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PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OF ORANGE (*Citrus sinensis*) PEEL

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ABSTRACT
This research work was carried out to evaluate the antibacterial activity and phytochemical analysis of *Citrus sinensis*, peels purchased from Dutsin-Ma Wednesday market in Dutsinma town, Katsina state, Nigeria. Thereafter shade-dried at room temperature, reduced to powder with electric blender and powder dried in an oven at 40^0C for 24 hours afterwards, water and methanol were used as solvents for the extraction of the active components using soxhlet extraction technique. Thereafter, sensitivity testing of each of *Staphylococcus aureus* and *E. coli* were carried out using agar diffusion method, while minimum inhibitory concentration of the extracts were determined for each of the isolate at varying concentrations of 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml Aqueous and methanol extract gave a yield of 19 and 15% respectively. The zone of inhibition for citrus peel extract using soxhlet extraction against *S. aureus* at concentration of 500mg/ml was 16mm; while that of *E. coli* was 10mm. Results of the phytochemical analysis of methanolic extract of *C. sinensis* revealed the presence of secondary metabolites such as tannin, flavonoids, reducing sugar, alkaloids, cardiac glycoside as well as the aqueous extract which showed the same thing. For the soxchlet extract it showed steroids, saponin, tannin, flavonoids, reducing sugar and alkaloids.

Keywords: Antimicrobial, Extracts, Phytochemical Medicinal Plants, *Citrus sinensis*,

INTRODUCTION

For a long period of time, plants has been a valuable source of natural products for maintaining human health, especially in the last decade, nowadays the use of phytochemicals for pharmaceutical purpose has gradually increased in many countries. The use of crude extracts of plants parts and phytochemicals of known antimicrobial properties can be of great significance in the therapeutic treatment of illness (Saadi *et al*., 2003). In recent years, a number of studies have been conducted in various countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to the secondary metabolites synthesized by the plants. These products are known by their active substances like, phenolic compounds which are part of the essential oils (Webber *et al*., 2003).

C. sinensis is an orange fruit basically and its shape is round its tree has a length of 9-10 m. Leaves of these trees are in oval shape their barks appear to be green or brown in color which is quite smooth. Leaves have a size of 4-10 cm if its length is taken in to account. The leaves of this tree are green. Leaves have smooth texture with a smell resemblance to the sweet orange. The flower of this tree consists of mainly five petals which smell same as saccharine (Webber *et al*., 2003). *C. sinensis* has seeds in between the parts where juices are present. The seeds are green or cream in color. The fruit's flesh is mostly made of the orange sweet juicy part. The peel has orange color (valiant *et al*., 2004). The endocarp is the palatable portion, partitioned into 10-14 sections segregated by thin septa, containing up to 8 seeds/septa, but it was appeared regularly with one. Each segment consists of juice vesicles ("pulp"), with long stalks attached to the juice containing outer wall (valiant *et al*., 2004). Citrus fruit extracts and citrus flavonoids exhibit a wide range of promising biological properties due to their phenolic profile and antioxidant properties (Middleton and Kandaswami, 1994). They have been seen to have wide range of antimicrobial activity on pathogenic microorganism. Citrus is the largest fruit crop in the world (100 million cubic tons per year) and the orange account for 60% (Oreopoulou, and Tzia, 2006). The remaining orange peel account for approximately 45% of the total bulk (Yeoh *et al*., 2008). Consequently, significant amounts of orange peel are available as a by-product. The orange peel, if treated as waste materials, may create environmental problems, particularly water pollution, due to the presence of biomaterials such as essential oil (Ferhat, 2008), pectin (Yeoh *et al*., 2008; Berna *et al* ., 2000) and sugar. This problem could be turned into an asset, if potentially marketable active principles such as essential oil could be extracted from the peel. After extraction, the peel could be a high protein stock feed in dry form, increasing the potential return for the orange juice industry and reducing the pollution (Yeoh *et al*., 2008).

