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Pharmacognostic and Antimicrobial Activity of Aqueous and Methanol Extracts of *Diospyros mespiliformis* Stem

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Abstract

Chewing sticks have proven to be effective in removing dental plaque due to mechanical cleaning and enhanced salivation. Chewing sticks from other plants have been shown to display antimicrobial activities against a broad spectrum of microorganisms. However, there is limited information available in Nigeria on the chemical composition, antibacterial properties of medicinal plants. Therefore, this study aimed to ascertain the pharmacognostic profile, phytochemical and antibacterial properties of Diospyros mespiliformisand correlate the results obtained to their ethno medicinal uses as chewing sticks. Powdered stem of Diospyros mespiliformis was exhaustively extracted using distilled water and methanol at room temperature for 48 hours. Antibacterial activities of the crude extracts were assessed using the agar well diffusion methods against the oral pathogens, Streptococcus mutans, Staphlococcus aureus and Streptococcus sallivarus. Chemomicroscopical evaluation revealed the presence of cellulose, tannins, starch, lignin, calcium oxalate, suberin, aleurone grain and mucilage with the exception of calcium carbonate. The average moisture contents, total ash, acid insoluble, water soluble ash, alcohol extractive value and water extractive values were 7.60%, 8.40%, 2.10%, 4.90%, 16.90% and 19.60% respectively. In addition, Fe, Mn, Ni, Pb, Zn, Cd and Cu were found to be within the safety limit. Carbohydrates, saponins, flavonoids and tannins, cardiac glycosides, triterpenes, alkaloids were observed to be present.The sensitivity test results showed that the test isolates were sensitive to the extract at 250mg/ml.Lethal dose(LD_{50}) of both extracts was above 5000 mg/kg and did not cause mortality in all the tested rats.Further research on isolation and characterization of the active compounds from fractions that showed antibacterial and anti-adhesive activity is recommended.

Keywords: Chewing sticks, *Streptococcus mutans, Staphlococcus aureus, Streptococcus sallivarus,* Phytochemical

INTRODUCTION

Dental diseases are among the major public health problems at the global level that affect mankind. The oral cavity is inhabited by microbial species and many intrinsic as well as extrinsic factors affect the composition, pathogenicity and metabolic activity of the highly diversified oral microbial flora. (Ishnava, 2018). Oral bacteria involved in the bloodstream have been linked to coronary artery disease, atherosclerosis and stroke (Ishnava, 2018). The main characteristic of Dental disease is destruction of supporting tissue of the tooth by microbial oral biofilm. Dental health is integral to general wellbeing and relates to the quality of life that extends beyond the functions of the craniofacial complex (Petkovic*et al.,* 2015; Ishnava, 2018). Antibiotic like tetracyclines and metronidazole, antiseptic like chlorhexidine have been used for a very long time, but many side effects observed to be associated with its use (Ishnava, 2018). Herbal medicine has an edge over conventional antibiotic treatment which suffer the limitation of low benefit to high risk as compared to herbal treatment that possess high benefit to low-risk ratio. Herbal medicine has been used for long period to prevent and treat different dental diseases (Ishnava, 2018). The major problem is the lack of the information and traditional knowledge about effects of medicine on dental disease. Thisnecessitates the urgent need to

reviewinformation for researcher to help infocusing herbal medicine in dental diseases treatment. Herbal medicine may vary in their effectiveness; therefore, it is necessary to select herbal medicine very carefully. Herbal medicine and their extract can be used as adjuvant in dental disease treatment. Different dental diseases treatable with herbal medicine are common intraditional health practice namely: dental caries, toothache, gingivitis, ulcerative gingivitis, mouth ulcers, swollen tonsil, oral thrush, tonsillitis and black tongue (Ishnava, 2018).

Furthermore, natural products, either pure compounds, or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Dangoggo *et al.,* 2012). In the past the wide range of antimicrobial agents from lower organisms and synthetic drugs sufficed in the treatment or control of infectious diseases, but currently there is a problem of microbial drug resistance and there is an increase of opportunistic infections especially with AIDS patients and individuals on immunosuppressive chemotherapy (Dangoggo *et al.,* 2012).

