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Research Article

Antibacterial Activity of Sweet Orange *Citrus sinensis* on some Clinical Bacteria Species Isolated from Wounds

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Keywords

• *Citrus sinensis; Staphylococcus aureus; Escherichia col; Pseudomonas aeruginosa;* Wound infection

Abstract

This study investigated the bacterial activity of the juice and peel extracts of *Citrus sinensis* against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* isolated from wound infections. The three bacterial wound isolates were identified after subjecting them to different biochemical tests. The dried and powdered plant materials were extracted using standard qualitative procedure and agarwell diffusion method was used for antibacterial assay. The MIC and MBC of the juice and peel extracts of C. *sinensis* were determined with values ranging from 0.6% to 10% (w/v). The juice and the peel extracts showed a remarkable inhibition against the three isolated pathogenic bacteria of wound with the juice having highest activity against infection. The juice exhibited the highest antibacterial activity against *S. aureus* (28.0 mm), whereas ethanol extract exhibited highest antibacterial activity against *E. coli* (22.0 mm) and the least affect was recorded with aqueous extract against *P. aeruginosa* (10.0mm). The results of the phytochemical screening of the juice and the peel revealed the presence of alkaloids, terpenoids, flavonoids, tannins, saponins, reducing sugar and amino acids. The result of this study suggests that the juice and peel extracts of C. *sinensis* can be beneficial in the treatment of wound infection.

ABBREVIATIONS

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bacetericidal Concentration; CFU: Colony Forming Unit

INTRODUCTION

Sweet orange is a small evergreen tree up to 7.5m high, and in some cases up to 15m. It originated from Southern China, where it has been cultivated for many years, but it is grown commercially worldwide in tropical and semi-tropical and some warm temperate regions to become the most widely planted fruit tree in the world [1]. Orangetree produce leathery and evergreen leaves of different shapes, ranging from elliptical to oblong to oval, 6.5-15 cm long and 2.5 -9.5 cm wide, often bearing narrow wings on the petioles. It bears fragrant either white flowers either singly or in whorls of 6, about 5 cm wide, with 5 petals and 20-25 yellow stamens. The small, white or purple scented hermaphroditic flowers produce nectar for pollination by insects. The fruit may be globose to oval which is about 6.5 to 9.5 cm wide and ripens to orange or yellow. The fruit consist of two distinct regions, the pericarp also called the peel, skin or rind and the endorcarp, or pilp and juice sacs. The skin consists of an epidermis of epicuticular wax with numerous small aromatic oil glands that gives it its peculiar smell.

Fruits are one of the oldest forms of food known to man. From the ancient time different cultures around the world have used herbs and plants as remedy indifferent diseases conditions and maintain health [2]. The chemical composition of citrus peels oil revealed that they are rich in nutrients and many phytochemicals [3,4]. The citrus peel is a rich source of flavonone and many polymethoxylated flavones, which are rarely found in other plants [5]. Citrus fruits are mainly used as dessert, but their peel produces an essential oil which has important economic value worldwide. It is used as flavouring agents to mask unpleasant taste in pharmaceutical industries [6]. They are used as flavor in food industries [7].

A wide number of plant extracts possess antimicrobial properties which are used as natural alternatives to treat several diseases. Scientific studies available on medicinal plants indicate that promising phytochemical can be developed for many health problems [8]. There is a great demand of fruit juices in the treatment of various illnesses such as arthritis, heart diseases, muscle aches and drug addictions [9]. Hamendra and Anand (2007) [10] reported its use as antidiabetic and also as an antimicrobial [11]. The medicinal potency of *C. sinensis* is due to its high content of Vitamin C which is believed to stimulate the production of white blood cells, primarily neutrophils which attack the foreign antigens such as bacteria and viruses. It does boost the immunity by production of antibodies and interferon, the proteins that helps protects against viral invaders and cancer cells [12]. Most wound infections are infected by Staphylococcus sp, Pseudomonas sp and *E. coli* [2,5]. This study was designed

to determine the antibacterial activity of *Citrus sinensis* against clinical strains of *S. aureus, E. coli* and *P. aeruginosa* from wound infection.

