

## Antimicrobial Activity of African Walnut (*Tetracarpidium conophorum*) Oil against Bacterial and Fungal Species Causing Food Spoilage and Food Poisoning Diseases

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### ABSTRACT

This study evaluated the antibacterial and antifungal activity of African walnut (*Tetracarpidium conophorum*) oil against species causing food poisoning and food spoilage. African walnut oil was extracted using the solvent extraction method. The fatty acid profile of the oil was determined using gas chromatography mass spectrometry (GC-MS). The agar disc diffusion method was used to determine the antibacterial activity of the oil before using it for further tests. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were also determined using the disc diffusion assay. The result indicates that palmitic (1.53%), linoleic (13.5%), linolenic 80.59%, stearic (4.41%), and eicosenoic acid

(0.42%) were present in the oil with higher polyunsaturated fatty acid values. African walnut oil showed remarkable activity against all the Gram positive organisms (*Bacillus cereus* ATCC 33018, *Staphylococcus aureus* ATCC 25923, *Clostridium perfringens* NCTC 8799, and *Listeria monocytogenes* ATCC 13932) with the inhibition zones ranging from  $21.6 \pm 0.27$  mm to  $26.3 \pm 0.23$  while the Gram negative organisms (*Pseudomonas fluorescens* ATCC 13883, *Pseudomonas fragi* ATCC 4973, *Escherichia coli* ATCC 12806, *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028) were less sensitive and resistant to the oil chromatography-mass with inhibition zones ranging from  $6.3 \pm 0.31$  to  $9.9 \pm 0.21$  mm. Out of the tested oil concentrations from 100 – 0.39 mg/mL, MIC obtained for the Gram positive organisms was 1.56 mg/mL while it was 3.13 mg/mL for Gram negative bacteria (except *L. monocytogenes*). The antifungal test reveals the MIC range of 100 - 0.78 mg/mL in all the fungal isolates tested. African walnut oil has proved to be potentially active against the Gram positive bacterial and fungal species tested indicating that it can be used as a natural alternative to control food spoilage and food poisoning diseases.

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**Keywords:** African walnut oil, Antifungal activity, Minimum bactericidal concentration, Minimum inhibitory concentration, Fatty acids, *Tetracarpidium conophorum*.

## INTRODUCTION

Food poisoning and food spoilage organisms have been considered as the main causes of diseases in humans and food quality deterioration in developed and developing countries across the globe. Food spoilage organisms are known to make foods undesirable and unacceptable for human consumption due to changes in sensory characteristics. To ensure the safety of foods and to extend the keeping quality of food products, the additions of chemical additives and preservatives into food products through physical, biological, or chemical means have been practiced in many food industries (Mith *et al.*, 2014). However, there are growing concerns about using both additives and preservatives in food products because their prolonged or excessive consumption may be linked with various health problems, and since most bacterial species after treatment still survive leading to the development of highly

resistant bacteria responsible for foodborne diseases (Mith *et al.*, 2014).

Foodborne diseases are responsible for morbidity and mortality globally (Bintsis, 2017). Numerous types of diseases can be transmitted to people through the consumption of contaminated food products. Out of the three main biological hazards causing acute foodborne diseases in humans, bacteria are the most important cause of foodborne diseases (Kirk *et al.*, 2017). Bacterial food contamination occurs at any point along the food chain (production, processing, and distribution) and through various sources such as soil, air, water, poor hygiene and sanitation practices in food industries (Nerin *et al.*, 2016; Kirk *et al.*, 2017). Many bacterial foodborne pathogens that cause serious human diseases include *Staphylococcus aureus*, *Escherichia coli*, *Campylobacter jejuni*, *Bacillus cereus*, *Clostridium botulinum*, *Listeria monocytogens*, and *Salmonella* sp. (Newell *et al.*, 2010). The majority of these pathogenic bacteria in food have become resistant to antibiotics leading to serious foodborne infections with complications in humans (Canica *et al.*,

2019). To curb the menace of foodborne pathogens, many pharmaceutical companies started producing new antibacterial drugs but the emergence of bacterial strains with multiple resistances hindered the activity of the new drugs leading to a global concern. However, because of the public health concern, the use of antibacterial in humans has been with caution. Consequently, there is a widespread search for plants with novel antimicrobials that could be used as alternative antibacterial compounds to control the growth of food spoilage and foodborne pathogens.

