JOPAT Vol 25(1) 3080 – 3098, Jan – June. 2026 Edition.
ISSN 2636 – 5448. https://dx.doi.org/10.4314/jopat.v25i1.27

ANTIMICROBIAL ACTIVITIES OF AFRICAN JOINT FIR (*GNETUM AFRICANUM* WELW.) ON BACTERIA ISOLATED FROM "WARA", A HIGHLY PRIZED AND CONSUMED LOCAL NIGERIAN CHEESE

Nwachukwu, Flourish Chinedu [2], Olumide Ekundayo Omotayo*[1], Idowu Arinola Obisesan[1], Jacob Olagbenro Popoola[1], Bukola Oyerinola Atobatele[2]

1. *Pure and Applied Biology Programme, College of Agriculture, Engineering and Science, Bowen University, P.M.B 284, Iwo, Osun State, Nigeria*
2. *Microbiology Programme, College of Agriculture, Engineering and Science, Bowen University, P.M.B 284, Iwo, Osun State, Nigeria.*

ABSTRACT

The potential of *Gnetum. africanum* extracts as natural antimicrobial agents in the production of "wara", a traditional and commonly consumed Nigerian snack, was investigated in this study. "Wara" samples were purchased from markets in Lagos and Osun States respectively. Serial dilution and pour plate techniques were used to isolate Gram-positive (73.3%) and Gram-negative (26.7%) bacteria of which the majority were susceptible to standard antibiotics used in the study. *Micrococcus halobius, Paenibacillus pectinilyticus,* and *Bacillus pumilus* however showed resistance to the drug samples. Phytochemical screening of *G. africanum* leaf extracts revealed the presence of several phytocompounds. Methanol extracts showed maximum antibacterial activity with 15 mm zone of inhibition while chloroform extracts showed the least value with a 9 mm zone of inhibition. *Citrobacter freundii* showed highest overall susceptibility to the applied *G. africanum* extracts. The results showed that *G. africanum* leaf extracts is a promising organic antibacterial agent useful in the preparation of the local cheese snack, "wara".

KEYWORDS: "Wara", *Gnetum africanum*, antimicrobial resistance, food safety, natural antimicrobial agents

*Corresponding author: olumide.omotayo@bowen.edu.ng; +234 802 379 9838

Introduction

Vegetables have been a part of the African staple diet since ancient times and they are known to provide many benefits to consumers including eating pleasure, nutrition, health, and longevity. One vegetable of considerable economic importance in Nigeria is *Gnetum africanum* Welw. It is an indigenous leafy green vegetable belonging to the Gnetaceae family, which comprises three genera including *Gnetum*, *Ephedra*, and *Welwitschia*. It is a transitional family between angiosperms and gymnosperms [1]. These genera exhibit certain traits parallel to angiosperms, like the existence of vessels in their secondary xylem and the formation of flowers, while also preserving characteristics typical of gymnosperms, such as the development of cones and exposed seeds. Early cladistic analysis based on morphological characteristics suggested that Gnetales was the sister order of the angiosperms [1].

In Nigeria, *G. africanum* is commonly known as "*Áfàng*" in Ibibio and Efik, "*Ọ̀kazị*" in Igbo, "*Ajaabaje* or *Ajakotale*" in Yoruba, while in Cameroon it is known as "*Eru*" [2]. It is a unique plant that is distributed across the tropical regions of West and Central Africa, displaying exceptional botanical characteristics. Characterized by a vine-like growth pattern, *G. africanum* is classified as a non-flowering gymnosperm and is dioecious, having individual plants as either male or female [3,4].

The plant *G. africanum* is a well-known and used African vegetable and has been reported for its different nutritional, medicinal, and economic importance [5-7]. The leaves of *G. africanum* are important in traditional cuisines where they impart a distinct flavour to various dishes. The leaves find frequent application in the preparation of dishes like áfàng soup, egusi soup, and oha soup. The leaves are also valued for their nutritional richness. In abundance, the leaves contain essential nutrients such as vitamin A, vitamin C, iron, calcium, and protein which enable them to enhance the nutritional variety in local diets [8]. Vitamin A plays a crucial role in vision and immune function, while vitamin C helps prevent iron deficiency, and boosts the immune system [9,10]. Also, iron is critical for cellular respiration and oxygen transport [11], and it is well known that calcium promotes teeth and bone health. Those are some of the health benefits that the consumption of *G. africanum* leaves in meals provides.

Furthermore, *G. africanum* is known to have medicinal properties and is traditionally utilised for those perceived health benefits [3]. Studies have shown that the plant possesses anti-inflammatory, antioxidant, and anticarcinogenic properties [3, 12], which make it a subject of interest in phytotherapy and traditional medicine. Research also indicates that the plant exhibits antimicrobial activity against various pathogens [13], which suggests its potential in combating infectious diseases. In rural communities, the harvesting and commercialization of *G. africanum* serve as a crucial income stream and are fundamental to sustaining community livelihoods and fostering economic stability [14]. For example, in regions like Cross River and Akwa Ibom states within Nigeria, *G. africanum* thrives abundantly, and residents engage in the collection and trading of the leaves to generate income for their families [12].

Traditional snacks are a mainstay source of nutrition for the majority of the African populace, particularly in large urban centers where busy work schedules and the fast pace of the lifestyle in city centers prevent many people from preparing home-made foods. One local snack that usually comes in handy in such situations is the local snack commonly known as "wara". "Wara" is a plant protein-based meat-like snack that is commonly found in different parts of Nigeria. It is prepared through the coagulation of fresh milk with the addition of a coagulant [15] and the milk that is used could either be fresh cow milk or fresh soybean milk although occasionally, fresh goat milk may be used in the preparation of "wara" [16]. The coagulants used in "wara" production may be *Calotropis procera* leaves, papaya leaf extracts, or lime juice, and along with other factors such as the heating and pressing processes, the choice of coagulant significantly affect the texture and flavour of the final "wara" product [17,18]. The preparation process of "wara" distinctly separates it from cheese, as cheese undergoes further ageing of the pressed milk curd, creating a product with different characteristics [19]. In essence, the starting material for "wara" could either be animal-based (such as cow milk or goat milk) or plant-based (such as soy milk from soybeans) [20,21].

"Wara" poses significant food safety concerns due to potential microbial contamination throughout its production process. Inadequate hygiene practices and limited regulatory oversight further exacerbate these risks, heightening the susceptibility of consumers to foodborne illnesses [22]. Synthetic preservatives are widely used in the food industry to preserve food quality [23] but there remains a need to explore the promise of natural antimicrobial agents such as *G. africanum* as bioenhancers in local snacks production. Thus, this study investigated both the microbial safety of "wara" and the antimicrobial efficacy of *G. africanum* extracts against pathogens isolated from "wara" to mitigate food safety risks and maintain the authenticity of this traditional snack. The outcome of this study holds significant implications for food safety and public health of local consumers of the highly nutritious snack.

Materials and methods

Sample Collection

The "wara" samples used in this study were obtained from vendors in two different locations. Fresh and fried "wara" samples were purchased from different vendors at Oja-Oba market, Iwo, Osun State. Fresh "wara" samples were also purchased at Oshodi market, Lagos state. These markets are major accessible

centers for wara purchase in both states of Nigeria. Fresh leaves of *G. africanum* were obtained from Oyingbo market, Lagos State, being a prime supply centre for fresh vegetables products for the local populace. The "wara" samples and *G. africanum* leaves were transported to the Microbiology laboratory of the Department of Biological Sciences, Bowen University, Iwo, and preserved under refrigerated conditions.

Preparation of Plant Extracts

Methanol, n-hexane, chloroform, ethanol, and water were selected as solvents for the extraction process. These are commonly used solvents for extracting various phytochemical compounds from leaves, specifically *G. africanum* [24,25]. After the leaves had been properly air-dried for two weeks, they were pulverized into fine powder using an electric blender and then placed in clean air-tight containers prior to the time for their use. The powdered leaf samples were then apportioned into five labelled containers according to the appropriate solvent. At least 100 ml of solvents were added respectively into each container until the plant material in the container was completely submerged. The containers were next properly sealed and allowed to sit for 24 - 48 hours with occasional shaking [26,27]. A Soxhlet rotary evaporator was used to concentrate the extracts [28] after which they were then preserved in the refrigerator until needed for further use.