Diospyros mespiliformis is known in Hausa as Kanya and in Yoruba as Igidudu, It is commonly called Jackal-berry or African ebony. It is found in Savanna and Northern low land forest. It is an ever green tree of 12-15 m height but sometimes reaching up to 20 m or more in the rain forest (Aliyu, 2006). The leaves are simple alternate dark green with small hairs on the underside of old leaves. Mature fruits are large yellow berries (NRC, 2008). The fruit of this plant is a traditional food of high nutritive value in Africa. The leaf extract is used against fever and syphilis. It is also used as an antihelmintic and as a wound dressing agent (NRC, 2008). Since the claimed pharmacological activities of this plant should be linked to one or more of its phytochemical constituents, there is the need to establish its pharmacognostic profile and antibacterial activity for the better prediction and easier confirmation of the medicinal value of this plant. This study was therefore necessary to establish the pharmacognosticprofile and antibacterial activity of this plant.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

The stems of *Diospyros mespiliformis*were collected from local farm in May, 2018 at Madobi Local Government Area, Kano state. The plant was identified and authenticated in the Herbarium of the Plant Biology Department of Bayero University, Kano and was compared with a voucher specimen numberBUKHAN121.

Preparation of Plant extracts

Fifty grams (50g) each of the powdered stem was added to 500ml each of methanol and distilled water respectively. Each was allowed to stand for 3days at room temperature (28 ±2^{o}C), with agitations at intervals. Each extract was sieved through a muslin cloth, filtered through a Whatman (no.1) filter paper, poured unto a clean evaporating dish and placed on a water bath at 50^{o}C until all the solvent evaporated.

Chemo-microscopic Studies of the Powdered Stem of *Diospyros mespiliformis*

Powdered sample (5g) of plant species was used for this study to detect the presence of cell wall materials and cell inclusions. Finely ground sample of plant was cleared in a test tube containing 70% chloral hydrate solution. It was boiled in a water bath for about thirty minutes to remove obscuring materials. The cleared sample was mounted with dilute glycerol onto a microscope slide. Using various detecting reagents the presence of cell wall materials and cell inclusions were detected in accordance to WHO (2011) guidelines.

Cell wall Materials

Test for Cellulose

A drop or two of iodised zinc chloride was added to the powdered sample and allowed to stand for a few minutes and observed under a microscope. It stained cellulose cell wall blue to blue-violet.

Test for Lignin

The powdered plant material was moistened on a slide with a small volume of phloroglucinol and allowed to stand for about two minutes or until almost dry. A drop of hydrochloric acid

was added and viewed under a microscope. Pink stained or cherry red was observed for the presence of lignin.

Test for Suberized or Cuticular cell walls
A drop or two of Sudan red was added to the cleared powdered sample and allowed to stand for few minutes and observed under a microscope. Orange red or red colour was observed presence of suberin or cutin on the cell (WHO, 2011).

Test for Gum and Mucilage
To a small portion of the cleared powdered sample of the plant, a drop of ruthenium red was added. Appearance of pink coloration was considered positive for gums and mucilage(WHO, 2011).

Cell Inclusions/ Cell Contents
Test for Starch grains
To a small portion of the cleared powder sample of the plant, N/50 iodine was added. Appearance of blue-black or reddish-blue coloration on some grains would be considered positive for starch(WHO, 2011).

Test for Calcium oxalates and Calcium Carbonates
To a small portion of the cleared powdered sample of the plant, HCl was added, dissolution of crystals in the powdered drug without effervescence was considered positive for calcium oxalate while slow dissolution with effervescence was considered positive for calcium carbonate(WHO, 2011).

Inulin
A drop of 1-naphthol and that of sulphuric acid was added to the powdered sample and viewed under the microscope. Spherical aggregations of crystals of Inulin turned brownish red and dissolve(WHO, 2011).

Test for Tannins
To a small portion of the cleared powdered sample of the plant, 5% ferric chloride solution was added. Appearance of greenish black colour was considered as positive for tannins(WHO, 2011).

Determination of Physicochemical Constants of the Powdered Stem of *Diospyros mespiliformis*
Some physicochemical parameters of the powdered sample were determined as described in the updated edition of quality control methods for medicinal plant materials (WHO, 2011).

Moisture Content
This is the quantity of moisture present in a plant material. Moisture content of the powdered sample will be determined by loss on drying method.

