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Evaluation of the Antibacterial and Phytochemical Activity of Ripe and Unripe Orange Peels (*Citrus sinensis* and *Citrus aurantium*)

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Abstract **Original Research Article**

Citrus fruits are rich in bioactive compounds such as vitamin C, carotenoids, flavonoids, vitamin B complex and minerals which are highly beneficial to human's health. This study was carried out to evaluate the invitro antibacterial and phytochemical activities of ripe and unripe orange peels extract on selected microorganisms. These microorganisms include *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus spp., Proteus spp.* and *Enterobacter spp*. isolated from urinary tract infections and wound swabs of patients that visited Irrua Specialist Teaching Hospital, (ISTH) Irrua, Edo State. A stock concentration of each successive orange peel extract was obtained using alcohol (95% ethanol + 5% methanol) and 100% methanol (absolute). These extracts along with positive and negative controls were tested for the presence of active phytochemicals. The result showed that the zones of inhibitions were significantly higher with methanol extract than alcohol extract against the test organisms. The zone of inhibition of all extracts against the test pathogenic organisms showed a significant increase as the concentration of the extract increased. The minimum inhibitory concentrations of the methanol and alcohol peel extracts ranged between 6.25-12.5 mg/ml while the minimum bacteriocidal concentrations ranged between 12.5- 25mg/ml. *Invitro* investigations confirmed the antimicrobial potential of *Citrus sinensis* and *Citrus aurantium* peels against pathogenic bacteria. It is therefore recommended that further *in vivo* studies should be done to determine the exact dosage and its effectiveness in practical situations along with toxicity studies.

Keywords: Antibacterial, phytochemical, Citrus, Peels, invitro, microorganisms.

INTRODUCTION

Over the years, fruits are regarded as an important aspect of diet and used extensively for the production of food products such as salads and fruit based drinks [1]. Fruits are equally known to contain some important bioactive ingredients such as vitamin C, carotenoids, flavonoids, minerals and vitamin B complex which play significant role in ameliorating the effect of some disease [2, 1].

Citrus belong to the Rutaceae family and consist of over 140 genera and 1,300 species where Citrus sinensis (sweet orange) and Citrus aurantium (sour orange) is included as one of the important fruits of the genus Citrus [3]. They are well known as one of the world's major food crops that are produced in tropical and subtropical countries such as Brazil, USA, Japan, China, Mexico, Pakistan and countries of the Mediterranean region [4, 2].

The citrus peel is seen as an important by product in the citrus processing industries where large amounts are produced and considered as wastes. However, citrus essential oil is one of the by-products of citrus where they are used to give flavor to drinks and foods. It is an integral component for the pharmaceutical industry for the preparation of drugs, soaps, perfumes and other cosmetics as well as for home cleaning products [5]. The citrus peels is divided into the epicarp (flavedo) and mesocarp (albedo) where the epicarp is the outermost surface of the peel while the mesocarp is the white, soft inner layer of the peel [6, 7]. The citrus peels contain high quantity of phenolic compounds such as flavonoids and is known to exhibit various antimicrobial and antioxidant activities [5, 8, 9].

There are several reports on the medical properties of citrus where its use in historical medical practice has been reported in various countries such as China, India, Japan and Africa [10-13]. In addition, plant extracts and phytochemicals with antimicrobial activity have been used as therapeutics over the years [14] and known to possess antibacterial activity [15-17].

Considering the various reports on the phytochemical and antibacterial properties of plant extracts, therefore study is aimed at evaluating the antibacterial and phytochemical activities of ripe (Citrus sinensis) and unripe orange peels (Citrus aurantium).

MATERIALS AND METHODS

Collection of Citrus Fruits

The ripe and unripe fruits of Citrus aurantium (bitter orange) and C. sinensis (sweet orange) that are free from insect infestation and other kinds of damages were collected from plant farms in Ekpoma Esan West Local Government Area, Edo state, Nigeria. The specimens were further identified and authenticated by Department of Botany, Ambrose Alli University, Ekpoma.