MATERIALS AND METHODS

Procurement of sweet orange

Fresh sweet orange (*C. sinensis*) was purchased from Gwari market in Minna metropolis, Niger state. The fruit was transported to Microbiology laboratory, of Ibrahim Badamasi University, Lapai. The fruits were washed with distilled water and sterilized using 70% alcohol and kept in a sterile zip polythene bag until ready for use.

Extraction of raw orange juice

The fruit was peeled off and hand squeezed to obtain the juice from the fruit. The peels were dried in an oven at 50**°C** for 48 hours and the peel was ground into a fine powder. The powdered material was stored in airtight jars in the refrigerator at 4**°C**.

Ethanol and aqueous extraction of the orange peels

The extraction was carried out using water and 95% ethanol using the methods previously described by [2] and [13]. Briefly, 25g of powdered material was dissolved in enough sterilized distilled water and another one in ethanol and make up 100ml to give extracts (25w/v). The mixture was kept undisturbed at room temperature for 24hours in a sterile flask covered with aluminium foil to avoid evaporation and subjected to filtration through sterilized Whatman no.1 filter. The filtrates were evaporated in water bath at 60**°C** until the solvents were completely evaporated.

Testing for presence of bioactive compounds present in plant extracts

The methods described by [14] and [15] were used to test for the presence of alkaloids, tannins, saponins, flavonoids, terpenoids, reducing sugar and amino acids.

Determination of alkaloids

The extract was prepared by macerating 3gm of the powdered sample in 50ml of methanol. The extract was evaporated to dryness. 0.5g of the residue was weighed and mixed with 10 ml of 1% aqueous hydrochloric acid in a water bath. 1ml of each from the mixture was treated with the following reagents; mayer's reagent, Dragendorrff's regaent, Wahner's reagent and 10% tannic acid solution. Turbidity or precipitation with all these reagents was regarded as an indication of the presence of alkaloids in the extract.

Determination of flavonoids

This was done by weighing 5g of the extracts and 10mls of the juice and completely detanned using acetone. The residue was extracted in warm water after evaporating the acetone on a water bath. The mixture was filtered and the filtrate was used for the following tests. 5ml of detanned extract and juice was added to 10% lead acetate solution. Precipitation is an indication of presence of flavonoids.

Sodium Hydroxide Test: - 5ml of 10% sodium hydroxide was added to an equal volume of the detanned water extract. A yellow solution indicated the presence of flavonoids

Determination of terpens

Ethanolic extraction of the plant sample was carried by macerating 5g of the sample with 50ml of 95% ethanol and filtered. The filtrate was evaporated to dryness, residue dissolved in 10ml of anhydrous chloroform and then filtered. The juice was giving the same treatment.

Liebermanm-Burchard Test for Terpens: The first portion of the chloroform solution from above was mixed with 1ml of acetic anhydride, followed by 1ml of concentrated sulphuric acid down the wall of the test tube to form a lower layer. The formation of reddish-violet colour at the interphase of the two liquids and a green colour in the chloroform layer were indications of the presence of terpens.

Determination of reducing sugar

To 3g of ach of the extracts and 5ml of the juice in beakers was added 50ml of distilled water on a water bath and boiled for 3 minutes. The mixture was filtered while still hot and the filtrate cooled and was used for the following tests.

Test for Reducing Sugar: This was carried out using fehling's test for free reducing sugars. To 2ml extract obtained above was added 5 ml of equal volumes of mixtures of fehling's solution A and B. This was boiled for 2 minutes on a water bath. A brick red colour formation was regarded as evidence for the presence of reducing sugar.

Determination of Amino acids

A small quantity of the extracts and juice was added to Ninhydrin reagent in separate tubes, mixed and heated gently. A blue colour shows the presence of protein.

