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Phytochemical Screening and Antibacterial Activity of *Citrus sinensis* (L.) *Osbeck* [Orange] *and Citrus aurantifolia* (Cristm.) Swingle [Lime] Stem from Bacteria Associated with Dental Caries

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MKN and BSO designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors MHD and AAI managed the analyses of the study. Authors SK, IB and FAU managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: The use of chewing stick as tooth cleanser by Arabs and now by most Muslims all around the globe has long been established. Stems of different trees have been used in this process. Stems of *Citrus sinensis* (Orange) and *Citrus aurantifolia* (Lime) are used in Nigeria in cleansing teeth. Few attempts were made to screen the antimicrobial activity of the stems of the trees on microorganisms isolated from teeth.
Aim of the Study: The aim was to determine the phytoconstituent and the antimicrobial activity of

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Citrus sinensis and *Citrus aurantifolia* on organism's isolated from human teeth.
Materials and Methods: Phytoconstituents of the aqueous and ethanolic extract of the stems of Lime and Orange tree were determined using standard methods. The antimicrobial activity of the extract against some microorganisms isolated from teeth was determined using agar well diffusion method. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) were determined using standard method.
Results: Phytochemical screening of stems of the two plants revealed the presence of alkaloids, flavonoids, steroids, anthraquinones and carbohydrates. Highest zone of inhibition of 7 mm and 10 mm was recorded on the ethanolic extracts of orange and lime tree stems on *Staphylococcus. aureus* respectively. No activity was recorded on both the aqueous and ethanolic extracts of the trees on *Pseudomonas aeruginosa*. MIC and MBC of 59 mg/ml and 100 mg/ml for the ethanolic extracts of lime tree stem on *S. aureus* and *Proteus mirabilis* were recorded. For the orange tree, MIC and MBC of 25 mg/ml and 100 mg/ml were recorded for the ethanolic extracts were recorded on *S. aureus*.
Conclusion: Aqueous and ethanolic extracts of *Citrus sinensis* and *Citrus aurantifolia* were shown to be active against some of the microorganisms isolated from human teeth.

Keywords: Antimicrobial activity; Phytochemical screening; Citrus sinensis; Citrus aurantifolia.

1. INTRODUCTION

Chewing stick also referred to as Miswak in Arabic has been known for centuries as tooth cleanser, the use of modern toothbrushes and inter-dental cleaners have made humans to ignore the most primitive oral hygiene tool, which is the chewing stick [1]. Several studies revealed that chewing sticks contain natural ingredients such as ascorbic acid, trimethylamine, chloride, fluoride, silica, resin and salvadorine, which are beneficial for oral health [2,3]. In addition, chewing stick also contains volatile oils, tannic acid, sulphur and sterols which attribute to antiseptic, astringent and bactericidal properties that help reduces plaques formation, provides anti-carious effects, eliminates bad odour and improves the sense of taste [4]. Some of the chewing sticks predominantly used in Northern Nigeria are: *Azadirachta indica* (Neem), *Psidium guajava* (Guava), *Citrus sinensis* (Orange) and *Citrus aurantifolia* (Lime)

Orange (*Citrus sinensis*) is a hybrid of pomelo (*Citrus maxima*) and mandarin (*Citrus reticulata*) [5]. It is an evergreen tree with 9-10 m in height, although in peculiar cases the orange tree reaches up to 15 m [6]. The leaves are ovate in shape with crenulated margins and are 4-10 cm long. The orange fruit is hesperidium, a type of berry [7]. The sweet orange does not usually occur in the wild, it is believed to have been first cultivated in southeastern Asia [8]. Sweet orange oil (Essential oil) is a byproduct of the juice industry produced by pressing the peel. It is used in the flavouring of foods, drinks and as a fragrance in perfume and aromatherapy [9].