Phytochemical analyses of *G. africanum* leaf extracts

Qualitative and quantitative analyses were purposively carried out on three of the leaf extracts namely the methanol, n-hexane, and aqueous extracts. Phytochemical screening of flavonoids, alkaloids, tannins and terpenoids was carried out according to the procedure of [29], phenols, saponins, anthraquinones conducted according to [30], with steroids and glycosides done according to the method described by Longbap *et al*. [31].

Isolation of microorganisms

Serial dilution was carried out on each sample and the pour plating method was used to isolate microorganisms from the "wara" samples. Subsequently, subculturing was done to obtain pure cultures.

Serial Dilution: Test tubes were filled with nine milliliters (9 mL) of water each, fitted securely with cotton wool, and autoclaved (Equitron 7431FA, Mumbai-India) for fifteen minutes at 121°C. The tubes were autoclaved and then allowed to cool before being utilized. For each sample, five test tubes were used and each tube was labeled 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, respectively. One gram of each sample was weighed and put into the 10^{-1} tube, after being pounded in the crucible. After that, one milliliter was transferred from the 10^{-1} tube to the 10^{-2} tube. The process was repeated up until the 10^{-5} tube for each sample. This was done according to method described by Cheesebrough [32].

Pour Plating Method: For each sample, one milliliter (1 mL) from the serial dilution test tubes labeled 10^{-2} and 10^{-4} was added to different Petri dishes that were appropriately labeled. Four different culture media (Nutrient Agar, MacConkey Agar, Mannitol Salt Agar, and Salmonella-Shigella Agar) were used to isolate organisms from the 10^{-2} and 10^{-4} tubes of each sample. Nutrient agar (NA) is a general-purpose agar used to isolate total heterotrophic bacteria, MacConkey agar (MCA) is applicable for isolation of total coliform bacteria such as *Klebsiella and E. coli*, Mannitol Salt agar (MSA) used for the isolation of *Staphylococcus aureus*, while Salmonella-Shigella agar (SSA) was used for the isolation of *Salmonella* and *Shigella*. 20 mL of the culture medium was placed respectively into each Petri dish that had previously inoculated with 1 mL culture inoculum. Each Petri dish was swirled to ensure that the contents were properly mixed and afterwards, were incubated for 24 hours at 37°C in an incubator. After incubation, the Petri dishes were examined for microbial growth after which distinct colonies were sub-cultured following the observation and recording of their macroscopic features. The isolated microorganisms were thereafter preserved on nutrient agar slants which were periodically sub-cultured to ensure continued viability of the microorganisms. Gram-staining and biochemical tests were used to identify the most probable organisms.

Antibiotic sensitivity test

The Kirby Bauer agar disc diffusion method was utilized to test the isolates for their susceptibility to various antibiotics. Isolates

were inoculated into peptone broth and incubated at 37 °C for 16 h. The suspension was standardized to 0.5 McFarland turbidity standard, and the standardized bacteria were seeded onto the surface of freshly prepared, dry-surfaced Mueller Hinton agar using sterile swabs according to the method described by Atobatele and Owoseni [33]. For Gram-positive bacteria isolates, the antibiotic discs utilized were pefloxacin (PEF) 10 µg, gentamycin (CN) 10 µg, ampiclox (APX) 30 µg, rocephin (R) 25 µg, ciprofloxacin (CPX) 10 µg, azithromycin (AZ) 12 µg, zinnacef (Z) 20 µg, amoxicillin (AM) 30 µg, levofloxacin (LEV) 20 µg, and erythromycin (E) 10 µg. For the Gram-negative isolates, septrin (SXT) 30 µg, chloramphenicol (CH) 30 µg, sparfloxacin (SP) 10 µg, ciprofloxacin (CPX) 30 µg, amoxicillin (AM) 30 µg, augmentin (AU) 10 µg, gentamycin (CN) 30 µg, pefloxacin (PEF) 30 µg, tarivid (OFX) 10 µg, and streptomycin (S) 30 µg make up the antibiotic discs used. Before the incubation of the plates for 24 hours at 37°C, the paper discs were firmly but carefully set on the inoculated plates. The zones of inhibition were measured in millimeters after incubation.

Susceptibility Tests of Leaf Extracts of *Gnetum africanum*

For each isolate, a pure 24-hour-old bacteria culture was suspended in 1 milliliter of sterile water inside test tubes and agitated until it was homogenized. To standardize microbial testing, the turbidity of the bacterial suspensions was adjusted to match 0.5 McFarland turbidity standard. A sterile swab stick was then inserted into each of the test tubes holding the bacterial suspension, and the soaked swab stick was used to swab the entire surface of the Mueller-Hinton agar plate. Two methods were used to carry out the susceptibility testing of the aqueous, methanol, n-hexane, chloroform, and ethanol leaf extracts on the isolates according to Balouiri *et al.* [34].

Agar-Well Diffusion Method: A 2 mm sterile cork borer was used to bore five holes in each of the inoculated Mueller-Hinton agar plates. The holes that were bored were appropriately labeled to represent each of the leaf extracts. For each of the extracts excluding the chloroform extract, the concentration of 300 mg/mL was used by weighing 600 mg of the stock extract and dissolving it in 2 mL of the solvent that was used for the extraction. For the chloroform extract, the concentration used was 150 mg/mL and it was obtained by weighing 300 mg of the stock extract and dissolving it in 2 mL of water. A different concentration was used due to the thickness of the chloroform stock extract compared to that of the other extracts. A micropipette was then used to dispense 50 µl of each extract into the respective holes that had been bored in the inoculated Mueller-Hinton agar plates. The plates were then incubated for 24 hours at 37°C. After incubation, the plates were observed for zones of inhibition. The zones of inhibition were measured in millimetres after incubation as described by Atobatele and Owoseni [33].

Paper Disc Diffusion Method: A perforator was used to cut out many pieces of filter paper into circular shapes. The papers were then put into a McCartney bottle which was then sterilized in an autoclave at 121°C for 15 minutes. The same concentration of the extracts was prepared as described above. The sterilized papers were divided into five parts for each extract and the papers were soaked for 30 minutes in the respective extracts which they were meant for. The inoculated Mueller-Hinton agar plate was appropriately labeled for each extract and a sterile forceps was then used to place the soaked papers onto the corresponding extract on each plate. After the paper discs had been carefully placed on the plates, the plates were incubated for 24 hours at 37°C in an incubator. The zones of inhibition were measured in millimetres after incubation as described by Balouiri *et al.* [34]

Results

Identification of bacterial isolates

Fifteen (15) bacterial isolates were obtained from the four "wara" samples considered in this study. ABIS online was used to identify the Gram-positive organisms while *Microrao* was used to identify Gram-negative isolates. Observed genera included *Bacillus*, *Citrobacter*, *Micrococcus*, *Paenibacillus*, and *Serratia* (Table 1). The percentage occurrence of each genus is represented in Figure 1 which shows the highest occurring genus to be *Bacillus* (46.7%) and the least occurring genus to be *Paenibacillus* (6.7%).

Phytochemical analyses of *G. africanum* leaf extracts

The extraction process followed after the leaves were properly dried (Plate 1) and qualitative phytochemical analysis was carried out on the methanol, n-hexane, and aqueous leaf extracts (Table 2). The screening showed that tannins, glycosides, anthraquinones, alkaloids, terpenes, and flavonoids were present in all the extracts, although to varying degrees. Phenols were absent in the methanol extract, saponins were absent in the n-hexane and aqueous extracts while steroids were absent in all three extracts. Tannins were found present in the methanol extract, while flavonoids were found present in the aqueous extract of the leaf. None of the phytochemicals were highly present in the n-hexane extract although some were moderately present, some were lightly present, and some were absent. Quantitative phytochemical screening was carried out on the aqueous and methanol extracts to determine the quantity of tannins, terpenoids, and anthraquinones in each of the extracts (Table 3). The aqueous extract had higher content of tannins (12 mg/100g) than the methanol extract (7 mg/100g). For the percentage of terpenoids and the value of anthraquinones, the methanol extracts had significantly higher values than the values of the aqueous extracts

