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EVALUATION OF PHYTOCHEMICAL COMPOSITION AND IN *VITRO* ANTIMICROBIAL ACTIVITY OF VARIOUS PARTS OF *CITRUS GRANDIS* OSBECK

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ABSTRACT

Citrus grandis Osbeck provides juicy, tasty and nutritious fruit with aesthetic qualities for human beings. The phytochemical composition was determined and the *in vitro* antimicrobial activities of leaf, stem, stem bark and ripe fruit peel extracts of *C. grandis* were evaluated using standard techniques. The significance of the results was evaluated using Duncan's multiple range test. The highest contents of alkaloids (2.76±0.06 mg/100g), flavonoids (3.41±0.12 mg/100g), phenols (1.64±0.11 mg/100g), sterols (0.82±0.03 mg/100g) and tannins (3.46±0.06 mg/100g) were detected in the peel. The peel and stem bark extracts had the lowest MIC values against *Staphylococcus aureus*, *Escherichia coli*, and *Rhizopus stolonifer*. This study disclosed that the extracts of *C. grandis* parts are rich with phytochemicals that are active against fungi and bacteria, hence, suggesting its pharmacological significance.

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Introduction

Today, several countries are looking for important compounds and plants that are useful in the remedy of diseases, or that can be utilized in different industries as a natural substance [1]. Medicinal plants have been utilized for centuries as a treatment for various human diseases [2] as they have hundreds, even thousands of active chemical compounds namely secondary metabolites [3]. *Citrus grandis* Osbeck is a perennial shrub commonly known as shaddock/pomelo. It is a member of the genus *Citrus* (family Rutaceae). It is regarded as food worldwide due to tasty, succulent, and nutritious fruit. It is of tropical origin–Malayan Archipelago [4]. This species is a member of three original species of the genus *Citrus* [5]. *Citrus* is cultivated in tropic and subtropic regions throughout the world. The members of this genus are small trees or large shrubs, reaching 5 to 15 meters tall, with spiny shoots and alternately arranged evergreen leaves with an entire margin [6, 7].
Pathogenic bacteria are the most common cause of ailments in human beings. Bacterial cells occur in the gut flora and a higher percentage on the skin [8]. On the other hand, harmful fungi also cause infections in humans. This normally happens when the immune system is weak or the microbes are too much for it to handle. Several regions of the world are endowed with different medicinal plants which are essentially used by the local populace in tackling their health problems. Investigation of the extracts of the whole plant and plant parts for antimicrobial activity is topical. This reveals that plants possess healing qualities that could be exploited for the development of new drugs. Hence, the objectives of this work were to evaluate the leaf, stem, stem bark, and ripe fruit peel of *C. grandis* for phytochemical composition and antimicrobial activity.

Materials and Methods

Collection of Sample

The leaf, stem, stem bark, and ripe fruit peels of mature *C. grandis* were collected in July from Enugwu-ukwu, Anambra State, precisely opposite General Hospital, Enugwu-ukwu town (6º10'N and 7º01'E). The samples were authenticated at the

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Herbarium of the Department of Botany, Nnamdi Azikiwe University, Awka, Nigeria, where the voucher specimen was stored.

Preparation of sample for analyses

The fresh plant parts were oven-dried at the temperature of 60 ^{o}C for 72 hours and then ground with a mechanical blender. The dried powdered samples were deposited in an air-tight container for further analyses.

Extraction of plant materials

An aliquot of 100 g of the plant sample was added into 70% w/v ethanol, with the view to get 100 mg/ml ethanol extract [9, 10].

Microbial analyses

Isolation of the test organisms

The colonies of test organisms including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Aspergillus flavus*, and *Rhizopus stolonifer* were collected with a wire loop. They were collected from pure cultures at the Pathology Laboratory of NRCRI Umudike, Abia State, Nigeria. All the clinical isolates were inspected for purity and were deposited in nutrient broth at 4 ^{o}C in the refrigerator until further research.

Antimicrobial test procedures

Preparation of stock solution

The initial concentration of each plant extracts (5 g) was diluted utilizing 50 ml of ethanol to obtain the stock culture. From this stock culture, 50, 100, and 150 mg/ml concentrations were got and stored in room temperature prior to use.