Determination of saponins

Test for Saponins was carried out using Froth Test. A small quantity of the plant extract and juice was added to 95% ethanol and boiled. The mixture was filtered and 2.5ml of the filtrate was added to 10ml of distilled water in a test tube. The test was stoppered and shaken vigorously for about 30 seconds. It was the allowed to stand for 30 minutes. The mixture was then shaken vigorously. Persistent Honey comb froth which persisted was taken as an evidence for the presence of saponins.

Determination of tannins

To 3g of each plants part in a beaker was added 50ml of distilled water on a hot plate and boiled for 3 minutes. The mixture was filtered while still hot and the filtrate cooled and was used for the following tests for tannins. The water extract was diluted with distilled water in a ratio 1:4 and few drops of 10% iron III chloride ($FeCl_3$) was added. Blue- Black or blue green coloration was taken as an indication of the presence of tannins.

Confirmation and characterization of the test organisms

The clinical isolates of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Eschrichia coli* from wound exudates were

collected from Microbiology unit of diagnostic laboratory of General Hospital Lapai on sterile peptone water and transported to the Department of Microbiology, Ibrahim Badamasi University Lapai. The species of the isolates were confirmed by streaking on Mannitol Salt Agar (MSA) and Eosine Methylene Blue (EMB). The MSA plates were incubated at 37**°C** for 24 hours and EMB plates at 44**°C** for 48 hours. The characterization of the isolates was confirmed by subjecting them to the following biochemical tests: These are catalase test, oxidase test, coagulase test, indole test, citrate test. They were tested for their ability to utilize glucose, sucrose, lactose, mannitol and fructose. The confirmed organisms were preserved on slants at 4**°C** in a refrigerator.

Preparation of inocula

Stock cultures were maintained on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loop from the stock cultures to test tubes of Mueller-Hinton broth (MHB) and incubated overnight at 37**°C** for reactivation. Cultures were diluted with fresh MHB and compared with 0.5 McFarland standard to achieve values corresponding to $2x10^6$ colony forming unit.

Antibiotic sensitivity test

All bacterial strains were subjected to antibiotic sensitivity test using Kirby-Bauer's method, on a Mueller- Hinton agar (MHA) medium as described by [15,16]. An aliquot of $2x10^6$cfu/mL (0.5 McFarland equivalents), from an exponentially growing culture was spread on to agar for development of lawn of all strain of bacteria tested. Further on lawn-agar of each plate, 8 high potency antibiotic-discs (ampicillin 30µg, benzyl-penicillin 30µg, streptomycin 10µg, ciprofloxacin 10µg, gentamycin 30µg, erythromycin 10µg, norfloxacin 30µg, and tetracycline 10µg) were placed at equal distances from one another. Plates were incubated for 24 hours at 37**°C** in a NAPCO 6500 incubator (Thermo Electron Corporation) after which diameter values of zones of inhibition were measured.

Antibacterial activity test

For determination of antibacterial activity of the extracts, the agar-well diffusion method [16] was used. An aliquot of $2x10^6$cfu/mL (0.5 McFarland equivalents) from an exponentially growing culture was spread on to agar for development of lawn of all strain of bacteria tested. The plates were incubated for 30 minutes inside the biosafety cabinet. Wells were punched using 6 mm sterile meter puncher on the plates and each well was based with 50 µL molten MHA. Further, wells were filled with 100 µL aliquots of 10 mg/mL solvent-extract (which were diluted from the original stock of plant extract of individual organic solvent by 10% DMSO to 30 mg/mL and that of the aqueous plant-extract sterile distilled water). Each of the plates were prepared in triplicates Plates were incubated at 37**°C** for 18-24 hours after which the results were read and as expressed as mean of zone of inhibition.

Determination of Minimum Inhibitory Concentration (MIC)

Extracts which exhibited high activities against the pathogenic isolates were further assayed for their minimum inhibitory concentration. A broth dilution susceptibility assay described by the National Committee for Clinical Laboratory Standards [17] was used to determine a minimum inhibitory concentration (MIC) of the juice and peel extract. The inocula of the bacterial strains were prepared from 10 hour broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity.

