the zone of inhibition produced by the isolates against the extracts. The statistical program SPSS 21.0 version (Statistical Package for the Social Science) was employed. The result was as a mean \pm standard deviation. The significance level for the differences was set at $p < 0.05$.

Result

Preliminary phytochemical screening

The result of phytochemical screening of the extracts is presented in Table 1. The results indicate the presence of alkaloid, flavonoid, glycoside, saponin, steroid, phenols, terpenoid and tannin in both aqueous and ethanol extracts.

Table 1: Phytochemical constituents of *C. aurantifolia* leave extracts.

Phytochemical	Aqueous extract	Ethanol extract
Alkaloids	+	+
Flavonoid	+	+
Glycosides	+	+
Reducing sugar	-	-
Saponin	+	+
Steroids	+	+
Phenols	+	+
Terpenoid	+	+
Tannin	+	+

Key: + = presence of phytochemical, - = absent of phytochemical

The result of antibacterial activity of aqueous leaf extract of *C. aurantifolia* is presented in Table 2. According to the result, the zone of inhibition recorded by the isolates depends on the concentration of the extract and the types of bacteria isolate. *Shigella* has the highest zone of inhibition (18.23±0.36mm) at 100mg/ml. The zone of inhibition of the control (Amoxicillin 50mg/ml) ranges from to 19.34-20.34mm.

Antibacterial activity of ethanol extract

The result of antibacterial activity of ethanol leaf extract of *C. aurantifolia* is presented in Table 3. According to the result, the zone of inhibition recorded by the isolates depends on the concentration of the extract and the types of bacteria isolate. *Klebsiella pneumoniae* has the highest zone of inhibition (19.27±0.31mm) at 100mg/ml. The zone of inhibition of the control (Amoxicillin 50mg/ml) ranges from to 19.34-20.34mm.

Table 3: Antibacterial activity of *C. aurantifolia* leaves aqueous extract.

Isolates	25	50	75	100	Control
<i>Salmonella typhi</i>	11.25±0.28 ^a	12.85±0.18 ^a	14.33±0.36 ^a	15.59±0.21 ^b	19.34
<i>Escherichia coli</i>	12.33±0.19 ^a	13.00±0.10 ^b	15.96±0.24 ^b	17.89±0.35 ^b	18.67
<i>Klebsiella pneumoneae</i>	12.32±0.12 ^a	16.15±0.22 ^a	17.69±0.28 ^b	19.27±0.31 ^b	19.67
<i>Shigella sp</i>	12.76±0.22 ^a	14.90±0.28 ^b	15.67±0.19 ^b	16.60±0.22 ^b	20.34

Key: Values having different superscript on the same row are considered significantly different at $p < 0.05$

MIC and MBC of the extract

Table 4 presents the minimum inhibitory concentration and minimum bactericidal concentration of aqueous and ethanol extract of *C. aurantifolia* leaves. According to the result, various

concentrations of the extracts were able to inhibit or kill the isolates. Lower MIC (3.125mg/ml) was shown by ethanol extract than aqueous extract. The ethanol extract exhibit MBC of between 12.5-50mg/ml.

Table 4: Minimum inhibitory concentration (MIC) and MBC of the extracts.

Isolates	MIC	MBC	MIC (mg/ml)	MBC (mg/ml)
	(mg/ml)	(mg/ml)		
<i>Salmonella typhi</i>	25	50	6.25	25
<i>Escherichia coli</i>	6.25	12.5	6.25	25
<i>Klebsiella pneumoneae</i>	12.5	50	6.25	50
<i>Shigella sp</i>	6.25	25	3.125	12.5

