Original Research Article

Phytochemical Screening and Antimicrobial Analysis of *Vernonia amygdalina* and *Psidium guajava* Stems on Bacteria Associated with Dental Caries

Abstract

Background: Chewing stick has long been used in many parts of Africa and the Middle East as a means of oral hygiene. Dry stems or Roots of different plants have been used in the process. Stems of *Vernonia amygdalina and Psidium guajava* are among the commonly used plants in Nigeria in cleansing teeth. Few attempts have been 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 Phytoconstituents and the antimicrobial activity of *Vernonia amygdalina and Psidium guajava* on Bacteria isolated from human teeth.

Materials and Methods: Phytoconstituents of the aqueous and ethanolic extract of the stems of Bitter leaf and Guava 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 and carbohydrates. Highest zone of inhibition of 9 mm and 10 mm was recorded on the ethanolic extracts of bitter leaf and Guava tree stems on *Staphylococcus aureus* respectively. MIC and MBC of 50 mg/ml and 100 mg/ml for the ethanolic extracts of Bitter leaf stem on *Klebsiella pneumonia, S. aureus* and *Proteus mirabilis* were recorded. For the Guava, MIC and MBC of 50 mg/ml and 100 mg/ml were recorded for the ethanolic extracts were recorded on *S. aureus* and *Proteus mirabilis*.

Conclusion: Aqueous and ethanolic extracts of both plants show potential antibacterial activity against the microorganisms isolated from human teeth.

Keywords: chewing stick; Phytoconstituents; Ethanolic extract; antimicrobial activity

1.0 Introduction

Vernonia amygdalina is commonly call bitter leaf because of its bitter taste. It is a member of the Asteraceae family and a small evergreen shrub that grows all over Africa. It is reported to be a

medicinal plant for diabetes and fever (Crellin *et al*., 1989). Bitter herbs are reportedly good for the body as they help tone the vital organs of the body like the kidney and liver. Ethnomedically, the leaves are consumed either as a vegetable (macerated leaves in soup) or aqueous extracts as tonics for the treatment of various illnesses (Igile *et al*., 1995). In the wild, chimpanzees have been observed to ingest the leaves when suffering from parasitic infections (Huffman *et al*., 1993). The roots of *V. amygdalina* have been used for gingivitis and toothache due to its proven antimicrobial activity (Ademola and Eloff, 2011). In North America, of the 17 species of *Vernonia* all have the same effective properties as a blood purifier, uterus toner and helps also to prevent atherosclerosis (Erasto *et al*., 2007; Nwanjo, 2005).

The apple guava (*Psidium guajava*) or common guava has nearly a global presence**.** It is an evergreen shrub or small tree native to Mexico, the Caribbean, and Central and South America. *Psidium guajava* is a common shade tree or shrub indoor-yard gardens in the tropics. The tree is easily identified by its distinctive thin, smooth, copper-colored bark that flakes off, showing a greenish layer beneath. Lozoya *et al*., 2002 reported that the phytochemical analyses of guava leaf, revealed the presence of more than 20 isolated compounds with quercetin as the main active substance. Spasmolytic and antidiarrheal effects are reported to be associated with its quercetin-derived, flavonoids and glycosides, which support use of this ancient leaf remedy in treating gastrointestinal disorders (Joseph and Priya, 2011). The plant is used in many different shampoo products for its scent. Local preparations made from the leaves and/or bark of *Psidium guajava* are reported to be useful in treatments of diarrhea, dysentery, sore throats, vomiting, stomach upsets and vertigo. They have also been found to be effective in regulating menstrual periods throughout the tropical Amazon and India (Holetz *et al*., 2002).