Antibiotic Sensitivity Test

Antibiotic susceptibility tests carried out on the Gram-positive isolates showed that six of the isolates were resistant to at least two antibiotics each with *Micrococcus halobius* (B5) and *Paenibacillus pectinilyticus* showing the highest level of resistance (Table 4). *Micrococcus halobius* (B5) was resistant to gentamycin, ampiclox, and zinnacef while *Paenibacillus pectinilyticus* was resistant to ampiclox, azithromycin, and amoxicillin. All other four isolates were resistant to two each of ampiclox, azithromycin, zinnacef, and levofloxacin. Most of the isolates were susceptible to all the antibiotics with the highest susceptibility, with ciprofloxacin inhibiting *Bacillus licheniformis* having an inhibition zone of 29 mm. although it was fully resistant to ampiclox and zinnacef. The antibiotic susceptibility testing of the Gram-negative isolates revealed that none of the isolates were totally resistant to any of the antibiotics (Table 5). The highest zone of inhibition was 25 mm for *Serratia marcescens* (B2) when both septrin and tarivid were tested. Interestingly, the least zone of inhibition was also seen in *Serratia marcescens* which was 7 mm for augumentin antibiotic. The percentage susceptibility and resistance of the Gram-positive isolates to each antibiotic showed that the isolates were totally susceptible to pefloxacin, rocephin, ciprofloxacin, and erythromycin antibiotics (Figure 2). The other antibiotics showed varying levels of resistance with the least resistance in Gentamycin and Amoxicillin. In Figure 3, out of all tested antibiotics, ciprofloxacin presented as the most effective antibiotic for the Gram-negative organisms tested.

Susceptibility Tests of Leaf Extracts of *Gnetum africanum*

Figures 4 and 5 showed the measurements of the zones of inhibition that were seen for each of the extracts, in the agar-well diffusion method and the paper disc diffusion method, respectively. For the agar-well diffusion method, only the methanol and ethanol extracts showed activity against the organisms, and of the fifteen isolates, only four isolates had activity. The methanol and ethanol extracts each had activity against two of the four isolates. The highest activity for the methanol extract was seen in *Bacillus endophyticus* (D2), while the lowest activity was in *Bacillus licheniformis* (D4) (Plate 2). For the ethanol extract, *Bacillus pumilis* (C3) had a higher activity than *Citrobacter freundii* (A1). In the paper disc diffusion method, the methanol extract generally had more activity than the chloroform and ethanol extracts. *Micrococcus halobius* (B5) was one of the organisms with a 12 mm zone of inhibition while *Bacillus endophyticus* (D2) had the lowest zone of inhibition which was 8 mm for the methanol extract (Plate 3). The chloroform extract was effective against only one organism which was the isolate A1. For the ethanol extract, the highest activity was seen in *Bacillus licheniformis* (D4) while the lowest activity was seen in isolate A1. Also, *Citrobacter freundii*

(A1) was the isolate with the most overall activity, while *Serratia marcescens* (B2) and *Micrococcus halobius* (B5) were the isolates with the least overall activity.

Discussion

The presence of beneficial and non-beneficial microorganisms in food samples, especially those that are milk-based, continues to generate research interest for useful applications in food microbiology. This study aimed at isolating and identifying microorganisms, including pathogens and indicator organisms from "wara", a traditional snack consumed by millions of local patrons in Nigeria. Bacteria successfully isolated from "wara" samples considered in this study included the genera *Bacillus*, *Citrobacter*, *Micrococcus*, *Paenibacillus*, and *Serratia*. This suggests microbial contamination of the analyzed samples by these microorganisms as they are not usually resident in milk or milk products. The resident microorganisms in milk and milk products include bacteria in the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and other Lactic Acid Bacteria (LAB) [35]. Similar to the results of this study, *Bacillus cereus*, *Citrobacter* spp., and *Serratia marcescens* have successfully been isolated from "wara" in other states of southwestern Nigeria [36-38].

The antimicrobial susceptibility testing on the isolates led to the identification of resistant microorganisms including *Bacillus pumilis*, *Bacillus cereus*, *Micrococcus halobius*, and *Citrobacter freundii*. Two different isolates that were identified as *Bacillus pumilis* were resistant to the same antibiotics which were azithromycin and levofloxacin. All the resistant organisms were from the Gram-positive isolates, as none of the Gram-negative isolates were resistant to any of the antibiotics. The isolation of resistant microorganisms from "wara" highlights the potential health risks associated with the snack, and the need for improved hygiene and antibiotic use practices in food production. Olasupo *et al*. [39] also isolated *Staphylococcus aureus* and *Klebsiella* spp. from "wara" and both organisms were 100% resistant to ampicillin. They were also all sensitive to gentamycin and that is similar to the result obtained in this work where all the isolates were susceptible to gentamycin, except *Micrococcus halobius*.

Among the Gram-negative isolates, no resistance pattern was observed, however, two isolates had considerably low zones of inhibition for augmentin. *Citrobacter freundii* had an inhibition zone of 9 mm while *Serratia marcescens* had a zone of 7 mm. The organisms isolated from "wara" by [40] showed a large percentage resistance of 94.3% to augmentin and antibiotic-resistant *Serratia marcescens* have been identified in commercialized dairy products and ready-to-eat food samples [41-42]. Ciprofloxacin had the highest zone of inhibition among all the antibiotics that were tested against the isolates, and this susceptibility was seen in *Bacillus licheniformis* with an inhibition zone of 29 mm. Figure 3 shows that ciprofloxacin was the most efficient antibiotic against the organisms tested, and this demonstrates the effectiveness of the antibiotic against the isolates from "wara". It also validates its use as a common antibiotic prescribed, which is usually well-tolerated [43]. Multidrug-resistant organisms that were resistant to three antibiotics each were *Micrococcus halobius* and *Paenibacillus pectinilyticus*. This highlights the fact that "wara" can be a source of microorganisms with multidrug-resistant phenotypes [37].

The qualitative phytochemical screening of *G. africanum* leaves revealed the presence of bioactive compounds including tannins, glycosides, anthraquinones, alkaloids, phenols, terpenes, and flavonoids. The presence of these phytochemicals in *G. africanum* leaves suggests potential medicinal and therapeutic benefits and also indicates the plant's potential for pharmacological and health-promoting properties. Similar to the results obtained by Ezekwe *et al.* [2], the aqueous leaf extracts of *G. africanum* were rich in tannins, alkaloids, glycosides, and flavonoids. However, although saponins and steroids were also seen by [2], the two compounds were absent in the analysis carried out in this research, for the aqueous extracts. This could be due to differences in analytical techniques, environmental factors of growth, or genetic variability of the plants. Steroid compounds were also observed to also be absent in the phytochemical analysis carried out on *G. africanum* leaves in this study. Similar results were also obtained by using water, ethanol, methanol, and n-hexane as the extraction solvents [24]. The presence of the

phytochemicals validates the medicinal nature of the plant and accounts for its antimicrobial, and anti-inflammatory properties [3].

In the antimicrobial assays carried out on the isolates using the *G. africanum* leaf extracts, the methanol, ethanol, and chloroform extracts showed some activity against the microorganisms and of the two methods that were used, the paper disc diffusion method was the most effective because it showed the most activity across the different isolates and extracts. The antimicrobial activity of the leaf extracts against microorganisms from "wara" highlights their potential use in developing natural preservatives for food safety. Using the agar well diffusion method, the highest zone of inhibition observed was 15 mm and it was observed in the methanol and ethanol extracts for *Bacillus endophyticus* and *Bacillus pumilis*, respectively. In like manner, the highest activity against *Pseudomonas aeruginosa* was shown by the ethanol extract of *G. africanum* leaves in the research by [26]. For the paper disc diffusion method, the highest zone of inhibition overall was again shown by the ethanol extract against *Bacillus licheniformis*. However, the methanol extract exhibited the highest activity against seven of the fifteen isolates that were tested, with the highest zone of inhibition being 12 mm and the lowest being 8 mm. In a study conducted by Akin-Osanaiye *et al*. [44], methanol extracts of *G. africanum* stem and root also displayed antimicrobial activities against *Staphylococcus aureus*. In this study, chloroform extracts of *G. africanum* showed the least activity compared to other extracts, having an effect against only *Citrobacter freundii*. Vlad *et al.* [45] also reported that chloroform extracts of *G. africanum* leaves did not have any activity against the microorganisms that were tested.