Plant oils have long constituted a natural source of antimicrobial compounds in food systems. They are extracted from plants and exhibit antibacterial and antifungal activity thus they are used to prevent the growth of food spoilage and food poisoning microorganisms resulting in a longer shelf life for food products. Many research studies have been carried out with a variety of foods such as meats, poultry, juices, vegetables, and ready-to-eat products and plant oils have exhibited antimicrobial activity against food spoilage and food poisoning

microorganisms. For example, a study conducted by Jana *et al.* (2015) found that lavender and rosemary oil inhibited the growth of *Pseudomonas* spp. Jayasena and Jo (2013) reported the oil extracted from thyme, rosemary, basil cloves, and fennel inhibited the growth of spoilage organisms such as *Pseudomonas*, *Enterobacter*, and *Acinetobacter* and food poisoning organisms such as *Salmonella* spp., *L. monocytogenes*, *Campylobacter jejuni*. Similarly, Javed *et al.* (2012) found that the clove and fennel essential oils inhibited the growth of spoilage organisms such as *Pseudomonas syringae*, *Bacillus subtilis*, *E. coli*, and *Staphylococcus* spp. and food pathogenic fungal organisms such as *Alternaria alternate*, *Fusarium oxysporum*, and *Aspergillus flavus*.

African walnut (*Tetracarpidium conophorum*) is used for therapeutic and culinary purposes worldwide (Udedi *et al.*, 2014; Ogunwusi and Ibrahim, 2016). African walnut oil has a high amount of phenols that can hinder the growth of both food spoilage and foodborne pathogens. In addition, it contains bioactive components like important minerals, saturated, monounsaturated,

and polyunsaturated fatty acids (Tchiegang *et al.*, 2001; Yangomodou *et al.*, 2020) African walnut oil is rich in linoleic (C18:2) and linolenic acid (C18:3) that help reduce cardiovascular and coronary heart diseases as well as Type 2 diabetes in people and promote healthy brain function, support good skin and hair, improve reproductive health and boost immune function (De Lorgeril and Salen, 2004).

However, limited information is available on using African walnut oil to control the growth of microorganisms causing food poisoning and food spoilage. Hence, the present study was conducted to evaluate the antibacterial and antifungal activity of African walnut oil against selected species causing food poisoning and food spoilage.

## MATERIALS AND METHODS

### *Plant Material*

Fresh African walnut (*Tetracarpidium conophorum*) was collected from walnut trees growing in Ogun State from September through October 2022 and a final sample of about 6 kg was randomly taken. The nuts were transported to the

Microbiology laboratory of Olabisi Onabanjo University, Ago-Iwoye, Ogun State in a paper bag where they were authenticated by a taxonomist.

### *Processing of Plant Material*

After harvesting and identification, African walnuts were sorted, and damaged ones were discarded. The nuts were preliminary washed three times under running tap water and rinsed twice with sterile distilled water to remove contaminants and other extraneous matter. After washing, the black husks were removed from the nuts manually and the nuts were again rinsed. The cleaned nuts were cut into small pieces and sundried in the shade for two weeks. Thereafter, the nuts were milled using a Marlex Excella mixer/ grinding machine (Amazon, UK) and sieved into a mean particle size distribution of less than 35-Mesh. The sieved samples were packed in air tight containers and kept in the refrigerator at 4°C for further processing. The method of Masoodi *et al.* (2022) was employed in the processing of African walnut plant materials.

### ***Extraction of African Walnut Oil***

The extraction was carried out using n-hexane solvent described by Jokic *et al.* (2014). Prior to extraction, the pulverized nut samples were kept in an oven at 105 °C for 1 h to remove any moisture that may still be present. Twenty grams (20 g) of the dried nut sample was wrapped in a white muslin cloth and put into a porous thimble of the Soxhlet extractor. Then, 200 ml of the n-hexane of HPLC grade with a boiling range of 40 - 60 °C was added. The Soxhlet coupled with a condenser and flask already filled with the set up was heated in a heating mantle at 65 °C to allow solvent boiling. In the process, the solvent vapour travels up a distillation arm and flows into the chamber housing the sample material. The extract seeps through the pores of the thimble and fills the siphon tube where it flows back down into the round bottom flask. The process was allowed to continue for 8 h until a clear solvent was obtained in the thimble chamber. At the end of the extraction, the resulting mixture of oil from the solvent was filtered with a 10 mm Syringe-driven filter of 0.45 µm to remove any impurities. The solvent was further

removed completely with a rotary-evaporator (Model N-1, Eyela, Tokyo Rikakikal Co., Ltd., Japan). The oil was stored in white bottles and tubes under nitrogen at 4 °C until analyzed.

