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Research Article

Antibacterial activity of Ethanolic extracts of *Citrus sinensis* peels on *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* isolated from Wound infections

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ABSTRACT

This study was aimed at assessing the extracts of peels of ripe and unripe *Citrus sinensis* as a veritable alternative therapy for treatment of wound infections. The streak plate technique was used to isolate the test organism while the Agar well diffusion technique was employed to determine the antibacterial activity of the peel extracts. The result obtained revealed the presence of five bacteria namely *Klebsiella* spp, *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Streptococcus pyogenes.* The antibacterial assay of the extracts showed that various concentrations 25mg/ml, 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml of the unripe *C. sinesis* peels produced zones of inhibition on *Staphylococcus aureus* and *Pseudomonas aeruginosa* ranging from 7.00mm to 16.63mm on *Staphylococcus aureus* to 7.50mm to 15.00mm on *Pseudomonas aeruginosa.* Extract from ripe *C. sinensis* did not show any zone of inhibition at all on any of the test isolates. Phytochemical analysis of the unripe peel extracts reveals the presence of alkaloids, flavonoids, cyanogenic glycoside, phenol, tannin and saponin. Since the unripe peel extract of *C. sinensis* were found in this study to have antibacterial activity against the isolated wound infecting organisms and are also compared to a conventional antibiotic (Gentamycin), its application will serve as a potent alternatives to antibiotics for effective treatment of bacterial wound infection.

Keywords: Inhibition, wound infection, antibacterial activity, phytochemicals, *Citrus sinensis* peels.

INTRODUCTION

The research into phytochemical and antimicrobial screening of compounds from natural sources has always been of great interest for scientists looking for new sources of useful drugs against infections and diseases [1].

The secondary metabolites present in plants have been linked with the healing properties of plant [2, 3]. In addition to their active ingredients, plants contains minerals, vitamins, volatile oils glycosides, alkaloids, bioflavonoids and other substances that are important in supporting a particular herb's medicinal properties [4]. It has been reported that plants used as medicine offers synergistic interactions between both known and unknown properties, since these medicinal plants have different actions for varied purposes. For example, herb's that play a role in the wound healing process encourages blood clothing, fights infections and accelerate the wound healing process in general [4, 5]. The skin is normally an effective barrier to pathogens, but the skin may be broken as a result of wounds, burns, surgery, bites etc. wounds may admit any of the variety of potential pathogens capable of causing systemic or localised disease. Bacterial pathogens can enter via bites [6].

Wound infections are those infections associated with wounds. Examples include burns, skin punctures and boils, accidental and surgical wounds. These wounds are quickly infected by micro-organisms such as

coliform bacilli, *Streptococcus faecales, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella* spp. The presence of these microorganisms in wounds significantly slows down the wound healing process [5].

In recent times, due to the growing resistance of wound pathogens to existing antibiotics, attention is now being turned to alternative sources of therapy. The nationwide use of plants as a sole source of traditional medicine provides promising opportunities for the search of ethno-botanical specimens based on traditional knowledge [7].

Citrus sinensis is a conventional fruit which belongs to the plant family Rutaceace and is commercially known as sweet orange. It originated from Southern-East Asia, is a tropic crop and are also an annual crop. *Citrus sinensis* is a spreading evergreen, sometimes spiny tree which could be 12m tall with oral elliptic leaves and rounded fruits that are up to 12 cm in diameter [8].

Generally, the fruit contains 80 to 90% sugar and acids, citric acid are the abundant acid in the sap. Pepsin in the juice gives it a cloudy, colloidal appearance *Citrus sinensis* contains mineral salts, glycosides, small amount of proteins and vitamins [9, 10].

C. sinensis peel, although not so juicy or tasty as the flesh, is edible and has higher contents of vitamin C and more fibre. It also contains citral, an aldehyde that antagonises the action of vitamin A particularly in an environment where resources are scarce[11]. Many of the highly nutritious compounds of *C. sinensis* are embedded in the peels. Amongst the health benefits of its peels include anti-cholesterol, avert cancer, relieve heart burn, combats digestive problems, high vitamin C content which wards off the common cold, combats respiratory conditions, and averts indigestion amongst other medical applications [12]. Away from the medicinal attributes of *C. sinensis* peels, other benefits are use as an air freshener, teeth whitening, and cleaning agent, comport, skin whitening, shields the skin from harmful ultraviolet rays, food cuisine, getting rid of bugs [12].