Chewing stick or Miswak in Arabic has been known for century as a tooth cleanser ignored for modern toothbrushes and inter-dental cleaners (Hyson, 2003). They are popular in many parts of Africa and the Middle-East as a means of oral hygiene. In using the stick, the end of the stick is chewed into a fibrous brush which is rubbed against the teeth and gum (Taiwo, 1999). Some of the chewing sticks predominantly used in Northern Nigeria are *Azadirachta indica* (Neem), *Psidium guajava* (Guava), *Citrus sinensis* (Orange) and *Citrus aurantifolia* (Lime) (Nata'ala *et al.,* 2018)

2.0 Materials and methods

2.1 Sample Collection and Processing

The fresh stems of *Psidium guajava* (Guava) was collected from Abdullahi Fodiyo Library Usmanu Danfodiyo University Sokoto (UDUS) and the fresh *Vernonia amygdalina* (bitter leaf) was purchased at Kasuwan nama, Sokoto. The plants were authenticated in the Botany unit, Biology Department of UDUS. The fresh stems were cut in to small pieces and air-dried at room temperature for three weeks. The dried stems were pounded and sieved to obtain fine powder, which was store in airtight bottle until needed.

2.2 Extraction of Plant Stems

Aqueous extract was obtained by dissolving 40g of each plant powder in 400ml of distilled water separately in a conical flask. 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 Whatmann No-1 filter paper. The filtrate was evaporated to dryness on a steam bath at 45°C. The extract was then recovered and weighed. The above procedure was repeated using 400 ml ethanol to obtain ethanolic extract.

2.3 Phytochemical Screening of Plant Stems Extract

The phytochemical analysis was conducted at the Department of Biochemistry, Usmanu Danfodiyo University Sokoto in accordance with the standard procedures as described by Harbone, [1973; 1998] as well as Trease and Evans (1989).

2.3.1 Test for Alkaloids

To 2 ml of each of the extract, 2 ml of 10% Hydrochloric acid was added and mixed. 1ml from the above mixture was treated with few drops of Wagner's reagent and another 1ml treated with few drops Maye`s reagent. Turbidity or precipitation with both of the two reagent was considered as an indication for the presence of alkaloids.

2.3.2 Test for Saponins

Four milliliter (4 ml) of each of the extract was placed in a test tube followed by 4 ml of distilled water. The mixture was shaken vigorously. Froth formation indicate the presence of Saponins.

2.3.3 Test for Tannins

Ferric Chloride solution (5%) was added drop by drop to 2 ml of each of the extract and the color produced was noted. Condensed tannins usually give a dark green color, while hydrolysable tannins give blue-black color.

2.3.4 Test for Flavonoid

To 3 ml of each of the extract, 1ml of 10%NaOH sodium hydroxide was added and mixed. Yellow color formation indicates the possible presence of flavonoid compounds.

2.3.5 Test for Glycosides

To 5 ml of each of the extract in a test tube, 2.5 ml of 50% H_2SO_4 was added and mixed. The mixture was heated in a boiling water for 15 minutes. After cooling, the mixture was neutralized with 10% NaOH. From the above mixture, 5 ml was mixed with 5 ml of Fehling's solution and the resultant mixture was boiled in a water bath. A brick-red precipitate indicate the presence of glycosides.

2.3.6 Test for Steroids (Salkowski)

Five milliliters (5 ml) of each of the extract was dissolved in 2 ml of chloroform. Two milliliters (2 ml) of concentrated sulphuric acid was carefully added down the side of the test tube to form two layers. Reddish-brown color at the interface indicates the presence of a steroidal ring.

2.3.7 Test for Anthraquinones

To 2.5 ml of each of the plant extract, 10 ml benzene was added and shaken followed by 5 ml of 10% ammonia solution. The mixture was shaken and the presence of a pink, red, or violet color in the lower phase indicates the presence of anthraquinones.

2.4 Isolation of Organisms

Swab sticks were used to collect sample from patient with dental carries from Specialist Hospital Sokoto. The swab sticks were transported to the Microbiology Department Laboratory. They were dipped in test tubes containing nutrient broth and incubated for 24 hours. They were then inoculated on nutrient agar and blood agar before being sub cultured on nutrient agar. Viability test of each isolate was carried out by resuscitating the organism in nutrient agar. The organism was preserved as stock culture for further used.

2.5 Media Preparation

2.5.1 Nutrient Agar Medium (NA)

Nutrient Agar Medium was prepared by dissolving 28 g of nutrient agar in 1000 ml of distilled water in a conical flask. The conical flask was plugged with cotton wool and wrapped with

aluminum foil. The medium was then heated to obtain the homogeneous solution. Later, the solution was sterilized in the auto clave at 121°C for 15 minutes. After sterilization, the medium was allowed to cool to 45°C after which it was dispensed into the sterilized petri dishes and allowed to solidify (Oyeleke and Manga, 2008).

