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Original Research Article

Phytochemical Screening and Antimicrobial Activities of Some *Citrus Spp.* Peel Extracts

Okon, Okon Godwin[1], Andrew, Nsikak Abraham[2], Udosen, Imo Joseph[2], Atoyebi, Babatunde[2], Asuquo, Nsini[2], Ezeh, Peace Amarachukwu[3]

[1]Department of Botany and Ecological Studies,
[2]Department of Microbiology, University of Uyo, Akwa Ibom - Nigeria.
[3]Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria - Nigeria.

Corresponding author: Okon, Okon Godwin

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ABSTRACT

Phytochemical constituents' analysis of fruit peel extracts of *Citrus reticulata, Citrus sinensis* and *Citrus limonum* and their antimicrobial activity by testing them against clinically important microorganisms such as: *Staphylococcus aureus, Bacillus subtilis, Shigella* sp., *Salmonella* sp and *Pseudomonas aeruginosa* was carried out. From this study, the fruit peel extracts of these *Citrus spp* revealed that alkaloids was highly present in *C. sinensis,* moderate in *C. limonum* and found in trace concentration in *C. reticulata.* Phenols were moderately present in all the *Citrus spp* analyzed. Cardiac glycosides was absent in *C. sinensis* and *C. reticulata* and in trace concentration in *C. limonum.* The various citrus peels extract had antibacterial effect on all the test isolates. Extracts of *C. limonum* showed the highest antibacterial effect, this was followed by extract of *C. reticulata* while that of *C. sinensis* had the least antibacterial effect. The result of this study supports the fact that extracts of certain tropical plants and crops can serve as an alternative antimicrobial agent as it had inhibitory effect on the test isolates which are commonly associated with human gastrointestinal infections.

Keywords: Antibacterial activity, *Citrus limonum, Citrus reticulata, Citrus sinensis,* peel extract and phytochemical screening.

INTRODUCTION

Certain plants have been known to be a phenomenal source of compounds of medicinal interest used extensively in traditional medicine to combat different kinds of emerging diseases and those already in existence. The antiseptic qualities of aromatic and medicinal plants and their extracts have been recognized since antiquity. [1,2] This is based on the belief that natural products are intrinsically less dangerous and can be obtained at a lower cost. [3] The antimicrobial activities of medicinal plant extracts have been linked to the presence of bioactive compounds which sometimes serve to protect the plants themselves against bacteria, fungi and viral infections as well as exhibiting their antimicrobial properties on these organisms. [4,5] Phytochemicals constitute one of the

most numerous and widely distributed groups of substances in the plant kingdom. [6]

Phytochemicals are certain non-nutritive plant chemicals which have some disease preventive properties. [7] They are not required by the human body for life sustenance, but they offer protection against pathogens. [8] It can stimulate certain enzymes; thereby reduce risk for breast cancer. [7] It may act as an anti-bacterial and hormonal-stimulant component. It may even act as binders which may prevent the adhesion of pathogens to the human cell walls. [9] Phytochemicals are already a part of our diet through vegetables and fruits. [7] Citrus fruits are found to be rich in phytochemical constituents. Citrus fruits, which belong to the family of rutaceae, are one of the main fruit tree crops grown throughout the world. Although sweet orange (*Citrus sinensis*) is the major fruit in this group accounting for about 70% of citrus output. [10] The group includes the species under investigation in this study; tangerine (*Citrus reticulata*), orange (*Citrus sinensis*) and lemon (*Citrus limonum*).

Therefore, the aim of this research is to ascertain the phytochemical constituents of fruit peel extracts of *Citrus reticulata, Citrus sinensis* and *Citrus limonum* and their antimicrobial activity by testing them against clinically important microorganisms such as: *Staphylococcus aureus, Bacillus subtilis, Shigella sp., Salmonella sp* and *Pseudomonas aeruginosa.*

MATERIALS AND METHODS

Collection and Extraction of Fruit extracts

Healthy disease free citrus fruits (*Citrus reticulata* – Tangerine, *Citrus limonum* - lemon and *Citrus sinensis* – Orange) were obtained from local farmers and fruit vendors at Akpan Andem Market in Uyo, Akwa Ibom State and authenticated by a plant taxonomist in the Department of Botany, University of Uyo, then brought to the laboratory for extraction. In the laboratory, the various fruits were washed and their epicarp peeled and chopped into tiny pieces separately. 100 g of each of the citrus peel was packed in a soxhlet apparatus and extracted exhaustively using acetone as organic solvent for 24 hours. Excess solvent was removed from the extraction using a rotator vacuum evaporator. The extractions were stored at 4 ^{o}C in amber colored airtight bottles.