3.0g each of the powdered sample was accurately weighed and placed in some clean, dried evaporating dishes of known weights. They were placed in an oven and heated at a temperature of 105^{o}C for 1 hour, then cooled in a dessicator and re-weighed. Heating and weighing were repeated until a constant weight was obtained. The weight loss on drying was computed following the formula below:

$$\% \text{ Moisture content} = \frac{\text{Initial Weight of Powder} - \text{Final Weight of Powder}}{\text{Initial Weight of Powder}} \text{X } 100$$

Total Ash Value

2g of powdered plant materials was accurately weighed and placed separately in a crucible of known weight. It was heated gently and the heat gradually increased until it is white indicating the absence of carbon. It was allowed to cool in a desiccator and weighed; this was repeated until a constant weight was obtained. The total ash value was determined as a percentage with the formula below

$$\% \text{ Ash Value} = \frac{\text{Weight of Residual Ash}}{\text{Original Weight of Powder}} \text{ X } 100$$

Acid-insoluble ash

This is the residue that remains after boiling the total ash with dilute hydrochloric acid.

This was determined for the powdered plant material. 25ml of dilute hydrochloric acid was added to the crucible containing ash. It was covered with a watch glass and gently boiled for 5mins. The watch glass was rinsed with 5ml of hot water and the liquid added to the crucible. The insoluble matter was collected on an ash less filter-paper and washed with hot water until the filtrate is neutral. The filter-paper containing the insoluble matter was transferred to the original crucible, dried in an oven and ignited to a constant weight. The residue was allowed to cool in a suitable desiccator for 30 minutes and then weighed without delay (Evans, 2002).

The acid-insoluble ash will then be calculated as a percentage for each of the two plants with the formula

$$\% \text{ Acid insoluble Ash} = \frac{\text{Weight of Residual Ash}}{\text{Original Weight of Powder}} \text{ X } 100$$

Water soluble ash

To the crucible containing the total ash, 25ml of water was added and boiled for 5 minutes. The insoluble matter was collected in a sintered glass crucible. It was then be washed with hot water and ignited in a crucible for 15 minutes at 105^{o}C. The weight of the residue was subtracted from the weight of the total ash. The content of water soluble ash per air dried powdered sample was calculated and recorded (WHO, 2011).

$$\% \text{ Water Soluble Ash} = \frac{\text{Weight of Total Ash} - \text{Weight of Residual Ash}}{\text{Original Weight of Powder}} \text{ X } 100$$

Alcohol-Soluble Extractive Value

This is the amount of extraction in percentage of a plant sample with alcohol.

4g of each of the plant material was separately weighed in a conical flask. 100ml of ethanol was added and macerated for 24 hours, during which the mixture was frequently shaken within the first 6hours using a mechanical shaker. It was filtered and 25ml of the filtrate transferred into an evaporating dish of known weight and evaporated to dryness on a water bath. It was dried to a constant weight, the percentage of alcohol-soluble extractive value was then determined for the plant as

$$\text{Alcohol-Soluble Extractive Value (\%)} = \frac{\text{Weight of Extract in 25ml X 4}}{\text{Original Weight of Powder}} \text{ X } 100$$

Water-Soluble Extractive Value

This is the amount of extraction in percentage of a plant sample with water. Same procedure as in alcohol-soluble extractive value was repeated here for the two plants, but solvent for extraction here was water.

$$\text{\% Water Extractive Value} = \frac{\text{Weight of Extract in 25ml X 4}}{\text{Original Weight of Powder}} \text{ X } 100$$

Elemental analysis of the Powdered Stem of *Diospyros mespiliformis*

The elemental analyses of the plant materials were carried out in Ahmadu Bello University Zaria, Multi-user Research Laboratory. Powdered plant material was digested using 2.5ml of hydrochloric acid (HCl) and 7.5ml Nitric Acid (HNO3). The concentration of Fe, Mg, Zn, Cu was read using the flame atomic absorption spectrophotometer (FAAS), AA 500 model, Atomic Emission Spectrophotometer. Atomic Absorption Spectrophotometer were used for other elements. Before determining the concentration of any element in the sample, calibration curve of the element in the sample was prepared using prepared standard stock solutions for the elements as reported by AOAC, 2000; 2005; Akpabio and Ikpe (2013).

Qualitative Phytochemical screening of the Aqueous and Methanolic extract of *Diospyros mespiliformis*Stem

The plant extracts (aqueous and methanol) were subjected to phytochemical screening in order to identify the phytochemical constituents of the plant using the method described below.