A serial two-fold dilution of the juice and peel extracts absolute ethanol was prepared to obtain concentration of the juice and peel extracts: 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 0.625 mg/ml. A volume of diluted extracts (500 µL) were transferred to the test tubes. Subsequently, a fixed volume (4 ml) of liquid culture medium was distributed into the test tubes and inoculated with 500µL of bacterial suspension (10^6 CFU/ml) and then incubated for 24 hours at 37**°C**. The MIC was determined by taking the lowest concentration of the extract which inhibited the organism. The experiment included three controls: the negative control tube containing culture medium and extracts only, the positive control tube containing culture medium and microorganisms, and the solvent control tube containing 10% DMSO, medium and microorganism. The MIC was taken as the minimum concentration of the dilutions that inhibited the growth of the test microorganism.

Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by sub-culturing the tubes from the MIC that do not show evidence of growth. The mixture was streaked on Nutrient agar plate in duplicate and incubated for 24 hours at 37**°C** and 48 hours at 44**°C** respectively. The plate with minimum concentration of the extract that do not allow the growth of the organisms were considered as Minimum Bactericidal Concentration of the extract.

RESULTS

Table 1 shows then result of phytochemical analysis of the juice, ethanol and aqueous extracts of the peel of sweet orange (*C.simensis*). The juice contains alkaloid, flavonoids, terpenoids, reducing sugars, saponins and tannins, while amino acid was present. The ethanol peel extract contains all the above mentioned metabolites except reducing sugar while the aqueous extracts contain all metabolites but no saponins and tannins. All the tested metabolites except the amino acids were found in the fruit juice.

The colonial cell morphologies and biochemical characteristics of the isolates are represented in Table 2. A Gram positive cocci with golden yellow colonies that were catalase positive, oxidase negative coagulase positive indole and citrate negative but ferments glucose, sucrose, lactose, mannitol and fructose was isolated and confirmed to be *Staphylococcus aureus*. Also a Gram negative single rods which exhibit green pigmentation on the culture media was isolated. The organism was catalase, oxidase, coagulase and citrate positive but indole negative. It only ferments glucose and lacotose which is an indication of the presence of *Pseudomonas aeruginosa*. A second Gram negative rods but with moist shiny green metallic colonies which can utilize catalase and indole but not oxidase, coagulase and citrate. It is able to utilize glucose lactose, mannitol and fructose but not sucrose which is confirmed as *E. coli.*

The results for the antimicrobial activities revealed that the three extracts have activities against the clinical isolates at the concentration of 10% w/v of the juice and 10mg/ml of ethanol and aqueous peel extracts. There was significant variation in the antibacterial activities of the various extracts which is shown by the diameter of inhibition zone. The juice exhibited the highest zone of inhibition of 28.0±2.1mm for *S. aureus* followed by 25.0±4.2mm for E. coli while *P. aeruginosa* had 22.0±2.50mm zone of inhibition. The ethanol extract showed the highest inhibition against *E. coli* with zone of inhibition of 22.2±3.1mm followed by *S. aureus* with zone of inhibition of 20.02±0.6mm and 18.0±00mm for *P. aeruginosa*. The aqueous extracts exhibited highest activity against *S. aureus* with zone of inhibition of 18.3±1.5mm followed by *E. coli* with 16.0±1.0mm and *P. aeruginosa* with 10.00±3.3mm (Table 3).

The isolates were tested against standard antibiotics. The results of the findings are presented in Table 4. Gentamycin, streptomycin, norfloxacin and tetracycline exhibited broad spectrum activity against the three isolates, while benzylpenicillin showed activity against *S. aureus*, *E. coli* but no activity against *P. aeruginosa*. Both *P. aeruginosa* and *E. coli* were susceptible to the effects of ciprofloxacin and Erythromycin while *S. aureus* was resistance to the effect of both of them. The result of the study showed that ampicillin has activity against E. coli only while *S. aureus* and *P. aeruginosa* were both resistant to the effect of ampicillin.

