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Evaluation of antibacterial activity of *Citrus aurantium* L. leaf extracts on bacteria isolated from blood of hepatitis B positive individuals in Ondo State, Nigeria

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ABSTRACT

Background: Concomitant bacterial pathogens associated with hepatitis B infection could further increase the stress on the liver of infected individuals thereby complicating the infection. **Objective:** This necessitates the need to evaluate the antibacterial activity of *Citrus aurantium* L. leaf extracts on bacteria isolated from blood of hepatitis B virus (HBV) positive individuals in Ondo State, Nigeria. **Method:** The leaf extracts of *Citrus aurantium* (aqueous, ethanol and N-hexane) were assessed for possible antibacterial activities against some isolated bacteria from blood samples of HBV positive individuals were conducted using agar well diffusion technique. **Results:** *Proteus mirabilis, S. typhi, S. aureus, Kl. pneumoniae, Ps. aeruginosa, E. coli, S. pneumoniae* and *Ch. Violaceum* were isolated. The most frequently encountered bacterial species was *S. aureus* (52%). The ethanol leaf extract exerted the highest growth inhibitory activity on *S. aureus, Kl. pneumoniae, Ps. auruginosa* and *E. coli.* The aqueous leaf extract exerted the highest inhibitory on *Ch. violaceum* while the N- hexane extract exerted the highest growth inhibitory activity on *P. mirabilis* and *Ps. aeruginosa.* The extracts compared favorably well with conventional antibiotic (Ciprofloxacin) which is the positive control. The minimal inhibitory concentration (MIC) of all the extracts ranged from 12.5 to 50.0 mg/ml while the minimal bactericidal concentration (MBC) ranged from 25 to 100mg/ml. The analysis of the phytochemicals revealed the presence of tannins, flavonoids, terpenoids, steroids, cardiac glycoside and saponin in all the leaf extracts except for the absence of saponins in N-hexane extract. **Conclusion:** This study showed that these extracts could be exploited to treat infection caused by these bacterial pathogens.

Introduction

Citrus aurantium L. (Rutaceae), which is commonly known as sour or bitter orange, is extensively consumed worldwide as marmalade and an additive agent [1]. The oils extracted from the plant have been recognized as generally safe for their broad usage as antibacterial, antifungal, anti-inflammatory, antioxidant [2-5], and have analgesic activity [6]. *Citrus aurantium* is a tree up to 6 m

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tallness, having weathered leaves and white fragrant blossoms [7]. In addition, its flavonoids have been reported to have anticancer, anti-diabetic and cell reinforcement properties [8].

Citrus aurantium L., is also known as *Fructus aurantii* [9,10]. The concentrate of the juvenile organic product or strip of *C. aurantium* L. have been broadly utilized in weight reduction, dietary enhancements and in sports execution products [11]. Similarly, *C. aurantium* L. is among the species that have been utilized for clinical purposes by virtue of the various bioactive compounds that it contains, for example, phenolic, fundamental oils, nutrients and flavonoids. Since the 19th century, most groups of antibiotics of great significance such as tetracyclines, cephalosporins, aminoglycosides and macrolides have experienced a setback losing their viability as a result of the expansion in microbial opposition [12]. Right now, its effect is extensive with treatment failure related with multidrug resistant microorganisms and this has become a worldwide worry to general wellbeing [13,14]. For this reason, the discovery of new antibacterial agents becomes imperative. Natural products are still one of the major sources of new drugs in today's science. They are derived from prokaryotic bacteria, eukaryotic microorganisms, plants and various animals. Microbial and plant products occupy the major part of the antimicrobial compounds discovered so far [15].

Hepatitis B virus (HBV) is one of the major leading infectious diseases in the world in terms of the number of morbidity and clinical significance, particularly in Asia and some other African countries [16]. Bacterial infections are often seen in patients with hepatitis, including HBV-induced hepatitis. Common examples include, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* which are all human pathogens associated with ascites, a serious complication of end-stage liver disorders. Although bacterial infections are treated with antibiotics, however the increase in antibiotic resistance by all known bacterial species against available antibiotics lends credence to the search for new effective drugs. It is against this background that this current study becomes imperative, evaluating the antibacterial activities of the leaf extracts of *Citrus aurantium* on bacteria isolated from individuals positive for Hepatitis B Surface Antigen in Ondo State, Nigeria for possible discovery of new effective drugs to manage complications associated with HB infections.