Tests for carbohydrates

Molish's (General) Test for Carbohydrates: To 1 ml of the filtrate, 1 ml of Molish's reagent was added in a test tube, followed by 1 ml of concentrated sulphuric acid down the test tube to form a lower layer. A reddish colour at the interfacial ring indicates the presence of carbohydrate (Evans, 2009).

Tests for Saponin

Frothing test: About 10ml of distilled water was added to a portion of the extract and was shaken vigorously for 30seconds. The tube was allowed to stand in a vertical position and was observed for 30mins. A honeycomb froth that persists for 10-15mins indicates presence of saponin.

Test for Flavonoids

Shinoda Test: A portion of the extract was dissolved in 1-2ml of 50% methanol in the heat metallic magnesium chips and few drops of concentrated hydrochloric acid were added. Appearance of red color indicates presence of flavonoids (Evan, 1996).

Test for Alkaloid

Wagner's Test: Few drops of Wagner's reagents were added to a portion of the extract, whitish precipitate indicates the presence of alkaloid (Evans, 1996).

Test for Steroid and Triterpenes

Liebermann-Burchard's test: To a portion of the extract, equal volume of acetic acid anhydride was added and mixed gently. 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer. A colour change observed immediately and later indicates the presence of steroid and triterpenes. Red, pink or purple colour indicates the presence of Triterpenes while blue or blue green indicates steroids (Trease and Evans, 1996).

Test for Cardiac Glycoside

Kella-killiani's test: A portion of the extract was dissolved in 1ml of glacial acetic acid containing traces of ferric chloride solution. This was then transferred into a dry test tube and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. Observed carefully at the interphase for purple-brown ring, this indicates the presence of deoxy sugars and pale green colour in the upper acetic acid layer indicates the presence of cardiac glycosides (Evans, 1996).

Test for Tannins

Ferric chloride test: To a portion of the extract, 3-5 drops of ferric chloride was added. A greenish black precipitate indicates presence of condensed tannins while hydrolysable tannins give a blue or brownish blue precipitate (Evans, 1996).

Test for Anthraquinones

Bontrager's test: To a portion of the extract in a dry test tube, 5ml of chloroform was added and shaken for at least 5mins. This was filtered and the filtrate shaken with equal volume of 10% ammonium solution, bright pink colour in the aqueous upper layer indicates the presence of free anthraquinones (Evans, 1996).

Antibacterial Susceptibility Test

Mueller Hinton Agar was used for the antibacterialsusceptibility testing. It was prepared according to manufacturer's instructions by suspending 38g of medium in 1000ml distilled water, sterilized at 121^{o}C, and cooled to room temperature prior to dispensing in Petri dishes.

Preparation of Extract Concentration

This was carried out according to the method described by Srinivasan*et al.,* (2009). Stock solution of the plant extracts were prepared by adding 0.5g of each crude plant extract in 1ml dimethyl sulphuroxide (DMSO). From each of the stock solutions, 250mg/ml, 125mg/ml,

62.5mg/ml and 31.25mg/ml concentrations were prepared using two-fold serial dilution method. These concentrations were labelled and kept in bijou bottles for subsequent use.

Preparation of Turbidity Standard

McFarland standard are used as a reference to adjust the turbidity of microbial suspension so that the number of bacteria will be within a given range. Firstly, $BaCl_2$(1%w/v) and H_2S0_4(1% v/v)were prepared by dissolving 1g of $BaCl_2$ in 100ml of sterile distilled water and 1ml of concentrated H_2S0_4 in 99ml of sterile distilled water respectively to serve as stock solutions for the preparation of the McFarland standard. From the stock solutions, 0.5McFarland scale was prepared by adding 9.95ml of (1%v/v) H_2S0_4 to 0.05ml of (1% w/v) $BaSO_4$ whose density is equivalent to 1.5×108 CFU/ml approximate cell density of bacteria. The barium sulphate suspension in 6ml aliquots were transferred in to screw-cap tubes, tightly sealed, and stored at room temperature in order to prevent loss by evaporation. This was subsequently used for comparison with the turbidity of the bacterial and fungal inoculum (Cheesbrough, 2010).

Standardization of the Inoculum.

Using inoculum loop, enough material from an over-night culture of the test organism was transferred in to a test tube containing normal saline until the turbidity of the suspension matched the turbidity of the 0.5 McFarland Standard as described by the National committee for clinical laboratory standard (NCCLS, 2008).