Methanol and ethanol extracts of *G. africanum* showed activity against some of the microorganisms that were resistant to the conventional antibiotics including *Bacillus cereus*, *Micrococcus halobius*, *Paenibacillus pectinilyticus*, and *Bacillus licheniformis*. Atwaa *et al*. [23] also demonstrated that methanolic extracts of several tropical spices showed most significant activity against contaminating microorganisms in food samples. This shows that in some cases, while conventional antibiotics may not be effective against food-borne pathogens and other such microorganisms, natural-based substances such as *G. africanum* leaf extracts could be potential solutions. This also highlights the need for further research into the application of the extracts as natural antimicrobials and for the enhancement of the food safety of "wara".

The findings of this study suggest that *G. africanum* could be integrated into food safety preparation strategies to enhance the microbial quality of "wara", however, practical implementation may face challenges. These include maintaining consistent extract quality, optimizing extraction procedures for industrial scalability [46], assessing shelf-life impact, and establishing safe concentrations that do not affect the organoleptic properties of the snack [47]. Additionally, further research and optimization techniques are required before safe and effective integration can be achieved in traditional food production systems [48]. The practical adoption of *G. africanum* extracts in food processing would require overcoming consumer skepticism and meeting regulatory standards. While natural preservatives are generally perceived to be safer options, their public acceptance could depend on factors like cultural familiarity, clear product labeling, and how consumers perceive their health benefits [49,50]. For the regulatory hurdle, approval from food safety authorities would be essential to ensure these extracts are safe, properly standardized, and legally suitable for use in traditional snacks like "wara" (National Agency for Food and Drug Administration and Control (NAFDAC) [51].

Conclusion

This study highlighted significant microbial contamination in "wara", a traditional Nigerian snack, emphasizing the need for improved food safety measures throughout its production process. The identification of various pathogenic and indicator organisms, including *Bacillus*, *Citrobacter*, *Micrococcus*, *Paenibacillus*, and *Serratia*, highlights the risk of foodborne illnesses associated with "wara". Furthermore, the study explored the potential of *G. africanum* extracts as natural antimicrobial agents. While the methanol, chloroform, and ethanol extracts exhibited some antimicrobial activity against the isolates, their overall efficacy varied, with methanol extracts showing the most promise. Improving food safety

protocols, such as employing organic antimicrobials, might reduce the likelihood of foodborne illnesses and preserve the genuineness of customary munchies like "wara."

Acknowledgments

The authors wish to acknowledge Bowen University Microbiology Laboratory, Bowen University Iwo, Nigeria for providing facility and technical assistance in the microbiological aspects of the study.

Declaration of Interest Statement

The authors declare that no competing conflict of interest exists with regards to the study.

Table 1: Morphological and Biochemical Testing of bacteria isolated from wara

S/N	Isolate	Citrate	Indole	MR	VP	Motility	Catalase	Starch	Gram	Glucose	Lactose	Mannitol	Sucrose	Most Probable Organism
1.	A1	+	-	+	-	+	+	-	-	+	+	+	+	*Citrobacter freundii*
2.	A2	+	-	+	-	+	+	+	+	g+	g+	g+	g+	*Micrococcus halobios*
3.	A3	+	-	+	-	+	+	-	+	g+	g+	g+	g+	*Bacillus pumilus*
4.	B1	+	-	+	+	+	+	-	+	g+	g+	g+	g+	*Bacillus pumilus*
5.	B2	+	-	-	+	+	-	+	-	-	-	-	+	*Serratia marcescens*
6.	B3	+	-	+	+	+	-	+	+	+	-	-	+	*Bacillus cereus*
7.	B4	+	-	-	+	+	+	+	+	-	-	-	+	*Bacillus cereus*
8.	B5	+	-	+	-	+	+	-	+	g+	g+	g+	g+	*Micrococcus halobios*
9.	C2	+	-	+	-	+	+	-	-	+	+	+	+	*Citrobacter freundii*
10.	C3	+	-	+	+	+	+	-	+	+	-	-	+	*Bacillus pumilis*
11.	C4	+	-	-	+	+	-	+	+	+	-	-	+	*Micrococcus luteus*
12.	D1	-	-	+	+	+	+	-	-	+	-	+	+	*Serratia marcescens*
13.	D2	+	-	+	-	-	+	-	+	+	-	+	+	*Bacillus endophyticus*
14.	D3	+	-	+	+	+	+	-	+	g+	-	g+	+	*Paenibacillus pectinilyticus*
15.	D4	+	-	+	+	+	+	+	+	g+	g+	g+	g+	*Bacillus licheniformis*

KEY:
MR = Methyl Red test, VP = Voges-Proskauer test, - = Negative, + = Positive,
g+ = Positive with gas production, Gram = Gram Reaction

Table 2: Phytochemical screening of *G. africanum* leaf extracts

Sample/Extract	Tannins	Steroids	Glycosides	Anthraquinones	Alkaloids	Terpenes	Saponins	Phenols	Flavonoids
Methanol	+++	-	+	+	++	++	+	-	++
N-hexane	+	-	++	+	++	+	-	+	++
Aqueous	++	-	+	++	++	++	-	+	+++

KEY: Highly present = (+++), moderately present = (++), lightly present = (+), Absent = (-)

Table 3: Quantitative Phytochemical Analysis of *G. africanum* extracts

S/N	COMPOUND	AQUEOUS EXTRACT	METHANOL EXTRACT
1.	**Tannins**	12 mg/100 g	7 mg/100 g
2.	**Terpenoids**	39%	45%
3.	**Anthraquinones**	11.34	25.1

Table 4: Antibiotic Susceptibility Testing of Gram-positive Isolates from wara

KEY: PEF = Pefloxacin, CN = Gentamycin, APX = Ampiclox, R = Rocephin, CPX = Ciprofloxacin,

ISOLATE/ANTIBIOTICS	PEF (mm)	CN (mm)	APX (mm)	R (mm)	CPX (mm)	AZ (mm)	Z (mm)	AM (mm)	LEV (mm)	E (mm)
***Micrococcus halobius* (A2)**	17	18	16	18	20	16	17	16	18	18
***Bacillus pumilis* (A3)**	18	17	18	16	19	-	18	15	-	10
***Bacillus pumilis* (B1)**	18	18	17	17	19	13	18	17	19	16
***Bacillus cereus* (B3)**	17	18	17	17	18	18	20	18	19	18
***Bacillus cereus* (B4)**	17	18	18	24	21	-	19	18	-	12
***Micrococcus halobius* (B5)**	18	-	-	18	17	17	-	10	18	18
***Bacillus pumilis* (C3)**	19	17	18	17	18	-	18	17	-	18
Micrococcus luteus	16	17	19	17	18	18	24	19	18	18
Bacillus endophyticus	17	17	21	18	18	16	10	23	17	17
Paenibacillus pectinilyticus	17	17	-	15	19	17	-	-	17	16
Bacillus licheniformis	23	17	-	15	29	14	-	16	22	16

AZ = Azithromycin, Z = Zinnacef, AM = Amoxicillin, LEV = Levofloxacin, E = Erythromycin, - = No zone of inhibition

Table 5: Antibiotic Susceptibility Testing Results for Gram-negative Isolates

ISOLATE/ANTIBIOTIC	SXT (mm)	CH (mm)	SP (mm)	CPX (mm)	AM (mm)	AU (mm)	CN (mm)	PEF (mm)	OFX (mm)	S (mm)
***Citrobacter freundii* (A1)**	10	18	20	24	13	9	16	22	22	18
***Serratia marcescens* (B2)**	25	16	22	24	16	7	17	21	25	17
***Citrobacter freundii* (C2)**	18	18	18	18	17	17	17	17	17	20
***Serratia marcescens* (D1)**	19	16	15	17	15	16	18	17	17	17

KEY: SXT = Septrin, CH = Chloramphenicol, SP = Sarfloxacin, CPX = Ciprofloxacin, AM = Amoxicillin, AU = Augmentin, CN = Gentamycin, PEF = Pefloxacin, OFX = Tarivid, S = Streptomycin