Materials and Methods

Collection, identification of plant leaves

The leaves of *Citrus aurantium* used in this study were harvested from the Botanical garden of Federal University of Technology Akure. After collection, the leaves were identified and authenticated by a botanist at the Biology Department of Federal University of Technology, Akure. The leaves were then rinsed with sterile distilled water and air dried at room temperature (30±2 °C) for 2 weeks. The dried leaves were made into fine powder under laboratory condition using sterile pestle and mortar. The powder was stored in dark and air tight container for further use.

Preparation of the leaf extracts

Three different solvents namely: (i) Sterile distilled water, (ii) 95 % Ethanol, (iii) N -hexane were used for the preparation of the leaf extracts. Two hundred grams (200 g) of the powered dried leaves was suspended in 2000 ml each of the 3 different solvents in 3 different containers. These were left to soak at room temperature (30±2 °C) for 3 days (72 hours) with agitation at intervals. The extracts from each solvent was filtered by passing through muslin cloth, and then with Whatman No. 1 filter paper [17, 18]. The ethanol and N- hexane filtrates were evaporated in a rotary evaporator to remove the solvent used. All the extracts were sterilized using 0.22 µg Millipore membrane filter [19]. The extracts sterility was confirmed by the method of **Sule and Agbabiaka** [20]. The extracts were labeled and transferred into sterile bottles and refrigerated at 4˚C until used. The ethanol and N – hexane filtrates were 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 200mg/ml as a stock concentration. The prepared extracts were refrigerated at 4 ºC for further use.

Bacterial isolates

The test bacteria were isolated from blood samples of hepatitis B surface antigen positive patients attending selected Government Hospitals in Ondo State, Nigeria. They were isolated and characterized to species level using standard laboratory procedures which include Culturomic, Gram's staining and Biochemical tests (Indole, Methyl red, Voges Proskauer, Catalase, Citrate utilization and coagulase tests) as described by **Holt et al.** [21] and **Cheesbrough** [22]. The bacterial isolates were maintained on Nutrient agar slants at 4 °C until needed for further use.

Phytochemical analysis of leaf extracts of *Citrus aurantium*

The phytochemical (qualitative and quantitative) analysis of the leaf extracts of *Citrus aurantium* was carried out as described by the method of **AOAC** [23].

Antibacterial assay of the leaf extracts

The antibacterial activities of the crude leaf extracts of C*itrus aurantium* were determined following the agar well diffusion method described by **Ogundare** [24]. Ciprofloxacin and Dimethyl sulfoxide (DMSO) were used as positive and negative controls respectively. The plates were incubated at 37 °C for 24h, and the zones of inhibition were measured using Vernier Caliper. Each test was done in triplicates.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the leaf extracts

The MIC and MBC were carried out using the agar dilution method [25,26]. They were done at different concentration of the extracts (100mg/ml, 50mg/ml, 25mg/ml, and 12.5mg/ml) which were reconstituted using 30 % DMSO. One ml of the extract concentration and 1 ml of 24 h broth culture of each test bacterium ($1.0x10^6$ CFU/ml) was inoculated into 8 ml of Mueller Hinton broth in a test tube. The seeded broth was incubated for 24 hours at 37 ^oC. A –non test bacterial seeded tube without addition of extract was used as negative control. The presence of turbidity was checked after 24 h of incubation. The lowest concentration of the extract that produced no visible growth when compared to the control was considered as the MIC. Determination of MBC of each extract, was carried out on the positive MIC tubes where detectable growth were observed [26].

Statistical analysis

Data analysis was carried out using the Statistical package for Social Sciences Version 16.0 (SPSS Inc. Chicago. 11.) The chi-square (X^2) test and New Duncan Multiple Range Test were used to determine significant differences and effect.

Results

Eight different bacterial species were isolated from the blood of positive individuals sampled. These are, *Proteus mirabilis, Salmonella typhi, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Pseudomonas aeruginosa, Escherichia coli* and *Chromobacterium violaceum* **(Table 1).** The most frequently encountered bacterial species was *S. aureus* (53%). **Table 2** showed the phytochemical profiles of the leaf extracts. All the leaf extracts had flavonoid, Terpenoid, Tannin and glycoside while only the aqueous and ethanol extracts had saponin. However, only the ethanol and N- hexane extracts had steroids. Alkaloid, Phlobatannin and Anthraquinone were absent in all the extracts. **Table 3** showed that N – hexane extract had the highest concentration of terpenoid (9.05 mg/g) which was followed by the aqueous extract (6.22mg/g). The highest concentration of flavonoid on the other hand was observed in the aqueous extract with 3.78mg/g which was followed by ethanol extract with 3.17mg/g. Also, the aqueous extract had the highest concentration of tannin, glycoside and saponin with 2.98mg/g, 2.02mg/g and .84mg/g followed by ethanol extract with 1.88mg/g, 2.02mg/g and 0.55mg/g respectively.

