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Phytochemical and antimicrobial activity screening of *Gnetum africanum* leaf extracts

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Abstract
The menace of antibiotic resistance and the antecedent evolution of innocuous microbes into superbugs is an epidemic of global concern. This study investigated the antimicrobial activity of water and ethanol extracts of *Gnetum africanum* leaves, a commonly consumed vegetable in most parts of Sub-Saharan Africa, on *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Leaves of *G. africanum* were extracted with water and ethanol using cold maceration technique; standardized chemical tests were employed for phytochemical screening and the disc-diffusion method used for antimicrobial analysis. Findings indicate that both extracts had dose-dependent inhibitory effect on the growth of *S. aureus*, with maximum inhibition zones of 13.30 and 13.10 mm at 200 mg/ml for aqueous and ethanolic extracts, respectively. For *C. albicans*, the ethanol extract proved to be more potent with a maximum inhibition zone of 13.30 mm at extract dose of 100 mg/ mL. However, the extracts did not show any activity against *E. coli*, suggesting its impotence against gram negative bacteria. The observed antimicrobial activity may be as a consequence of the rich phytochemistry of the leaves, as preliminary phytochemical screening revealed the leaves to contain saponin, flavonoid, cardiac glycoside, alkaloids, tannin and anthraquinones. The findings of this study further reinforces the importance of *G. africanum* leaves in traditional healthcare practice and its use in culinary. Further investigation is however needed to isolate and purify the bioactive antimicrobial principles for potential development into generic antimicrobials.

Keywords: *Gnetum africanum*, antimicrobial resistance, *S. aureus*, *E. coli*, *C. albican,* phytochemisty, ethanol and aqueous extract

1. Introduction
Over the past two decades, the incidence antimicrobial resistance has been on a steady rise, with modest statistics stating that over 50,000 thousand persons die from AMR resistant-related diseases in the US and Europe alone, with even more casualties projected in poorer countries [1]. If this trend is left unchecked, high-level estimates by KPMG and RAND Europe projects that over 10 million persons will die from AMR related disease every year by 2050; and that global production and GDP will shrink by 2-3% as a consequence [2]. This increasing resistance is attributable to a plethora of reasons, including selective pressure on microbes, indiscriminate use of antibiotics, use of antibiotics for agricultural purposes, and lack dedicated research effort on the part of big pharmaceuticals on the development of new antimicrobial agents owing to economic and regulatory obstacles [3]. In fact, the number of new antibiotics developed and approved by the US FDA has declined consistently over the past five decades [4].

The increasing reliance by big pharmaceuticals on screening synthetic chemical libraries for the development of new antibiotics as against the traditional method of isolating potential antimicrobial leads from natural products have also contributed to the slow-pace of new antibiotic discoveries [5]. Nonetheless, scientists are beginning to rethink a solution to the epidemic, and recently research on plant extracts or endophytes with antibiotic potentials has gained prominence [6, 7], especially in Africa where a sizeable population rely on plants for medicinal purposes [8]. Moreover**,** the use of plant as medicine is being reinvigorated in different countries of the world, with China, India and the USA leading the pack. The Chinese herbal medicine industry was estimated to worth over 2 billion USD as of 2002, and continues to grow [9]. However, in Africa, the over-reliance on orthodox drugs produced in developed nations for the treatment and control of diseases has generally led to stagnation in the scientific exploration of traditional medicine in Africa. Also, there are very few studies on African traditional plants including their physiochemical and phytochemical properties and safe doses of use. It is only recently that the need to source for locally available medicine because of the increasing cost of orthodox medicines and drug failure became a prominent issue in African research [10].

Gnetum africanum (Gnetaceae) commonly called "Salad" in Nigerian pidgin English,

Afang in Efik, Okazi in Ibo, Eru in Western Cameroon pidgin English and Koko in French [11] is a popular vegetable that is highly priced and collected more from the wild (rather than domesticated) across the tropics in Asia, South America and in Central Africa. In Nigeria, the leaf of *G. africanum* is used in the treatment of splenomegaly, sore throats and as a cathartic [12]. In Ubangi (DR Congo), it is used to treat nausea and is considered to be an antidote to some forms of poison [12]. Studies on oil extracted from the leaves of the plant revealed it to be rich in phytochemicals such as tannins, polyphenols, saponins, flavanoids, alkaloids, O- and C-glycosides [13]; compounds whose therapeutic properties have been empirically ascertained by various research effort.