Phytochemical Screening

The experiment was carried out in the Department of Pharmacognosy and Natural Medicine, University of Uyo, Uyo. The phytochemical screening involves the simple chemical test to detect the presence of secondary metabolites. The methods of [11-13] were used for phytochemical screening. The phytochemical test include: tests for saponins, tannins, flavonoids, alkaloids, cardiac glycosides and phenol.

Test for Alkaloids

12 ml of chloroform was stirred with peel extracts separately with a few drops of dilute hydrochloric acid and filtered. The filtrates were divided into four equal portions and tested with various alkaloidal reagents namely Mayer's reagent (cream precipitate), Dragendorff's reagent (orange brown precipitate), Wagner reagent (reddish brown precipitate), and Picric acid (white precipitate). [12,13]

Dragendorff's reagent: Three drops of Dragendorff's reagent were added to first portion of the filtrate and observed for formation of the characteristic orange yellow or brown coloured precipitates.

Wagner's reagent: Three drops of Wagner reagent were added to second portion of the filtrate and observed for formation of the characteristic reddish brown precipitates.

Mayer's reagent: Three drops of Mayer's reagent were added to third portion of the

filtrate and observed for formation of the characteristic cream coloured precipitates.

Test for Saponins

Frothing test: 0.5 g of the extract was weighed and dissolved in 10 ml of sterile distilled water. The test tube was stopped and shaken vigorously for 30 seconds. It was then allowed to stand for half an hour. [12]

Foaming test: 0.5 g of the extract was weighed and stirred in 20 ml of sterile distilled water and boiled in a water bath for 5 minutes and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion. [13]

Sodium bicarbonate test: 0.5 g of the extract was weighed and dissolved in 10 ml of sterile distilled water. 3 drops of sodium bicarbonate solution were added and shaken vigorously for two minutes, then observed for the formation of emulsion. [13]

Test for Flavonoids

Ammonia test: Filter paper strips were dipped in the ethanolic solutions of the extract and ammoniated. The filter paper changed its colour to yellow which indicates the presence of flavonoids. [14]

Shinoda's test: To 1 ml of the each extract, a piece of metallic magnesium was added followed by addition of 2 drops of concentrated hydrochloric acid. A red colour confirmed the presence of flavonoids in the extract. [14]

Sodium hydroxide test: Aqueous sodium hydroxide was added to 1 ml of the extract solution, dilute sulphuric acid was added slowly. A yellow colour which turned cream on addition of dilute sulphuric acid confirmed the presence of flavonoids. [14]

Test for Tannins

Ferric chloride test: About 0.5 g of the extract was stirred with 2 ml of distilled water and filtered. The filtrate was treated with five percent (5 %) ferric chloride reagent. A blue-green precipitation was taken as evidence for the presence of tannins. [15]

Bromine water test: About 0.5 g of the extract solution was dissolved in distilled water in a test-tube. 5 drops of bromine water was added gently. Decolouration of the bromine water confirmed the presence of tannins. [15]

Test for Cardiac Glycosides

Three grammes (3 g) of the extract were hydrolysed with dilute hydrochloric acid for 2 hours in water bath and allowed to cool well on ice for 2 minutes and filtered. The filtrate was subjected to different test for cardiac glycosides: Liebermann-burchard's, Keller-killani, and Salkowski tests. [12,13]

Keller-Killani Test: One millilitre (1 ml) of glacial acetic acid containing traces of $FeCl_3$ and one millilitre (1 ml) of concentrated H_2SO_4 was added to the extract carefully. The formation of brown ring at the interface indicated the presence of cardiac glycosides.

Salkowski test: To another portion of the solution, one millilitre (1 ml) of sulphuric acid was added carefully to form lower layer. A red brown colour at the interface indicated the presence of cardiac glycosides.