Susceptibility Test of Clinical isolates to *Diospyros mespiliformis*extracts.

The antibacterial activity of *Diospyros mespiliformis* crude extracts (Aqueous and Methanolic) against the test organisms was evaluated using agar well diffusion method of susceptibility test (Srinivasan*et. al.*, 2009*)*. Mueller-Hinton agarplates were inoculated with 0.1ml of standardized inoculum of each bacterium (in triplicates) using 0.1ml pipette and spread uniformly with sterile swab sticks. Wells of 6mm size were made with sterile cork borer into the inoculated agar plates. Using micropipette, 0.1ml volume of the various concentrations; 250mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml each of the crude extracts were dispensed into wells of inoculated plates. The prepared plates were then left at room temperature for10minutes, allowing the diffusion of the extracts into theincubation at 37 ^oC for 24 hrs. The diameter of inhibition zones (DIZ) were measured and expressed in millimetres after incubation. The mean values of the diameter of inhibition zones were calculated to the nearest whole number. DMSO was used as negative control. Commercially available standard antibiotic, Ciprofloxacin (10mg) was used as positive control parallel with the extracts.

Determination of Minimum Inhibitory Concentration (MIC)

Extracts that exhibited activity against the test organisms were further assayed for their minimum inhibitory concentrations (MIC).The minimum inhibitory concentration (MIC) of the test organisms was determined using the test tube dilution technique. Nine milliliter (9ml) of the nutrient broth was pippeted in to various test tubes containing concentrations of 2000mg/ml, 1000mg/ml, 500mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml, 31.25mg/ml, 15.625mg/ml and 7.8125mg/ml of the extract, the remaining two as negative (containing only

nutrient broth and positive control (nutrient broth and organism) respectively. The overnight culture of the test organisms (0.1ml) aseptically obtained was added to the test tubes with exception of negative control and incubated at 37^{o}C for 24hrs. The least concentration of the extract that did not indicated any visible growth of the incubated organisms in broth culture was taken as the minimum inhibitory concentration (MIC) (Abalaka *et al.,* 2012).

Determination of Minimum Bactericidal Concentration

The bactericidal and bacteriostatic effect of the extract was determine by sub-culturing the well that showed no growth on the fresh medium and incubated it for 24hrs. If the growth is observed, the extract is considered to have only bacteriostatic effect while on the other hand, if the growth is observed, the extract is said to have bactericidal effect (Abalaka*et al.,* 2012).

RESULTS

Chemo-microscopical studies on stem of *Diospyros mespiliformis* were found to have cellulose cell wall, lignin, calcium oxalate crystals, tannins, starch and mucilage but calcium carbonate was absent and is presented in Table 1.

Table 1. Chemo-microscopic Studies of Powdered Stem of *Diospyros mespiliformis*

Constituents	Inference
Starch	+
Gum and Mucilage	+
Cellulose cell walls	+
Lignin	+
Aleurone grain	+
Calcium oxalate crystals	+
Calcium carbonate	-
Suberized/Cuticular cell wall	+
Inulin	+

Key: + Present, - Absent

The result of average moisture contents using loss on drying method was calculated to be 7.60% and the percentage yield of total ash, acid insoluble and water soluble matter were recorded in percentage values as 8.40%, 2.10% and 4.9% respectively. The extractives obtained were 16.60% and 19.60% for alcohol and water solvents respectively (Table 2).

Table 2. Physicochemical Constituents of Powdered Stem of *Diospyros mespiliformis*

Parameters	Values (%w/w) ± SEM*	B.H.P Standard
Moisture content	7.60±0.33	10-12%
Ash content	8.40±0.58	6-19%
Acid insoluble ash	2.10±0.33	>1
Water soluble ash	4.90±0.42	-
Water extractive value	19.60±0.33	-
Ethanol extractive value	16.90±0.58	-

*Average values of three determinations.

Key: BHP- British Herbal Pharmacopeia

Elemental analysis of *Diospyros mespiliformis* powdered stem is presented in Table 3. Trace metals which include Fe, Mn and Ni detected in *Diospyros mespiliformis* powdered stem were below the FAO/WHO (1984) permissible limit for edible plants.