This research is part of an effort conceived to screen plants that are locally used by traditional medical practitioners for antimicrobial activity against microbes commonly associated with diseases. We believe that our study will have the dual benefit of discovering new antimicrobial leads while also encouraging the bio-conservation of indigenous African herbs. For this effort, *Gnetum africanum* was investigated for its antimicrobial activity.

2. Materials and Method

2.1 Plant material collection and Identification:

Fresh leaves of *G. africanum* were bought from Eke-Awka market, a popular vegetable market in the capital city of Anambra State, Southeastern Nigeria. The leaves were identified by a taxonomist with the Department of Botany, Nnamdi Azikiwe University Awka, Nigeria, and voucher specimen stored in a herbarium.

2.2 Extract Preparation

The leaves of the plant were properly washed in tap water and rinsed in distilled water. The rinsed leaves were air-dried for 3 days. The dried leaves were pulverized using pestle and mortar to obtain a powdered form which was stored in airtight plastic containers until needed for the experiment. The powdered leaf (120 g) was separately macerated in 1L of ethanol (70 % w/v) in an Erlenmeyer flask for 24h, and the mixture agitated mechanically at intervals. The mixture was filtered afterwards using Whatman No. 1 filter paper in a Buchner funnel. The filtrate obtained was concentrated in water bath (Chem-index, WB500E, USA) at 70ºC to obtain a gel-like concentrate. The concentrate obtained was stored in refrigerator at 4ºC until needed for analysis. The water extract was similarly prepared, except that 1L of distilled water was used for the maceration.

2.3 Preliminary Phytochemical screening

Preliminary phytochemical screening was carried out using standardized procedures. The tests are briefly described below:

Anthraquinones: This was carried out as described by Akinjogunla. 0.5g of each extract was shaken with 10 mL of benzene and filtered. 5mL of 10% ammonia was added to the filtrate and was shaken for a few minutes. The presence of pinkish-red or violet colour indicates the presence of anthraquinones [14].

Saponins: *(Frothing test).* Distilled water (5 mL) was added to 200 mg of each extract. 0.5 mL filtrate was diluted to 5 mL with distilled water and shaken vigorously for 2 minutes. Formation of stable froth head indicates the presence of saponins [15].

Tannin: The ferric chloride test was employed. A quantity of each extract (200 mg) was heated with 20 mL of water for 5 min in appropriately labeled test-tubes. Each solution was allowed to cool and then filtered. 1mL of each filtrate was diluted with 5mL distilled water in a test tube; few drops of 0.1% ferric chloride solution were added. A characteristic blue, blue-black, green or blue-green colour and precipitate indicate the presence of tannin? Ref.

Flavonoids

Lead acetate test: A quantity of each extract (500 mg) was dissolved in water and filtered. To 5 ml of each of the filtrate, 3 ml of lead acetate solution was then added. Appearance of a buff-coloured precipitate indicates the presence of flavonoids [16].

Sodium hydroxide test: A quantity of the each portion was dissolved in water and filtered; to this 2 mL of the 10% aqueous sodium hydroxide was added to produce a yellow colouration. A change in colour from yellow to colourless on addition of dilute hydrochloric acid was an indication for the presence of flavonoids [16].

Alkaloids: Each extract (200 mg) was stirred with 5 mL of 1% aqueous HCl on water bath and then filtered. Of the filtrate, 1 mL was taken individually into 2 test tubes. To the first portion, few drops of Dragendorff's reagent were added; occurrence of orange-red precipitate was taken as positive. To the second 1 mL, Mayer's reagent was added and appearance of buff-coloured precipitate will be an indication for the presence of alkaloids [17].

Cardiac Glycoside: The Salkowski test was employed for this. The extracts (0.5 g) were dissolved in 2 mL of chloroform. Sulphuric acid was then carefully added to form a lower layer. A reddish-brown colour at the interface will indicate the presence of a steroidal ring (i.e. aglycone portion of the cardiac glycoside)? Ref.