The orange peels are usually considered as waste materials, which may create environmental problems for local

communities because of the presence of biomaterials in orange peel. Every ton of food waste means *4.5* ton of CO2 emissions. (Adamu *et al*., 2006).There is a great need for development of new and environmental friendly design processing techniques which could be turned into an asset. Also, the development of drug resistance by some microbial strain to commercial antibiotics has posed concerns to scientist which is a serious problem.

The problem of environmental pollution also can be reduced considerably, though there are several reports on antioxidant and antibacterial effect of juice and edible parts, there are meager literature on the wastes of citrus fruit besides this, the development of bacterial resistance to currently available antibiotics is still a threat to contend with, this necessitated the search for new antibacterial agent. Therefore, this work was done to determine the minimum inhibitory concentration and minimum bactericidal concentration of *Citrus sinensis* waste on bacterial isolates and to compare the antibacterial activity of the citrus waste against a standard antibiotics.

MATERIALS AND METHOD

Plant materials

Citrus sinensis, was purchased from wednesday market in Dutsinma Town, Katsina State, Nigeria. The peels were diced to smaller pieces after which the peels were shade-dried at room temperature (30-35ºC). 100g of peels of oranges were coarsely powdered using a mortar and pestle and were further reduced to powder using an electric blender. The powder was dried in an oven at 40°C for 24 h.

Extraction Procedure

The dried and powdered plant materials (15 g) were extracted successively with 200 ml of each solvent separately by using soxhlet extractor for 5h (Lin *et al*., 1999). The solvents used for the study was ethanol and water. The extracts were filtered and then concentrated to dryness using a steam bath at 37ºC. Yield of the extract obtained was calculated as

$$\text{Yield \%} = \frac{weight\,of\,extract\,recovered}{weight\,of\,dried\,powder} \times 100$$

Each extract were transferred to glass vials and kept at 4º C before use. The extracts were dissolved in 25% aqueous dimethyl sulfoxide (DMSO) to produce a stock solution of 100 mg/ml

Aqueous Extraction

The method of Adams *et al*. (2006) was adopted for extraction with little modification, 15g of the powdered plant were soaked separately in 200 ml of distilled water at ambient temperature for 24 hour at 130 rpm (revolution per minute). The extract was then filtered using Whatman filter paper No 1 .Each extracts were transferred to glass vials and kept at 4 ºC before use.

Preparation of media

The media was prepared according to manufacturer's specifications. Mueller Hinton agar was prepared by weighing 39g of the powdered agar into 1000mls of distilled water in a clean conical flask. It was swirled until it became a mixture. It was then covered with a foil and was autoclaved at 121ºC, 115 atmospheric pressure for 15 minutes. The medium was cooled at 47ºC and 20ml of the molten medium was poured into a sterile glass petri dish and allowed to solidify.

Microorganisms

Clinical isolates including *Staphylococcus aureus* and *Escherichia coli* were obtained from the stock culture at New Biology Laboratory, Faculty of Science, Federal University Dutsinma, Katsina state, Nigeria. The identity of the test organisms were confirmed by biochemical and morphological characteristics were confirmed by standard methods (Cheesbrough, 2000). The test organism was maintained at 37ºC on nutrient agar slant and sub-cultured before use.

Antimicrobial Activity

Overnight cultures of the Gram positive strains *S. aureus*, and the Gram negative strains *E. coli were* prepared on nutrient broth plates. All bacterial isolates were suspended in saline to a turbidity equivalent to 0.5 McFarland (1.5 x 108 CFU/ml) and a sterile swab stick was dipped into the test tube containing the organisms and it was used to seed the organism on the solidified Nutrient agar(MHA, pH 7.3 ± 0.1, Difco).Then 6 mm wells were prepared. In these wells 3.125ug, 6.125 ug, 12.50 ug, 25, 50 ug and 100 ug of the solvent extract of *Citrus sinensis* peel was added and allowed to stand for 30 minutes for pre-diffusion. The plate was incubated overnight at 37ºC for 24hours. After incubation the zones of inhibition were measured and recorded (Bonev, 2008; Yagoub, 2005).