Table 3. Elemental analysis of *Diospyros mespiliformis* Powdered Stem

Elements	Concentration (ppm)	FAO/WHO (1984) limit* (ppm)
Iron(Fe)	3.574	20.00
Copper (Cu)	0.081	3.00
Lead (Pb)	0.544	0.43
Zinc (Zn)	0.270	27.40
Nickel (Ni)	0.456	1.63
Manganese (Mn)	0.144	2.00
Aluminum (Al)	1.744	-
Cadmium (Cd)	0.000	0.21
Selenium (Se)	1.218	-
Chromium (Cr)	0.019	-
Arsenic (As)	-0.322	-

Phytochemicals which include flavonoids, alkaloid, saponins, tannins, glycosides, triterpenes and steroid, anthraquinones were detected in both aqueous and methanolic extracts of *Diospyros mespiliformis* as presented in Table 4.

Table 4. Qualitative Phytochemical screening of Aqueous and Methanolic Stem extracts of *Diospyros mespiliformis*

Metabolite	Inference	
	Aqueous	Methanol extract
Alkaloid	+	+
Flavonoid	+	+
Saponins	+	+
Cardiac glycoside	+	+
Tannins	+	+
Steroid	+	+
Triterpenes	+	+
Phenol	+	+
Anthraquinones	+	+
Carbohydrate	+	+

Key; + Present, - Absent

Table 5: Thin layer Chromatography result ofMethanolic Stem extracts of *Diospyros mespiliformis*

Plant species	Solvent system	Distance travel by spot	No of spot	Rf-value
Diospyros mespiliformis	DCM: ME (2:3)	4.1	6	0.14,0.32,0.54,0.70,0.75,0.95
	DCM:ME(1:1)	4.2	6	0.09,0.23,0.42,0.54,0.71,0.92

Key: DCM- Dichloromethane, ME- Methanol

Table 6. Acute toxicity study of Methanolic Stem extract of *Diospyros mespiliformis*

Plant species	Group	Number of Animals	Dose (mg/kg)	Mortality recorded after 24hrs
Phase I	I	3	10	0/3
	II	3	100	0/3
	III	3	1000	0/3
Phase II	I	1	1500	0/1
	II	1	2250	0/1
	III	1	3250	0/1
	IV	1	5000	0/1

The result for activity of the stem extracts on clinical isolates of *Streptococcus mutans, Streptococcus salivarius*and *S. aureus* with methanol extract having the highest activity with mean inhibition zone range of 32, 26 and 27mm at 250mg/ml respectively.

Table 7. Sensitivity/Inhibitory Results of Aqueous and Methanolic extracts of *Diospyros mespiliformis* Stem

Extract	Test organism	Diameter zone of inhibition(mm)/ Concentration (mg/ml)					
		250mg/ml	125mg/ml	62.5	31.25	Ciprofloxacin	DMSO
Aqueous	*Streptococcus mutans*	23	20	17	12	44	0
	Streptococcus salivarius	28	25	21	16	40	0
	Staphylococcus aureus	20	16	13	10	38	0
Methanol	*Streptococcus mutans*	32	26	22	17	44	0
	Streptococcus salivarius	26	21	17	11	40	0
	Staphylococcus aureus	27	23	20	17	38	0

Table 8: Minimum Inhibitory (MIC) and Bactericidal Concentrations(MBC) of Crude Aqueous and Methanolic Extract of *Diospyros mespiliformis*on Clinical Isolates.

Clinical isolates	MIC		MBC	
	Aqueous	Methanol	Aqueous	Methanol
Streptococcus mutans	15.625	15.625	31.25	31.25
Streptococcus salivarius	15.625	15.625	31.25	31.25
Staphylococcus aureus	15.625	15.625	31.25	31.25

DISCUSSION

Chemo-microscopical examination of the powdered stem of *Diospyros mespiliformis*revealed the presence of cellulose, tannins, starch, lignin, calcium oxalate, suberin, aleurone grain and mucilage but calcium carbonate was absent. The chemo-microscopic features are most valuable in the identification of powdered drug as their identification is largely based on the form, the presence or absence of certain cell types and cell inclusions. These are very important diagnostic pharmacognostic parameters for the identification and authentication of crude drugs especially in powdered plants (Chanda, 2011).