Reducing Sugars: To 10 mL of each extract, a few drops of Fehling's solution A and B were added; an orange-red precipitate suggests the presence of reducing sugar?Ref.

2.4 Antimicrobial Studies

2.4.1 Collection of Microbial Samples

Pure microbial cultures of *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923) and *Candida albicans* (ATCC 10231) were obtained from Our Saviour's Laboratory and Diagnostic Centre Awka, Anambra State Nigeria, in agar slants.

2.4.2 Preparation of Nutrient Agar

Dehydrated powder of Nutrient agar (Sigma-Aldrich, USA) with composition (gL^{-1}): meat extracts 1.0, yeast extract 2.0, peptone 5.0, sodium chloride 5.0, agar 15.0; pH 7.4 was used as the microbial culture media. It was prepared following manufacturers instruction by suspending 28 g in 1 L of distilled water, boiling the mixture to obtain a consistent liquid broth and subsequently sterilized by autoclaving at 121°C for 15 min.

2.4.3 Inoculation and Incubation of Agar

The prepared nutrient agar was poured into sterile, labeled 150 mm petri-dishes and allowed to set for 24 h. A sterile swab was dipped into the broth culture containing the microorganism, gently removed and used in streaking the

surface of the nutrient agar to form a microbial lawn.For the disc diffusion assay, 7 mm discs were saturated with different concentrations (100, 200 and 300 mg/mL) of the extracts, allowed to dry and was introduced on the top of seeded agar plates using flame-sterilized forceps. The plates were incubated overnight at 37 °C. Microbial growth was determined by measuring the diameter of zone of inhibition. The result was obtained by measuring the zone diameter. The experiment was done three times and the mean values were presented.

3. Results

3.1 Qualitative Phytochemical Analysis

The result of the preliminary phytochemical study carried on aqueous and ethanolic extracts of *G. africanum* to determine the presence of medicinally important phytochemicals in the leaves revealed the presence of various phytochemicals such as tannins, saponins, flavonoids, alkaloids, cardiac glycosides, and reducing sugars in both extracts of the leaves (Table 1).

Table 1: Qualitative Phytochemical analysis of the aqueous and ethanolic extracts of G*. africanum.*

S. No.	Phytochemical	Test	Inference	
			EtOH	Water
1	Anthraquinones		+	+
2	Saponin	Emulsification	+	+
		Frothing Test	+	+
3	Tanins	Bromine water	+	+
		Lead acetate	+	+
		Acid test	+	+
4	Flavonoids	Ferric chloride	+	+
		Lead acetate	+	+
		Sodium hydroxide	+	+
5	Alkaloids	Meyer's reagent Dragendorff's	+ +	+ +
6	Cardiac glycoside	Huppert-Salkowski's	+	+
7	Reducing compounds	Fehling's reagent	+	+

Note: Present (+), Absent (-), Ethanol extract (EtOH), Aqueous extract (water)

3.2 Antimicrobial studies

As the pharmacological action of any plant cannot be accurately ascertained by the result of preliminary phytochemical studies only, thus, the antimicrobial activity of the extract against select microbes was also evaluated. The present investigation showed the efficacy of the extracts in inhibiting the growth of *S. aureus* and *C. albicans* (Table 2).

Table 2: Antimicrobial activities of ethanol and aqueous extracts of *G. africanum* leaves

Organism	Concentration of extract (mg/mL)	Zone of inhibition (mm)	
		Water	EtOH
Escherichia coli			
	100	0.00	0.00
	200	0.00	0.00
	300	0.00	0.00
Staphylococcus aureus			
	100	8.30	8.60
	200	13.30	13.10
	300	11.40	11.20
Candida albicans			
	100	0.00	13.30
	200	9.21	13.20
	300	13.42	12.90

Note: Inhibition zones are the mean including disc (7 mm) diameter. Ethanol extract (EtOH), Aqueous extract (water).