Determination of minimum inhibitory concentration (MIC)

The MIC of the extracts were determined for each of the isolate at varying concentrations of 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25mg/ml as described by (Sanchez *et al*., 2005 and Musa *et al*., 2018). To obtain this concentration 1ml of the containing double strength of concentration was placed in test tubes. One milliter (1mL) was added and then a loopful of the test organism previously diluted to 0.5McFarland turbidity stand were introduced into the tubes. All the tubes were incubated at 37ºc for 24 hours after which they were examined for MIC.

Determination of minimum bactericidal concentration (MBC)

From the test tubes that show no visible growth in MIC determination, a loopful of broth was collected from the test tubes, which did not show any visible sign of growth and inoculated on sterile nutrient agar by streaking. Nutrient agar

plates were streaked with the test organisms only to serve as control. The plates were then incubated at 37ºC for 24 h. After incubation the concentration at which no visible growth was seen was noted as the minimum bactericidal concentration.

Control experiment using standard antibiotics and dimethylsulphoxide (DMSO)

Standard antibiotics was used as the positive control in order to compare the diameter of zone of clearance from the extracts it was carried out aseptically (Oyagede *et al*., 1993). This is to ensure the prescription of either antibiotics or plant herbs for antibacterial activities. Antibiotics (280mg) bottle with stock solution 80mg/2ml was used by diluting 1ml in 19mls of distilled water that is, 1:20 dilution (1+19mls) giving a final concentration of 2mg/ml. Dimethlysulphoxide (DMSO) was used as a negative control, 50% DMSO was placed on the inoculated culture medium along the extract.

Phytochemical Screening

Tannins Test

One gram (1 g) of the extract was dissolved in 25 ml of distilled water and filtered, 2 to 3 drops of 10% of FeCl3 was added to 3 ml of the filtrate. The production of a blackish-blue or blackish-green colouration was indicative of tannins. To another conical flask, 3 ml of the filtrate was added 2 ml of bromine water. A precipitate was taken as positive for tannins.

Flavonoids Test

About 0.4 g of the extract was dissolved in 3 ml of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of three (3) drops of concentrated HCL. The occurrence of a red or orange colouration was indicative of the flavonoids.

Test for Alkaloids

About 0.5g of the extract was dissolved in 5ml of 1% HCL and was in boiling water bath. One milliliter of filtrate was dropped in Mayers reagent, Precipitate observed indicated the presence of alkaloids

Test for saponin

Precisely 0.5g/ml of each sample was stirred with water in a test-tube Frothingpersiston warming was taken asevidence for the presence of Saponin.

RESULTS

Table 1 below present's result of the percentage yield of the crude extractaqueous crude extract gave the highest yield (19%) while methanol extract produced the lowest (14%). Table 2 presents results of phytochemicals analysis with alkaloids, flavonoids, tannins, cardiac glycosides

Table 1. Percentage yield of the crude extract *Citrus sinensis*

Plant species	Extract type	Weight of pulverized sample (g)	Weight of Extract (g)	Percentage yield of extract (%)
Citrus sinensis	Aqueous	25	4.75	19
	Methanol	25	3.6	14
	Methanol (soxchlet extract)	20	3.0	15

Table 2. Preliminary phytochemical analysis of *Critussinensis*

Extract	Flavonod	Saponin	Tanin	Cardiac Glycoside	Alkaloid	Steroids
Aqueous	+	-	+ +	+ +	+	-
Methanol	+ +	-	+ +	+ +	+	+
Soxchlet	+	+	+	+ +	-	+ +

\+ = Present
++ = Markedly present
--=Absent

Table 3 presents the diameter of inhibition of the various extracts on selected bacteria specieswith the negative control Ciproflaxin having the highest inhibition in diameter on both *E.coli* and *S. aureus* and aqueous extract. Table 4 and 5 presents results of the MIC and MBC of the powdered orange peel respectively, while table 5 present results of the MBC of the powered orange peels with no bacteriacidal concentration in aqueous extract, while soxhlet extraction provided 100mm in the two test organisms.