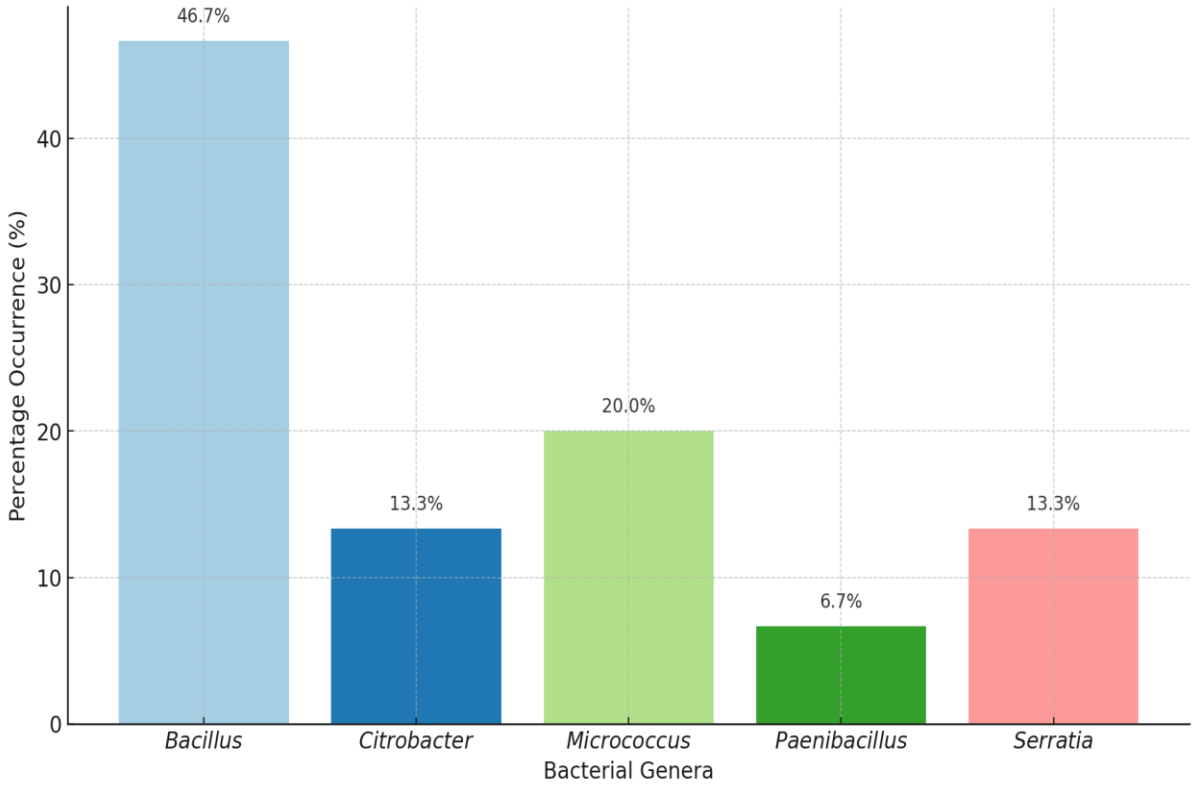

Figure 1

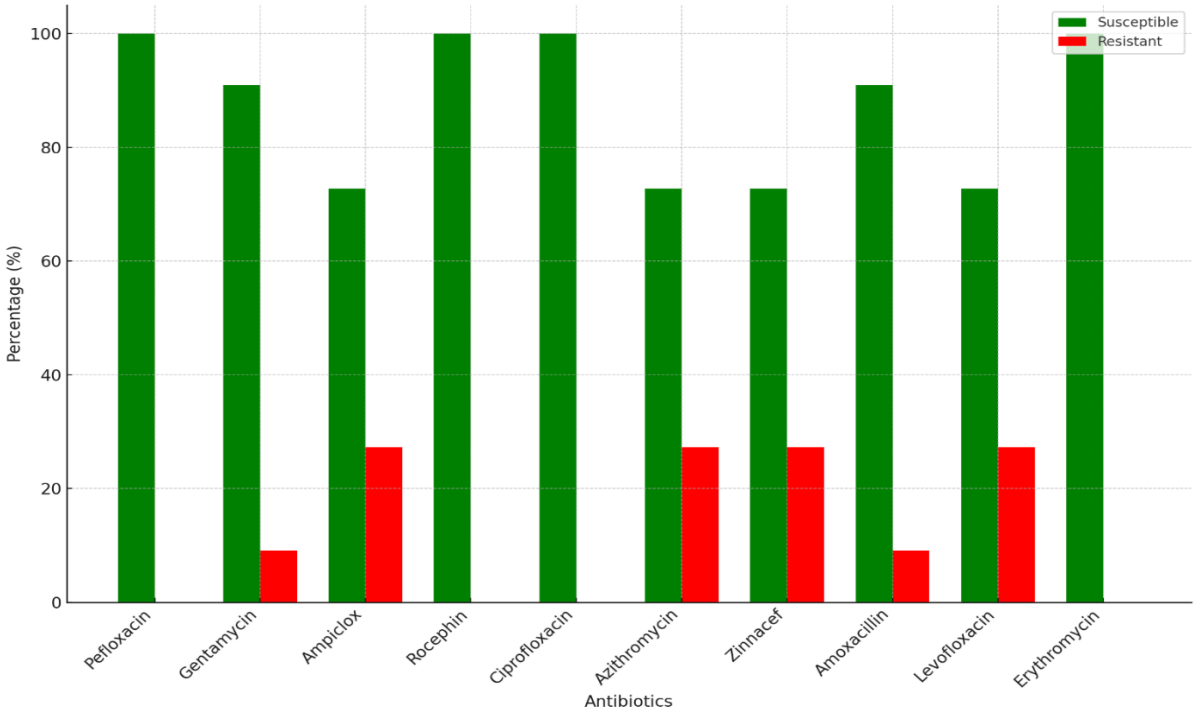

Figure 2

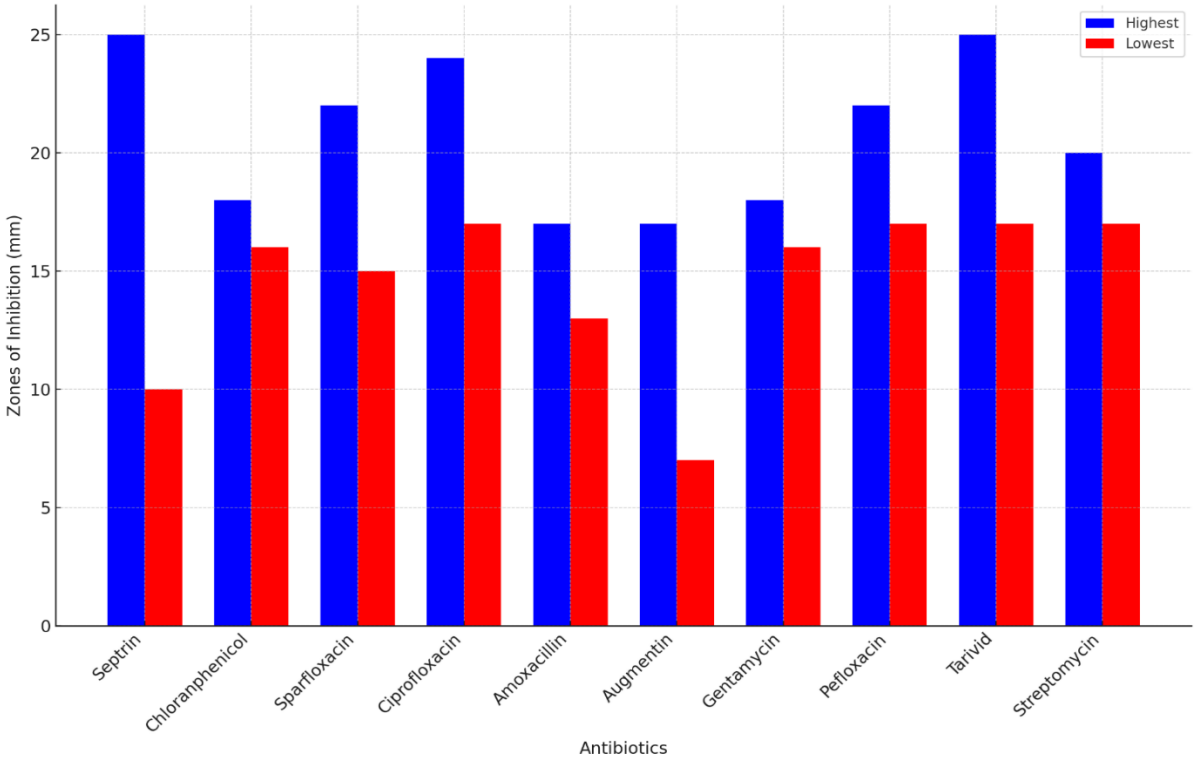

Figure 3

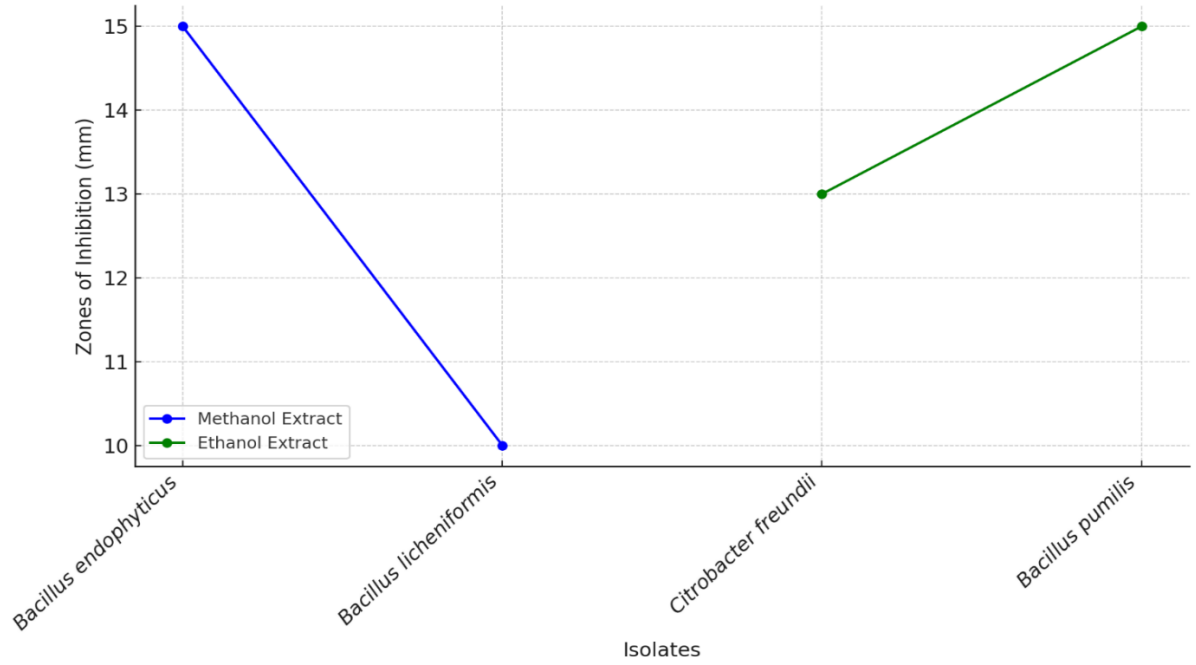

Figure 4

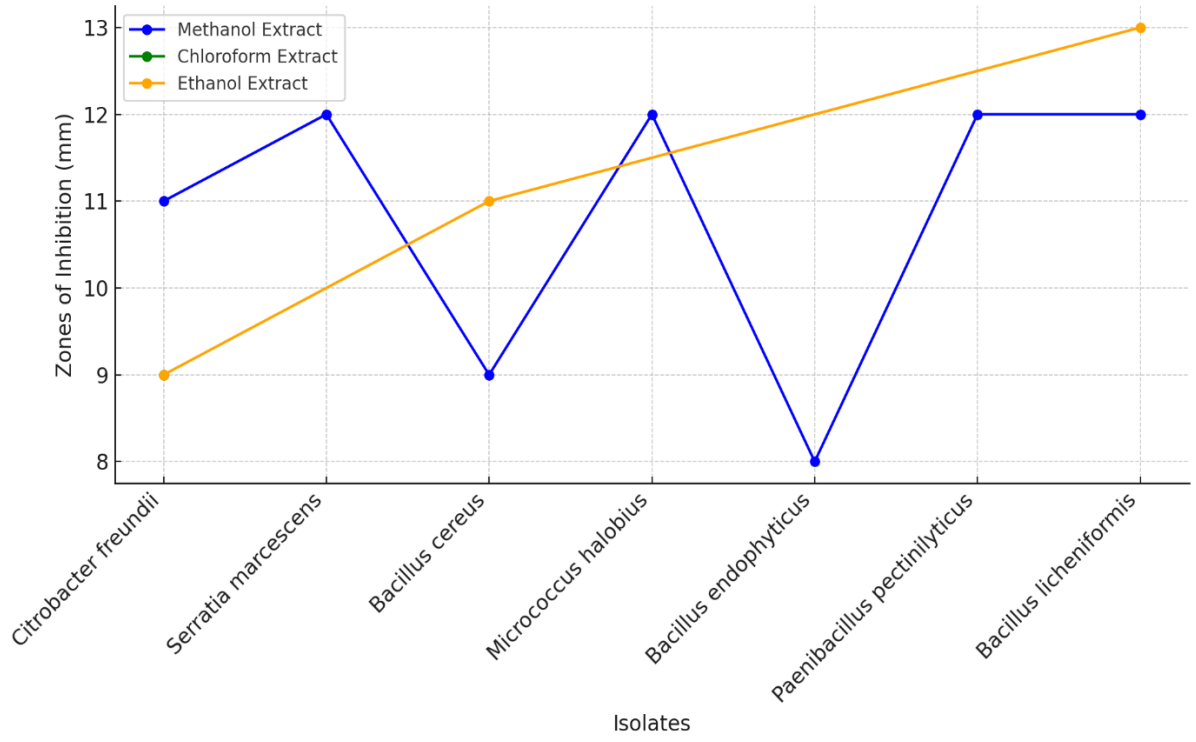

Figure 5

Plate 1

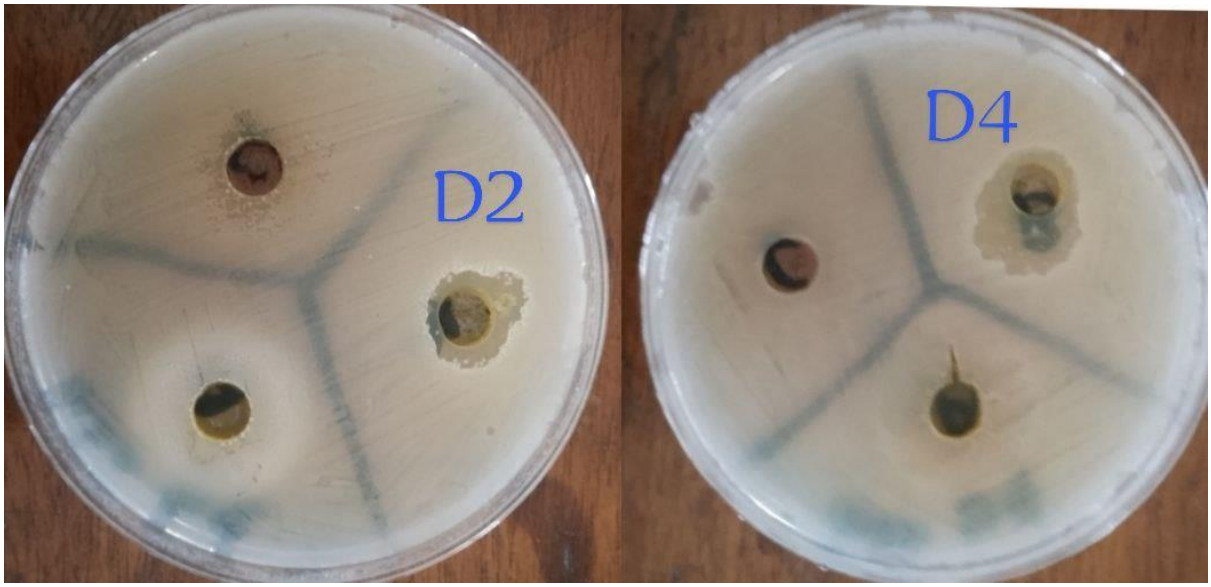

Plate 2

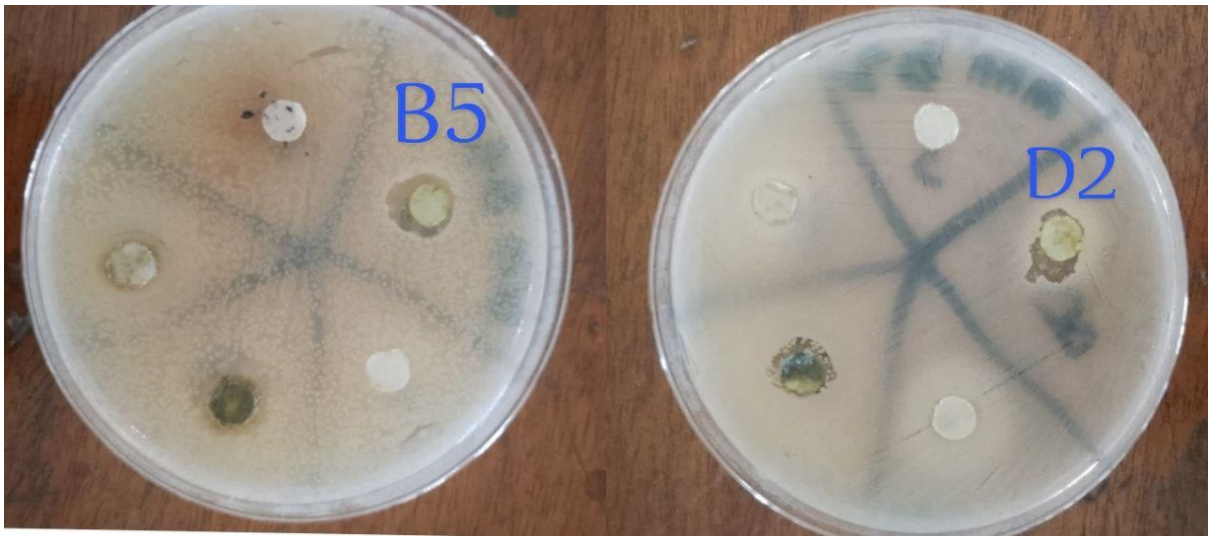

Plate 3

FIGURE (AND PLATE) CAPTIONS

Figure 1: Percentage Occurrence of Bacterial Genera in "Wara" Samples
Figure 2: Percentage Resistance and Susceptibility of Gram-positive Isolates to Antibiotics
Figure 3: Percentage Resistance and Susceptibility of Gram-negative Isolates to Antibiotics
Figure 4: Result of Antimicrobial Susceptibility Test Using the Agar-Well Diffusion Method
Figure 5: Antimicrobial Susceptibility Test Using the Paper Disc Diffusion Method

Plate 1: Shredded *Gnetum africanum* Leaves Being Air Dried

Plate 2: Zone of inhibition of methanol leaf extracts against *Bacillus endophyticus* (D2) and *Bacillus licheniformis* (D4) using the agar well diffusion method

Plate 3: Zone of inhibition of methanol leaf extracts against *Micrococcus halobius* (B5) and *Bacillus endophyticus* (D2) using the paper disc diffusion method

References

[1] Wang XQ, Ran JH. Evolution and biogeography of gymnosperms. Molecular Phylogenetics and Evolution. 2014; 75:24–40. https://doi.org/10.1016/j.ympev.2014.02.005.

[2] Ezekwe AS, Ugwuezumba PC, Nwankpa P, Egwurugwu JN, Ekweogu, CN, Emengaha F, Akukwu D. Qualitative Phytochemical Screening, GCMS Studies and *In-Vitro* Anti-Oxidative Properties of Aqueous Leaf Extract of *Gnetum africanum*. Journal of Drug Delivery and Therapeutics**.** 2020; 10(1): 11-17. http://dx.doi.org/10.22270/jddt.v10i1.3813