The medicinal potency of the *C. sinensis* is due to its high content in vitamin c, which is believed to stimulate the production of white blood cells, primary neutrophilies which function solely in combating foreign antigens such as bacteria and viruses. It also boosts the body's production of antibiotics and interferon that functions in protection from viral invaders and cancer cells [13]. The *C. cinensis* peels contain volatile essential oils which are said to be effective in inhibiting bacterial growth and disinfecting wounds [14].

Several scientists have studied the ethnobotanical, phytochemical and antibacterial activities of several medicinal plants, but this work focuses on the phytochemical and antibacterial activity of *C. sinensis* peels on some selected wound pathogens.

MATERIALS AND METHODS

Collection of plant materials:

Fresh unripe and ripe *Citrus sinensis* were plucked for a particular tree at the National Root Crop Research Institute, Umudike. The unripe *C. sinensis* were plucked at 6.30 am in the morning when the ripening enzymes have not been activated by sunlight. This way, the unripe *C. sinensis* stays unripe for at least 24 hours.

Preparation of plant materials:

The ripe and unripe *C. sinensis* were carefully and properly washed, peeled and dried separately on a sterilised stainless tray. The peels were dried for 8 days under mild sun. After drying, the peels were separately grounded into fine powder using an industrial milling machine disinfected with 95% ethanol before use. The powders were transferred separately into a sterile round bottom flask.

Extraction procedure:

Both flasks containing the grounded peels were separately submerged in 95% ethanol. The two flasks were labelled according to the contents, stoppered and allowed to stand for 24 hours in an undisturbed place.

After 24 hours, the extracts were carefully filtered with the Whatman No 1^R filter paper into two different sterilised beakers and labelled according to content. The filtrates, both of the ripe and unripe were then subjected to evaporation at 80^0C for the complete evaporation of the extraction solvent, ethanol. The resultant residue was dried in a hot air oven and stored in sterile universal bottles.

Preliminary phytochemical analysis of plant extracts:

These were carried out according to the methods described for determination of alkaloids, tannins, saponins, flavonoids, cyanogenic glycosides and phenols[15].

Collection of wound samples and isolation of test organisms:

Wound swap samples were collected on consent from the accident and emergency ward of Federal Medical Centre, Umuahia. A total of 11 wound swabs were collected and immediately taken to the laboratory for the isolation of test organisms.

The collected swabs were immediately cultures on the MacConkey agar and blood agar. The 11 swabs sticks were inoculated individually on both the MacConkey agar and blood agar using the streak plat technique and incubated at 37^0C for 24 hours. After incubation the plates were read and isolates were identified using some standard biochemical methods and recorded.

EVALUATION OF ANTIBACTERIAL ACTIVITY

Preparation of stock solutions of extracts:

Exactly 0.8g of the resultant residue was reconstituted in 4ml of 20% Dimethylsuphoxide to give a concentration of 200mg/ml. therefore two fold serial dilutions was made to get concentration of 100mg/ml, 50mg/ml, 25mg/ml. also a concentration of 150mg/ml was made by dissolving 0.30g of the resultant residue in 2ml of 20% Dimethylsuphoxide. The tubes containing the various concentrations was labelled and stored in a refrigerator at 5^0C until they were needed for the various experiments. The tubes were stored away from a light source [16].

Sensitivity testing:

The agar well diffusion technique was employed as described by Esimone CO[17] and Osadebe PO[18]. The Mueller-Hinton agar was used. Four holes each measuring about 6mm were exceptionally bored on the Mueller-Hinton agar plates using a sterile cork bones. About 0.04ml of different concentrations of the extracts was transferred into the holes using a sterile Pasteur pipette. The plant extracts were thereafter allowed to stand for one hour for a pre-diffusion of the extracts [17] and were incubated at 37^0C for 24 hours.

After incubation, the plates were collected and the zones of growth inhibition were measured.

Minimum Inhibitory Concentration (MIC):

1g of the extract was dissolved in 4ml of Mueller-Hinton broth, thus resulting in 250mg/ml. thereafter, two folds serial dilutions were made from the original stock according to the method of Egorov NS using the Mueller-Hinton broth to obtain the concentrations of 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml[19]. A loopful of the organisms was inoculated into the various tubes containing the different dilutions [20]. The tubes were incubated at 37^0C for 24 hours. The lowest concentration of each of the test extracts that inhibited the growth of the microorganism was the MIC.