Liebermann's test: To another portion of the solution, one millilitre (1 ml) of hydrochloric acid was added carefully to form lower layer. A green colour with a ring formation on top while blue-green colour indicated the cardiac glycosides.

Test for phenols

An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu reagent and 4 ml of sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 minutes at 40 ˚C for colour development. An appearance of blue colour indicates the presence of phenols.

Preparation of Disc for antibacterial activities

Sterile filter papers were perforated and divided into three groups. The first group was treated with extract from *C. reticulata*, the second with extracts from *C. limonum* and the third with extract from *C. sinensis.* The three set were allowed to absorb the extract for one hour. The impregnated prepared discs were then dried in controlled temperature to remove excess of moisture before being used for antibacterial activity. [16]

Source of Microbial Test Organisms

Bacterial isolates used in this study were clinical isolates obtained from the University of Uyo Health Center. They include two Gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) three Gram negative (*Shigella sp. Salmonella sp* and *Pseudomonas aeruginosa*). The morphological, biochemical and fermentative characteristics of the bacterial isolates were confirmed using standard microbiological techniques as described by. [17] The obtained characteristics were compared with those given by. [18,19]

Standardization of Inoculums

The obtained isolates were subcultured into fresh plates of Mueller-Hinton medium and incubated at 37 ^{o}C for 24 hours. Colonies from this plate were suspended into Mueller-Hinton broth and incubated until turbidity matched the 0.5 MacFarland standard.

Screening for Antibacterial activity of the various citrus extracts

The Kirby-Bauer modified disc diffusion technique as recommended by National Committee for Clinical Laboratory Standards (NCCLS) and described by [16] was used to determine the susceptibility of the test organisms to the various citrus peel extracts. Using a sterile swab stick, each for the different isolates, the test isolates were obtained from the standardized broth (turbidity = 0.5 MacFarland) and inoculated by spreading on the surface of a freshly prepared Mueller-Hinton agar. The plates were allowed for 5 minutes then after which the various impregnated paper disc were placed on the surface of the inoculated plates. All the plates were incubated at 37 ^{o}C for 24 hours after then observed for zones of inhibition which was measured in millimeters and interpreted using the by National Committee for Clinical Laboratory Standards (NCCLS).

RESULTS AND DISCUSSION

The results of the phytochemical screening on the fruit peel extracts of *C. reticulata, C. limonum* and *C. sinensis* revealed that they were rich in phytochemical activity, as shown in Table I. Alkaloids was highly present in *C. sinensis,* moderate in *C. limonum* and found in trace concentration in *C. reticulata.* Phenols were moderately present in all the *Citrus spp* analyzed. Cardiac glycosides was absent in *C. sinensis* and *C. reticulata* and in trace concentration in C. limonum. This is in line with the works of [7,10] who reported the presence of these phytochemicals at different concentrations in the peel extracts of the *Citrus spp* under investigation in this study.

Table I: Phytochemical analysis of some *Citrus spp* peel extracts

Phytochemicals	*C. reticulata*	*C. limonum*	*C. sinensis*
Alkaloids	+	++	+++
Saponins	+	-	+
Tannins	+	+	++
Flavonoids	-	++	+
Cardiac glycosides	-	+	-
Phenols	++	++	++

+++ = Highly present, ++ = moderately present, + = Trace Concentration, - = absent.

The result of the morphological, biochemical and fermentative screening of the isolates is given in Table II. The characteristics were in agreement with those given by. [18]

Table II: Biochemical Characteristics and Fermentative Properties of the Test Bacterial Isolates

S/n	Grams stain Gram s stain	Cell shape	Motility	Catalase Catalase	Coagulase	Citrate Citracitratete	Urease	Methyl red Mr	Voges proskauer	Indole	Sugar Fermentation					Probable organism
											Glucose	Sucrose	Maltose	Lactose	Mannose	
1	-	R	+	-	-	+	-	+	-	-	AG	-	A	-	AG	*Salmonella* sp.
2	-	R	+	-	-	+	-	+	-	-	A	A	A	-	AG	*P. aeruginosa*
3	-	R	-	-	-	-	+	-	-	-	AG	-	A	-	-	*Shigella* sp.
4	+	R	+	+	-	+	-	+	+	-	AG	-	AG	A	AG	*Bacillus subtilis*
5	+	S	-	+	+	+	-	-	+	+	AG	AG	AG	AG	AG	*S. aureus*

Key: R - Rod; S - Spherical; + - Positive; – - Negative; A - Acid; AG - Acid and Gas.