2.5.2 Preparation of Mueller Hinton Agar

This Medium was prepared by dissolving 38g in 1000 ml of distilled water. It was heated with frequent agitation for one minute to completely dissolve the medium. The medium was sterilized in an autoclave at 121^0C for 15 minutes. It was then allowed to cool to 45^0C.

2.6 Gram Staining

This was done by making a smear on a clean glass slide which was allowed to air dry. Crystal violent was added and allowed to stand for 1 minute before being washed off with water. The preparation was then treated with Iodine as with Crystal violet above. The preparation was decolorized using methanol for 10 seconds and washed off with distilled water followed by the addition of safranin. After 1 minute, the preparation was washed off with water and air-dried. The slide was observed under light microscope with x100 objective lens after oil immersion (Oyeleke and Manga, 2008).

2.7 Identification of Bacteria by Biochemical Characterization

In order to identify and characterize the isolated bacteria, the colony character and cell morphology have been supplemented with routine biochemical tests, as described by Oyeleke and Manga (2008) as well as (Cheesbrough, 2002). The procedures are briefly described below,

2.7.1 Indole Test

This was done by growing the organism in 5 ml of nutrient broth for 24 hours. After 24 hours of incubation, 3 drops of kovacs indole reagent was added and shaken gently. Development of red color within one minute was taken as positive.

2.7.2 Coagulase Test

About 2-3 colonies of test bacteria was emulsified with 0.5 ml of saline in a test tube and 1ml of human plasma was added and incubated at 35^0C for 4hrs. Increase in viscosity or clotting of the plasma after 4hrs of the incubation was checked. Then the incubation continues over night at 35^0C.

2.7.3 Catalase Test

A drop of 3% hydrogen peroxide was dropped on a glass slide. Using a wire loop, a growth of bacteria from a solid medium placed on the slide. Bubbling was taken as positive.

2.7.4 Triple Sugar Iron (TSI) Test

Using a sterile wire loop, bacterial colony was inoculated into the surface of TSI, which was slanted and stabbed at the butt of the media 2 to 3 times. The cap was closed loosely and incubated at 35^0C for 24 hours.

Blackening at the whole butt, yellowish at the butt or yellowish at the slant and the butt was taken as positive for hydrogen sulphide, glucose or sucrose and lactose respectively.

2.7.5 Methyl Red (MR) and Voges-Proskauer (VP) Test

The bacterial colony was suspended in the MR/VP medium and incubated at 37^0C for 48 hours. About 2-3 drops solution of methyl red was added. The presence of red color was taken as positive.

Another colony of the bacteria was suspended in VP/MR medium and incubated at 37^0C for 48 hours. About 0.2 ml of 40% Potassium hydroxide and 0.6 ml of the alpha-naphthol solution was added. A pink color was taken as positive (Manga and Oyeleke, 2008).

2.7.6 Urease Test

A bacterial colony was inoculated in a urea agar slanted and incubated at 37^0C for 24 hours. A bright pink or red color development was taken as positive.

2.7.7 Citrate Test

A bacterial colony was inoculated in to a Simmon citrate agar. Then, it was slanted and incubated at 37^0C for 24 hours. A deep blue color development was taken as positive.

2.8 Sensitivity Test of the plant stems Extract

The susceptibility test of each bacterial isolate to the plant extract was assessed as described by (Aliyu *et al.,* 2009). Each bacterial isolate from slant was cultured on nutrient agar at 37^0C for 18 hours. It was suspended in saline solution (0.85% NaCl) and adjust to match a turbidity of 0.5 McFarland standard. Fifteen milliliter (15 ml) of sterile Mueller Hinton agar was poured into

each sterile Petri dish of equal sizes and allowed to solidify. An aliquot (0.1 ml) 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. Four wells on each Petri dish was created using a sterilized cork borer (5mm in diameter). Small amount of plain agar was poured into the designated well to seal the bottom of the well. Zero point two milliliter (0.2 ml) of different concentration of the plant extract (500mg/ml, 375mg/ml, 250mg/ml and 125mg/ml) was poured into the designated wells. The plates were allowed to stand for 15 minutes before incubating at 37^0C 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.

