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Phytochemical analysis and antibacterial activity of leaf and stem bark extracts of *Citrus sinensis* (sweet orange)

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Abstract

Background and objective: Resistance of microorganisms to chemical agents is a challenge in the treatment of various infections. Therefore, there is a need to find new substances with efficient antimicrobial potential. The aim of this study was to determine phytochemical composition and antimicrobial activity of *Citrus sinensis* extract obtained from its leaf and bark.

Materials and methods: Leaves and stem barks of *Citrus sinensis* were collected from University of Agriculture, Makurdi, Benue State, Nigeria. Bacterial isolates of *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus* were prepared in the microbiology laboratory. The leaves and stem barks were extracted with ethanol and water using maceration technique. Agar well diffusion method was used to determine antibacterial activity of the extracts. Broth dilution method was used to determine minimum inhibitory concentration (MIC) of the extracts. Minimum bactericidal concentration test was done by culturing the MIC positive broth on nutrient agar plates. Steroids, phenols, flavonoids, saponins, alkaloids, tannins, and glycosides were determined according to standard methods in the laboratory.

Results and conclusion: Phytochemical screening showed the presence of phenols, flavonoids, alkaloids, tannins, steroids, and glycosides. Ethanolic leaf extract had the highest antibacterial activity against *S. typhi* with inhibition diameter of 25 mm, followed by 20 mm for *E. coli* and 12 mm for *S. aureus*. In comparison, ethanolic stem bark extract showed the highest inhibition against *E. coli* with diameter inhibition of 25 mm, while diameter inhibition of 22 mm and 20 mm were determined for *S. aureus* and *S. typhi*, respectively. Aqueous extract of leaf showed inhibition zone of 16 mm for *S. typhi*, and 10 mm for *E. coli* and *S. aureus*. Inhibition zone of 16, 11, and 10 mm was observed for *S. typhi*, *E. coli*, and *S. aureus* respectively, after treatment by aqueous extract of stem bark. Minimum inhibitory concentration and minimum bactericidal concentration of both extracts were in the range of 6.25-50.00 mg/ml. This study revealed that the leaf and stem bark of *Citrus sinensis* contain several bioactive compounds with antibacterial potential (i.e., steroids, phenols, flavonoids, saponins, alkaloids, tannins, and glycosides) which can be used for treatment of microbial infections.

Keywords: Antibacterial, *Citrus sinensis*, Minimum bactericidal concentration, Minimum inhibitory concentration, phytochemical

1. Introduction

Medicinal plants have been used for therapeutic goals. It has been estimated that around 80% of the world population use herbal medicines to treat

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illnesses especially infectious diseases. Herbal medicine is more popular in developing societies than developed countries [1]. Healing power of medicinal plants was known in the past when people used them as poultice for wound healing [2]. Many infectious diseases have been treated by herbal remedies. Indeed, herbal products either as pure compounds or standardized extracts have been used for development of new drugs [3]. It has led to the need for discovering a new antimicrobial compound and finding the mechanisms of action for curing the emerging infectious diseases [4]. According to the World Health Organization (WHO), medicinal plants are the best source to formulate a variety of drugs. Therefore, plant sources should be investigated scientifically to characterize their compounds, and evaluate their effectiveness and safety for human use [5].

Citrus sinensis plant is a perennial tree and belongs to the family of Rutaceae. Its common name is sweet orange. In Latin America, the fruit is called "naranja deChina". *C. sinensis* is widely distributed in tropical regions. The fruit was originated from southern China, north eastern India, and southern Asia formerly Indochina. Entire part of *C. sinensis* tree is useful medically [2,6,7]. *C. sinensis* has been found to be a valuable source of essential oil. The components include bioflavonoids, carbohydrates, and terpenoids. Orange peel oil has lethal effect on fleas, fire ants, and houseflies due to its high limonene content (90-95%) [8]. In this study, we aimed to evaluate phytochemical composition and antibacterial activity of ethanolic and aqueous extracts of leaf and stem bark of *C. sinensis* (sweet orange).

2.Materials and methods

2.1. Sample collection

Leaves and barks of *C. sinensis* was collected in University of Agriculture, Makurdi Benue State, Nigeria. They were authenticated by the experts in Department of Biological Sciences, University of Agriculture Makurdi, Benue State, Nigeria, where the voucher specimens were deposited.

2.2. Extraction

Fresh plants were carefully washed by tap water followed by sterile distilled water. Then, they were air-dried at room temperature (30 ºC) for two weeks, and were grinded further. The fine powders were stored in air-tight bottles until analysis [2].