### ***Determination of Fatty Acid Content of African Walnut Oil***

A preliminary investigation of the active component of African walnut oil was carried out. The active components of African walnut oil were identified using gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS was used to identify the fatty acids present in the oil that could be responsible for its antimicrobial activity. In doing this, walnut oil obtained from a screw press was first trans-esterified into fatty acid methyl esters (FAME) by Boron trifluoride methanolysis-BF<sub>3</sub>-MeOH. This was done following the guidelines of the International Olive Oil Council (IOOC, 2006). After trans-esterification, the upper hexane layer that contained methyl esters was decanted into amber Agilent vials and stored at -20°C for GC analysis. The sample was thereafter sent to GlycoAnalytics Services, San Diego, United States for accurate analysis. African walnut methyl ester sample was

pre-analyzed by injecting 1  $\mu\text{L}$  and profiling of fatty acids was done using an Agilent 6890N Gas Chromatography device (Agilent Technologies, Wokingham, United States). Profiling and quantification of fatty acids were achieved using Restek-5MS (30 m x 30 mm x 0.25  $\mu\text{m}$ ) column. The column temperature was programmed at 80  $^{\circ}\text{C}$  for 3 min and then raised to 220  $^{\circ}\text{C}$  at 4  $^{\circ}\text{C}$   $\text{min}^{-1}$ , and then held for 3 min. The carrier gas was helium with a column flow rate of 1.12  $\text{cm}^3$  and the Mass detector transfer line temperature of 280  $^{\circ}\text{C}$ . The fatty acids were identified and quantified by comparing sample peak retention times with those of known mixed supelco 37 standards of known composition and mass fragmentation pattern of a standard mixture of FAME (Supleco, UK). Also, peaks were identified by comparing the mass spectra with the mass spectral database in NIST Library 2008. The fatty acid profile of African walnut oil was determined as corresponding methyl esters.

### ***Antimicrobial Activity***

#### *Bacterial Strains and Culture Conditions*

To determine the antibacterial properties of African walnut oil, eight strains responsible for food poisoning and spoilage were used. Bacterial strains are composed of four Gram positive bacteria (*B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringes* NCTC 8799, and *L. monocytogenes* ATCC 13932) and Gram negative (*P. fluorescens* ATCC 13883, *P. fragi* ATCC 4973, *E. coli* ATCC 12806 and *Salmonella typhimurium* ATCC 14028). All the bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC Manassas, VA, USA). Bacterial strains were kept alive using the short and long-term maintenance methods described by Vitko, (2013). The bacterial strains were maintained in three ways; firstly, as stocks in a -80  $^{\circ}\text{C}$  freezer, secondly, they were supplemented with 20% to 30% (v/v) glycerol at -30  $^{\circ}\text{C}$  and lastly, they were maintained on nutrient agar (NA) and tryptic soy agar (TSA) (Oxoid, Hampshire, UK) slants at 4  $^{\circ}\text{C}$ .

### *Preparation of Bacterial Inoculums and Standardization*

Before performing the disc diffusion assay, standardized inoculums for the disc diffusion assay were prepared following the guidelines of the Clinical Laboratory Standards Institute, CLSI (CLSI, 2013) and National Committee for Clinical Laboratory Standards NCCLS (2012). Briefly, using aseptic techniques, a loop full of each bacterial strain maintained on nutrient agar and tryptic soy agar (Oxoid, UK) slants at 4 °C was cultured in 10 mL of Mueller Hinton Broth (MHB) (Oxoid, UK) overnight. From the overnight cultures, using the streak plate method, a loop full of each bacterial strain was streaked across the Mueller Hinton Agar (MHA) (Oxoid, UK) plate and incubated at 37 °C for 18 - 24 h. From the incubated MHA culture plates, inoculums were prepared by making a direct broth suspension of two to three well-isolated colonies of the same morphological type of bacterial strain into a freshly prepared 10 mL of Mueller Hinton Broth (MHB), and incubated overnight in a rotatory shaker (Camlab, UK) at 37 °C, 200 rpm for 18 h to obtain an optical density of >2.5 (OD<sub>600</sub> >2.5).

The bacterial suspensions were adjusted to achieve a turbidity equivalent to a 0.5 McFarland,  $1.0 \times 10^8$  to  $1.5 \times 10^8$  colony forming units per milliliter CFU/mL. Furthermore, the inoculum suspensions were used within 30 min of standardization, which is a very important factor in avoiding any change in the size of inoculums or loss of their viability.

### *Antibacterial Activity of African Walnut Oil*

African walnut oil was tested for antibacterial activity against Gram positive bacteria (*B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799, and *L. monocytogenes* ATCC 13932) and Gram negative (*P. fluorescens* ATCC 13883, *P. fragi* ATCC 4973, *E. coli* ATCC 12806, *S. enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028) using the agar disc diffusion method described by Maharaj *et al.* (2022) with minor modifications. Briefly, after adjusting for the turbidity of the inoculum suspensions with McFarland standards, 100 µL of the adjusted suspensions of each bacterial strain were separately smeared uniformly over the surface of the MHA plates (15 mL) with a sterile cotton swab. Thereafter, the lid of each

plate was partly left open for about 5 min in a safety cabinet to get rid of excess moisture. Then after about 10 min, sterile 6 mm diameter discs already impregnated with 40  $\mu$ L of the extracted oil. Discs impregnated with African walnut oil were aseptically placed on the agar surface. The plates were left on the slab at 4 °C for 1 h before incubating them at 37 °C for 24 h. After incubation, the diameter of growth inhibition zones around each disc was measured with a millimeter ruler (in mm). Discs impregnated with autoclaved sterile 10 % DMSO were used as negative control and streptomycin (100  $\mu$ g/mL) as positive control. The tests were carried out in triplicate and data were presented as mean  $\pm$  standard deviation.