Determination of inhibitory activity

Inhibitory activity of ethanol extract of leaf, peel, stem, and stem bark of *C. grandis* and antibiotics (positive control) were determined using the disc diffusion method. In 5 mm discs, concentrations of 1000 mg/ml of the ethanol extract and 100 mg/ml of antibiotics were inserted.

Nutrient agar medium was poured in the sterile Petri dishes and was allowed to solidify. One milliliter (1 ml) of the test organisms were placed all over the surface of the solidified agar. The locally prepared sterile discs were soaked in the water extract for several hours, and placed on the surface of the agar by a sterile forcep. The plates were incubated at 37 ^{o}C for 24 hours. The organisms' sensitivity to the plant extract was recorded by determining the zone of inhibition around each paper disc in millimeters (mm) [11].

Determination of minimum inhibitory concentration

Minimum inhibitory concentrations (MIC) for the absolute (stock) concentrations were determined by agar well diffusion method. Nutrient agar was poured in sterile Petri dishes and was allowed to solidify. One milliliter (1 ml) of the test culture was dropped on the solidified agar and the organism was spread all over the surface of the agar using a spreader. A sterile cork borer was used to make wells of 5 mm in diameter on the surface of the agar medium. The plates were turned upside down and the wells tagged with a marker. 0.2 ml of the extract was poured in each well. The plates were incubated aerobically at 37 ^{o}C for 24 hours. The lowest concentration of ethanol extracts with a clear zone of inhibition was considered as the MIC [12].

Determination of minimum bactericidal/fungicidal concentration

The plates with the MICs were further incubated for 24 hours at 35 ^{o}C to test which organism would grow on the zones of inhibition. After 24 hours, those plates in which the organisms were completely killed and clear zones maintained; were referred to as bactericidal for bacteria and fungicidal for fungi [13].

Statistical analysis

The experiment was carried out using a completely randomized design. Analysis of Variance (ANOVA) using SPSS version 21 was employed and Duncan's Multiple Range Test (DMRT) was used to measure the test of significance. The data were expressed as mean± standard deviation of triplicate determinations.

Results

The quantitative phytochemical composition of extracts of leaf, peel, stem, and stem bark of *Citrus grandis* was shown in Table 1. It was revealed that the peel extract had the highest contents of alkaloids (2.76±0.06 mg/100g), flavonoids (3.41±0.12 mg/100g), phenols (1.64±0.11 mg/100g), sterols (0.82±0.023 mg/100g) and tannins (3.46±0.06 mg/100g), while the stem extract had the highest composition of cardiac glycosides (0.75±0.03 mg/100g) and saponins (1.39±0.01 mg/100g).

In the *in vitro* antimicrobial assessment of the leaf, peel, stem, and stem bark extracts of *C. grandis*, the plant showed inhibitory activity against all tested bacterial and fungal pathogens (Tables 2 and 3 and Figures 1–4). At 50 mg/ml concentration, the peel had the highest inhibitory actions against *S. aureus* (6.43±0.03 mm), *S. typhi* (4.33±0.02 mm), and *E. coli* (5.67±0.10 mm). The leaf extract showed the highest restriction on the growth of *A. flavus* (5.23±0.02 mm) and *R. stolonifer* (6.12±0.26 mm). At 100 mg/ml concentration, the leaf extract had the highest restraint on *S. typhi* (8.78±0.04 mm). The peel extract exhibited the highest inhibitory activity against *S. aureus* (8.73±0.04 mm) and *E. coli* (7.22±0.02 mm). The inhibitory actions of the leaf extract (9.63±0.25 mm) and the peel extract (10.15±0.42 mm) against *A. flavus* that were the highest at that concentration had no significant difference. Moreover, at 150 mg/ml concentration, there was no significant difference between the inhibitory activities of the leaf extract (11.82±0.05 mm) and the peel extract (11.68±0.04 mm) against