2.2.1. Ethanolic extraction

20 g of each powder (leave and stem bark) was added to 100 ml ethanol in conical flask for 24 h followed by heating on water bath at 37 ºC for 30 min under occasional shaking, and left constantly for additional 24 h. The mixture was filtered by Whatman paper no.1, and ethanol was removed by Soxhlet apparatus. The remained ethanol was evaporated by using steam bath at 100 ºC [2,9].

2.2.2. Aqueous extraction

20 g of each powder (leave and stem bark) was added to 100 ml distilled water in conical flask for 24 h followed by heating on water bath at 37 ºC for 30 min under occasional shaking, and left constantly for additional 24 h. The mixture was filtered by Whatman paper no.1 [2], and the water content was evaporated by using steam bath at 100 ºC [2].

2.3. Microbial isolates

Pure cultures of clinical strains of *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus* were obtained from the microbiology laboratory of Benue State University Teaching Hospital Makurdi (Nigeria). Bacteria isolates were transferred to the research laboratory in sterile bottles and stored at 4 ºC until analysis.

2.4. Microbial analysis

The isolates were cultured on selective or differential medium, followed by sub-culturing on nutrient agar. Then, they were identified according to their morphology and the results of biochemical analysis [10].

2.5. Antimicrobial assay

Antibacterial activity of the extracts was evaluated by agar well diffusion assay by using Mueller-Hinton Agar medium (Himedia, India) [11,12]. A sterile

cotton swab was dipped into the microbial suspension, rotated several times, and pressed firmly on the inside wall of the tube above the fluid to remove the excess inoculum. Then, surface of Mueller Hinton Agar plate was streaked thoroughly by the swab. After streaking, 5 mm diameter wells were punched into the medium by a sterile borer. The plates were left 3-5 min to dry. In the next step, 50 µl of each extract was added to each well. Ampicillin was used as positive control for bacterial isolates and clotrimazole was used as positive control for fungal isolates. In addition, dimethyl sulfoxide (DMSO) was used as negative control. The plates were incubated at 37 ºC for 24 h. Diameter of the inhibition zones was measured by a ruler and a pair of dividers. Inhibition diameter was reported in millimeter (mm). Percentage of growth inhibition was determined according to below equation:

$$\text{Growth inhibition (\%)} = \frac{\text{Inhibition diameter of (control} - \text{sample)}}{\text{Inhibition diameter of control}} \times 100$$

2.6. Minimum Inhibitory Concentration (MIC)

MIC is defined as the concentration giving the least inhibitory activity, below which no inhibition is seen. Briefly, 1 ml of reconstituted extract at concentration of 50 mg/ml was added to a test tube containing 1 ml prepared broth to reach a final concentration of 25 mg/ml. It was diluted more three times to reach the fourth dilution. A test tube containing the prepared broth and free of the extracts was served as negative control. Then, 1 ml of 18 h grown culture of each bacterial strain at concentration of 1.5×10^8 CFU/ml was added to each tube and mixed by vortex mixer thoroughly. The tubes were incubated at 37 ºC for 18 h. Bacterial growth was monitored by development of turbidity. The test tube at the lowest concentration of the extracts with no detectable growth by visual inspection was considered for MIC calculation [12].

2.7. Minimum Bactericidal Concentration (MBC)

For determination of MBC, 50 µl of the suspension in MIC tubes (the tubes without visible growth) was added to nutrient agar by streaking. Nutrient agar plates inoculated with the test organisms were considered as control. The plates were incubated at 37 ºC for 24 h. After incubation, the concentration with no visible growth was determined as MBC [13].

2.8. Phytochemical analysis

2.8.1. Tannins

0.5 g of each powder was boiled in 20 ml distilled water followed by filtration. Then, 0.1% $FeCl_3$ was added to the filtrate, and the mixture was monitored up to a brownish-green or blue-black color was appeared, which shows the presence of tannins [14].

2.8.2. Saponins

2 g of each powder was boiled in 20 ml distilled water followed by filtration. Then, 10 ml of filtrate was mixed with 5 ml distilled water and shaken vigorously to obtain stable froth. The froth was mixed with three drops of olive oil, and observed for formation of emulsion, which indicated the presence of saponins [15].

2.8.3. Flavonoids

First, 5 ml distilled water was added to 0.5 g of each extract, followed by mixing with magnesium ribbon fragments and drops of concentrated hydrochloric acid. Formation of orange, red, pink or purple color indicated the presence of flavonoids [16].