The physicochemical constants of *Diospyros mespiliformis* stem determined include the moisture content, total ash value, acid insoluble ash, water soluble ash, alcohol (ethanol) extractives value and water extractives value. These values are useful as criteria to evaluate the identity and purity of crude drugs (Evans, 2009; WHO, 1996). It also indicates the presence of various inorganic materials like carbonate, oxalate and silicate in plant materials.

The average moisture content of the powdered plant material using loss on drying method was found to be 7.60%, and this value is within the permissible limits because British Herbal Pharmacopeia, (1990) and WHO, (2011) recommend the percentage of moisture content in any crude drug to be within 12-14 %. Low or permissible moisture in crude drugs may discourage the growth of bacteria, yeast, mould and fungi and will stand for long period of time during storage without spoilage or suggesting better stability against degradation of product (WHO, 1996). Ash values obtained include total ash as 8.40%, acid insoluble ash 2.10% and water soluble 4.90%. These Ash values indicate the presence of various impurities such as carbonate, oxalate, sand and silicate in plant materials (Kaneria and Chanda, 2011).

From the ash values mentioned above, the total ash value represented both the physiological and non-physiological ash from the crude drugs upon incineration. The non-physiological ash is the inorganic residues in water soluble ash after the plant drug is burnt while the acid insoluble ash indicated that the plant was in good physiological condition and it contained little extraneous matters compared to the total ash content. The total ash value is used as a standard to assess the identity and purity of crude drugs (WHO, 1996, WHO, 2011).

The alcohol and water extractive values were 16.90% and 19.60% respectively. It was observed that water had a higher extractive value (19.60%). This is because water is a universal solvent that has high polarity and is able to extract more phytochemical constituents than alcohol that has less polarity. This verified why water is mostly used as solvent by traditional medical practitioner and individuals in preparation of dosage forms (Ajazuddin and Shailendra, 2010). Despites alcohol's low extraction capacity, it is sometimes more preferred than water especially in researches that deals with natural products because it serves as preservative against microbial growth and easy to evaporate and handle. Preliminary phytochemical screening of the extracts of *Diospyros mespiliformis* revealed the presence of some phytochemicals such as carbohydrate, alkaloids, tannins, flavonoids, cardiac glycosides, saponins, steroids/ triterpenes. These primary and secondary metabolites in plants have numerous functions. Crude, pure and isolated alkaloids and their synthetic derivatives have been used as analgesic, antispasmodic and bactericidal agents (Stary, 1998; Okwu and Okwu, 2004). Flavonoids have been shown to provide antibacterial, anti- inflammatory, antiallergic, antimutagenic, antiviral, antineoplastic, anti-thrombotic and vasodilatory activity (Alan and Miller, 1996). Flavonoid also has immense antioxidant and anti-inflam-matory activity because of its ability to scavenge hydroxyl radicals, super oxide anions and lipid peroxy radicals (Okwu, 2004; Okwu and Josiah, 2006). Tanins have been used in the treatment of wounds especially those emanating from varicose ulcers and hemorrhoids (Njoku and Akumufula, 2007) and is able to stop bleeding during circumcision (*Edeoga et al.,* 2005). The phytochemical constituents especially the secondary metabolites could

be useful as guide to chemotaxonomic markers (Jonathan and Tom, 2008) that will aid in chemo taxonomical classification system and further phylogenetic studies in Fabaceae family.

The thin layer chromatographic analysis of the methanol extract was carried out on TLC plate precoated with analytical silica gel and developed in suitable solvents systems at different ratios, and it gave distinct and good degree of separations of phytochemicals. The hexane extract when developed in Dichloromethane: Methanol (2:3), (1:1) and sprayed with *p*-Anisaldehyde/H2SO4 reagent for visualization, it gave six spots. The Thin Layer Chromatography chemical screening is usually done to target isolation of new or very important constituent present in the plant extracts which has marked pharmacological activities and also serves as an important tool in recognizing how metabolite for isolation behaved and can be purified; hence, channeling scientific efforts towards the desired compound(s) and prevent waste of resources and time (Patra*et al.,* 2012). The success of separation of bio-molecules by chromatographic technique is markedly influence by the suitability of the separating solvent systems largely influences the successful and also rely upon an ideal range of partition coefficient (k) for each target compound(s) (Ito, 2005).