#### *Determination of Minimum Inhibitory Concentrations (MIC's) of African Walnut Oil*

The minimum inhibitory concentration of African walnut oil was tested against both Gram positive and Gram negative strains using the agar disc diffusion method described by Maharaj *et al.* (2022) with minor modifications. Different concentrations of African walnut oil (0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100

mg/mL) were first prepared separately by dissolving 100 mg/mL of the oil in 1 mL of 10% DMSO and sterile 6 mm diameter discs were loaded with 50  $\mu$ L each of the respective concentrations. Mueller Hilton agar (15 mL) was poured into sterile plates and left to solidify. After solidification, the adjusted suspensions (100- $\mu$ L) of each bacterial strain were separately smeared uniformly over the surface of the Mueller Hilton agar plates with a sterile cotton swab. Thereafter, the lid of each plate was partly left open for about 5 minutes in a safety cabinet to get rid of excess moisture. Then sterile 6 mm diameter discs already impregnated with different concentrations of African walnut oil were aseptically placed on the inoculated agar plates. The plates were left on the clean slab at 4 °C for 1 h before incubating them at 37 °C for 24 h. After incubation, the diameter of growth inhibition zones around each disk was measured with a millimeter ruler (in mm). Discs impregnated with autoclaved sterile 10% DMSO were used as the negative control. One positive control was used; streptomycin (100  $\mu$ L/disc) served as the positive control (for Gram positive bacteria). The tests were carried out in



triplicates and data were presented as mean  $\pm$  standard deviation.

#### *Determination of Minimum Bactericidal Concentrations (MBCs) of African Walnut Oil*

Bacterial streaks were taken from the last three lowest concentrations (0.78, 1.56, and 3.13) of African walnut oil plates showing invisible growth (as observed from the inhibition zone of Minimum Inhibitory Concentration (MIC) plates) and sub-cultured onto sterile Tryptone soya agar (TSA) plates. The plates were incubated at 37 °C for 24 h and examined for bacterial growth in corresponding African walnut oil concentration. MBC was considered the lowest concentration that did not exhibit any bacterial growth on the TSA plates.

#### *Antifungal Activity*

##### *Fungal Strains and Culture Condition*

To determine the antifungal properties of African walnut oil, three fungal species *Aspergillus niger*, *Penicillium oxalicum*, and *Trichoderma viride* associated with food spoilage were used. These strains were

isolated from different types of fried meat and fish (chicken, beef, goat meat, titus fish, croaker) and bread brands purchased in Ogun State. The samples were all neatly kept at room temperature and allowed to undergo spoilage for a period of three to five days. Spoilage of each sample was confirmed by changes in the organoleptic properties of the samples and spoilage organisms were then isolated using the method described by Oseghale *et al.* (2020).

##### *Isolation of Fungi*

A total of 50 fried meat and fish and 10 loaves of bread were purchased from food sellers in Ogun-State. The food samples were transported to the laboratory in sterile plastic bags. The food samples were left at room temperature (25°C - 30°C) to spoil. The direct plate method described by Oseghale *et al.* (2020) was used for culturing the individual mold specie observed on the food samples. Each food sample was transferred with sterile forceps into plates containing sterilized Sabouraud dextrose agar (SDA) and Potato Dextrose Agar (PDA) and the plates were incubated at 28 °C for 5 days.

Pure cultures were obtained and maintained by sub-culturing each of the colonies that grew onto both the SDA and PDA plates and incubated at 28 °C for 5 days.

#### *Identification of Isolated Fungi*

The fungal isolates were identified using cultural and morphological characteristics such as conidial morphology and pattern of growth of the colony. The method described by Mailafia *et al.* (2017) was used for the identification of the isolated fungi. The identification of the isolated fungi was conducted using lactophenol cotton blue stain. This was achieved by placing a drop of the stain on a clean slide with a mountain needle, where a tiny portion of the aerial mycelia from the representative fungi cultures was removed and placed in a drop of lactophenol. The mycelium was properly spread on the slide with the needle and a cover slip was gently placed to eliminate air bubbles. The slide was mounted and viewed under the light microscope with ×10 and ×40 objective lenses. The appearance of the fungal organisms and morphological

characteristics were identified following the procedure of Samuel *et al.* (2015).