Discussion

Phytochemical screening of aqueous and ethanol leaves extracts of *Citrus aurantifolia* indicate the presence of alkaloid, flavonoid, glycoside, saponin, steroid, phenols, terpenoid and tannin. The presence of such secondary metabolites in plant parts was responsible for its antibacterial efficacy. The saponin and tannin are known to possess antimicrobial activities, and as result, the tannin play an important role in wound healing [22-24]. The alkaloid is known to consist of a large group of nitrogenous compounds which are widely used as anesthetic, anticancer and central nervous stimulants. In addition to that, alkaloids are known to play some metabolic roles which include interference with cell division and antimicrobial properties [21], hence, the presence of alkaloid in the leaves extracts of *Citrus aurantifolia* could account for their use as antibacterial agent. According to the presence study, the plant leaves extracts demonstrated antibacterial efficacy against the test isolates. The findings showed that ethanol leaves extract exhibit higher activity than aqueous extract. The ethanolic leaves extract had highest zone of inhibition of 19.27mm at

100mg/ml against *Klebsiella* while 17.89mm for *Escherichia coli* while aqueous extract had highest zone of inhibition of 18.23mm at the same concentration for *Shigella* while 16.41mm against *Escherichia coli* at 100mg/ml. Higher activity of ethanol may be attributed to better solubility of the secondary metabolites in the ethanol extract which lead to better efficacy of the ethanol extract. However, Doughari et al. [25] stated that the anti-microbial effect of the plant could be due to the bioactive compounds such as the Phytochemicals constituent present in the plant. The finding of this study showed that the efficacy of *Citrus aurantifolia* leaves extracts on the test isolates had different hierarchy of susceptibility among the organisms. The findings of this study indicated that; higher concentration of the extract shows a greater zone of inhibition; this result agrees with that of Bisno and Stevens [26] which states that the higher the concentration of antibacterial substance, the higher it shows an appreciable zone of inhibition. The result of antibacterial activity of *Citrus aurantifolia* leaves extracts in the present study was in conformity with several studies conducted on the same extracts. Antibacterial assessment of *Citrus aurantifolia* aqueous ethanol, acetone, chloroform, ethanol and petroleum ether leaves

extract conducted by Pathan R, et al. [14] against various pathogen showed significant activity against *Staphylococcus aureus*, *Pseudomonas* spp, *Klebsiella pneumonia* along with antifungal activity against *Mucor* spp., *Aspergillus fumigates* and *Aspergillus niger*. This is in conformity with the result of the present study. The finding of this study was also in conformity with that of Kandpal et al. [15] who isolated actinomycetes from *C. aurantifolia* and tested for antibacterial activity against various pathogens. In their study, five actinomycetes isolated from the plant exhibited antibacterial activity against various pathogens including *S. aureus*, *E. coli*, *K. Pneumonia* and *S. typhi*. Minimum inhibitory concentration of aqueous and ethanol extracts of *Citrus aurantifolia* leaves showed dilutions of various concentrations of aqueous and ethanol of the extracts can inhibit and/or kill the isolates. Lower MIC (3.125mg/ml) was shown by ethanol extract than aqueous extract. The ethanol extract exhibit MBC of between 12.5 - 50mg/ml.

Conclusion

Phytochemical screening of leaves extracts of *Citrus aurantifolia* indicated the presence of alkaloid, flavonoid, glycoside, saponin, steroid, phenols, terpenoid and tannin in both aqueous and ethanol extracts. The antibacterial efficacy of the extracts against the test isolates (*S. typhi*, *E. coli*, *Klebsiella pneumoniae* and *Shigella*) showed that the leaves extracts demonstrated an antimicrobial effect against the isolates. The Minimum inhibitory Concentration (MIC) of aqueous and ethanol extract showed dilutions of various concentrations of the extracts can inhibit and/or kill the isolates. The finding of this study supported the use of *Citrus aurantifolia* leaves extracts for medicinal purpose.

Acknowledgement

The authors wish to acknowledge to the staff of Microbiology Laboratory of Murtala Muhammad Specialist Hospital for provision of Samples. Thanks to the Management of Kano University of Science and Technology Wudil and School of Technology, Kano State Polytechnics for the use of laboratory facilities.

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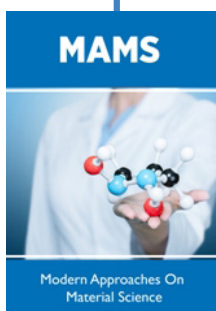
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DOI: [10.32474/MAMS.2018.01.000107](https://doi.org/10.32474/MAMS.2018.01.000107)



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