[3] Ali F, Assanta M, Robert C. *Gnetum africanum*: A Wild Food Plant from the African Forest with Many Nutritional and Medicinal Properties. Journal of Med. Food. 2011; 14:1289-97. 10.1089/jmf.2010.0327.

[4] Doungous O, Minyaka E, Medza-Mve SD, Medueghue AF, Ngone MA, Simo C, Nsimi AM. Improving propagation methods of *Gnetum africanum* and *G. buchholzianum* from cuttings for rapid multiplication, domestication and conservation. Agroforestry Systems. 2019;93(4):1557–65. https://doi.org/10.1007/s10457-018-0269-8

[5] Biye EH, Balkwill K, Cron GV. A clarification of *Gnetum* L. (Gnetaceae) in Africa and the description of two new species. Plant System. and Evol. 2014;300(2):263–72. https://doi.org/10.1007/s00606-013-0879-6https://doi.org/10.1007/s00606-013-0879-6

[6] Dada TE, Otitoloju K, Adjonu R, Crockett J, Nwose EU. Nutritional and medicinal values of common green leafy vegetables consumed in Delta State, Nigeria: A review. Intl J of Comm. Med. and Pub. Health. 2021;8(5):2564. https://doi.org/10.18203/2394-6040.ijcmph20211789

[7] Cole AT, Omole FO, Lawal IK, Adedunmola PB, Olasunkanmi BT, Olowoyeye OJ. Assessment of medicinal importance and application of wild edible vegetables in Ikom Ethnic Group of Cross River State. Assessment. 2022;6(5):229–236. ISSN 2643-9603.

[8] Nonye B. Health Benefits and Uses of Ọkazị Leaves. Agric4Profits. 2024 [accessed 2024 Jan 9]. Available from https://agric4profits.com/health-benefits-and-uses-of-ọkazị-leaves/

