INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

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PHYTOCHEMICAL CONSTITUENTS AND ANTIMICROBIAL EFFECT OF UNRIPE EPICARP OF ORANGE FRIUTS (*Citrus sinensis*) AGANIST *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT
This study investigated the antibacterial activity of extracts of unripe epicarp of *Citrus sinensis* against *Escherichia coli* and *Staphylococcus aureus*. The dried and powdered plant materials were extracted using soxhlet extractor whereas paper disc diffusion method was used for antibacterial assay. The crude extract showed a remarkable inhibition against *S. aureus* (21 ± 0.01mm) compared to *E. coli* (14 ± 0.02mm).The acetone fraction exhibited the highest antibacterial activity against *E. coli* (14.13 ± 0.06mm) whereas dichloromethane fraction exhibited the highest antibacterial activity against *S. aureus* (12.00 ± 0.01mm). N-hexane fraction had no antibacterial activity against the tested organisms. The result of the phytochemical screening revealed the presence of tannins, saponins, alkaloids, terpenoids, flavonoids and amino acids. The results of this study suggests that unripe epicarp of *C. sinensis* can be beneficial in developing a novel antibiotics.

KEYWORDS: Antimicrobial activity, phytochemical constituents, acetone, *Citrus sinensis*, *Escherichia coli*.

INTRODUCTION

An orange fruit (*Citrus sinensis*) of the genus Citrus which belongs to the family Rutaceae is among the most important fruit crops grown in all continents of the world with over 80 million tons produced yearly (Mar´ın *et al*., 2007). Citrus fruits are mainly used as dessert, but their peel produces an essential oil which has important economic value worldwide (Bourgon *et al*., 2012). The citrus peel oils are primarily used as flavour by food industries (Kumar *et al*., 2011) and as flavouring agent to mask unpleasant taste in pharmaceutical industries (Bourgou *et al*., 2012).The chemical composition of citrus peels oil revealed that they are rich in nutrients and many phytochemicals (Droby *et al*., 2008; Chutia *et al*., 2009; Espina *et al*., 2011). The citrus peel is a rich source of flavanone and many polymethoxylated flavones, which are rarely found in other plants (Ahmed *et al*., 2006; Dhiman *et al*., 2012). Hamendra and Anand (2007) reported its use as an anti-diabetic. It can also be used as antimicrobial (Caccioni *et al*., 1998), antifungal (Stange Jr *et al*., 1986; Okwu *et al*., 2007), antioxidant (Proteggente *et al*., 2003; Kanaze *et al*., 2008), carminative, insect repellent, larvicidal, antiviral, antimicrobial, uricosuric, anti-yeast, anti-hepatotoxic and anti-mutagenic agent (Han, 1998). Rehman *et al*. (2007) reported that citrus peel essential oil exhibit antifungal, antibacterial, antiviral and anti-parasite properties. Bacterial diseases are widespread and its treatment is largely based on the use of antibiotics. Recently, a number of antibiotics have lost their effectiveness due to the expression of resistant genes (Bakhru, 2001). In addition, antibiotics are sometimes linked to adverse health effects such as hypersensitivity, allergic reactions and immune suppressions (Ahmed and Beg, 2001). Hence, the need for novel antibacterial drugs that is safe for the treatment of infectious diseases. According to Bhardwaj and Laura (2009), plants possess various secondary metabolites that exhibit inhibitory effect against the growth of most pathogens. In earlier studies, antimicrobial activity was reported of *Citrus sinensis* peel extracts using acetone, petroleum ether, ethanol, ethyl alcohol, methanol and water against several microbial species, but no report of unripe epicarp extracts of *Citrus sinensis* using dichloromethane and n-hexane as solvent. This study is therefore aimed at screening the phytochemical composition and antibacterial effect of unripe epicarp extracts of *Citrus sinensis* using acetone, methanol, dichloromethane, n-hexane and water as solvent.

MATERIALS & METHODS

Collection of plant materials:

The unripe orange fruit (*Citrus sinensis*) used in this study were purchased from retailers at Afikpo, Ebonyi State, Nigeria. They were peeled and sundried for 2 weeks. Exactly 20g of the citrus peels were ground to fine powder using mortar and pestle and hand grinder. The powder was transferred into closed container.

Extraction and fractioning of extracts

The dried powdered plant materials (100 g) were extracted with equal volume of petroleum ether and acetone (100 ml) using soxhlet extractor for 5 hours at a temperature not exceeding the boiling point of the Solvent (Lin *et al*., 1999). The obtained extract was filtered through Whatman No. 1 filter paper and the solvent was removed by evaporation using water bath at 45 ◦C to a constant mass and concentration of 40.8g. The resulting crude extract was then stored at 4 ◦C until further analysis. A portion of the crude extracts (10 g) was fractioned by filter column

chromatography over 100 g silica gel 60 (S) (Santos *et al*., 2009), and eluted with approximately 1 L of the solvents n-hexane, dichloromethane, methanol, and acetone, until a clear extract was obtained at the end of the elution. Pump pressure at approximately 5 bar was applied to accelerate the elution of the solvents. Elutes were collected in 1-L Erlenmeyer flasks and each fraction was subjected to evaporation under reduced pressure in a rotary evaporator (Martins *et al*., 2013. Fractions were stored at 4 ◦C until assayed.