#### *Preparation of Fungal Spores*

The fungal suspensions used were prepared by the method of Al-Garadi *et al.* (2023) with minor modifications. Briefly, two to five days old isolated cultures of the fungal strains *A. niger*, *P. oxalicum*, and *T. viride* were grown on potato dextrose agar at 35 °C to prepare inoculum suspensions. The colonies of each strain in the plates were separately covered with 1 mL of sterile distilled water with 0.1 % Tween 20 already added. The conidia were separately collected with a sterile cotton swab and transferred to a sterile tube. Then, for about 15 seconds a rotary vortex blender (Camlab, UK) at 2,000 rpm was used to uniformly blend the inoculum. Conidia of each sample were carefully examined for hyphae and clumps and were diluted with sterile distilled water before counting in a hemocytometer (Camlab, UK). In cases where a significant number of hyphae or clumps were more than 5 % (> 5 %), the inoculums were filtered using a sterile nylon grid filter with an 11 mm pore size (Thermo Fisher Scientific, UK).

After this, the suspensions were standardized by adjusting them with sterile distilled water to  $2-5 \times 10^8$  CFU/mL. The adjustment was made by diluting each suspension at a ratio of 1:10 with sterile distilled water to obtain a final working concentration of  $2-5 \times 10^8$  CFU/mL. Confirmations of the adjusted suspensions were quantified by plating on sabouraud dextrose agar (SDA) (Oxoid, UK) plates. Miconazole nitrate (20 mg/g) was used as a control standard for antifungal activity. It was prepared by dissolving it in DMSO at a concentration of 20 mg/mL. The final concentration of DMSO in the reaction mixture was less than 1 % (v/v).

#### *Determination of Antifungal Activity of African Walnut Oil*

The disc diffusion method was used to determine the inhibitory activities of African walnut oil against *Aspergillus niger*, *P. oxalicum*, and *T. viride* according to the method of Garadi *et al.* (2023). Briefly, 50  $\mu$ L of a standardized suspension of *A. niger*, *P. oxalicum*, and *T. viride*. was spread evenly on potato dextrose agar plates. The inoculum plates were all placed in a safety cabinet for

about 10 minutes to dry at room temperature. Then, sterile 5 mm blank discs were impregnated with 50  $\mu$ L of the prepared African walnut oil concentrations (0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 mg/mL) and aseptically applied to the inoculated agar plates. The agar plates were sealed with parafilm and incubated at 35 °C for 48 – 72 h of incubation. The diameter of growth inhibition zones around each disc was measured with a millimeter ruler (in mm). Discs impregnated with autoclaved sterile 10% DMSO and Miconazole nitrate 20 mg/g were used as negative control and positive control respectively. Experiments were performed in triplicates

#### **Statistical Analysis**

The diameters of inhibition zone data were analyzed by SAS program using one-way ANOVA. The results are expressed as mean ( $\pm$  SD).

## RESULTS AND DISCUSSION

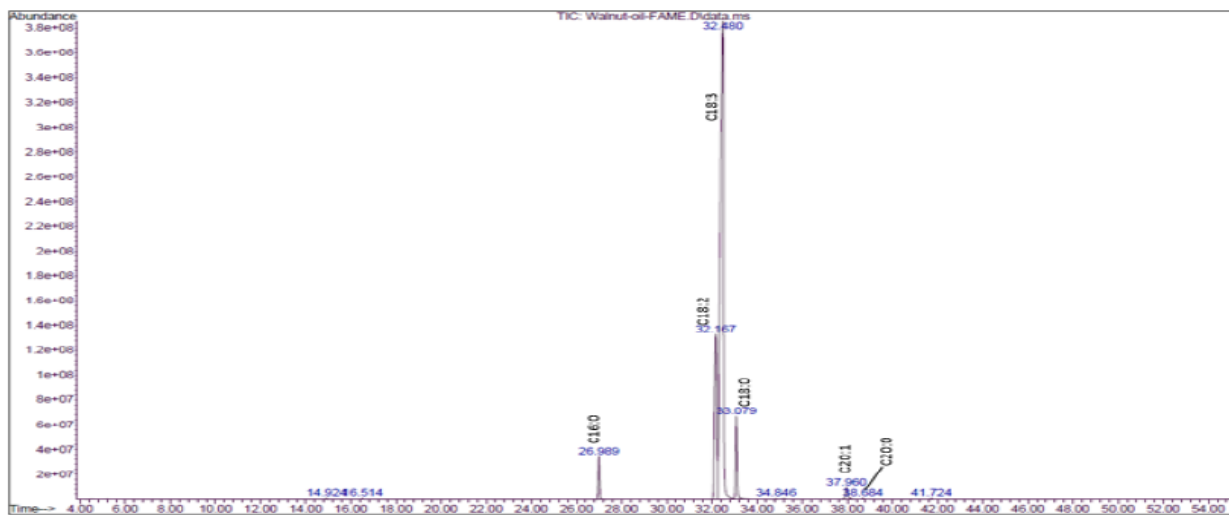
### *Fatty Acid Composition (GC-MS results of African walnut oil FAME)*