The results of the antibacterial assay of the leaf extracts showed that all the leaf extracts inhibited the growth of all the test bacteria with the ethanol extract exerting the greatest effect on four of the eight test bacteria **(Table 4).** The growth inhibition of the extracts was superior to that of ciprofloxacin which served as control. The ethanol extract had the lowest MIC of 12.5mg/ml against *S. aureus* (12.5 mg/ml) while the aqueous extract had the lowest MIC of 12.5 mg/ml against *Strep. pneumoniae* and *Ch. violaceum.* The ethanol and aqueous extracts had the lowest MIC of 25 mg/ml against *Kl. pneumoniae* and *P. mirabilis.* The lowest MIC of the aqueous extract was observed to be 25 mg/ml against *S. typhi* **(Table 5). Table 6** shows that the lowest MBC of the aqueous extract against *Ch. violaceum* is 25 mg/ml. However, the MBC of the aqueous extract was 50 mg/ml against *S. typhi, Kl. pneumoniae* and *S. pneumoniae* while the lowest MBC of the ethanol extract against *Ch. violaceum, S. pneumoniae, P. aeruginosa* and *S. aureus* is 50mg/ml respectively.

Table 1. Frequency of occurrence of bacterial isolates from HBsAg positive individuals.

S/N	Bacterial Isolates	Frequency (%)
1	*Staphylococcus aureus*	13(52)
2	*Salmonella typhi*	3(12)
3	*Escherichia coli*	3(12)
4	*Klebsiella pneumoniae*	2(8)
5	*Proteus mirabilis*	1(4)
6	*Chromobacterium violaceum*	1(4)
7	*Streptococcus pneumoniae*	1(4)
8	*Pseudomonas aeruginosa*	1(4)
	Total	25 (100)

Table 2. Qualitative phytochemical profile of the leaf extracts of C*itrus aurantium***.**

Properties	Aqueous	95% Ethanol	N - Hexane
Saponin	+	+	-
Tannin	+	+	+
Phlobatannin	-	-	-
Flavonoid	+	+	+
Steroids	-	+	+
Terpenoid	+	+	+
Alkaloid	-	-	-
Anthraquinone	-	-	-
Glycosides	+	+	+

Key: + = positive; - = negative

Table 3. Quantitative Phytochemical profile of Leaf Extracts of *Citrus aurantium.*

Properties (mg/g)	Aqueous	Ethanol	N-Hexane
Saponin	0.84 ± 0.10^{c}	0.55 ± 0.00^{b}	0.00 ± 0.00^{a}
Tannin	2.98 ± 0.01^{c}	1.88 ± 0.01^{b}	1.16 ± 0.01^{a}
Glycoside	2.02 ± 0.03^{c}	1.79 ± 0.04^{b}	0.38 ± 0.04^{a}
Flavonoid	3.78 ± 0.02^{c}	3.17 ± 0.02^{b}	1.33 ± 0.02^{c}
Steroid	0.00 ± 0.00^{a}	1.82 ± 0.01^{b}	0.93 ± 0.01^{c}
Terpenoid	6.22 ± 0.03^{b}	4.43 ± 0.03^{a}	9.05 ± 0.03^{c}

Values carrying the same alphabet in the same row are not significantly different (p >0.05) while different alphabets denote significant difference ($p< 0.05$) .

Table 4. Antibacterial activity of crude leaf extracts of *Citrus aurantium* (100mg/ml) on bacteria isolated from HBV patients.

Bacterial Isolates	**Crude Extracts (mg/g)**				
	Aqueous	**Ethanol**	**N –Hexane**	**Ciprofloxacin**	**DMSO**
Staphylococcus aureus	12.75±0.24^{b}	15.25±0.36^{c}	11.02±0.30^{a}	12.56±0.13^{b}	0.0±0.0
Escherichia coli	12.07±0.84^{a}	16.43±0.17^{c}	12.83±0.40^{a}	15.70±0.71^{b}	0.00±0.00
Salmonella typhi	11.53±0.18^{a}	17.11±0.29^{c}	13.17±0.31^{b}	19.09±0.14^{d}	0.00±0.00
Klebsiella pneumoniae	13.55±0.25^{b}	14.70±0.32^{c}	12.50±0.22^{a}	13.57±0.99^{b}	0.00±0.00
Pseudomonas aeruginosa	12.00±0.00^{b}	13.00±0.00^{b}	13.00±0.00^{b}	11.40±0.10^{a}	0.00±0.00
Proteus mirabilis	13.00±0.00^{a}	12.00±0.00^{a}	16.00±0.00^{b}	13.30±0.10^{b}	0.00±0.00
Streptococcus pneumoniae	12.00±0.00^{b}	14.00±0.00^{c}	10.30±0.00^{a}	16.30±0.15^{d}	0.00±0.00
Chromobacterium violaceum	15.00±0.00^{c}	12.00±0.00^{a}	12.00±0.00^{a}	13.20±0.12^{b}	0.00±0.00