Test Microorganism

Clinical isolates of *Escherichia coli* and *Staphylococcus aureus* were collected from the laboratory of Mater Misericordiae hospital Afikpo, Ebonyi State, Nigeria. The isolates were identified and preserved on nutrient agar at 4^0C in a refrigerator.

Antibacterial analysis

The disc used in this study was prepared by punching a Whatman No. 1filter paper using a perforator. They were put into vial bottles and sterilized using hot air oven at 150 ^{0}C for 15 minutes. The stock culture of *E. coli* and *S. aureus* were subcultured in nutrient broth and incubated at 37 ^{0}C for 24 hours. The overnight cultures were suspended in 0.9% saline to achieve a turbidity equivalent to 0.5 McFarland standard (10^8 cfu/ml). Thereafter, 0.1 ml standardized inoculums suspension was swabbed uniformly on Mueller Hington agar. The prepared discs containing the various fractions were carefully and aseptically placed on the inoculated plates using a sterilized forceps. It was kept to incubate for 24 hours at 37 ^{0}C. Standard antibiotic disc containing ciprofloxacin (5µg) was used as control. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone formed around the disc. The assay was performed in triplicate and the mean calculated.

Phytochemical analysis

Phytochemical analyses of the extracts were performed according to the methods described by Sofowora (1994) and Harborne and Harborne (1998).

Test for alkaloids

Exactly 1 ml of 1%HCl was mixed with 2 ml of extract and about 5 drops of Mayor's reagent was added. A creamy or pale yellow precipitate indicated the presence of alkaloiods.

Test for tannins

About three drops of 0.1% ferric chloride was added to 1 ml of extract. A brownish green or a blue black colour indicated the presence of tannins.

Test for saponins

Froth Test: To 1g of extract in a conical flask, 10 ml of sterile distilled water was added and boiled for 5 minutes. The mixture was filtered and 2.5 ml of the filtrate was mixed with 10 ml sterile distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 seconds. It was then allowed to stand for half an hour. The observation of Honeycomb froth indicated the presence of saponins.

Test for flavonoids

Exactly 2 ml filtrate was added to concentrated HCl and magnesium ribbon. Pink-tomato red colour indicated the presence of flavonoids.

Test for terpenoids

This was carried out by Salkowski test: 5 ml of each extract was mixed in 2 ml of chloroform and 3 ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colour at the interface indicated the presence of terpenoids.

Test for amino acids

Few drops of Ninhydrin reagent was added to 1 ml of extract. The development of purple colour shows the presence of amino acids.

RESULTS

The result of the antibacterial activity obtained for the crude petroleum/acetone extract and fractions of acetone, dichloromethane, methanol, and n-hexane of unripe epicarp of *Citrus sinensis* with commercially available antibiotics is presented in Table 1. The crude extract showed a remarkable inhibition against *S. aureus* (21 ± 0.01mm) compared to *E. coli* (14 ± 0.02mm). The acetone fraction showed the highest antibacterial activity against *E. coli* (14.13 ± 0.06mm) followed by the methanol fraction (13.02 ± 0.02mm), and dichloromethane fraction (9.05 ± 0.01mm). Dichloromethane fraction demonstrated the highest antibacterial activity against *S. aureus* (12.00 ± 0.01mm), followed by acetone (11.01 ± 0.01mm) and methanol fraction (10.05 ± 0.01mm) was the least. The fraction of n-hexane did not inhibit the tested organisms.

TABLE 1: Antimicrobial activity of unripe epicarp extracts of *Citrus sinensis*

Microorganisms	Mean diameter of inhibition zone (mm)					Standard antibiotic (ciprofloxacin 5 µg)
	Crude extract	Acetone fraction	Dichloro-methane fraction	Methanol fraction	n-Hexane fraction	
Escherichia coli	14 ± 0.02	14.13±0.06	9.05±0.01	13.02±0.02	-	28
Staphylococcus aureus	21 ± 0.01	11.01±0.01	12.00±0.01	10.05±0.01	-	20

Observations are expressed as mean ± standard deviation, (-) represents no inhibition
Disc concentration= 34mg/ml

The phytochemical screening result presented in Table 2 revealed the presence of tannins, saponins, alkaloids, terpenoids, flavonoids and amino acids with the different solvents.