Extraction

The fruits were washed several times with distilled water and were then peeled off carefully using a sharp knife avoiding any damage of the oil glands. Peels were separated, cut into small pieces, dried under shade and grinded into powder [18]. A known quantity of each peel powder (1g) was added into separate sterile bottle containing 10ml of the various diluents; water (hot and cold), alcohol (95% ethanol + 5% methanol), ether and 100% methanol (absolute), and left for 48 hours with occasional stirring. The content of flask was filtered through sterile Whatman No. 1 filter paper and evaporated to dryness. The condensed peel extracts were used for determining antimicrobial activity [19].

Phytochemical Analysis

A stock concentration of 1 % (W/ V) of each successive extract obtained using alcohol (95% ethanol + 5% methanol) and 100% methanol (absolute) were prepared using the respective solvents. These extracts along with positive and negative controls were tested for the presence of active phytochemicals such as alkaloids, cardiac glycosides, flavonoids, phlobatannins, reducing sugars, saponins, starch/polysaccharide, steroids, tannins, and terpenoids following standard methods [20, 21]:

Source of Bacterial Isolates

The different test organisms were isolated from urine and wound swab from clinical samples of patient that visited Irrua Specialist Teaching Hospital (ISTH) Irrua, Edo State. They were analyzed in the Medical Diagnostic Laboratory, College of Medical Sciences, Ambrose Alli University, Ekpoma, Edo State within a period of four months (May 2019 - August 2019). The test organisms isolated for the study include; Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Streptococcus species, Pseudomonas aureginosa, Proteus spp and Enterobacter species.

Identification of Test Organisms

All isolates were identified by their colonial appearance on the media, gram staining reaction and biochemical tests [22]. Catalase test was done on Gram positive cocci. Catalase negative Gram positive cocci in chains were identified as Streptococcus species while the catalase positive cocci in clusters were identified as Staphylococcus species. Coagulase test was carried out on all the catalase positive cocci. The coagulase positive organisms were identified as Staphylococcus aureus while the coagulase negative organisms were identified as Staphylococcus albus. For the Gram negative bacilli, overnight broth cultures were made by adding the colonies to sterilized peptone water and incubated for 24 hours at 37^0C and motility test was done. For non-lactose fermenting Gram negative bacilli that are motile, oxidase test was done. For lactose fermenting Gram negative bacilli that are motile indole test was done. For those colonies that were lactose fermenters and oxidase negative, urease test was performed on them [22].

Preparation of Test Organisms

The different organism isolated; Staphylococcus aureus, Klebsiella pneumonia, Streptococcus species, Proteus species, Pseudomonas aeruginosa and Escherichia coli and Enterobacter spp were sub-cultured into peptone water overnight before antibiogram extract testing.

Antimicrobial (Antibiogram) Activity Test

The antibacterial properties of the extracts was tested against each isolate comprising of both Gram-positive and Gram-negative organism using disc diffusion method as described by Kirby-Baur [23].

Sensitivity Testing of Citrus Peel Extract

The sensitivity testing of the plant extract was determined using the disc diffusion method as described by Kirby-Bauer [23]. The antimicrobial disc were locally prepared by punching out disc 6mm in diameter from a good quality Whatman no 1 filter paper and was sterilized for 15 minutes with autoclave. The bacterial isolates were first inoculated in peptone water and incubated for 8 hours and sub-cultured on nutrient agar, excess broth was tilled off and allowed to dry. Using a sterile forceps, the prepared sterile disc was picked and impregnated with the various extracts in 1mg/ml and the disc placed on the inoculated agar plate equidistant from each other alongside with a locally prepared antibiotic disc (Gentamycin) as positive control and diluents as negative control. It was incubated at 37^0C for 24 hours in an incubator and inhibition zone (IZ) was measured by using ruler calibrated in millimeters.