Table 1: Phytochemical content of the juice, ethanol and aqueous extracts of the peel of *C. simensis.*

Components	Extracts		
	Juice	Ethanol	Aqueous
Alkaloid	+	+	+
Flavonoid	+	+	+
Terpenoid	+	_	+
Reducing sugar	+	+	+
Amino acid	_	+	+
Saponin	+	+	_
Tannins	+	+	+

+ = present, — = absent

Table 2: Colonial, cell morphology and biochemical characteristics of bacterial cultures isolated from the wound samples.

	Isolate 1	Isolate 2	Isolate 3
Colony characteristics	Smooth, circular and golden yellowish	Smooth shiny with green pigments	Smooth, moist and shiny, metallic green
Gram Reaction and morphology	Positive cocci in bunches	Negative small single rods	Negative small single rods
Biochemical Tests			
Catalase	+	+	+
Oxidase	-	+	-
Coagulase	+	+	-
Indole	-	-	+
Citrate	-	+	-
Sugar Fermentation			
Glucose	+	+	+
Sucrose	+	-	-
Lactose	+	+	+
Mannitol	+	-	+
Fructose	+	-	+
Isolated Bacteria	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*

Table 3: Inhibition of the juice and extracts against tested bacteria.

	Diameter of zone of inhibition (mm) (mean values ± SD)		
	S.aureus	*P. aeruginosa*	*E. coli*
Juice	28.0±2.1	22.0±2.50	25.0±4.2
Ethanolic extract	20.0±0.6	18.0±0.00	22.2±3.1
Aqueous extract	18.3±1.5	10.0±3.3	16.0±1.0

Table 4: Mean inhibition zones of antimicrobial activity of standard antibiotics against test microbes..

Type of standard antibiotics	Isolates		
	S. aureus	P. aeruginosa	E. coli
AP	-	-	6.3±0.14
B	8.17±0.12	-	15±0.12
ST	6.63±0.03	5.7±0.17	14.9±0.21
CI	-	8.21±0.15	23.03±0.15
GM	14.43±0.18	12±0.12	15.3±0.21
ER	-	6.63±0.07	12.6±0.21
NF	18.33±0.22	11.27±0.07	24.11±0.25
TE	17.43±0.05	7.07±0.18	13.3±0.16

The results are expressed as mean inhibition zones ± SEM of triplicates
KEY: AP: Ampicilin (25µg); BP: Benzylpenicillin (µg); S: Streptomycin (25µg); CI: Ciprofloxacin (5µg); GM: Gentamicin (10µg); ER: Erythromycin (15µg); NF: Norfloxacin (10µg); TE: Tetracycline (100µg)

The minimum inhibition concentration and minimum bactericidal concentration was carried out on the juice and the two extracts. Table 4 represented the MIC and MBC of the juice and the extracts against the bacterial isolates. The MIC of the juice for *S. aureus* was 0.625%, 1.25% for *E. coli* and 2.5% against *P. aeruginosa*. The ethanol extract of the orange peel had MIC of 0.625mg/ml for *S. aureus*, 1.25mg/ml for *E. coli* and 2.5mg/ml for the *P. aeruginosa*. The aqueous extract of the peel had MIC of 5mg/ml for the three organisms.

The minimum bactericidal concentration of the fresh juice of *C. sinensis* was 2.5% for *S. aureus*, 5% for *E. coli* and 2.5% for *P. aeruginosa*. The ethanolic extract of *C. sinensis* fruit peel, was 10mg/ml for *S. aureus*, 5mg/ml for *E. coli* and no antibactericidal activity showed against *P. aeruginosa*. The aqueous extract of the peel showed no bactericidal activity against the three isolates.

DISCUSSION

Natural and environmentally friendly antimicrobials, antibiotics, antioxidants have become the priority search for pharmaceutical industries. Citrus is one of the most important commercial fruit crops grown in all continents of the world.

Table 5: The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Juice and the Orange peels.