Citrus aurantifolia is a genus of flowering plants in the family Rutaceae (orange family) and a common name for edible fruits of this genus and sometimes-related genera. Lime is a small shrub-like tree ranging from 3.5 to 9 m in height and 2.5 to 7.5 m in width. The fruit is typically round, green to yellow in color and about 3-6 cm in diameter [10]. Lime (*Citrus aurantifolia*) juice has been shown to have both medicinal and cosmetic values. Studies have shown that lime juice destroys both human immunodeficiency virus (HIV) and sperm cells [11]. The high acidity of the lime juice is probably responsible for the destruction of the HIV and sperm cells [12]. In the current study, we evaluate the phytochemical and antibacterial activities of *Citrus sinensis* and *Citrus aurantifolia* on some microorganisms isolated from human teeth.

2. MATERIALS AND METHODS

2.1 Sample Collection and Processing

Fresh stems of *Citrus sinensis* and *Citrus aurantifolia* were collected from Abdullahi Fodiyo Library Usmanu Danfodiyo University Sokoto (UDUS). The plants were authenticated in the Botany unit, Biology Department of UDUS. After the collection of the four plant stems, they were cut into small pieces and dried at 28±2ºC for three weeks. The dried stems were pounded and sieved using a sieve with a mesh size of 0.5 mm to obtain a fine powder.

2.2 Extraction of Plant Stems

The two solvents that were used to extract the plants are distilled water and ethanol.Forty grams

of each plant powder was dissolved it in 400 ml of distilled water in a conical flask. Similarly, 40 g of each plant powder was dissolved in 400 ml of ethanol. The mixture was vigorously stirred with a sterile glass rod, it was then allowed to stand for 45 minutes and the mixture was filtered using Whatman No1 filter paper [13]. The filtrate was poured into a sterile bottle and placed on a steam bath at 45°C for evaporation to dryness. The extract was then recovered and weighed [14]. The extract was transferred into a sterile polyethene bag and stored under appropriate temperature.

2.3 Phytochemical Screening of Plant Stems Extract

The phytochemical analysis was carried out in the Department of Biochemistry, Usmanu Danfodiyo University Sokoto. This analysis was conducted in accordance with the standard procedure by Trease and Evans [15].

2.3.1 Test for alkaloids

Two millilitres of the extract was mixed with two millilitres of 10% Hydrochloric acid. One millilitre of the solution was treated with little drops of Wagner's reagent and the other one millilitre was treated with Maye`s reagent. Turbidity and precipitation with the two reagents were observed, which indicate the presence of alkaloids [16].

2.3.2 Test for saponins

Five millilitres (5ml) of the extract solution was placed in a test tube and 5ml water was added to it and shaken. Then observe the froth formation, which indicates the presence of Saponins [16,17].

2.3.3 Test for tannins

Ferric Chloride solution 5% was added drop by drop to 2-3 ml of the extract and the color produced was observed. Condensed tannins usually give a dark green color, hydrolysable tannins give blue-black color [15,16].

2.3.4 Test for flavonoid

Three (3) millilitre aliquot of the filtrated was mixed with one millilitre of 10%NaOH sodium hydroxide, yellow color was developed, which indicate the possible presence of flavonoid compounds [16,17].

2.3.5 Test for glycosides

Two and a half millilitre of 50% H_2SO_4 was added to 5cm^3 of the extracts in a test tube. The mixture was heated in boiling water for 15minutes, then cooled and neutralized with 10% NaOH, and 5ml of Fehling's solution was added, the mixture was then boiled. A brick-red precipitate was observed which indicated the presence of glycosides [16].

2.3.6 Test for steroids (salkowski)

This was carried out according to the method of Harbone [16]. Five millilitres of the extract was dissolved in two millilitres of chloroform. Two millilitres of sulphuric acid was carefully added to form a lower layer. Reddish-brown colors at the interface indicated the presence of a steroidal ring.