Table 3: Diameter of inhibition of various extracts as well as controls in millimeter on selected bacteria species

Bacteria	**Aqueousµl**			**Methanolµl**			**Soxchlet µl**			**Ciproflaxinµl**	**DMSO**
Concentration	**500**	**300**	**100**	**500**	**300**	**100**	**300**	**200**	**100**	**100**	
Escherichia coli	10	7	5	19	12	10	16	10	7	27	0
Staphylococcus aureus	16	13	6	22	19	10	20	16	10	28	0

Table 4: MIC values of *Citrus sinensis* powdered peel extract in millimeter on selected bacteria species.

	Concentration	**In ml**	
Bacteria	Aqueous	Methanol	Soxchlet
Escherichia coli	50	30	30
Staphylococcus aureus	50	12.5	30

Table 5: MBC values of *Citrus sinensis* extract on different bacteria species

	Concentration	**In ml**	
Bacteria	Aqueous	Methanol	Soxchlet
Escherichia coli	__	__	100
Staphylococcus aureus	__	100	100

DISCUSSION

Three different extract of citrus peel were tested for their antibacterial activities against two isolates of Gram-negative and Gram-positive bacteria. Citrus peel extract showed antibacterial activity against all the test organisms. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body (Akinmoladun *et al.*, 2007). The preliminary phytochemical investigation revealed the presence of various constituents of citrus peel as shown in table 2. Different solvent showed different class of phytochemicals. flavonoids, saponins, alkaloids and cardiac glycosides were detected while steroids were not detected in both the citrus peels extracted by maceration method. These constituents may be responsible for the antibacterial activity but it is difficult to correlate their action to a specific phytochemical.

The methanol extracts exhibited inhibitory activities that were found to be a little higher than aqueous extract on all the test organisms. Although with a slight difference it can therefore be inferred that the active principles of the plant may be more soluble in ethanol as employed in ethnomedicine (Sofowora, 1984).Methanol extract showed a maximum zone of inhibition against *E. coli* and *S. aureus* whereas the aqueous extract did not show such high antibacterial activity. Aqueous extract showed far less antibacterial activity when compared to other solvents.*S. aureus* was more susceptible to the extract than *E.coli*, thus it indicates that the extract may have diverse antibacterial agent that has different modes of action or the bacteria may have a special metabolism to overcome or adapt to its activity.

The zone of inhibition for citrus peel extract by using soxhlet extraction against *S. aureus* at concentration of 500mg/ml was 16mm; *E. coli* was 10mm respectively. This is value is similar to that ofGulay *et al* (2009). Amandeep *et al* (2009) showed 14mm and 12mm against *S. aureus* and *E. coli* respectively. Ekwenye and Edeha (2010) reported antibacterial activity of the ethanol and aqueous extract of *Citrus sinensis* leaf against the test organisms taken in their study (*S.aureus*: aqueous extract 7mm, methanol extract 3mm; *E.coli*: aqueous extract 1mm, methanol extract 2mm). Similarly, this study shows that *Citrus sinensis* peel extract (*E.coli*: aqueous extract -7mm, methanol extract 17mm; *S.aureus*: aqueous extract 10mm, methanol extract 19mm) has high degree of antibacterial activity as compared to the leaf extract.

MIC and MBC of different solvent extract of citrus peel are shown in table 4 and table 5 respectively the extract showed significant activity. A low level of activity at a low extract concentration may suggest that the concentrations of the active constituent in the extracts are too low for any appreciable antibacterial activity (Ashebir and Ashenati, 1999). MIC by broth dilution showed positive results compared to disc diffusion method as there maybe problem with the diffusion of the biological component into the agar or the hydrocarbon component may remain at the surface or evaporate. [Griffin *et al*. (2000], the positive control (ciproflaxin) had the widest zones of inhibitionon all the organisms where the Dimethyl sulphoxide (DMSO) (negative control) had no effect on all the test organisms.

CONCLUSION

Citrus sinensis peels extracts obtained from different solvents shows antibacterial activity against the test organisms (*E. coli and S. aureus)*, with the orange peels providing similar MIC and MBC to the negative control Dimethyl sulphoxide (DMSO) phytochemical analysis carried out revealed the presence of flavonoids, saponins, alkaloids and cardiac glycosides while steroids were not detected,.

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