Minimum Inhibitory Concentration (MIC)

The tube dilution method described by Cowan and Steel [24] was used. The minimum inhibitory concentration gave the lowest concentration of the aqueous extract that can inhibit the growth of bacteria isolates. 1ml prepared nutrient broth was dropped into the test tube 2 to 10, 1ml of the extract was added to tube 1 and 2. Serial dilutions were made resulting to decreasing concentration of the aqueous extract. The extract in tube 3 was diluted until tube 9 from which 1ml was discarded. 1ml of the test bacteria was added to all the tubes from tube 2 to tube 10. Tube1 which contain nutrient broth and organism serves as control tube [25]. The entire procedure was done for the isolates that were susceptible to the extracts with zone of inhibition above. The tubes were thoroughly mixed and incubated at 37^0C for 24 hours after which they were examined for visible growth which is seen as turbidity. The MICs reported as the lowest concentration of the plants extracts that prevent visible growth [22].

Minimum Bactericidal Concentration (MBC)

The minimum bactericidal of Citrus peel extract was determined with little modification, by obtaining samples in the MIC assay and Sub-cultured onto freshly prepared nutrient agar medium and incubated at 37^0c for 24 hours. The MBC was taken as the lowest concentration of the extract that did not show any visible bacterial growth on the surface of the agar plate [22].

Antimicrobial Activity Test

The antimicrobial properties of the extracts were tested against each isolate comprising of both gram-positive and gram-negative organism using disc diffusion test (Kirby-Bauer) method.

RESULTS

The results showed that the hot and cold aqueous extracts of Citrus sinensis and Citrus aurantium showed no antimicrobial activity against any of the test organisms while the methanol and alcohol extract shows significant antimicrobial activities against Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa., Klebsiella pneumoniae, Streptococcus spp., Proteus spp. and Enterobacter spp. Also the ether extract of both fruit samples only showed significant antimicrobial activities against Klebsiella pneumonia (Table-1).

The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of C. sinensis and Citrus aurantium peel extracts (Methanol and alcohol) was shown to be between 6.25 – 12.5mg/ml and 12.5 - 25mg/ml respectively (Tables 2 and 3).

The phytochemical analysis of the peel extracts of C. sinensis and C. aurantium revealed the presence of alkaloids, reducing sugars, steroids and terpenoids amongst others (Table-4).

Table-1: Antimicrobial activities of Citrus sinensis and Citrus aurantium extracts against the test organisms

Fruit sample		Sample Extracts	Zones of inhibition (mm) against test organisms						
			Escherichia coli	Staphylococcus aureus	Pseudomonas aeruginosa	Klebsiella pneumonia	Streptococcus spp.	Proteus spp.	Enterobacter spp.
Citrus sinensis	Ripe	Methanol	13	0	15	15	12	17	16
		Alcohol	14	0	14	0	12	11	13
		Ether	0	0	0	13	0	0	0
		Cold water	0	0	0	0	0	0	0
		Hot water	0	0	0	0	0	0	0
	Unripe	Methanol	0	0	14	11	9	12	14
		Alcohol	17	0	17	0	11	12	10
		Ether	0	0	0	0	0	0	0
		Cold water	0	0	0	0	0	0	0
		Hot water	0	0	0	0	0	0	0
Citrus aurantium	Ripe	Methanol	0	0	0	14	9	11	15
		Alcohol	0	17	10	10	0	10	0
		Ether	0	0	0	0	0	0	0
		Cold water	0	0	0	0	0	0	0
		Hot water	0	0	0	0	0	0	0
	Unripe	Methanol	0	0	0	0	0	0	0
		Alcohol	0	0	0	0	0	0	0
		Ether	0	0	0	13	0	0	0
		Cold water	0	0	0	0	0	0	0
		Hot water	0	0	0	0	0	0	0
Control	Gentamicin		23	18	20	23	25	17	20

Table-2: The Minimum Inhibitory Concentration (MIC) of Citrus sinensis and Citrus aurantium extracts (methanol and alcohol) against test organisms