2.3.7 Test for anthraquinones

Five millilitres each of the plant extract was shaken with 10 ml benzene and 5 ml of 10% ammonia solution was added. The mixture was shaken and the presence of a pink, red, or violet color in the ammonia Cal (lower) phase indicates the presence of anthraquinone [15].

2.4 Isolation of Microorganisms from Teeth

Swab sticks were used to collect sample from patients with dental caries in Specialist Hospital Sokoto. The swab sticks were transported to the laboratory of the Department of Microbiology UDUS. The swabs were dipped in test tubes containing nutrient broth and incubated for 24 hours, then inoculated on nutrient agar and blood agar later subcultured on nutrient agar. Viability test of each isolate was carried out by resuscitating the organism in nutrient agar. The organisms were preserved as stock culture at 4ºC for further used.

2.5 Microscopic Characterization of the Isolated Organisms

Gram staining was done by making a smear on a clean glass slide and allowed to air dry. After crystal violent was added, it was allowed to stand for 1 minute before it was washed off with distilled water. Grams iodine was added, allowed to stand for 1 minute and washed off with water. The preparation was decolorized with methanol for 10 seconds and washed off with water. It was then flooded with safranin which was allowed to stand for 1 minute and washed up with water.

The stained slide was air dried and oil immersion was applied, and then observed under a light microscope with x100 objective lens [18].

2.6 Biochemical Characterization of the Isolated Organisms

In order to identify, characterized a bacteria, the colony character and cell morphology have to be supplemented with routine biochemical test, as described by Manga and Oyeleke [18], which are:

2.6.1 Indole test

This was done by growing the organism in 5 ml of nutrient broth for 24 hours. After 24 hours of incubation, 3-8 drops of Kovacs indole reagent was added and shaken gently. A positive test result is indicated by the development of red color within one minute. A negative test result was indicated by the development of yellow color [18].

2.6.2 Coagulase test

Two colonies of test bacteria were emulsified in 0.5 ml of saline in a test tube and 1 ml of human plasma was added and incubated at 35ºC. It was checked after 4hrs of incubation for an increase in viscosity and clotting of the plasma after 4 hrs of the incubation. The tube was then incubated overnight at 35ºC and observed the increased viscosity and clotting indicated a positive tube coagulate test while the absence of viscosity and clotting indicated a negative coagulase test [18].

2.6.3 Catalase test

A drop of 3% hydrogen peroxide was added to a glass slide. A growth of bacteria from a solid medium was removed using a wire loop and placed on the slide. Positive test was indicated by bubbling and negative test indicated with no bubbles [18].

2.6.4 Triple sugar iron (TSI) test

With a sterile wire loop, bacterial colony was inoculated into the surface of TSI slant and stab at the butt of the media 2 to 3 times. The cap was closed loosely and incubated at 35ºC for 24 hours. The following reactions were observed:

Positive result for gas formation is the presence of one or several bubbles which may result in the cracks at the butt. Negative result is that no bubbles. Positive result for hydrogen sulphide is the presence of blackening at the whole butt. Negative result is that no blackening at the butt. Positive result of the glucose fermented is due to the presence of yellowish at the butt. Negative result is that no change in color. Positive result for the sucrose and lactose fermented is the presence of yellowish at the slant and the butt. Negative result is that no change in color [19].

2.6.5 Methyl red (MR) and voges-Proskauer (VP) test

The bacterial colony was suspended in the MR/VP medium and incubated at 37ºC for 48 hours. Three drops of methyl red was added. Positive test result was indicated by the appearance of red color. Negative test result showed no change in color [18]. One colony of the bacteria was suspended in VP/MR medium and incubated at 37ºC during 48 hours. About 0.2 ml of 40% potassium hydroxide and 0.6 ml of the alpha-naphthol solution was added. Positive test results showed color change to pink, while negative test result showed no color change [18].