[9] Raman R. 7 Impressive Ways Vitamin C Benefits Your Body. Healthline. 2020 [accessed 2024 Oct 22]. Available from https://www.healthline.com/nutrition/vitamin-c-benefits

[10] Gürbüz M, Aktac Ş. Understanding the role of vitamin A and its precursors in the immune system. Nutrition Clinique Et Métabolisme 2022;36(2):89–98. https://doi.org/10.1016/j.nupar.2021.10.002

[11] Charlebois E, Pantopoulos K. Nutritional Aspects of Iron in Health and Disease. Nutrients. 2023;15:2441. https://doi.org/10.3390/nu15112441

[12] Ogbonnaya EC, Chinedum EK. Health promoting compounds and in vitro antioxidant activity of raw and decoctions of *Gnetum africanum* Welw. Asian Pacific Journ. of Trop. Dis. 2013;3(6):472–79. https://doi.org/10.1016/S2222-1808(13)60103-6

[13] Ilodibia CV, Ugwu RU, Nwokolo OL, Chukwuma MU, Akachukwu EE. Phytochemical screening, antifungal and antibacterial activity of aqueous and ethanol leaf and stem extracts of *Gnetum africanum* Welw. Research Journal of Med Plants. 2015;9(6):275–83.

[14] Umoh IU, Nkeme KK, Ekanem JT. Understanding the capabilities of *Gnetum africanum* production for improved livelihood and sustainable development among rural farmers in Akwa Ibom state Nigeria. Journal of Agricultural Economics, Extension & Rural Development. 2019; 2(1):21-29.

[15] Adetunji VO, Alonge DO, Singh RK, Chen J. Production of Wara, a West African soft cheese using lemon juice as a coagulant. LWT - Food Science and Technology. 2008;41(2): 331-36. https://doi.org/10.1016/j.lwt.2007.02.012.