Test organism	Extracts		Concentration of extracts (mg/ml)						
			100	50	25	12.5	6.25	3.125	MIC (mg/ml)
Escherichia coli	Alcohol	Ripe C. sinensis	-	-	-	-	-	+	6.25
		Unripe C. sinensis	-	-	-	-	-	+	6.25
Staphylococcus aureus	Alcohol	Ripe C. aurantium	-	-	-	-	-	+	6.25
Pseudomonas aeruginosa	Alcohol	Unripe C. sinensis	-	-	-	-	-	+	6.25
		Ripe C. sinensis	-	-	-	-	+	+	12.5
		Ripe C. aurantium	-	-	-	-	+	+	12.5
Klebsiella pneumonia	Methanol	Ripe C. aurantium	-	-	-	-	-	+	6.25
		Ripe C. sinensis	-	-	-	-	-	+	6.25
Streptococcus spp.	Alcohol	Ripe C. sinensis	-	-	-	-	+	+	12.5
		Unripe C. sinensis	-	-	-	-	+	+	12.5
Proteus spp.	Methanol	Ripe C. sinensis	-	-	-	-	+	+	12.5
		Unripe C. sinensis	-	-	-	-	-	+	6.25
		Ripe C. aurantium	-	-	-	-	+	+	12.5
Enterobacter spp.	Methanol	Ripe C. sinensis	-	-	-	-	-	+	6.25
		Unripe C. sinensis	-	-	-	-	-	+	6.25
		Ripe C. aurantium	-	-	-	-	+	+	12.5

Key: - = No turbidity, + = Turbidity

Table-3: The Minimum Bacteriocidal Concentration (MBC) of Citrus Sinensis and Citrus aurantium extracts (methanol and alcohol) against test organisms

Test organism	Extracts		Concentration of extracts (mg/ml)						
			100	50	25	12.5	6.25	3.125	MBC (mg/ml)
Escherichia coli	Alcohol	Ripe C. sinensis	-	-	-	+	+	+	25
		Unripe C. sinensis	-	-	-	-	+	+	12.5
Staphylococcus aureus	Alcohol	Ripe C. aurantium	-	-	-	-	+	+	12.5
Pseudomonas aeruginosa	Alcohol	Unripe C. sinensis	-	-	-	-	+	+	12.5
		Ripe C. sinensis	-	-	-	+	+	+	25
		Ripe C. aurantium	-	-	-	+	+	+	25
Klebsiella pneumonia	Methanol	Ripe C. aurantium	-	-	-	+	+	+	25
		Ripe C. sinensis	-	-	-	+	+	+	25
Streptococcus spp.	Alcohol	Ripe C. sinensis	-	-	-	+	+	+	25
		Unripe C. sinensis	-	-	-	+	+	+	25
Proteus spp.	Methanol	Ripe C. sinensis	-	-	-	+	+	+	25
		Unripe C. sinensis	-	-	-	+	+	+	25
		Ripe C. aurantium	-	-	-	+	+	+	25
Enterobacter spp.	Methanol	Ripe C. sinensis	-	-	-	-	+	+	12.5
		Unripe C. sinensis	-	-	-	-	+	+	12.5
		Ripe C. aurantium	-	-	-	+	+	+	25

Key: - = No Growth, + = Significant Growth.

Table-4: The Phytochemical Constituents Present in extracts of ripe and unripe Citrus sinensis and Citrus aurantium

Phytochemical constituent	Citrus sinensis				Citrus aurantium			
	Ripe		Unripe		Ripe		Unripe	
	Alc	Meth	Alc	Meth	Alc	Meth	Alc	Meth
Alkaloids	+	+	+	+	+	++	+	+
Reducing sugar	++	++	++	++	++	++	++	++
Phlobatannin	-	-	-	-	-	-	-	-
Saponins	-	-	-	+	-	+	-	-
Steroid	++	++	++	++	++	++	++	++
Starch	+	+	+	+	-	-	+	+
Flavonoids	-	+	+	++	+	+	-	+
Terpenoids	+	+	+	+	++	++	+	+
Hydrolysable tannin	+	+	+	-	+	+	+	+
Condensed tannin	+	+	+	+	+	+	+	+
Cardiac glycoside	+++	++	+++	++	++	++	++	++

Key: Alc = Alcohol, Meth = Methanol, - = Absent, + = Fairly Present, ++ = Moderately Present, +++ = Highly Present.