As shown on Table III, the various citrus peels extract had antibacterial effect on all the test isolates. Extracts of *C. limonum* showed the highest antibacterial effect, this was followed by extract of *C. reticulata* while that of *C. sinensis* had the least antibacterial effect. The result obtained from this study showed that extract of *C. limonum* had the highest antibacterial activity with the highest inhibitory effect (20mm) of *Salmonella* sp. The extract had equal effect on (15 mm) on *P aeruginosa* and *Shigella* sp. and the least effect was seen on *Bacillus subtilis* with diameter of clear zone of 14 mm. Extract of *C. reticulata* had its highest antibacterial effect on *Shigella* sp (18 mm), this was followed by *P aeruginosa* (14 mm) while *B. subtilis and S. aureus* showed the least sensitivity (12 mm). *C. sinensis* extract which showed the least antibacterial activity in general had its highest sensitivity on *S. aureus* (15 mm), followed by *Shigella* sp. (13 mm) while *P. aeruginosa* and *B. subtilis* had the same level of sensitivity (10 mm). The result of these studies agrees with the results obtained by. [20-22] Figure I and II shows an illustration of the antibacterial effect of the various extracts and inhibitory levels of different *Citrus spp* on the microbial test isolates.

Table III: Antimicrobial Activity of the Various Citrus Extracts

Test Isolates	Zones of Inhibition (mm)		
	Citrusreticulata	Citruslimonum	Citrussinensis
Salmonella sp.	13	20	12
P. aeruginosa	14	15	10
Shigella sp.	18	15	13
Bacillus subtilis	12	14	10
S. aureus	12	16	15

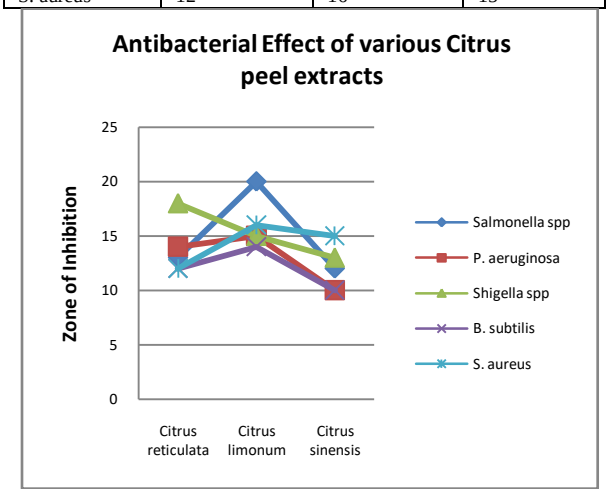

Figure 1: Antibacterial Effect of Various Citrus peel Extracts

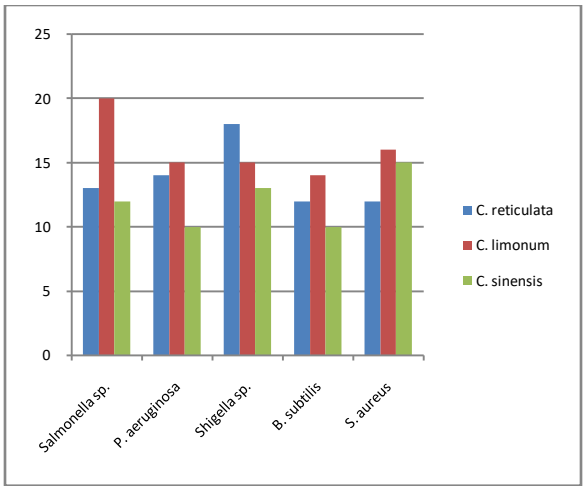

Figure II: Inhibitory levels of different Citrus spp on the microbial test isolates

CONCLUSIONS

This study reveals the antibacterial activity of *Citrus* fruit peel acetone extract. The result of this study supports the fact that extract of certain tropical plants and crops

can serve as an alternative antimicrobial agent as it had inhibitory effect on the test isolates which are commonly associated with human Gastrointestinal infections.

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