[16] Hassan A. Awara: The Nigerian snack that smacks of goodness. TRT Afrika. 2023 [2024 Feb 20]. Available from https://www.trtafrika.com/lifestyle/awara-the-nigerian-snack-that-smacks-of-goodness-13643288

[17] Ayeni AO, Adeeyo O, Obanla, O. The production of Wara cheese from locally sourced coagulants and its nutritional evaluation. IOSR Journal of Envtl Sci Toxicol. and Food Tech. 2014;8:55-57. 10.9790/2402-081025557

[18] Atuonwu A. Effect of Selected Coagulants on the Production Of "Wara": A West Africa Soft Cheese. 2022 [2024 Oct 23]. Available from https://repository.mouau.edu.ng/work/view/effect-of-selected-coagulants-on-the-production-of-wara-a-west-africa-soft-cheese-7-2

[19] Omotolani. This local delicacy, wara, is not cheese. 2020 [2024 Feb 18]. Available from https://www.pulse.ng/lifestyle/food-travel/wara-this-local-delicacy-is-not-cheese/x8mpf4m\

[20] Nwadozie J. How to Make Wara. Guardian:Life. 2024 [2024 Feb 18]. Available from https://guardian.ng/life/how-to-make-wara/

[21] Adetola MA. Nigeria's popular wara cheese has a short shelf life: We've found a way to keep it fresh for longer. The Conversation. 2024 [2024 Feb 18]. Available from https://theconversation.com/nigerias-popular-wara-cheese-has-a-short-shelf-life-weve-found-a-way-to-keep-it-fresh-for-longer-220968

[22] Akanbi BO, Usoh EA. Safety of Street-Vended Soy Wara in Nigeria. Journal of Food Protection. 2016;79(1):169–73. https://doi.org/10.4315/0362-028X.JFP-15-136

[23] Atwaa ESH, Shahein MR, Radwan HA, Mohammed NS, Aloraini MA, Albezrah NKA, Alharbi MA, Sayed HH, Dauod MA, Elmahallawy EK. Antimicrobial Activity of Some Plant Extracts and Their Applications in Homemade Tomato Paste and Pasteurized Cow Milk as Natural Preservatives. Fermentation. 2022;8: 428. https://doi.org/10.3390/ fermentation8090428

[24] Okerulu I, Onyema C. Comparative Assessment of Phytochemicals, Proximate and Elemental Composition of *Gnetum africanum* (Okazi) Leaves. Ame Journal of Analyt Chem. 2015; 6:604-609. doi: 10.4236/ajac.2015.67058.

[25] Dhakal S, Aryal P, Aryal S, Bashyal D, Khadka D. Phytochemical and antioxidant studies of methanol and chloroform extract from leaves of *Azadirachta indica* A. Juss. in the tropical region of Nepal. Journal of Pharmacognosy and Phytotherapy. 2016;8(12):203-208.

[26] Ayuk EL, Oforji CF, Ugwu F, Aronimo SB, Njokunwogbu A. Determination of secondary metabolites and biological potential of *Gnetum africanum* (Okazi) Leaves. The Pharm and Chem. Journal. 2017;4(4):115-122.

[27] Udeh NE, Anaga A, Asuzu IU. Acute and sub-chronic oral toxicity studies on methanol leaf extract of *Gnetum africanum* Welw. in wistar rats. Am J Res Med Sci. 2018;3(1): 7-14.

[28] Handa S, Khanuja, S.P., Longo, G. and Rakesh, D.D., 2008, Extraction Technologies for Medicinal and Aromatic Plants. United Nations Industrial Development Organization and the International Centre for Science and High Technology.

[29] Junaid RS, Patil, MK. Qualitative tests for preliminary phytochemical screening: An overview. Int J Chem Stu 2020; 603 – 608.

[30] Banu KS, Cathrine L. General techniques involved in phytochemical analysis. Int J Adv Res Chem Sci. 2015;2(4): ISSN 2349–039X.

[31] Longbap C, Ushie OA, Ogah E, Kendenson AC, Nyikyaa, JT. Phytochemical screening and Quantitative determination of phytochemicals in leaf extracts in *Hannao undulata*. Intl J of Med Plants and Nat Prd 2018;4(2): 32–38 doi: 10.20431/2454-7999.0402005

[32] Chessbrough, M. (2000). Bacteriological Testing of water. Cambridge low price edition, Cambridge University Press. 434.

[33] Atobatele, B.O and Owoseni, A.A (2023). Distribution of multiple antibiotic-resistant Gram-negative bacteria in potable water from hand-dug wells in Iwo, Nigeria. *H2Open Journal* Vol 6 No 1, 40 doi: 10.2166/h2oj.2023.043

[34] Balouiri M, Sadiki M, Ibnsouda SK (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

[35] Hickey CD, Sheehan JJ, Wilkinson MG, Auty ME. Growth and location of bacterial colonies within dairy foods

using microscopy techniques: A review. Frontiers in Microbiology 2015;6(99):1 – 7. https://doi.org/10.3389/fmicb.2015.00099

[36] Oguntoyinbo FA, Kintum A. Toxigenic *Bacillus cereus* isolated from Nunu and Wara, two Nigerian fermented dairy foods. Nigerian Food Jour 2017;33(2):39-45.

[37] Amosun EA, Agbato AO, Daodu OB, Ojo OE. Evaluation of Bacteria and Antibiotic Resistance Profiling of Wara (White Soft Cheese) in Oyo-State, South-West, Nigeria. Nig. Vet. J. 2017;38(4):319-330. https://doi.org/10.4314/nvj.v38i4.7

[38] Adebayo A, Olubukola O, Oluseye A, Juliet C, Sukurah O, Waheed O, Fowora M, Okesina AB. Molecular characterization and antibiotic susceptibility patterns of bacteria isolated from wara (West African cheese) sold in Osun state, Nigeria. Innovative Romanian Food Biotech. 2014;5:23-30.

[39] Olasupo NA, Smith SI, Akinsinde KA. Examination of the microbial status of selected indigenous fermented foods in Nigeria. Journal of Food Safety. 2007;22(2):85-93. https://doi.org/10.1111/j.1745-4565.2002.tb00332.x

[40] Omoshaba EO, Ojo OE, Sofela OO, Onifade OI. Prevalence and antibiotic resistance patterns of methicillin-resistant Staphylococcus aureus in raw milk and soft cheese (wara) sold in Abeokuta, Nigeria. Sokoto J of Vet Sci. 2018;16:1-8.

[41] Melo DH, Souza, BV, Nascimento JS, Amorim AM, Medeiros LM, Mattoso JM. A reddish problem: Antibiotic-resistant Serratia marcescens in dairy food commercialized in Rio de Janeiro. Intl Food Res J. 2018;25(2):880

[42] Roy S, Wangkheimayum J, Chaudhury SR, Das BJ, Mazumda PB, Bhattacharjee A. Occurrence of virulent *Serratia marcescens* with co-existing antibiotic resistance determinants in ready-to-eat food samples. J of Microbiol and Infectious Dis. 2023; 13(3):118-24. 10.5455/JMID. 2023.v13.i3.3

[43] Thai T, Salisbury BH, Zito PM., Ciprofloxacin. StatPearls - NCBI Bookshelf. 2023. Available from https://www.ncbi.nlm.nih.gov/books/NBK535454/

[44] Akin-Osanaiye BC, Ekpeyong EJ, Olobayotan IW. Phytochemical and antimicrobial screening of *Gnetum africanum* stem and root. Intl J of Path Res. 2019;3:1–11. https://doi.org/10.9734/ijpr/2019/v3i230090

[45] Vlad CS, Laia L, Vlaia V, Dumitrașcu V, Filimon MN, Popescu R, Cimperoscu A, Dehelean C, Onwubiko, CE, Daliborca, CV. Chromatographic analysis and antibacterial potential of extracts of *Gnetum africanum*. Farmacia. 2019; 67(6):1083-90. 10.31925/farmacia.2019.6.22

[46] Gyawali R, Ibrahim SA. Natural products as antimicrobial agents. Food Control. 2014; 46:412–29. https://doi.org/10.1016/j.foodcont.2014.05.047

[47] Tiwari BK, Valdramidis VP, O'Donnell CP, Muthukumarappan K, Bourke P, Cullen PJ. Application of natural antimicrobials for food preservation. Journal of Agric and Food Chem. 2009;57(14):5987–6000. https://doi.org/10.1021/jf900668n

[48] Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. International Journal of Food Microbiology. 2004;94(3):223–253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022

[49] Mesías F, Martín A, Hernández A. Consumers' growing appetite for natural foods: Perceptions towards the use of natural preservatives in fresh fruit. Food Res Intl. 2021;150: 110749. https://doi.org/10.1016/j.foodres.2021.110749

[50] Perito MA, Chiodo E, Serio A, Paparella A. Fantini A. Factors Influencing Consumers' Attitude Towards Biopreservatives. Sustainability. 2020;12(24):10338. https://doi.org/10.3390/su122410338

[51] National Agency for Food and Drug Administration and Control NAFDAC. Food Additives Regulations 2019. Policy-Vault Africa. 2019. Available from https://policyvault.africa/policy/nafdac-food-additives-regulations-2019/