2.6.6 Urease test

A bacterial colony was inoculated into a urea agar slant and incubated at 37ºC for 24 hours. A positive test result was indicated by the development of a bright pink or red color. Negative test result is that which no color change was observed [18].

2.6.7 Citrate test

A bacterial colony was inoculated into a Simon citrate agar slant and incubated at 37ºC for 24 hours. Positive test result developed a deep blue color. Negative test result was that which no color change was observed [18].

2.7 Sensitivity Test of the Plant Stems Extract

The susceptibility test of each bacterial isolate to the plant extract was assayed as described by [20]. Each bacterial isolate from slant was cultured on nutrient agar at 37°cfor 18 hrs. It was suspended in 0.85% NaCl and adjusted to match a turbidity of 0.5 McFarland standards.15 ml of sterile Mueller Hinton agar was poured into each sterile Petri dish of equal sizes and allowed to solidify. A sample portion 0.1 ml aliquot of each of the standardized bacterial cell suspension was transferred onto the surface of the dried agar

plate and spread evenly using a sterile swab stick. A cork borer of 5 mm in diameter was sterilized by flaming and used to create four wells on each Petri dish. Small amount of plain agar was poured into the designated well to seal the bottom of the well. A sample portion measuring 0.2 ml of different concentrations of the plants, which includes 500 mg/ml, 375 mg/ml, 250 mg/ml and 125 mg/ml, was poured into the designated wells that carry the concentration of the extract. The plates were allowed to stand for 15 minutes before incubating at 37ºC for 24 hours. At the end of the incubation period, the diameter of the zone of inhibition was measured in millimeter (mm) using a meter rule.

2.8 Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) was determined as the least concentration that showed an inhibitory effect on test organisms using the broth macro dilution method. A total of 24 test tubes per extract and 12 per isolate were used. One milliliter of Mueller Hinton broth was dispensed into test tubes 2-12 each for each of the extracts for an isolate. A stock solution of the extracts was prepared by dissolving 10g of the extract in 50 ml of distilled water in a conical flask giving a final concentration of 200mg/ml. One milliliter each of the stock solutions was dispensed into test tube 1 and 2. Serial dilutions were carried out using 1ml transfer through to the 10^{th}test tube. One milliliter was pipetted out of the 10^{th} tube and discarded. 1:100 dilution of the broth culture (of the test organism) was prepared and 1ml each was dispensed into test tubes 1-12 with the exception of test tube 11. 1ml of sterile Mueller Hinton broth was added into test tube 11. The test tubes were observed for growth after incubation at 37ºC for 24 hrs.[21].

2.9 Determination of Minimum Bactericidal Concentration (MBC)

No growth/turbidity (MIC and higher dilutions) was detected from all test tubes, loopfuls were inoculated onto sterile Mueller Hinton agar (Accumix – Verna, India) plates by streak plate method. The plates were then overnight incubated at 37°C. The least concentration that did not show any growth of tested organisms was considered as the MBC [21].

2.10 Statistics

No statistical analysis employed.

3. RESULTS

The phytochemical analysis revealed the presence of some secondary metabolites namely; Alkaloids, steroids, flavonoids, carbohydrates, and anthraquinones in the stems of the two trees of lime and orange as presented in Table 1.

Table 1. Phytochemical analysis of sweet orange and lime plant stem extracts

Phytochemical	Orange	Lime
Carbohydrates	+	+
Glycosides	-	-
Alkaloids	+	+
Flavonoids	+	+
Steriods	+	+
Tannins	-	-
Saponins	-	-
Anthraquinone	+	+

Shown in Table 2 are the microscopic and the biochemical characteristics of the organisms(*S. aureus, P. aeruginosa, P. mirabilis* and *K. pneumoniae*) isolated from teeth of the patients.

The sensitivity test of the crude extract of the *Citrus sinensis* showed the activity of both the aqueous and ethanolic extract on *S. aureus*. The ethanolic extract also showed activity on the *K. pneumonia*e No activity was recorded by both extracts on *P. mirabilis* and *P. aeruginosa* as shown in Table 3.