S. typhi that were the highest. There was also no significant difference among the inhibitory activities of the leaf extract (8.46±0.27 mm), peel extract (8.52±0.38 mm), stem extract (8.16±0.23 mm), and stem bark extract (8.34±0.09 mm) against *R. stolonifer* at 100 mg/ml concentration as well as at 150 mg/ml. The leaf extract had the highest inhibitory action against *A. flavus* (12.82±0.06 mm) while the peel extract had the highest inhibitory activity against *E. coli* (9.53±0.11 mm). The stem extract had the highest inhibitory activity against *S. aureus* (10.82±0.06 mm). The leaf extract exhibited relatively high MIC against the entire test organisms (Table 3). Furthermore, the study revealed that *A. flavus* showed the highest susceptibility to the leaf and peel extracts while *E. coli* was the most resistant in 100 and 150 mg/ml concentrations (Figures 1 and 2). *Aspergillus flavus* was the most susceptible to the stem extract in 150 mg/ml concentration (Figure 3). *Salmonella typhi* was the most resistant to the stem bark extract, while *R. stolonifer* was the most susceptible in 100 and 150 mg/ml concentrations (Figure 4).

Table 1: Mean quantitative phytochemical composition of the ethanol extracts of *Citrus grandis* leaf, peel, stem, and stem bark**.**

Composition (mg/100g)	Plant Parts			
	Leaf	Peel	Stem	Stem Bark
Alkaloids	1.67±0.02^{b}	2.76±0.06^{c}	1.31±0.01^{a}	1.24±0.09^{a}
Cardiac glycosides	0.64±0.12^{b}	0.49±0.01^{a}	0.75±0.03^{c}	0.61±0.03^{b}
Sterols	0.75±0.03^{c}	0.82±0.03^{d}	0.35±0.04^{a}	0.44±0.02^{b}
Phenols	1.47±0.02^{c}	1.64±0.11^{d}	0.84±0.01^{b}	0.65±0.04^{a}
Saponins	0.75±0.02^{a}	1.05±0.02^{b}	1.39±0.01^{d}	1.16±0.05^{c}
Tannins	1.83±0.06^{c}	3.46±0.06^{d}	0.79±0.01^{a}	1.06±0.02^{b}
Flavonoids	2.79±0.02^{c}	3.41±0.12^{d}	1.25±0.00^{a}	1.75±0.12^{b}

Results are in Mean± Std of triplicate determinations. Means with the same letter in a column are not significantly different (p>0.05).

Table 2. Effects of ethanol extracts of *Citrus grandis* leaf, peel, stem, and stem bark on pathogens.

Concen-tration (mg/ml)	Pathogens	Mean Zone of Inhibition (mm) ± SD				
		Control	Leaf	Peel	Stem	Stem Bark
50	*Staphylococcus aureus*	10.52±0.1^{e}	5.83±0.11^{c}	6.43±0.03^{d}	4.43±0.25^{a}	5.27±0.09^{b}
	Salmonella typhi	9.33±0.01^{e}	3.35±0.07^{b}	4.33±0.02^{d}	2.83±0.04^{a}	3.82±0.03^{c}
	Escherichia coli	9.11±0.33^{d}	3.93±0.04^{b}	5.67±0.10^{c}	3.68±0.12^{b}	2.48±0.02^{a}
	Aspergillus flavus	10.55±0.14^{d}	5.23±0.02^{c}	4.43±0.04^{b}	4.36±0.11^{b}	3.85±0.07^{a}
	Rhizopus stolonifer	11.31±0.78^{c}	6.12±0.26^{b}	5.32±0.023^{b}	4.53±0.33^{a}	4.94±0.30^{a}
100	*Staphylococcus aureus*	13.6±0.26^{e}	7.46±0.06^{c}	8.73±0.04^{d}	5.91±0.04^{a}	6.71±0.22^{b}
	Salmonella typhi	11.67±0.02^{e}	8.78±0.04^{d}	7.34±0.09^{c}	6.53±0.12^{b}	4.88±0.04^{a}
	Escherichia coli	11.33±0.11^{e}	6.63±0.39^{c}	7.22±0.02^{d}	4.82±0.06^{a}	5.57±0.10^{b}
	Aspergillus flavus	14.88±0.03^{c}	9.63±0.25^{b}	10.15±0.42^{b}	7.43±0.11^{a}	6.87±0.05^{a}
	Rhizopus stolonifer	13.29±0.72^{b}	8.46±0.27^{a}	8.52±0.38^{a}	8.16±0.23^{a}	8.34±0.09^{a}
150	*Staphylococcus aureus*	13.72±0.04^{d}	10.41±0.13^{b}	9.77±0.04^{a}	10.82±0.06^{c}	9.63±0.25^{a}
	Salmonella typhi	15.10±0.03^{d}	11.82±0.05^{c}	11.68±0.04^{c}	9.54±0.20^{b}	8.20±0.28^{a}
	Escherichia coli	12.32±0.03^{e}	8.43±0.04^{a}	9.53±0.11^{d}	9.10±0.14^{c}	8.66±0.06^{b}
	Aspergillus flavus	13.01±0.30^{c}	12.82±0.06^{c}	11.77±0.02^{b}	11.38±0.11^{b}	9.63±0.33^{a}
	Rhizopus stolonifer	17.30±0.05^{b}	10.57±0.12^{a}	10.38±0.11^{a}	10.35±0.07^{a}	10.55±0.44^{a}