The fatty acid profile of African walnut oil is shown in Table 1. The fatty acid profile of walnut oil was determined as corresponding methyl esters, using gas chromatography. A Chromatogram of fatty acid methyl esters identified in walnut oil is presented in Figure 1. The chromatogram clearly shows that there were five (5) major peaks with retention times ranging from 18.68 to 32.39 min. Figure 1 also shows the presence of five (5) major fatty acids between C16:0 and C20:1 which include palmitic, linoleic, linolenic, stearic, and eicosenoic acids as listed in Table 1. In the chromatogram, the peak with the greatest abundance is shown by the highest peak with a retention time of 32.39 min and it is Linolenic (C18:3). Results in Table 1 indicate that five fatty acids were identified in African walnut oil and these include saturated, monounsaturated and polyunsaturated fatty acids. Two saturated fatty acids (palmitic acid (1.53%) and stearic acid (4.41%)), one monounsaturated fatty (eicosenoic acid

0.42%), and two polyunsaturated fatty acids (dominantly linoleic (13.5 %) and linolenic acid 80.59 % otherwise called  $\alpha$  linolenic acid)) were identified in African walnut oil. The percentage values of the saturated fatty acids in African walnut oil were low and this shows that the oil has a plethora of health beneficial effects such as reducing the risk of heart diseases, lowering cholesterol levels, aiding better metabolism and digestion, lowering chances of cancer, and providing omega-3 fatty acids to the human body (Shahidi and Ambigaipalan, 2018). The results obtained in this study, further indicate that African walnut oil compares favorably well with the findings of El-shazly *et al.* (2017) who reported low saturated fatty acid content in pomegranate seed oil. In addition, the presence of monounsaturated and polyunsaturated fatty acids in the oil corresponds to the report of Kumar *et al.* (2016) which states that vegetable oils are a source of edible fatty acids which play an important role in cellular metabolism, thereby storing energy. Thus, African walnut oil could constitute an important component of the human diet providing nutritional, health, and industrial benefits.

**Table 1.** Fatty acid composition of African walnut oil.

Peak No	Fatty acid methyl ester	Area %	Molecular weight (g/mol)	Retention times
1	Palmitic acid (C16:0)	1.53	270	26.98
2	Linoleic acid (C18:2)	13.05	294	32.16
3	Linolenic acid (C18:3)	80.59	292	32.39
4	Stearic acid (C18:0)	4.41	298	33.07
5	Eicosenoic acid (C20:0)	0.42	325	18.68



**Figure 1.** GC-MS spectrum of fatty acid methyl esters identified in walnut oil with their retention times.

### ***Antibacterial Activity***

Table 2 shows the inhibitory effect of the extracted African walnut oil against food spoilage and food poisoning bacterial species including; Gram positive bacteria (*B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799 and *L. monocytogenes* ATCC 13932) and Gram negative (*P. fluorescence* ATCC 13883, *P.*

*fragi* ATCC 4973, *E. coli* ATCC 12806, *S. enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028). Generally, it was observed that African walnut oil had a significant effect on the growth of Gram positive species than the Gram negative species. This is observed in the significant differences ( $p < 0.05$ ) between the inhibition zones exhibited by the species. African walnut oil

exhibited the most inhibitory effect against four Gram positive bacteria (*B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799, and *L. monocytogenes* ATCC 13932) at 40  $\mu$ L concentration. African walnut oil exhibited significantly ( $p < 0.05$ ) larger inhibition zones against *B. cereus* ATCC 33018 ( $22.3 \pm 0.23$  mm), *S. aureus* ATCC 25923 ( $18.9 \pm 0.46$  mm), *C. perfringens* NCTC 8799 ( $17.6 \pm 0.37$  mm) and *L. monocytogenes* ATCC 13932 ( $15.6 \pm 0.27$  mm) while others species such as *P. fluorescens* ATCC 13883 ( $6.3 \pm 0.31$  mm), *P. fragi* ATCC 4973 ( $8.9 \pm 0.21$  mm), *E. coli* ATCC 12806 ( $7.0 \pm 0.23$  mm), *S. enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028 ( $6.8 \pm 0.19$  mm) had smaller inhibitory zones respectively as comparable with the positive control.

