The Pharmaceutical and Chemical Journal, **2017, 4(4):115-122**

Available online www.tpcj.org

Research Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

Determination of Secondary Metabolites and Biological Potential of *Gnetun africanum* (Okazi) Leaves

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Abstract The ethanol, methanol, n-hexane and aqueous extracts of Gnetum *africanum* leaves were investigated for phytochemical studies and antibacterial assay using *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, *Penicillium chrysogenum* and *Aspergillus fumigatus*. The phytochemical analysis revealed the presence of tannins, saponins, glycosides, steroids in varying amount and the absence of flavonoids and anthraquinones. The antimicrobial assay for the bacterial strains showed that, n-hexane extracts exhibited the highest zone of activity against *Escherichia coli*, aqueous extract showed the highest activity against *Staphylococcus aureus* while the ethanol extract showed the highest activity against *Pseudomonas aeruginosa* at a minimum inhibitory concentration (MIC) of 62.5mg/ml respectively. Aqueous extract exhibited the highest against *P. aeruginosa* and the lowest zone against *P. aeruginosa*. There was relatively high antifungal activity, particularly against *Penicillium chrysogenum* for all the solvent extracts except for the n-hexane extracts. Generally, the methanol extract exhibited more activity than the others. The study justifies that *Gnetum africanum* leaves possess pharmacological properties and therefore can be use as medicinal plants for treatment of microbial infections and diseases.

Keywords *Gnetun africana*, Secondary metabolites, Antimicrobial activity, Minimum inhibition concentration, Microbial infections.

Introduction

The treatment of ailments with medicinal plants is an age long practice by which local herbalists have depended on as a reliable means of healing or minimizing disease infections [1-3]. Locally known as ***eru or okazi,*** the fresh leaves of *Gnetum africanum* are consumed locally as vegetable in soups by the Igbos of South Eastern Nigeria, some parts of the Republic of Cameroon, Central African Republic, Gabon, Democratic Republic of Congo and Angola. The leaves can be chewed raw for the management of excessive urination by infantile diabetic patients as a traditional medicine [4-5]. Available literature also reported that both the leaves and the seeds have medicinal efficacy in the treatment of enlarged spleen, sore throats, reduction of pains during child-birth, antidotes to some forms of poison and snake bite. The seeds are specially used as fungicide for dressing fresh and septic wounds, [6]. Medicinal plants are known to be the starting materials for the production of modern medicine. Consequently, herbal remedies are obtained from a wide variety of natural resources including plant leaves, bark, berries, flowers, and roots, [7]. The use of herbal medicine as an alternative medicine has remain very popular in the developing countries and even throughout China, the Far East, and nowadays is growing in popularity and acceptance in the

United States and the United Kingdom, [8]. While many of these medicinal plants are used as spices and food, they are also sometimes added as supplements to food in order to handle some health problems. The importance of medicinal plants cannot be overemphasized due to gap they cover and their role in the health of individuals and communities [9]. The efficacy of these plants is as a result of the presence of some chemical compounds that produce physiological activity in the human body. These bioactive chemical compounds include alkaloids, tannins, flavonoids, saponins, glycosides and other phenolic compounds [10]. This paper reports the fundamental scientific facts obtained from one of the Nigeria medicinal plant (*Gnetum africanum*) by determining the presence of the crude phytochemical constituents in this plant that play an important role in the treatment and healing processes of diseases [11].

Material and Methods

Collection of Plant Materials

Fresh leaves of *Gnetum africanum* (Okazi) were collected from Ikot Udo –Ekpat of Akwa Ibom State, Nigeria. The plant was identified as *Gnetum africanum* by Mr. Peter Onah a Botanist in the Department of Biological Sciences, Faculty of Natural and Applied Sciences, Godfrey Okoye University, Ugwuomu-Nike, Enugu, Enugu State, Nigeria.

Preparation of Plants Material

The fresh leaves of *Gnetum africanum* (Okazi) leaves were removed from their stalks, rinsed with tap water and air dried at room temperature for 30 days. The dried leaves were pulverized to fine powder using laboratory electric blender. The pulverized leaves were stored in air tight containers for extraction.