Values are in Mean± Std of triplicate determinations. Means with the same letter in a column are not significantly different (p>0.05).

Table 3. Effects of ethanol extracts of *Citrus grandis* leaf, peel, stem, and stem bark on pathogens.

Pathogens	MIC (mg/ml)			
	Leaf	Peel	Stem	Stem bark
Staphylococcus aureus	12.5	6.5	12.5	6.5
Salmonella typhi	50.0	25.0	25.0	12.5
Escherichia coli	25.0	6.5	12.5	6.5
Aspergillus flavus	50.0	12.5	25.0	12.5
Rhizopus stolonifer	25.0	6.5	12.5	6.5

Results are in Mean± Std of three different determinations.

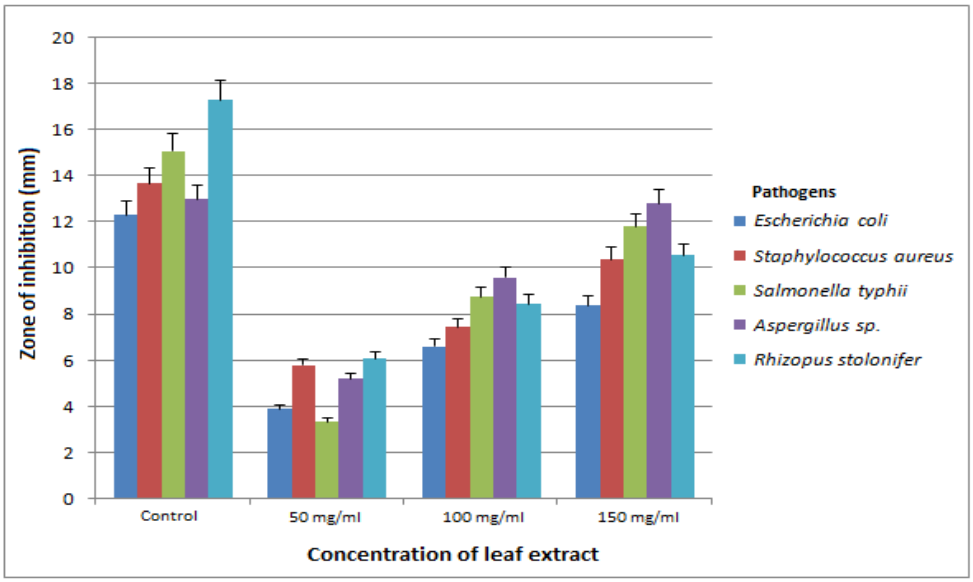

Figure 1: Zone of inhibition (mm) of pathogens by the leaves' extract of *Citrus grandis*.