The elemental analysis revealed some of the elements that are present in the stem bark of *Diospyros mespiliformis*. The elements are rich sources of macro and minor elements that aid in the growth of plants, and as well in human body functions such as muscle contraction, bone formations, growth, metabolism, osmotic balance, regulatory processes activation and other organic bimolecular activities (Rabia *et al.,* 2012). The concentrations of elements gotten from this study were within FAO/WHO (2004) permissible limits for edible plants. Zinc (Zn) is an essential component of a number of enzymes present in animal tissue including alcohol dehydrogenase, carbonic anhydrase, procarboxy peptidase and aids in normal growth, reproduction, tissue repair and wound healing. Zinc deficiency causes growth retardation and skin lesions (Chatterjee and Shinde, 1995).Throughout the world, there is increasing interest in the importance of dietary minerals in the prevention of several diseases (Jeremiah *et al.,* 2019). Minerals are of critical importance in the diet, even though they compromise only 4-6 % of the human body. However, lack of full understanding of the amount and type of elements found in medicinal plants can cause a lot of danger to consumers as some of these plants may contain toxic elements in high quantities (Jeremiah *et al.,* 2019). Again, proper dose rate of many of these medicinal plants is not established and makes it difficult for users to take them appropriately. The probability of taking overdose to facilitate healing processes is high and these can cause serious problems for users because they are ignorant of the dangers involved.

In order to determine the safety margin of drugs and plant products for human use, toxicological evaluation was carried out in experimental animals using Lorke's method to predict toxicity and to provide guidelines for selecting a "safe'' dose in animals and also used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs (Olson *et al.,* 2000; Rang *et al*., 2012). In this study, median lethal dose (LD_{50}) of the extracts (aqueous and methanol) of the *Diospyros mespiliformis* stem bark was carried out orally in rats. The LD_{50} was found to be greater than 5000 mg/kg when administered orally in rats and all the animals remain alive and did not

manifest any significant visible signs of toxicity at these doses. These studies showed the extracts *Diospyros mespiliformis* stem bark of are practically non-toxic when administered using the oral route. This is based on the toxicity classification which states that substances with LD_{50} values of 5000 to 15,000 mg/kg body weight are practically non-toxic (Loomis and Hayes, 1996).

From the result obtained in this study, it was observedthat the assayed chewing sticks extracts demonstrated antimicrobial activities against the selected organisms. This was reflected in the varying zones of inhibition of the individual extracts on the oral pathogens *in- vitro.*This is in agreement with previous studies reporting the antimicrobial and inhibitory effects of chewing sticks on oral micro flora (Adeniyi*et al.,* 2010).*Diospyros mespiliformis*extracts investigated in this study displayed a good inhibitory effect on the investigated bacteria (*S. mutans, Streptococcus salivarius* and *S. aureus*). It could therefore be deduce that the level of the secondary metabolites detected in the extract of *Diospyros mespiliformis*may be too high to demonstrate antibacterial activities against the isolates. *Streptococcus mutans* is known to be the chief pathogen responsible for the formation of dental plaque which normally results to caries (Adeniyi*et al.,* 2010). Its susceptibility to *Diospyros mespiliformis*in this study confirms the use of this plant as a chewing stick locally in removing dental plaque. Studies have shown that frequent users of chewing sticks show less carious lesion than people who use toothbrush (Adeniyi*et al.,* 2010). *Staphylococcus aureus* was also susceptible to *Diospyros mespiliformis*in this study, thus confirming the potency of the plant as it is been used among the traditional medicine in treatment of various ailments and infections (Adeniyi*et al.,* 2010). The antibacterial activity displayed by *Diospyros mespiliformis*could be due to the presence of secondary metabolites revealed in its phytochemical screening. The presence of alkaloids, tannins, saponins have been associated with antimicrobial activities of plants (Edeoga, 2005, Adeniyi *et al.,*2010). The observed rate of kill displayed by *Diospyros mespiliformis* against *S. aureus, S.mutans* and *S. salivarius* correlate with its bactericidal activity.

CONCLUSION

From the results obtained, *Diospyros mespiliformis*extractspossess secondary metabolites which includes alkaloids, tannins, flavonoids, cardiac phenols and saponins. The values of Fe, Mn and Ni in the plant were below the FAO/WHO (1984) permissible limit for edible plants.The facts in this study confirms the use and potency of *Diospyros mespiliformis*as an effective medicinal plant whose active principles could serve as a potential chemotherapeutic agent. Further studies are hereby advised on the antibacterial activities of *Diospyros mespiliformis*using a different approach and methodology.

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