2.8.4. Cardiac glycosides

First, 5 ml of each aqueous extract (as described in section 2.8.3) was mixed with 2 ml glacial CH_3COOH containing one drop of $FeCl_3$. The mixture was added to 1 ml concentrated H_2SO_4. Appearance of greenish-blue color indicated the presence of glycosides [3].

2.8.5. Alkaloids

To determine the presence of alkaloids, 200 mg of each powder was added to 10 ml methanol followed by filtration. Then, 2 ml filtrate was mixed with 2 ml HCl 1%, and the mixture was heated at 37 ºC for 5 min. In the next step, *Dragendroff* reagent was added, and formation of orange precipitate indicated the presence of alkaloids [2].

2.8.6. Phenol

Presence of phenol was determined by addition of 3 ml of aqueous or ethanolic extracts to 5% $FeCl_3$ solution. Formation of deep blue-black color indicated the presence of phenol [15].

2.8.7. Steroids

First, 2 ml chloroform and 10 drops of acetic acid were mixed in a test tube. Then, 0.5 ml of each plant extract was added to the test tube followed by addition of 2 ml concentrated sulfuric acid. Color change from red to blue or green indicated the presence of steroids [2,17]

3. Results and discussion

Ethanolic and aqueous extracts of *C. sinensis* leaf and stem bark were evaluated for phytochemical content and antibacterial activity against *E. coli*, *S. typhi*, and *S. aureus*. Table 1 shows yield of extraction for both leaf and stem bark. As shown in the table, ethanolic extraction of the leaf had the highest yield.

Table 1- Extraction yield of leaf and stem bark of *Citrus sinensis* by polar and non-polar solvents

Plant Part	Solvent	Sample weight (g)	Extract weight (g)	Yield (%)
Leaf	Ethanol	25	4.80	19.2
	Water	25	4.0	16
Stem bark	Ethanol	20	3.5	18
	Water	25	4.2	16.8

Results of phytochemical analysis is presented in Table 2. Presence of phenols, flavonoids, saponins, alkaloids, and tannins were confirmed by both method of extraction. In detail, steroids were just present in ethanolic extract of leaf and stem bark, and no glycoside was detected in aqueous extract of stem bark.

Table 2- Monitoring of phytochemical agents in the extracts of *Citrus sinensis* leaf and stem bark

Plant part	Solvent	Phenols	Flavonoids	Saponins	Alkaloids	Tannins	Steroids	Glycosides
Leaf	Ethanol	++	++	++	++	++	+	+
	Water	+	+	+	++	++	_	+
Stem bark	Ethanol	++	++	++	++	++	+	+
	Water	++	++	+	+	+	-	-

+ = Positive; - = Negative; ++ = Highly present

Table 3 shows the antibacterial potential of the ethanolic and aqueous extracts. As observed, ethanolic extract of leaf had the highest antibacterial activity against *S. typhi* with zone inhibition of 25 mm, followed by *E. coli* and *S. aureus*, respectively. Similar result was observed for aqueous extract of leaf against *S. typhi*, but lower inhibition against *E. coli* and *S. aureus* was observed compared to the ethanolic extract. Ethanolic extract of stem bark had the highest antibacterial activity against *E. coli*, followed by *S. aureus* and *S. typhi*, and its aqueous extract showed the highest antibacterial activity against *S. typhi*, followed by *E. coli* and *S. aureus*. However, all extracts were not as efficient as Ampicillin as positive control.

Table 3- Inhibition zone (mm) of bacterial isolates in the presence of *Citrus sinensis* leaf and stem bark extracts

Plant part	Bacteria	Ethanolic extract	Aqueous extract	Ampicillin	DMSO*
Leaf	*E. coli*	20 (31%**)	10 (66%**)	29	0
	S. typhi	25 (24%**)	25 (24%**)	33	0
	S. aureus	12 (59%**)	10 (66%**)	29	0
Stem bark	*E. coli*	25 (14%**)	11 (62%**)	29	0
	S. typhi	20 (40%**)	16 (52%**)	33	0
	S. aureus	22 (24%**)	10 (66%**)	29	0

* Dimethyl sulfoxide

** Inhibition percent compared to ampicillin

Results of MIC are presented in Table 4. Accordingly, ethanolic leaf extract had lower MIC than aqueous extract. In comparison, MIC of aqueous stem bark extract was lower than the ethanolic extract for *S. typhi* and *S. aureus*. The lower MIC means the higher antibacterial potency against the tested bacteria.