2.9 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 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 50ml of distilled water in a conical flask giving a final concentration of 200mg/ml. Each of the stock solutions (1ml) was dispensed into tube 1 and tube 2. Serial dilutions were carried out using 1ml transfer through to the 10^{th} 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 tubes 1-12 with the exception of tube 11. A sterile Mueller Hinton broth (1ml) was added into tube 11. The tubes were incubated at 37^0C for 24 hours before being examined for growth (Aamer *et al*., 2015).

2.10 Determination of Minimum Bactericidal Concentration (MBC)

A loopfuls from all tubes that showed no visible signs of growth/turbidity (MIC and higher dilutions), were inoculated onto sterile Mueller Hinton agar (Accumix – Verna, India) plates by streak plate method. The plates were then incubated at 37°C overnight. The least concentration that did not show any growth of tested organisms was considered as the MBC (Aamer *et al*., 2015).

3.0 Results and Discussion

The medicinal value of plants lies in some chemical substances that have a definite physiological action on the human body. Different phytochemicals have been found to possess a wide range of Pharmacological and biochemical activities, which may help in protection

against chronic diseases. For example, alkaloids protect against chronic diseases. Saponins protect against hypercholesterolemia and antibiotic properties. Steroids and triterpenoids show the analgesic properties. The steroids and saponins were responsible for central nervous system activities (Mir, 2016; Iwu *et al.,* 1999).

Table 1: Preliminary Phytochemical Analysis of Bitter Leave and Guava.

Phytochemical	Bitter leaf	Guava
Carbohydrates	+	+
Glycosides	+	-
Alkaloids	+	+
Flavonoids	+	+
Steriods	+	+
Tannins	-	-
Saponins	-	-
Anthraquinone	+	-

+ = present; - = absent

The phytochemical screening of *Vernonia amygdalina* (bitter leaf) and *Psidium guajava* (guava) show the presence of Alkaloids, steroid, Flavonoids and carbohydrates while Saponins and tannins were absent in all the study plants. Glycosides and Anthraquinone was found in bitter leaf only (Table 1). The result of the study contradict the finding of Chollom *et al* (2012) who found the presence of Glycosides, Tannins and Saponins from Aqueous leaf extract of *Psidium guajava*. Likewise, Imaga and Bamigbetan (2013) show the presence of Tannins and Saponins from Aqueous extract of *Vernonia amygdalina*.

Table 2: Antibacterial Activity of the Aqueous and Ethanol Extract of the Stem of *Vernonia amygdalina and Psidium guajava* against Test Isolates

Isolates	Bitter leaf						Guava					
	Aqueous Concentration (mg/ml)			Ethanol Concentration (mg/ml)			Aqueous Concentration (mg/ml)			Ethanol Concentration (mg/ml)		
	30	60	90	30	60	90	30	60	90	30	60	90
Klebsiella pneumoniae	NA	NA	NA	1mm	1.5mm	2mm	NA	NA	NA	1mm	2mm	3mm
Staphylococcus aureus	2mm	3mm	6mm	3mm	5mm	9mm	1.3mm	1.7mm	2mm	4mm	3mm	10mm
Proteus mirabilis	NA	NA	NA	2mm	3mm	4mm	NA	NA	NA	2mm	3mm	4mm
Pseudomonas aeroginosa	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Key: mm = Millimeter, NA = No Activity, mg = Milligram, ml = Milliliter

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 carries (Khushbu and Satyam, 2016).

The antibacterial activities tests of the aqueous (water) and ethanolic extracts of bitter leaf and guava were assayed *in vitro* by agar well diffusion method against the four (4) isolated bacterial species. The result showed that, the ethanolic extract of bitter leaf was effective against *Klebsiella pneumonia*, *Staphylococcus aureus* and *Proteus mirabilis* at tested concentrations while the aqueous extract shows activity against *S. aureus* only (Table 2). This could be due to the inability of the aqueous extract to fully extract all the bioactive ingredients. Moreover, the ethanolic extract of bitter leaf was ineffective on *Pseudomonas aeruginosa* probably because it is resistance to the extracts, this correspond with the finding of Ndukwe *et al*. (2018).