	Juice		Ethanol extract		Aqueous extract	
	MIC	MBC	MIC	MBC	MIC	MBC
Staph. aureus	0.625	2.5	0.625	10	5	0
E. coli	1.25	5	1.25	5	5	0
P. aeruginosa	2.5	2.5	2.5	0	5	0

The orange peel extract contains phytochemical constituents like tannins, saponin, flavonoid, terpenoid, cardiac glycosides, alkaloids and phenols. The result of this present study showed the presence of alkaloid, flavonoid, terpenoid, reducing sugars, amino acids, saponin and tannin. These phytochemical compounds obtained from these extracts have also been reported to occur in other parts of the plant [18]. The result of the phytochemical analysis from this study is in agreement with the findings of [19]. However, the report of this study showed the absence of terpen and saponin in the ethanol and aqueous extracts respectively and contrary to the findings of [20] that detected both Terpens and saponin in both the ethanol and aqueous extracts.

In the present study antibacterial activity and phytochemical analysis of *Citrus sinensis* fruit juice and the peel extract against 3 organisms isolated from wound infection which have also been reported to be associated with wound infection [21,22]. These include a gram positive and two Gram negative bacteria.

In this study, the juice of *C. Sinensis* and the ethanol and aqueous extracts of the peel showed good antibacterial activities against tested organisms. *S. aureus* is most susceptible to the fruit juice and aqueous extracts of the peel followed by *E. coli* which is also the most susceptible to the ethanol extracts. Similar effects were also reported by [23] where ethanol extracts exhibited higher antibacterial activity than the aqueous extracts. Melendez and Capriles (2006), [24] reported that juice and extracts from the peels of *C. Sinensis* possess *Invitro* antibacterial activity against many bacteria with zone of inhibition of between 11-31mm. However, the activity of aqueous extract in this study showed (zone of inhibition of (18.3±1.5mm), this was higher than the activity reported from the study by [25]. *P. aeruginosa* is generally known to be resistant to many antibacterial agents [26] and this is in agreement with findings of this study. It is of note that *P. aeruginosa* tested, showed resistance to 2 known antibiotics (Ampicillin and Benzyl penicillin) but inhibited by the juice and ethanol extracts of the peel. When comparing the eight commercial standard antibiotics, the antibacterial potentials of the juice against the bacteria isolates were better than all the standard antibiotics. The ethanol extracts also showed a higher potency than 6 of the standard antibiotics while the aqueous extracts produced higher effects than 4 antibiotics. The activity of the juice and ethanol extracts agains *S. aureus* were found to be higher compared to all the tested standard antibiotics, even the aqueous extracts was comparable to the Norfloxacin that has the highest activity against *S. aureus.* The three extracts showed much higher activities against *P. aeruginosa* compared to all the tested standard antibiotics. Ciprofloxacin and Norfloxacin had higher activities against *E. coli* compared to the three extracts while the remaining standard antibiotics have less activity compared to the extracts.

The ability of these extracts to inhibit the growth of the tested bacteria in varying degrees is an indication of the presence of the active principles for antimicrobial actions. Ogueke et al. 2006 [27], and [28] attributed their observed antimicrobial effects of plants extracts to the presence of secondary metabolites. The sensitivity pattern of the organism to these extracts was comparable to the values obtained by [29]. It has been reported earlier that the juice, ethanol and aqueous extracts of *C. sinensis* fruit contain antimicrobial substances [28]. The results of activity of the juice as obtained in this study, is similar to the result of the study by [13] but higher than the results from the study by [20].

CONCLUSION

The results obtained in this study showed that juice extracts, ethanol and aqueous peel extracts of *C. sinensis* have varying degree of antimicrobial activity against *S. aureus, E. coli* and *P. aeruginosa*. This suggests that extracts of *Citrus sinensis* can be beneficial in developing a novel antibacterial agent that can be used in treating superficial infections. This can be of commercial interest to pharmaceutical industries in search of new leads that are cheap with readily available raw materials especially in the present threat of resistance by many pathogenic bacteria. The antimicrobial activity of these extracts may not be unconnected to the phytochemical constituents. Therefore, further studies to relate their action to specific phytochemicals are suggested.

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