Presented in Table 4 is the zone of inhibition recorded on the two extracts of Citrus aurantifolia on the organisms isolated from teeth. Activity was seen in almost all the extracts with a similar trend being observed. No activity was recorded on all the extracts against *P. aeruginosa*.

MIC and MBC of 50 mg/ml and 100 mg/ml of the ethanolic extract of *Citrus sinensis* were recorded against *S. aureus*, which was found to be the lowest. So also, a MIC and MBC of 50 mg/ml and 100 mg/ml against an ethanolic extract of *Citrus aurantifolia* was recorded against *S. aureus* and *P. mirabilis* which was also found to be the lowest as shown in Table 5 and 6.

Table 2. Biochemical characterization of bacteria isolated from patient with dental caries

S/No.	Gram rxn	Shapes	Citrate	Indole	MR	Urease	Catalase	Glucose	Sucrose	Lactose	Motility	H2S	Gas	Gas	*Organism Identified*
1	-	Rod	-	-	+	+	-	+	+	+	-	-	+	-	*Klebsiella pneumoniae*
2	+	Cocci	+	-	-	-	+	+	+	+	-	-	+	-	*Staphylococcus aureus*
3	-	Rod	+	-	+	+	+	+	+	+	-	-	+	-	*Proteus mirabilis*
4	-	Rod	+	-	+	-	+	-	+	+	+	-	+	-	*Pseudomonas aeruginosa*

Table 3. Antibacterial activity of the aqueous and ethanol extract of the stem of *Citrus sinensis* (orange) against test isolates

Source	Isolate	Extract	Concentration (mg/ml)		
			30	60	90
Orange	*Klebsiella pneumoniae*	Aqueous	NA	NA	NA
Orange	*Klebsiella pneumoniae*	Ethanol	1.3mm	2mm	2.5mm
Orange	*Staphylococcus aureus*	Aqueous	2mm	3mm	4mm
Orange	*Staphylococcus aureus*	Ethanol	3mm	5mm	7mm
Orange	*Proteus mirabilis*	Aqueous	NA	NA	NA
Orange	*Proteus mirabilis*	Ethanol	NA	NA	NA
Orange	*Pseudomonas aeruginosa*	Aqueous	NA	NA	NA
Orange	*Pseudomonas aeruginosa*	Ethanol	NA	NA	NA

Key: mm = millimeter, NA = No Activity, mg = milligram, ml = milliliter

4. DISCUSSION

Phytochemicals are biologically active plant constituents that are naturally present in plants [22]. It is believed that phytochemicals take part in resisting diseases formation in plants [23]. The phytochemical screening of *Citrus sinensis* stem extract revealed the presence of carbohydrates, alkaloids, flavonoids, steroids and anthraquinones. The presence of these compounds gives an insight into the medicinal importance of the stems of the tree, as flavonoids were reported to have antibacterial and antimicrobial properties [24]. Similarly, the phytochemicals detected in *C. sinensis* were the same found in *Citrus aurantifolia* stem. This does not completely agree with the findings of Abdallah [25] who reported the presence of anthraquinones and the absence of saponins and tannins in the methanolic extract of the leaf of *C. aurantifolia*.

The bacteria isolated from patient with dental caries were *Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus,* these organisms are normal flora of the oral cavity, which are opportunistic and are found to play important role in tooth plaque, gingivitis and dental caries, this is in line with the findings of Khushbu and Satyam [21].

Our result shows that the ethanolic extract of *Citrus sinensis* stem has antibacterial activity against *K. pneumoniae* and *S. aureus* with the aqueous extract being active only against *S. aureus*. Hany *et al*. [22] also reported antibacterial activity of *Citrus sinensis* on *S. aureus*. No antibacterial activity was recorded on by both extracts (aqueous and ethanolic) of the orange tree stem. The extracts that showed promising potentials have their MIC and MBC and assayed, which shows that 50 mg/ml and 100 mg/ml of the ethanolic extract were the minimum concentration of the extract that can inhibit the growth of the *S. aureus* as well as the minimum concentration of the extract that can kill the bacteria.