4. Discussion

Phytochemical screening indicated that both aqueous and ethanol leaf extracts of *G. africanum* contained alkaloid, tannin, saponin, anthraquinones, flavonoids, and cardiac glycoside (Table 1). Phytochemical screening indicated that both aqueous and ethanol leaf extracts of *G. africanum* contained alkaloid, tannin, saponin, anthraquinones, flavonoids, and cardiac glycoside (Table 1). This is in agreement with phytochemicals studies on the leaves previously carried out by other researchers [18-20]. Phytochemicals have been extensively studied and their medicinal properties documented. Tannins possess astringent taste and help in healing of wounds and inflamed mucous membrane [21]. Tannins are also potent metal ion chelator, proton precipitating agents and biological antioxidant [22]. Flavonoids are most commonly known for their antioxidant activities and act as detoxifiers with the ability to modify a cell's reactions to carcinogens, viruses and allergens. They possess anti-microbial, anti-cancer, anti-inflammatory, and anti-allergy activities [23] and may, therefore, be of therapeutic importance [24]. Similarly, saponins which are a special class of glucosides have been found to possess antifungal activity [25]. Saponins have been reported to have a wide range of pharmacological and medicinal activities, and do not pose serious risk of toxicity in humans, even in large quantities [26]. Plants containing saponins are used in wound healing in folkoric medicine [27] because they are good hemagglutinins [28].

The results of the antimicrobial study showed both ethanol and aqueous extracts of *G. africanum* leaves to possess antimicrobial activity against two of the test organisms employed in this study- *C. albicans* and *S. aureus*. However, there was no detectable activity against *E. coli*, suggesting that the extracts may not have bactericidal effect on gram negative bacteria strains. Both extracts induced similar inhibition zones that were dose-dependent on *S. aureus*, with maximum inhibition zones (mm) of 13.30 and 13.10 for the water and ethanol extract respectively, at a concentration of 200 mg/ml. This result agrees with that of Enyi-Idoh *et al.* [29], who studied the antibacterial activity of *G. africanum* on diarrhoegenic bacteria, including *S. aureus*.

The extracts also displayed antimicrobial properties against *C. albicans*, with a noticeable difference in the optimum inhibitory concentrations between the two extracts. While the water extract showed no activity at low concentration (100 mg/ml), it produced a maximum inhibitory zone of 13.42 mm at 300 mg/ml. On the other hand, the ethanol extract displayed inhibition of (13.30 mm) at a concentration of 100 mg/ml, with no significant change in activity at increased concentration. This suggests that ethanol was better than water at eluting the antifungal principle from the leaves.

S aureus and *C albicans* are opportunistic pathogens indicating that they cause infections mainly in tissues and sites with lowered host resistance. *S. aureus* is the cause of superficial infections, subcutaneous and submucuous abscesses, osteomyelitis, bronchiopneumonia especially post influnenzal, pyelonephritis, lymphangitis, lymhadenitis, bactericemia, septicaemia, pyaemia, acute bacterial endocarditis, staphylococcal food poisoning associated with vomiting and diarrhea [30].

5. Conclusion

There is an inexorable development of resistance to a given antibiotic once it is in widespread clinical use, hence a pressing and recurrent need for new molecules with antibiotic properties. Historically, two lines of discovery have been

fruitful: natural products with antibiotic activity and synthetic antibacterial agents. Penicillins, cephalosporins, vancomycin, tetracycline, and aminoglycosides are in the first category, and fluoroquinolones, sulfonamides, and oxazolidinones are in the second. Natural and synthetic molecules are both likely to remain important sources for new antibiotics but offer distinct challenges. In the present study, the extracts of *Gnetum africanum* leaves inhibited microbial growth and is thus a potential lead in the development of new antibiotic agents.

Interestingly, its effectiveness against *Candida albicans* can be exploited in developing safer antifungal drugs, since the raw leaf is consumed as food. Currently, only a handful of antifungal agents are tolerated systematically, with the most widely used being amphotericin B, a polyene class antifungal well known for its severe and potentially lethal side effects. Again, as this leaf is widely consumed in various parts of Africa as vegetable, the possibility of serious toxicity resulting from antimicrobial agents isolated from this leaf would be very minimal.

The effectiveness of the extracts against *S. aureus* and *C. albican* may result from the disruption membrane function in these organisms. This may as well be the reason why the extracts were ineffective against *E. coli*; as most gram negative microbes are not inhibited by membrane-disrupting class antibiotics.

Further investigations, however, need to be conducted to isolate and purify the bioactive components of the extracts as well as characterize the structures of these compounds, with the hope that they can be developed into safer antimicrobial drugs.

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