Preparation of the Extracts

The pulverized leaves (70.0 g) were packed in a Soxhlet extractor and extracted with 300 cm^3 *n*-hexane at 60 ^oC. The n-hexane extracts were evaporated to dryness using a water bath to give a light oily brown crude extract.

The same procedure was repeated with ethanol 400 cm^3 and methanol 300 cm^3 using the same quantity of the plant material respectively and the extracts were then concentrated using a water bath and evaporated to dryness to give dark brown oily crude extracts.

70g of the pulverized leaves were also extracted using distilled water (1500cm^3) in tightly closed container. The content of the container was shaken vigorously and left to stand for 48 hours at room temperature to obtain the water (aqueous) extract. The extract was concentrated using a water bath and evaporated to dryness to give a dark brown crude extract.

All the extracts were labelled accordingly and were used for qualitative phytochemical, antimicrobial activity and minimum inhibitory concentration (MIC) analyses.

Phytochemical analysis

The phytochemical analysis carried out to ascertain the presence of the secondary metabolites present in each of the extracts was based on standard procedures as described by the authors [12-13]. The methanol, n-hexane, aqueous and ethanol extracts respectively were tested for the presence tannins, saponins, alkaloids, flavonoids, glycosides, steroids and anthraquinones.

One gram of the crude extract of each was dissolved in 100 ml of each of the solvent (methanol, ethanol, n-hexane and water) to obtain the stock solution and was used for the phytochemical screening as described below.

Test for alkaloids

5.0 cm^3 of each of the extracts was added to 5 % HCl (2.0 cm^3), followed by Dragendorff's reagent (1.0 cm^3). An orange precipitate was produced immediately which indicated the presence of alkaloids.

Test for steroids

2.0 cm^3 of acetic anhydride was added to 0.5g of each of the extracts containing 5% H_2SO_4 (2.0cm^3). The colour change from violet to blue or green indicated the presence of steroid.

Test for glycosides

0.5 g of each of the extracts was dissolved in 5.0 cm^3 of distilled water, and 2.0 cm^3 of glacial acetic acid containing 1.0 cm^3 of ferric chloride solution was added, followed by the addition of 1.0 cm^3 of concentrated sulphuric acid. A brown ring at the interface of the two solutions was observed which indicated the presence of glycoside.

Test for Anthraquinones

0.5 g of the crude extract each was boiled with 5 % H_2SO_4 (10.0 cm^3) and filtered while hot. The filtrate was shaken with 5 cm^3 of chloroform. The chloroform layer was pipette out into another test-tube and 1.0 cm^3 of dilute ammonia was added, the resulting solution was observed for colour change. There was no colour change and this indicated the absence of anthraquinones.

Test for Saponins

0.5 g of each of the crude extracts was boiled with distilled water (5.0 cm^3) and filtered; to the filtrate 2 drops of olive oil were added. Formation of emulsion showed the presence of saponins.

Test for Tannins

To 5.0 cm^3 of the extract each, a few drops of 1 % lead acetate was added, yellow precipitate was formed, which indicated the presence of tannins.

Test for Flavonoids

To 1.0 cm^3 of the extract each, a few drops of dilute sodium hydroxide was added. The absence of an intense yellow colour after the addition of few drops of dilute HCl showed the absence of flavonoids.

Microbial Assay

Microorganism Collection

The clinical isolates were collected from the stock organisms in the Department of Biological Sciences Laboratory, Godfrey Okoye University. These microorganisms used are; Bacteria: *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Fungi: *Aspergillus fumigatus* and *Penicillium chrysogenum.*

Preparation of Culture Media

28 g of nutrient agar was dissolved in 1000 cm^3 of distilled water. The prepared nutrient agar was distributed in 15.0 cm^3 portion using Mac Conkey bottles capped and then sterilized in an autoclave at 121 ^oC for 15 minutes. The seeded agar plates were prepared by pouring 15.0 cm^3 of the molten nutrient agar into sterile Petri-dish which 0.1 cm^3 of the test microorganisms was added.

Preparation of the Standard drugs used

In preparing the standard drugs used, the materials used were the same as those of the stock solution of the extract. But the concentration of the standard drug was not the same as the crude extract. The standard drug for the bacteria was Amoxicillin while that of the fungi was fluconazole.