TABLE 2: Phytochemical constituents of unripe epicarp extracts of *Citrus sinensis*

Phytochemicals	Solvents used for fractioning				
	Acetone	Dichloro-methane	Methanol	n-Hexane	Crude extract
Tannins	+	-	+	-	+
Saponins	+	+	+	-	+
Alkaloids	-	+	+	+	+
Terpenoids	+	+	+	+	+
Flavonoids	+	+	+	-	+
Amino acids	-	-	+	-	+

Key: + indicates presence, - indicates absence

DISCUSSION

The rapid increase in multidrug resistant pathogenic bacteria in human and animals, and the undesirable side effect of certain antibiotics has necessitated the search for safe antimicrobial drug of plant origin. Plants are being looked at as having great potential for therapeutic treatment of various bacterial diseases. In the present study, *Citrus sinensis* unripe epicarp extracts showed antimicrobial activity against the tested organisms. The crude extract showed a remarkable inhibition against *S. aureus* (21 ± 0.01mm) compared to *E. coli* (14 ± 0.02mm). Gram negative bacteria have been reported as being more resistant to antimicrobial agents due to the presence of an outer-membrane permeability barrier, which limits access of the antimicrobial agents to their targets in the bacterial cell (Vaara, 1992; Martins *et al.*, 2013). Notably, the inhibition zone of the crude extract against *S. aureus* is higher than the control which corroborates the potentials of plant extracts for antibacterial therapy. The antimicrobial activity against *E. coli* (gram negative) and *S. aureus* (gram positive) bacteria used in this study is an indication of its broad spectrum activity. This observation is similar to earlier reports of Doughari and Manzara, 2008; Kumar *et al*., 2011; Dhiman *et al*., 2012). Doughari and Manzara (2008) suggested that it can be used in the development of safe antibiotics in the treatment of bacterial infections. Acetone fractions demonstrated the highest antimicrobial activity against *E. coli* (14.13±0.06mm), while dichloromethane fractions showed the maximum antimicrobial activity against *S. aureus*. This observation is in agreement with the report of Kumar *et al*., (2011) who reported a maximum zone inhibition (16mm) against *E. coli* with acetone extract of *Citrus sinensis* peels. The variation in the antimicrobial activity of the various extracts (Table 1) showed that different extracts may have varying antimicrobial agents with different modes of action and bacteria susceptibility or that not all phytochemicals that are responsible for antibacterial activity are soluble in a single solvent (Badar *et al*., 2008; Kumar *et al*., 2011). The lack of antibacterial activity of n-hexane extract may be an indication that the phytochemicals that are responsible for antibacterial activity are insoluble in n-hexane. Dhiman *et al*. (2012) reported that methanol extract of Citrus *Sinensis* showed a zone of inhibition of 12 ± 0.5mm for *E. coli* and 11.6 ± 0.5mm for *S. aureus* whereas this study recovered a higher zone of inhibition for *E. coli* (14.13 ± 0.06mm) and lower zone of inhibition for *S. aureus* (11.05 ± 0.01mm). The phytochemical screening of unripe epicarp extracts of *Citrus Sinensis* revealed the presence of tannins, saponins, alkaloids, terpenoids, flavonoids and amino acids. There were variations in the phytochemical constituents; this may be due to its solubility in the solvents used for extraction. The antibacterial activity observed in this study may be attributed to the phytochemical constituents of the extracts. Phytochemicals are secondary metabolites produced by plants that fight with microorganisms in their environment (Pattarawadee, 1976; Sham *et al*., 2010). Flavonoids, among others are known to be produced by plants in response to microbial infection. Flavonoids are hydroxylated phenolic compounds that occur as C_3-C_6 unit linked to an aromatic ring. According to Dhiman *et al*. (2012), flovonoids activity may be due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. Cowen (1999) reported that lipophilic flavonoids may disrupt microbial membrane. Ahonkhai *et al*. (2009) reported that volatile oils have antimicrobial effects against bacteria and fungi. *Citrus sinensis* peels have high quantity of saponin with haemolytic activity and cholesterol binding properties (Kumar *et al*., 2011).

CONCLUSION

The results obtained in this study showed that acetone, dichloromethane and methanol fractions of unripe epicarp of *Citrus sinensis* have varying degree of antimicrobial activity against *E. coli* and *S. aureus*. This suggests that unripe epicarp extracts of *Citrus sinensis* can be beneficial in developing a novel antibacterial agent that can be used in treating bacterial infections. The antimicrobial activity may not be unconnected to the phytochemical constituents. Therefore, further studies to correlate their action to specific phytochemical are suggested to enhance possible drug development.

ACKNOWLEGEMENT

We would like to thank all the staff of Microbiology department, Akanu Ibiam Federal Polytechnic Unwana, Ebonyi State, Nigeria who contributed in one way or the other to the success of this work.

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