Minimum Bacteriocidal Concentration (MBC):

The tubes which showed no visible growth from the MIC test were sub-cultured onto sterile Mueller-Hinton Agar, and incubated at 37^0C for 24 hours. The lowest concentration of the extract that yields no growth was recorded as the MBC.

RESULTS

The characterisation and identification test on the isolates reveals them to belong to the genera *Klebsiella* spp, *Staphylococcus* aureus, *Escherichia coli*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* were used for the antibacterial activity testing as shown in Table 1.

Table 2 shows the effects of the Ethanolic Extract of the ripe *C. sinensis* peel on the isolates. No concentration of the ripe peel extract could inhibit the growth of any of the 3 test organisms.

Table 3 shows the antibacterial activity of the Ethanolic Extracts of the unripe *C. sinensis* peel on the test organism. The highest concentration of the extract (200mg/ml) exhibited an inhibitory effect against *Pseudomonas aeruginosa* with 12.75mm zone diameter while the lowest concentration (25mg/ml) showed weak inhibition potential against *Staphylococcus aureus* with 7.00mm diameters. *E.coli* was not inhibited by any of the concentrations.

Table 4 shows the zone diameter of inhibition of the crude ethanolic extract of the ripe and unripe *C. sinensis* peel, Gentamycin, and distilled water against the test organisms. No inhibitory effect was observed with the crude ripe peel extracts and distilled water. *E.coli* was not inhibited by any of the extracts. Undiluted unripe peel extract and Gentamycin produces the same zone diameter of inhibition (15.00mm) against *Pseudomonas aeruginosa*. The crude unripe peel extract showed great inhibitory qualities on *Staphylococcus aureus* showing a diameter zone of inhibition of 16.63 mm.

Tables 5 show the Minimum Inhibitory Concentration (MIC) and the Minimum Bacteriocidal Concentration (MBC) of the unripe *C. sinensis* peel extracts on the test isolates. The MIC of the unripe peel extracts required to inhibit the growth of *Staphylococcus aureus* was 25mg/ml and a MBC of 50mg/ml while the MIC required to inhibit the growth of *Pseudomonas aeruginosa* was 100mg/ml and MBC of 150mg/ml.

The result of the preliminary phytochemical screening of the unripe peel extract is shown in table 6. Saponins, tannins, flavonoids, alkaloids, phenol and cyanogenic glycosides were present.

DISCUSSION

This study was to determine the antibacterial activity of ripe and unripe *citrus sinensis* peels against bacterial isolates from wound infection. Five bacterial genera were isolated amongst which 3 where Gram negative (*Klebsiella* spp, *E. coli* and

Pseudomonas aeruginosa). It is most possible that the type of environment and the state of the wound at any particular time influences the type and prevalence of organisms isolated from a given wound sample. The result obtained from this study showed that the extracts inhibited the growth of the bacterial isolates except *E. coli*. This result was in agreement with the work of Lawal DI, Gulay KF, Vivek VK and Jacob A [20, 21, 22, 23]. That the extracts inhibited the growth of the isolates is an indication that they contain substance(s) that are active against bacterial species [24, 25, 18]. That the extract did not inhibit the growth of *E. coli* maybe due to the fact that the bacterium posses mechanisms for dexotoxifying or removing the active principles. The observed antibacterial activities of the extracts may be due to tannins, alkaloids, flavonoids, saponins, phenols and cyanogenic glycosides identified in the extracts [26].

However, the unripe *C. sinensis* peels extract showed strong inhibition on the isolates. Its highest inhibition was on *Pseudomonas aeruginosa* (12.75mm), followed by *S. aureus* (11.00mm), but no effect on *E. coli*. The ripe peel of *C. sinensis* had no effect whatsoever on any of the wound pathogens while the positive control antibiotics, Gentamycin inhibited all the isolates moderately with the most successful being *S. aureus* and the least successful being *E. coli*. This result however has proved that Gram negative organisms are generally more resistant to antimicrobial agents, probably due to their complex cell wall structure as well as possession of antibiotic resistance plasmids and production of enzymes called Extended Spectrum Beta Lactamases (ESBL) by organisms such as *E. coli* [27].