Key: DMSO = Dimethyl sulfoxide

Values carrying the same alphabet in the same row are not significantly different (p>0.05), while different alphabet denotes significance difference (p<0.05).

Table 5. Minimum inhibitory concentration (MIC) (mg/ml), of leaf extracts of *Citrus aurantium* on Bacterial Isolated from hepatitis B surface antigen positive individuals.

Isolates	**Aqueous**	**Ethanol**	**N –Hexane**
Staphylococcus aureus	25	12.5	50
Escherichia coli	25	25	50
Salmonella typhi	25	50	50
Klebsiella pneumoniae	25	25	50
Pseudomonas aeruginosa	25	25	50
Proteus mirabilis	50	25	50
Streptococcus pneumoniae	12.5	12.5	25
Chromobacterium violaceum	12.5	12.5	50

Table 6. Minimum bactericidal concentration (MBC) (mg/ml), of leaf extracts of *Citrus aurantium* on Bacterial Isolated from hepatitis B surface antigen positive individuals.

Isolates	**Aqueous**	**Ethanol**	**N –Hexane**
Staphylococcus aureus	100	50	100
Escherichia coli	100	100	100
Salmonella typhi	50	100	100
Klebsiella pneumoniae	50	100	100
Pseudomonas aeruginosa	100	50	100
Proteus mirabilis	100	100	100
Streptococcus pneumoniae	50	50	100
Chromobacterium violaceum	25	50	100

Discussion

Citrus aurantium generally known a**s** bitter orange has been reported to have several therapeutic potentials in folklore medicine. In this study, the aqueous, ethanol and N – hexane extracts of the leaves of *C. aurantium* revealed the presence of saponin, tannin, flavonoid, terpenoid and glycoside except for saponin which was absent in the N – hexane extract. The results of this study corroborates the study of **Nirmala et al.** [27] who reported the

presence of tannins, flavonoids terpenoids, and the absence of saponins and phlobatannins in the aqueous and ethanol extracts of *C. auratium*. The study is also in agreement with the study of **Khudhair et al.** [28] who reported the presence of flavonoids, saponins and tannins in the aqueous extract of *C. auratium*. Furthermore, the presence of flavonoids in the study is in agreement with the report of **He et al.** [29] who reported the presence of flavonoids and the types of flavonoids present. Several studies have reported that flavonoids have natural potentials that positively affect human wellbeing as an ideal group of secondary metabolites in citrus products that may have natural potential and affect human wellbeing as antimicrobial, anti-inflammatory,anti-diabetic, anti-cholesterolemic, anti-inflammatory and anti-carcinogenic [29-33].

In this present study, the leaf extracts of *C. aurantium* using ethanol, aqueous and N – hexane as solvents revealed high antimicrobial activities. However, the ethanol extract exhibited the highest antibacterial activities against all the test bacteria except for *Proteus mirabilis* where the N- hexane extract had the highest antibacterial activity. This agrees with the study of **Rich and Ma** [34] in Philippines that all the leaf extracts of three citrus plants namely *Citrus microcarpa* (*calamansi*), *Citrus aurantium* (*dalandan*) *and Citrus maxima* (*pomelo*) had high antimicrobial activities against *Staphylococcus aureus.* However, the report of this study disagree with their observation that the extracts are not effective on *E. coli* because all the leaf extracts used in this study inhibited the growth of *E. coli* with ethanol extract exhibiting the highest growth inhibition of the organism. The study of **Tumane et al.** [35] on the other hand reported marked inhibitory effect of the ethanol and methanol leaf extracts of the plant against different groups of bacteria.

Conclusion

In conclusion, the results of this study demonstrated that the leaf extracts of *C. aurantium* have antimicrobial properties with different degrees of antimicrobial abilities. The greater *in vitro* antimicrobial activity of bitter orange leaf extracts than ciprofloxacin could be attributed to the presence of phytochemical inherent in the plant. These biological properties can be exploited in the development of new drugs that can be used to replace the ones that are no longer effective. Further study however is suggested to compare the phytochemicals in the different parts of the plants for proper harnessing.

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