DISCUSSIONS

It is without doubts that there have been various reports on the medicinal importance of plants, but however there is still need to standardize these medicinal plants according to the modern parameters to ensure their activity and efficacy.

In this study, the methanol and alcohol extracts of Citrus sinensis and Citrus aurantium showed greater antibacterial activity as compared to the water extracts. Also, ether extracts of both fruit samples only showed significant antimicrobial activities against Klebsiella pneumonia, Staphylococcus aureus which were found to be resistant to all extracts except the alcohol ripe Citrus aurantium extracts. Moreover, minimum inhibitory concentrations of the alcohol extracts were lesser than the methanol extracts. These observations are line with the studies of Ellof [26] and Nagarajappa et al., [27]. Nair et al., [28] and Nisha et al., [29] also reported better antibacterial activity with orange peel extract prepared in organic solvent.

The zones of inhibitions were significantly higher with Methanol extract than with Alcohol extract against the test organisms. However, Nisha et al., [29] reported that the potency of citrus fruit peel is enhanced by the type of solvent used where he indicated that there are some active ingredients in orange peel which have high antimicrobial effect but which would not be released except when orange fruit peel is used in conjunction with a particular solvent. The zones of inhibition of all extracts against the test organisms increased significantly as the concentration of the extract increased. This is in agreement with the studies of Vivek et al., [30] and Lawal et al., [31] where they reported similar trend of the result.

The minimum inhibitory concentrations of Citrus sinensis and Citrus aurantium methanol and alcohol extracts tested ranges between 6.25-12.5 mg/mL, while the minimum bacteriocidal concentration ranges between 12.5 – 25mg/ml. The MIC values obtained are much lower than that obtained by Lawal et al., [31] against Salmonella typhi, Salmonella paratyphi and Aeromonas hydrophila. The difference in findings may be due to the differences in the type of isolates tested. In this study, none of the organisms were sensitive to the cold and hot water extracts for both the Citrus sinensis and Citrus aurantium. Klebsiella pneumonia was the only organism sensitive to Citrus sinensis ether extract and Staphylococcus aureus was only sensitive to Citrus aurantium alcohol extract in the study. E. coli was only sensitive to ripe Citrus sinensis methanol and alcohol extract and unripe Citrus sinensis alcohol extract in the study. Pseudomonas aeruginosa was only sensitive to ripe Citrus sinensis methanol and alcohol extract, unripe Citrus sinensis methanol and alcohol extract and Citrus aurantium alcohol extract. Enterobacter spp. was only sensitive to Citrus sinensisripe methanol and alcohol extract, Citrus sinensis unripe methanol and alcohol extract and Citrus aurantium methanol extract.

These antimicrobial potencies of the citrus plants could be attributed to the presence of tannins, saponins, phenolic compounds, essential oils and flavonoids [32]. Tannin as observed in Citrus sinensis and Citrus aurantium extract have been found to form irreversible complexes with proline rich protein [33] resulting in the inhibition of cell protein synthesis. Parekh and Chanda [34] reported that tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues. Another secondary metabolite observed in the alcohol extract was alkaloid. The biological property of alkaloids is to exhibit toxicity against cells of foreign organisms which have been employed in the reduction and elimination of human cancer cell lines [35]. Just et al., [36] revealed the inhibitory effect of saponins on inflamed cells and was found to be present in the extracts of Citrus sinenesis and Citrus aurantium peel. Flavonoids was reported to be present in the citrus extracts and known to possess biological activities like anti-inflammatory, antimicrobial, anti-angionic, analgesic, cytostatic, antioxidant properties and anti-allergic functions [37].

CONCLUSION

The results of this study gave substantial evidence that the extracts were active against Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus spp., Proteus spp. and Enterobacter spp. The minimum inhibitory concentrations of the methanol and alcohol peel extracts ranged between 6.25-12.5 mg/ml while the minimum bacteriocidal concentrations ranged between 12.5-25mg/ml. The phytochemical analysis revealed the presence of alkaloids, tannins, reducing sugars, steroids, flavonoids and terpenoids.

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