Table 4- Minimum inhibitory concentration of the extracts against the bacterial isolates

Plant part	Bacteria	Ethanolic extract (mg/ml)	Aqueous extract (mg/ml)
Leaf	*E. coli*	25	37
	S. typhi	25	25
	S. aureus	18	50
Stem bark	*E. coli*	25	50
	S. typhi	19.37	12.50
	S. aureus	18.15	6.25

Similar to MIC, ethanolic extract of leaf showed lower MBC than the aqueous extract. Moreover, lower MBC was observed for ethanolic extract of stem bark against the bacteria except for *S. aureus* which was killed by equal concentration of ethanolic and aqueous extracts of stem bark (Table 5).

Table 5- Minimum bactericidal concentration of the extracts against the bacterial isolates

Plant part	Bacteria	Ethanolic extract (mg/ml)	Aqueous extract (mg/ml)
Leaf	*E. coli*	12.50	25
	S. typhi	9.37	25
	S. aureus	25	50
Stem bark	*E. coli*	25	50
	S. typhi	6.25	19.00
	S. aureus	6.25	6.25

Our study revealed that leaf and stem bark of *C. sinensis* contain phenols, alkaloids, saponins, flavonoids, steroids, and tannins, and glycosides. It is in agreement with results of Ekwenye and Edeha [3] that reported the presence of alkaloids, tannins, saponins, flavonoids, steroids, and terpenes in extract of sweet orange. Similar findings were reported by Hany et al. [18] and Ngele et al. [19] for *C. sinensis*. In agreement, Sumathi and Janarthanam [20] reported that different extracts of *Punica granatum* were rich in secondary metabolites including tannins, saponins, flavonoids, cardiac glycosides terpenoids, phenols, steroids, and alkaloids.

Presence of secondary metabolites or bioactive phytochemical constituents in the extracts of *C. sinensis* is associated with their considerable antimicrobial potential. It is corroborated by findings of Ngele et al. [20] in which *C. sinensis* unripe epicarp extracts showed antimicrobial activity against the tested organisms. The crude extracts showed a remarkable inhibition against *E. coli*, *S. typhi*, and *S. aureus*. Gram-negative bacteria are more resistant to antimicrobial agents due to the presence of outer membrane in cell structure, which limits the access of antimicrobial agents to their target in bacterial cell.

Antimicrobial activity of the extracts against *E. coli* (gram-negative), *S. typhi* (gram-negative), and *S. aureus* (gram-positive) in our study refers to their broad-spectrum activity. Our observation is similar to the report of Kumar et al. [21] who suggested that the herbal extracts can be used in development of safe antibiotics for treatment of bacterial infections.

In general, ethanolic extract of leaf and stem bark of *C. sinensis* showed better antimicrobial activity compared to the aqueous extract. It was also reported by Shetty et al. [22] by studying the antimicrobial potency of Citrus fruit peel. The authors found that antimicrobial activity is closely associated with type of solvent and some active ingredients with high antimicrobial effects are extracted by specific solvents. In this regard, Musa et al. [9] and Gupta et al. [13] stated that most of herbal antibiotic compounds are aromatic or saturated organic molecules which can be dissolved easily in organic solvent.

Aqueous extract of leaf and stem bark of *C. sinensis* showed less antibacterial activity compared to ethanolic extract. However, *S. typhi* was more susceptible to the aqueous extract than *E coli* and *S. aureus*. It was also observed by Hany et al. [18] when studied *C. sinensis* peel. It indicates that the bioactive compounds such as alkaloids, flavonoids, saponins, tannins, glycosides, saponins, and steroids in herbal extracts may have different modes of action, or the bacteria

may have special defensive mechanism when faced with specific antimicrobial agent [2,13]. MIC of the extracts varied at different concentrations against the pathogens. It is in agreement with findings of Gurusiddappa et al. [23] who reported that *C. limon* peel extracts had various MIC against different microorganisms. On the other hand, the lower MIC of ethanolic extracts in our study was in accordance with the MIC observed in study of Hussain et al. [2] who studied *C. sinensis* peel. Similar results were observed by Kumar and Gitika about MBC of ethanolic and aqueous extracts of *Psidium guajava* leaves [24].

4. Conclusion

Crude extracts of *C. sinensis* (leaf and stem bark) showed good antimicrobial activity against both gram-positive and gram-negative bacteria. Higher antimicrobial activity was observed for ethanolic extracts compared to aqueous extracts. It is assumed that antimicrobial activity of the extracts is due to the presence of phytochemicals including alkaloids, flavonoids, saponins, tannins, steroids, glycosides, and phenols. MIC and MBC of the extracts ranged from 6.25 to 50.00 mg/ml. Our findings support the use of *C. sinensis* leaf and stem bark in traditional medicine.

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6. Conflict of interest

The authors declare no competing interest.

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