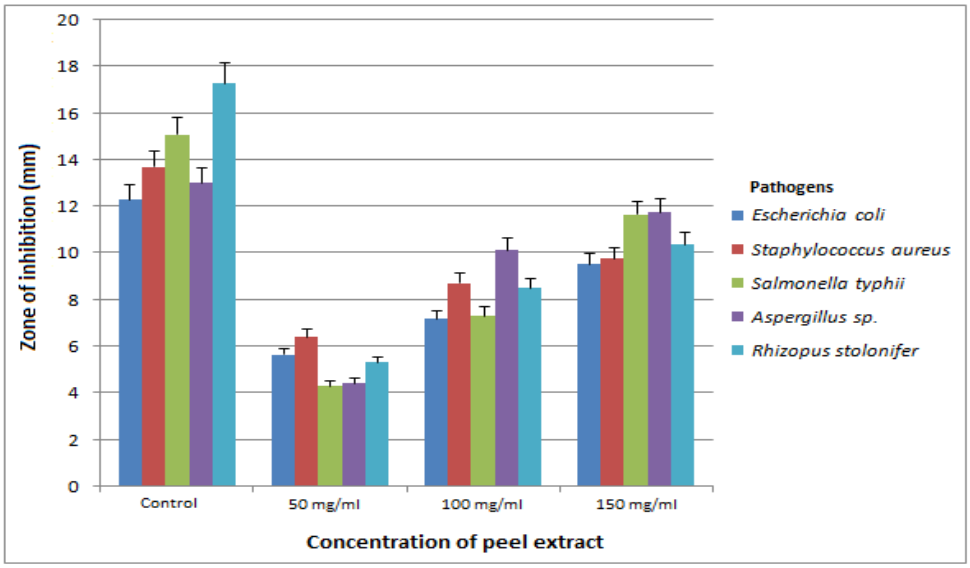

Figure 2: Zone of inhibition (mm) of pathogens by the peels' extract of *Citrus grandis*.

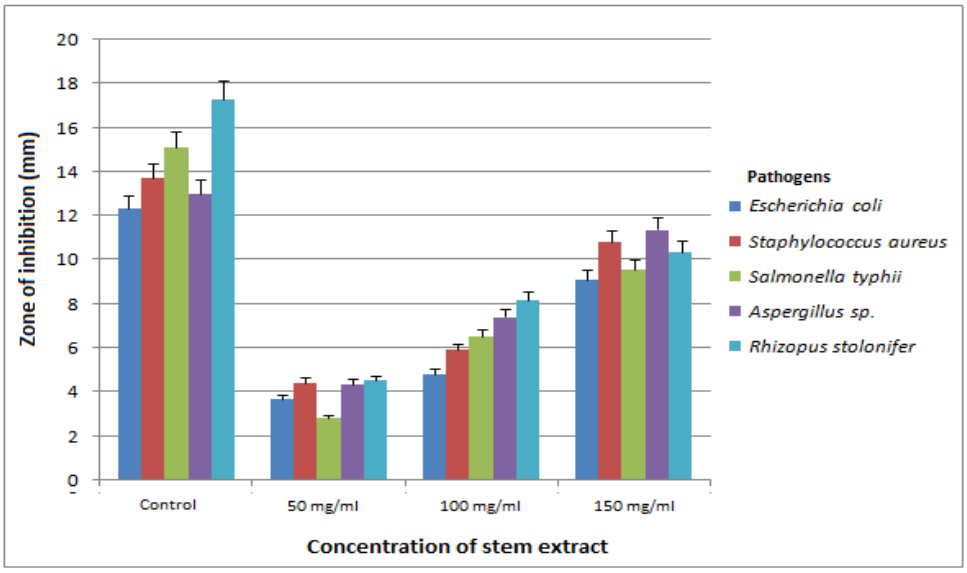

Figure 3: Zone of inhibition (mm) of pathogens by the stem extract of *Citrus grandis*.