One capsule Amoxicillin containing 500.0 mg was dissolved in 1.0 ml of DMSO to give 500.0 mg/ml using the conversion factor below;

$$500.0mg/ml = 50000.0\mu g/ml$$
$$x = 1000.0\mu g/ml$$
$$x = \frac{500 \ x \ 1000}{500000} = 1mg/ml$$

1.0 mg/ml of the Amoxicilin was dissolved in 1.0 ml of DMSO to give 1000.0 ug/ml concentration.

For the fluconazole, one capsule contains 150.0mg and was dissolve in 1.0 ml DMSO to give 150.0 mg/ml, employing the same formula as described above;

$$150.0mg/ml = 150000.0\mu g/ml$$
$$x = 1000.0\mu g/ml$$
$$x = \frac{150 \ x \ 1000}{150000}$$

1.0mg/ml of the fluconazole was dissolve in 1.0ml of DMSO to give 1000.0ug/ml concentration

Preparation of Stock solution of the extract

0.2 g of each extract was carefully weighed and transferred into the sterilized test-tube. DMSO (2.0 cm^3) was added to each of the test-tubes containing the extract and was dissolved completely to get the stock.

Sensitivity Test

The method used for sensitivity test was as described [14]. The seeded agar plates of various test organisms were prepared as discussed above. Wells were made at the respective plates using the Cork-borer. Each plate contains three wells. Three drops of each of the extract were transferred into their respective wells, and three drops of stock solution of the drug were transferred into the wells respectively. However, distilled water served as the negative control and the drug as the positive control.

The extracts and the drugs were allowed to diffuse for 30minutes; these were then incubated at 37 ^oC for 24 hours. In response to the test, the zones of inhibition were then taken after the incubation period using a graduated ruler and then recorded.

Determination of Minimum Inhibitory Concentration (MIC)

5ml of the nutrient broth was dispensed into well labelled separate test-tubes, according to the following concentrations, 500, 250, 125, and 62.5 mg/ml. 1.0 ml of each of the extracts was then transferred into test-tubes containing the different concentrations of nutrient broth above. 2 ml of each concentrations above was then transferred to four different test-tube serially were allowed to stand for 30 minutes before incubation. After incubation, the lowest concentration which showed no turbidity in the test-tube was recorded as the MIC. The extract without the microorganism served as control.

Result and Discussion

Qualitative Phytochemical Screening

Result of the phytochemical screening of the methanol, ethanol, aqueous and n-hexane extracts of *Gnetum africanum* leaves is given in Tables 1. From the analysis, alkaloids was present only in n-hexane, flavonoids and anthraquinones were absent in all the extracts while saponins and steroid were observed to be present in all extracts. Glycosides were present in only aqueous and methanol extracts.

Table 1: Result for phytochemical analysis

Test	*n*-Hexane extract	Ethanol extract	Aqueous extract	Methanol extract
Tannins	+	-	+++	++
Saponins	++	+++	+	+++
Alkaloids	++	-	-	-
Flavonoids	-	-	-	-
Glycosides	-	-	++	+++
Steroids	++	+++	-	+
Anthraquinones	-	-	-	-

Keys: +++ = abundantly present, ++ = moderately present, + = present, - = absent

From the phytochemical screening analysis carried out with methanol, ethanol, water and *n*-hexane extracts of *Gnetum africanum* respectively the following bioactive compounds were observed to be present in the above extracts, namely; tannins, saponins, glycosides, alkaloids and steroids in varying amounts. The qualitative phytochemical analyses of aqueous, methanol, ethanol and n-hexane extracts of *Gnetum africanum* revealed the presence of saponins, in all the four extracts, tannin was absent only in ethanol extract while steroid was absent in the aqueous extract. Flavonoids and anthraquinones were absent in all the extracts. However, cardiac glycosides were absent in n-hexane and ethanol extract and present in the rest while alkaloids were present in only the n-hexane extract (Table 1). This could be attributed to the solubility of the metabolites in the solvents used. While some were very soluble and were able to be extracted others were not. From the above result, it can be seen that methanol was the best amongst the four solvents used, followed by water.