The ripe extract of *C. sinensis* peels did not show any antimicrobial activity throughout the study. This therefore may be due to the higher concentration of aliphatic aldehydes and oxygen containing monoterpenes and sesqui-terpenes which little antimicrobial potentials than the peels of the unripe *C. sinensis*.

The pH of the unripe *C. sinensis* was 4.8 while the pH of the ripe *C. sinensis* was 6.8. The acidic nature of the unripe peel extract could be responsible for the antibacterial activity evident by the work of Almajano NP et al [28] where they observed that of caffeic acid with pH 4.0 was enough to inhibit the growth of some of the studied microorganisms' whole pH requirements range from 5.0 to 7.0.

As observed from the preliminary phytochemical screening result, the unripe peel extracts contains alkaloids, tanins, saponins, phenol, cyanogenic glycosides and flavonoids. Tannins have been reported to reversibly form complexes with proline-rich proteins resulting in the inhibition of cell protein synthesis as well as production of typical tanning effect which is important in treating inflamed or ulcerated tissues, burns, wounds, pneumonia and dysentery [29]. Saponins and flavonoids in plant materials exert antibacterial properties, together with alkaloids and tannins in synergistic manner, are responsible for growth inhibition of the pathogens [15]. Flavonoids in *C. sinensis* have anti-inflammatory and bacterial actions.

CONCLUSION

This work has revealed the need to recommend that orthodox medicine and herbal medicine should come together to harness the full potentials of medicinal plants for medical and pharmaceutical development. In this work, the importance of utilising the *C. sinensis* peels as a pharmaceutical option is seen in the sensitivity pattern on *Pseudomonas aeruginosa* against the unripe peel extract. The undiluted unripe extract showed a zone of inhibition of 15.00mm against *Pseudomonas aeruginosa* as opposed to Gentamycin which was the positive control and also had a diameter zone of inhibition of 15.00mm. This however proves that the unripe peels of the *C. sinensis* having met the antibacterial requirements of Gentamycin could be substituted for therapeutic treatments of wound infections. This approach however would go a long way in combating the rising tide of antibacterial resistance.

Table 1
Bacteria isolated from wound specimen

Gram positive	Gram negative
Klebsiella spp	**Staphylococcus aureus*
**Escherichia coli*	*Streptococcus pyogenes*
**Pseudomonas aeruginosa*	

Key: * Indicates those bacteria used for the antibacterial activity testing

Table 2
The effect of the ethanolic extract of the ripe *Citrus sinensis* peels with pH 6.8

Mean diameter zone of inhibition (in mm)			
Concentration of extract (mg/ml)	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***
25	0.00	0.00	0.00
50	0.00	0.00	0.00
100	0.00	0.00	0.00
150	0.00	0.00	0.00
200	0.00	0.00	0.00

Table 3
The antibacterial activity of the ethanolic extract of the unripe *Citrus sinensis* peels with pH 4.8

Mean diameter zone of inhibition (in mm)			
Concentration of extract (mg/ml)	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***
25	7.00	0.00	0.00
50	8.50	0.00	0.00
100	9.75	0.00	7.50
150	9.88	0.00	9.00
200	11.00	0.00	12.75

Table 4
Zone diameter of inhibition (in mm) of the crude ethanolic extract of the ripe and unripe *Citrus sinensis*, Gentamycin and distilled water

Men diameter zone of inhibition (in mm)				
Test organisms	**Undiluted ripe peel extract**	**Undiluted unripe peel extract**	**Gentamycin**	**Distilled water**
Staphylococcus aureus	0.00	16.63	20.00	0.00
Escherichia coli	0.00	0.00	10.20	0.00
Pseudomonas aeruginosa	0.00	15.00	15.00	0.00

Table 5
Minimum inhibitory concentration and Minimum Bacteriocidal Concentration of the unripe *Citrus sinensis* extract (mg/ml)

	mg/ml	
Test organism	**MIC**	**MBC**
Staphylococcus aureus	25	50
Escherichia coli	Nil	Nil
Pseudomonas aeruginosa	100	150

Key: Nil means no MIC or MBC

Table 6
Phytochemical components of the unripe *Citrus sinensis* peel extract

S.No	Phytochemical	Result
1	Alkaloid	+
2	Saponins	+
3	Tannins	+
4	Flavonoids	+
5	Cyanogenic glycosides	+
6	Phenol	+

Key: + means positive

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