Similarly, ethanolic and aqueous extract of *Citrus aurantifolia* were found to have antibacterial activity on *C. pneumonia, S. aureus* and *P. mirabilis*. No activity was seen in *P. aeruginosa*. MIC and MBC of 50 mg/ml of the ethanolic

Table 4. Antibacterial activity of the aqueous and ethanol extract of the stem of *Citrus aurantifolia* (lime) against test isolates

Source	Isolate	Extract	Concentration (mg/ml)		
			30	60	90
Lime	*Klebsiella pneumoniae*	Aqueous	NA	3mm	5mm
Lime	*Klebsiella pneumoniae*	Ethanol	1.7mm	2mm	3mm
Lime	*Staphylococcus aureus*	Aqueous	3mm	5mm	7mm
Lime	*Staphylococcus aureus*	Ethanol	5mm	7mm	10mm
Lime	*Proteus mirabilis*	Aqueous	NA	NA	2.5mm
Lime	*Proteus mirabilis*	Ethanol	1mm	2mm	3mm
Lime	*Pseudomonas aeruginosa*	Aqueous	NA	NA	NA
Lime	*Pseudomonas aeruginosa*	Ethanol	NA	NA	NA

Key: mm = millimeter, NA = No Activity, mg = milligram, ml = milliliter

Table 5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *Citrus sinensis* (orange)

Isolates	Plant extract	Extract concentration (mg/ml) for MIC									MBC (mg/ml)
		200	100	50	25	12.5	6.25	3.125	1.56	0.78	
Klebsiella pneumoniae	Ethanol	-	-	+	+	+	+	+	+	+	200
Staphylococcus aureus	Aqueous	-	-	+	+	+	+	+	+	+	200
Staphylococcus aureus	Ethanol	-	-	-	+	+	+	+	+	+	100

Key: (-) = absence of growth; (+) = presence of growth, mg = milligram, ml = milliliter
MIC= Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration

Table 6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *Citrus aurantifolia* (lime)

Isolates	Plant extract	Extract concentration (mg/ml) for MIC									MBC (mg/ml)
		200	100	50	25	12.5	6.25	3.125	1.56	0.78	
Klebsiella pneumoniae	Ethanol	-	-	+	+	+	+	+	+	+	200
Staphylococcus aureus	Aqueous	-	-	+	+	+	+	+	+	+	200
Staphylococcus aureus	Ethanol	-	-	-	+	+	+	+	+	+	100
Proteus mirabilis	Ethanol	-	-	-	+	+	+	+	+	+	100

Key: (-) = absence of growth; (+) = presence of growth, mg = milligram, ml = milliliter
MIC= Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration

extracts of the stem of the lime tree were recorded against *S. aureus* and *P. mirabilis*. Abdallah [25] reported the antibacterial activity of the methanolic extract of the leaf of C. arantifolia on *K. pneumoniae, S. aureus and P. aeruginosa*. Pathan *et al*. [26] also reported antibacterial activity of the hydroalcoholic leaf extract on *S. aureus, K. pneumonia*e, *Escherichia coli and Pseudomonas* species.

5. CONCLUSION

Citrus sinensis and *Citrus aurantifolia* are important trees with many domestic uses and ethnopharmacological importance. There fruits stems, as well as the waste of the fruits, are consumed and employed for various applications. This study conducted to determine the phytoconstituents and antibacterial activity of the extracts of their stems revealed the presence of different bioactive compounds and the extract were active against most of the organisms associated with dental caries. Further studies are required to understand the nature of the antibacterial compounds present and also other biological importance of the stem extract and other parts of the plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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