At concentrations of 30mg/ml, 60mg/ml and 90mg/ml (1mm, 2mm and 3mm), only the ethanolic extract of the guava was effective against *Klebsiella pneumonia*. This could be due to the ability of the ethanol to fully extract most of the bioactive ingredients from the study plant. The extracts were effective against *S. aureus* at concentration of 30mg/ml, 60mg/ml and 90mg/ml (3mm, 5mm and 7mm) for aqueous and (4mm, 6mm and 10mm) for ethanolic extract. However, only the ethanolic extract of the guava was effective against *Proteus mirabilis* at concentration of 30mg/ml, 60mg/ml and 90mg/ml (2mm, 3mm and 4mm). This may be due to the inability of the aqueous extract to fully extract all bioactive ingredients. It also remain ineffective on *Pseudomonas aeruginosa* due to its high resistance to the extracts. Flávia *et al*, (2008), also reported similar finding.

Several studies were conducted to find a suitable alternative to synthetic antibiotics for the management of diseases caused by bacteria. The antibacterial activity was tested by minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) against the isolate (Table 3).

The result of antibacterial assay from this study confirm that the ethanolic extract of Bitter leaf had antibacterial potential against all the isolates. This is shown by the complete inhibition of the isolate at 50mg/ml MIC and 100mg/ml MBC (Table 3). At these concentrations, there was no observable growth in any of the isolate. However, the aqueous extract of the plant shows antibacterial activity against *Staphylococcus aureus* only at 100mg/ml MIC and 200mg/ml MBC (Table 3).

Table 3: MIC and MBC for *Vernonia amygdalina* (Bitter leaf) and *Psidium guajava* (Guava)

Source	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	
***Vernonia amygdalina* (Bitter leaf)**	*Klebsiella pneumonia*	Ethanol	-	-	-	+	+	+	+	+	+	100
	Staphylococcus aureus	Aqueous	-	-	+	+	+	+	+	+	+	200
	Staphylococcus aureus	Ethanol	-	-	-	+	+	+	+	+	+	100
	Proteus mirabilis	Ethanol	-	-	-	+	+	+	+	+	+	100
***Psidium guajava* (Guava)**	*Klebsiella pneumonia*	Ethanol	-	-	+	+	+	+	+	+	+	200
	Staphylococcus aureus	Aqueous	-	-	-	+	+	+	+	+	+	100
	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.

This is probably because not all the bioactive ingredients was fully extracted by the aqueous solution.

This result support the finding of Taiwo et al 1999, who show that Bitter leaf extract has antibacterial potential against wide spectrum of bacteria significant to periodontal disease. This is not surprising considering the phytochemical composition found in the plant extract (Table 1).

Ethanolic extract of Guava has also demonstrated antibacterial potential against the isolate (except for *Klebsiella pneumonia*) at 50mg/ml MIC and 100mg/ml MBC (Table 3). The inactivity of the extract against *Klebsiella pneumonia* may be due to its insusceptibility to the extract. However, unlike Bitter leaf, aqueous extract of Guava was active against *Staphylococcus aureus* at 50mg/ml MIC and 100mg/ml MBC (Table 3). Several studies indicated remarkable antibacterial activity of Guava against common diarrhea – causing bacteria such as *Staphylococcu, Shigella, salmonella, Bacillus, E. coli, Clostridium* and *Pseudomonas* (Chollom, 2012). The antimicrobial activity of Guava is attributable to Guajaverine and Psydiolic acid (Berdy *et al*., 1981).

4.0 Conclusion

This study was conducted to determine the phytochemical constituents and antibacterial activity of the extracts of the stems of bitter leaf and Guava against bacteria associated with dental carries. Different bioactive compounds were found present in the extracts. Moreover, the extracts of both plants show potential antibacterial activity against the organisms tested which justify ethnomedicinal uses of the plants.

COMPETING INTERESTS

There was no competing interests exist.

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