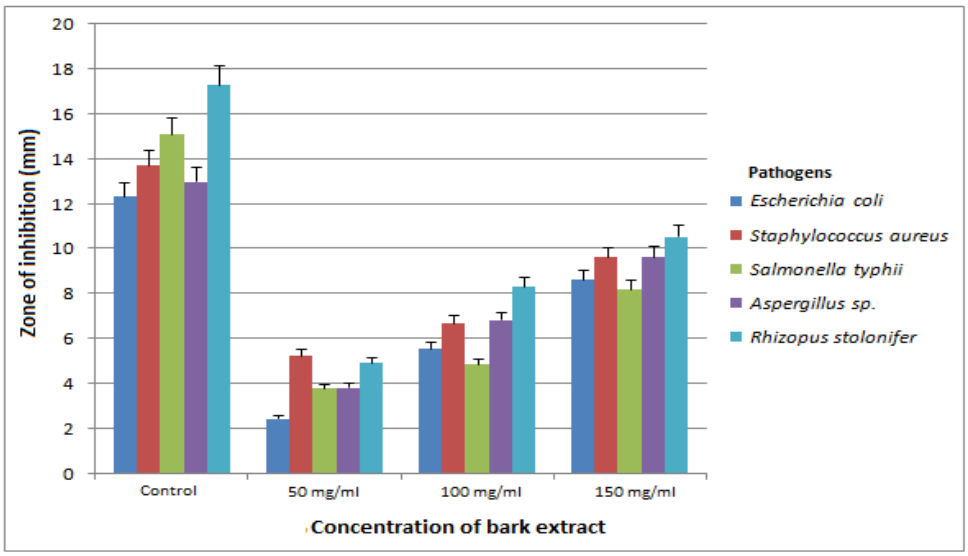

Figure 4: Zone of inhibition (mm) of pathogens by the stem bark extract of *Citrus grandis*.

Discussion

Considerable high concentrations of alkaloids, flavonoids, saponins, and tannins were detected in the leaf, peel, stem, and stem bark of *C. grandis*. These active compounds of plant origin exert pharmacological actions in animal and human systems. Hence, their antimicrobial properties have been reported. Alkaloids isolated from *Stephania glabra* hindered the growth of *S. aureus*, *S. mutans*, *Microsporum gypseum*, *M. canis*, and *Trichophyton rubrum* [14]. Flavonoids isolated from moss species showed antibacterial actions [15]. Saponins extracted from *Vitex doniana* and *Pentaclethra macrophylla* exhibited great effect and a wide range of actions against both gram-positive and gram-negative bacteria [16]. Tannins extracts of *Solanum trilobatum* had observable harmful activities against bacteria [17].

The greatest inhibitory activities of various parts of *C. grandis* extracts against the entire test isolates were observed at the highest concentration (150 mg/ml). The inhibitory actions of the leaf extract (11.82±0.05 mm) and peel extract (11.68±0.04 mm) against *S. typhi* were significant. This implied that a high dose of leaf and peel extracts would have a noticeable effect against *S. typhi*, a causative agent of typhoid fever. In addition, the leaf extract had the highest inhibitory action against *A. flavus* (12.82±0.06 mm). This indicated that it might be used to treat aspergillosis, a disease caused by the fungus *Aspergillus*. The peel extract was the most active against *E. coli* (9.53±0.11 mm) while the stem bark extract (2.48±0.02 mm) was the least effective, followed by the stem extract. The low inhibitory activities of the stem and stem bark extracts might be as a result of low percentages of alkaloids (1.31±0.01 and 1.24±0.09), flavonoids (1.25±0.00 and 1.75±0.12) and tannins (0.79±0.01 and 1.06±0.02), respectively. *Escherichia coli* is usually resistant to stem extracts of plants and this report tallied with the previous studies [18, 19]. The more activeness of the extracts at higher doses could be attributed to the larger concentration of active compounds in the extracts, indicating that the extracts of *C. grandis* would inhibit the growth of these pathogens at high concentrations.

Moreover, the MIC values of leaf, peel, stem, and stem bark extracts of *C. grandis* against the test organisms ranged between 12.5 and 50.0 mg/ml for the leaf, 6.5 and 25.0 mg/ml for the peel, 12.5 and 25.0 mg/ml for the stem, and 6.5 and 12.5 mg/ml for the stem bark, respectively. High MIC values exhibited by the leaf against the entire test organisms presented it as the poorest active extract against them while the peel and stem bark exhibited the greatest activities.

Conclusion

The findings of this study showed that the phytochemicals present in the extracts of *C. grandis* parts are active against all the test pathogens, while the peel and stem bark had the greatest activities. Moreover, *C. grandis* peel and stem bark extracts are of crucial importance in the treatment of infections caused by *S. aureus*, *E. coli*, and *R. stolonifer*. Therefore, the use of extracts of these parts of *C. grandis* in primary health delivery as well as in the development of antibacterial and antifungal drugs is suggested, particularly the peels which are normally discarded.

Conflict of Interest Statement

The authors declared no conflict of interest.

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