Although their specific functions were not investigated in this study, however, it has been reported that the presence of theses active ingredients in plants is responsible for many of the pharmacological and biological activity of plants [15]. For example, plants that contain tannins have been used for the treatment of intestinal disorders such as diarrhoea and dysentery [16]. In addition to this, tannins have been discovered to possess astringent properties that hasten the healing process of wounds and inflamed mucous membranes. Flavonoids are important antioxidants, and promote several health effects. Aside from antioxidant activity, these molecules provide the following beneficial effects: which include, anti-viral, anti-cancer, anti-inflammatory, and anti-allergic, [17] . Some studies have shown that flavonoids intake is inversely related to heart disease, these compounds inhibit the oxidation of low-density lipoproteins and therefore reducing the risk of atherosclerosis development [18]. Studies have also that revealed saponins have health benefits which include; cholesterol reduction, antitumour and anti-multagenic activities and can lower the risk of human cancer, immunity booster, reduces bone loss etc [19]. The medicinal values of glycosides have been reported as well; they are used as cardiac drugs, laxatives, counter irritant, analgesics, they also prevent renal infections and they exhibit anti-rheumatic, anti-inflammatory, expectorant and antispasmodic effects [20]. Steroids have been reported to possess antibacterial and antineoplastic properties as well as hormonal effects in human [21]. Alkaloids have been reported to exert inhibiting activity against most bacteria, and also reduce high blood pressure [22].

Antibacterial activity of the crude extracts was determined by measuring the diameter of zone of inhibition on some members of enterobacteriaceae and the results are presented as shown in (table 2 -5) below respectively. All the test organisms were susceptible to *Gnetum africanum* (Okazi) extracts though to varying degrees. This is because the susceptibility of bacteria to plant extract on the basis of inhibition zone diameters varied according to it species. The bacteria used in this study were gram-positive and gram-negative.

Gram-negative bacteria are known to be resistant to the action of most antimicrobial agents including plant based extracts. The gram-positive bacteria do not have outer cell membrane found in gram-negative bacteria. The cell wall of gram-positive bacteria is high in peptidoglycan which is responsible for retaining the crystal violet dye.

Table 2: Zone of Inhibition (mm) observed when *n*-haxane extract was used

Organisms	**Concentration of extract (mg/ml)**					
	500	**250**	**125**	**62.5**	**Amoxicillin 500mg/ml**	**Fluconazole 150mg/ml**
Staph-aureus	15.0	12.0	10.1	8.1	19.0	_
E. coli	16.1	14.0	12.0	10.1	20.0	_
P. aeruginosa	12.0	8.1	8.7	7.1	18.0	_
P. chrysogenum	18.1	12.0	8.1	5.0	_	21.1
A. fumigatus	11.3	9.0	7.2	7.0	_	15.0

Key: 0mm = no inhibition, 0-10mm = moderate sensitivity, 10-20mm = sensitive, 20mm and above = very sensitive.

Table 3: Zone of Inhibition (mm) observed when ethanol extract was used

Organisms	**Concentration of extracts (mg/ml)**					
	500	**250**	**125**	**62.5**	**Amoxicillin 500mg/ml**	**Fluconazole 150mg/ml**
Staph- aureus	20.2	18.1	15.0	11.1	22.0	_
E. coli	24.1	12.0	11.1	10.0	17.1	_
P. aeruginosa	18.0	15.1	14.0	12.0	20.0	_
P. chrysogenum	21.0	18.1	15.0	12.2	_	18.0
A. fumigatus	20.0	17.1	15.1	11.0	_	18.0

Key: 0mm = no inhibition, 0-10mm = moderate sensitivity, 10-20mm = sensitive 20mm and above = very sensitive.

Table 4: Zone of inhibition (mm) observed when methanol extract was used

Organisms	Concentration of extract (mg/ml)					
	500	**250**	**125**	**62.5**	**Amoxicillin 500mg/ml**	**Fluconazole 150mg/ml**
Staph. aureus	14.0	12.1	11.0	10.0	22.0	_
E. coli	13.0	12.0	11.1	10.0	17.0	_
P. aeruginosa	24.0	26.1	32	35.0	37.0	_
P. chrysogenum	24.1	20.5	18.0	15.4	_	25.2
A. fumigatus	12.0	10.1	8.0	6.3	_	15.1

Key: 0mm = no inhibition, 0-10mm = moderate sensitivity, 10-20mm = sensitive 20mm and above = very sensitive.