The inhibitory effect of African walnut oil on Gram positive bacterial species comparable to Gram negative bacterial species as observed by the exhibited zones explains the difference in resistance of Gram positive bacteria (*B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799 and *L. monocytogenes* ATCC 13932) and Gram negative bacteria (*P. fluorescens* ATCC 13883, *P. fragi* ATCC 4973, *Escherichia coli*

ATCC 12806, *S. enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028) to African walnut oil which could be related to differences in the bacterial cell membranes. According to Abu-zaid *et al.* (2021), the outer membrane of Gram negative bacteria functions as a selective barrier allowing only the internalization of valuable nutrients. In addition, their membranes are composed of hydrophilic molecules with small ion channels that do not permit the entry of plant seed oils, while Gram positive bacteria outer membranes possess thick peptidoglycan layers that contain teichoic and lipoteichoic acids. Therefore, the results obtained in this study agree with those of Vahid-Dastjerdi *et al.* (2014), El-Shazly *et al.* (2017), and Mostafa *et al.* (2018). This study revealed that African walnut oil had antibacterial activity on food spoilage and food poisoning bacteria.

The antibacterial activity of African walnut oil on Gram positive bacteria showed that active fatty acid compounds such as linolenic acid and linoleic acids present in the oil could be responsible for its antimicrobial activity.

**Table 2.** Antimicrobial activity of African walnut oil against bacterial species causing food spoilage and food poisoning diseases.

Microorganisms	African walnut oil 40 µL/disc	Streptomycin 100 µg/mL /disc
<i>Gram positive bacteria (+ve)</i>		
Inhibition zones (mm)		
<i>Bacillus cereus</i> ATCC 33018	26.3 ± 0.23 <sup>b</sup>	27.5 ± 0.35 <sup>a</sup>
<i>Staphylococcus aureus</i> ATCC 25923	25.9 ± 0.46 <sup>c</sup>	26.9 ± 0.24 <sup>a</sup>
<i>Clostridium perfringens</i> NCTC 8799	24.1 ± 0.37 <sup>c</sup>	25.8 ± 0.62 <sup>a</sup>
<i>Listeria monocytogenes</i> ATCC 13932	21.6 ± 0.27 <sup>d</sup>	22.4 ± 0.45 <sup>a</sup>
<i>Gram negative bacteria (-ve)</i>		
<i>Pseudomonas fluorescense</i> ATCC 13883	6.3 ± 0.31 <sup>c</sup>	8.7 ± 0.11 <sup>a</sup>
<i>Pseudomonas fragi</i> ATCC 4973	9.9 ± 0.21 <sup>b</sup>	19.3 ± 0.19 <sup>a</sup>
<i>Escherichia coli</i> ATCC 12806	9.0 ± 0.23 <sup>b</sup>	17.3 ± 0.26 <sup>a</sup>
<i>Salmonella typhimurium</i> ATCC 14028	6.8 ± 0.19 <sup>c</sup>	7.04 ± 0.12 <sup>a</sup>

Data are means of three readings (± SD) (n=3). Values with different superscripts in the same column are significantly different (p < 0.05).

#### *Minimum Inhibitory Concentrations (MICs) of African walnut oil*

The effect of different African walnut oil concentrations on growth inhibition in selected species is reported in Table 3. The inhibition zones exhibited by the species significantly ( $p < 0.05$ ) varied between concentrations. African walnut oil exhibited the highest activity against all the Gram positive organisms investigated with a MIC range of 100 – 1.56 mg/mL. Both 0.39 mg/ml and 0.78 mg/mL of African walnut nut oil concentrations had no inhibition effect on

any of the selected organisms (Gram-positive and Gram negative). African walnut oil started inhibiting the growth of Gram positive bacterial strains at 1.56 mg/mL with inhibition zones of  $14.9 \pm 0.32$  mm,  $13.9 \pm 0.32$  mm,  $12.8 \pm 0.22$  mm, and  $11.5 \pm 0.22$  mm against *B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799 and *L. monocytogenes* ATCC 13932 respectively. At 3.13 mg/mL, African walnut oil inhibited the growth of Gram positive strains with inhibition zones of  $17.5 \pm 0.21$  mm,  $15.5 \pm 0.21$  mm,  $14.1 \pm 0.18$  mm, and  $13.6 \pm 0.18$  mm against *B. cereus* ATCC