Table 5: Zone of Inhibition (mm) observed when aqueous extract was used

Organism	Concentration of extracts (mg/ml)					
	500.0	**250.0**	**125.0**	**62.5**	**Amoxicillin 500mg/ml**	**Fluconazole 150mg/ml**
Staph- aureus	29.0	25.0	23.0	15.0	27.0	-
E. coli	17.0	15.0	11.0	10.0	22.0	-
P. aeruginosa	13.0	12.0	10.0	9.0	15.0	-
P. chrysogenum	19.0	17.5	16.1	13.5	-	15.2
A. fumigatus	16.0	16.0	14.3	11.4	-	17.5

Key: 0mm = no inhibition, 0-10mm = moderate sensitivity, 10-20mm = sensitive 20mm and above = very sensitive

The minimum Inhibitory Concentration (MIC) of the extracts was also determined. For bacteria, various concentrations of methanol, ethanol, aqueous and *n*-haxane (62.5-500mg/ml) were used to inhibit the test organisms using broth dilution methods. The lowest MIC (62.5mg/ml) was found in aqueous extract against *Staphylococcus aureus* (Table 7). (125mg/ml) was recorded against *E. coli* exhibited by methanol extract (Table 6) and the same concentration (125mg/ml) was recorded against *Pseudomonas aeruginosa* exhibited by ethanol extract (Table 8).

For fungi, the lowest concentration (500mg/ml) was exhibited by ethanol and aqueous extracts against *A. fumigatus* respectively. 500mg/ml was recorded against *P. chrysogenum* exhibited by ethanol extract (Table 8).

Table 6: Minimum inhibitory concentration of methanol extract of *Gnetum africanum*

Micro-organism	Turbidity at various concentration of the extract (mg/ml)			
	500	**250**	**125**	**62.5**
Staph- aureus	-	-	-	+
E. coli	-	-	-	+
P. aeruginosa	-	-	+	+
P. chrysogenum	+	++	++	+++
A. fumigatus	+	++	++	+++

Key: - = no growth, + = slight turbidity, ++ = moderate turbidity, +++ = very turbid.

Table 7: Minimum inhibitory concentration of aqueous leaf extract of *Gnetum africanum*

Micro-organism	Turbidity at various concentration of the extract (mg/ml)			
	500	**250**	**125**	**62.5**
Staph- aureus	-	-	-	-
E. coli	-	-	+	++
P. aeruginosa	-	+	+	+++
P. chrysogenum	+	+	++	+++
A. fumigatus	-	+	++	++

Key: - = no growth, + = slight turbidity, ++ = moderate turbidity, +++ = very turbid.

Table 8: Minimum inhibitory concentration of ethanol extract of *Gnetum africanum*

Micro-organism	Turbidity at various concentration of the extract (mg/ml)			
	500	**250**	**125**	**62.5**
Staph- aureus	-	+	+	++
E. coli	-	-	+	++
P. aeruginosa	-	+	-	+++
A. fumigatus	-	+	++	+++
P. chrysogenum	-	+	++	+++

Key: - = no growth, + = slight turbidity, ++ = moderate turbidity, +++ = very turbid.

Table 9: Minimum inhibitory concentration of n. hexane extract of *Gnetum africanum*

Micro-organism	Turbidity at various concentration of the extract (mg/ml)			
	500	**250**	**125**	**62.5**
Staph- aureus	-	+	++	+++
E. coli	-	+	++	++
P. aeruginosa	-	+	++	+++
A. fumigatus	+	+	++	+++
P. chrysogenum	-	++	+++	+++

Key: - = no growth, + = slight turbidity, ++ = moderate turbidity, +++ = very turbid.

Conclusion

The results obtained above from the phytochemical screening, antimicrobial and MIC analysis of *Gnetum africanum* put together, justify its use as an antimicrobial agent. The efficacy of the extracts are probably due to the identified secondary metabolites and therefore the plant can be used to source for oral antibacterial drugs that can treat infection caused by susceptible gram-negative and gram-positive bacteria. The phytochemicals determine the medicinal values of these edible vegetable leaves and also could serve as stating materials for the synthesis of new drugs in pharmaceutical industries. If it is well harnessed and modified, the plant leaves may serve as an agent for antimicrobial drugs whose activity will produce little or no side effect in the body system.

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