33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799 and *L. monocytogenes* ATCC 13932 while for Gram negative strains (*P. fluorescens* ATCC 13883, *P. fragi* ATCC 4973, *E. coli* ATCC 12806, *S. enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028), it was not able to inhibit their growth. However, small inhibition zones of  $7.1 \pm 0.18$  mm and  $6.2 \pm 0.08$  mm were observed for *P. fragi* ATCC 4973 and *E. coli* ATCC 12806. At 6.25 mg/mL, the oil had diameter of inhibition zones of  $19.2 \pm 0.16$  mm,  $17.2 \pm 0.16$  mm,  $16.7 \pm 0.41$  mm, and  $15.3 \pm 0.30$  mm against *B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799 and *L. monocytogenes* ATCC 13932 respectively. At 12.5 mg/mL, the diameter of inhibition zones were  $20.3 \pm 0.09$  mm,  $18.3 \pm 0.09$  mm,  $17.4 \pm 0.23$  mm, and  $17.0 \pm 0.26$  mm against *B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799 and *L. monocytogenes* ATCC 13932. At 25 mg/mL, the diameter of inhibition zones were  $21.0 \pm 0.10$  mm,  $20.0 \pm 0.10$  mm,  $19.0 \pm 0.21$  mm, and  $18.1 \pm 0.38$  mm against the against *B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799

and *L. monocytogenes* ATCC 13932. At 50 mg/mL, the diameter of inhibition zone observed were  $24.0 \pm 0.12$  mm,  $22.0 \pm 0.12$  mm,  $21.0 \pm 0.39$  mm, and  $20.9 \pm 0.43$  mm against *B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799 and *L. monocytogenes* ATCC 13932. At 100 mg/mL, the diameter of inhibition zone observed were  $25.4 \pm 0.20$  mm,  $24.3 \pm 0.20$  mm,  $23.2 \pm 0.32$  mm, and  $20.9 \pm 0.35$  mm against *B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799 and *L. monocytogenes* ATCC 13932.

Overall, when increasing the concentration of African walnut oil (6.25 mg/mL, 12.25 mg/mL, 25 mg/mL, 50 mg/mL, and 100 mg/mL) the diameter of the inhibition zone was increased in all the tested organisms (Table 3).



**Table 3.** Minimum inhibition concentration of African walnut oil against bacterial species causing food poisoning diseases.

African walnut oil Conc. (mg/mL)	Inhibitory Zone (mm)					
	Gram – Positive Bacteria			Gram-Negative Bacteria		
	<i>B. cereus</i>	<i>S. aureus</i>	<i>C. perfringens</i>	<i>L. monocytogenes</i>	<i>P. fragi</i>	<i>E. coli</i>
0.39	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.78	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.56	14.9 ± 0.32 <sup>a</sup>	13.9 ± 0.32 <sup>b</sup>	12.8 ± 0.22 <sup>c</sup>	11.5 ± 0.22 <sup>a</sup>	0.0 ± 0.0	0.0 ± 0.0
3.13	17.5 ± 0.21 <sup>a</sup>	15.5 ± 0.21 <sup>b</sup>	14.1 ± 0.18 <sup>c</sup>	13.6 ± 0.18 <sup>a</sup>	7.1 ± 0.18 <sup>b</sup>	6.2 ± 0.08 <sup>c</sup>
6.25	19.2 ± 0.16 <sup>a</sup>	17.2 ± 0.16 <sup>b</sup>	16.7 ± 0.41 <sup>c</sup>	15.3 ± 0.3 <sup>a</sup>	7.6 ± 0.21 <sup>b</sup>	6.5 ± 0.15 <sup>c</sup>
12.5	20.3 ± 0.09 <sup>a</sup>	18.3 ± 0.09 <sup>b</sup>	17.4 ± 0.23 <sup>c</sup>	17.0 ± 0.26 <sup>a</sup>	8.0 ± 0.34 <sup>b</sup>	7.1 ± 0.25 <sup>c</sup>
25	21.0 ± 0.10 <sup>a</sup>	20.0 ± 0.10 <sup>b</sup>	19.0 ± 0.21 <sup>c</sup>	18.1 ± 0.38 <sup>a</sup>	8.6 ± 0.23 <sup>b</sup>	7.9 ± 0.25 <sup>c</sup>
50	24.0 ± 0.12 <sup>a</sup>	22.0 ± 0.12 <sup>b</sup>	21.0 ± 0.39 <sup>c</sup>	20.9 ± 0.43 <sup>a</sup>	9.0 ± 0.42 <sup>b</sup>	8.0 ± 0.55 <sup>c</sup>
100	25.4 ± 0.20 <sup>a</sup>	24.3 ± 0.20 <sup>b</sup>	23.2 ± 0.32 <sup>c</sup>	20.0 ± 0.35 <sup>a</sup>	9.2 ± 0.54 <sup>b</sup>	8.5 ± 0.35 <sup>c</sup>

Data are means of three readings ( $\pm$  SD) (n=3). Values with different superscripts in the same row are significantly different ( $p < 0.05$ ).

The present study has demonstrated that African walnut oil effectively inhibited the growth of all Gram positive organisms investigated. While it was only able to mildly inhibit the growth of Gram negative organisms. For the Gram negative strains, 50 mg/mL and 100 mg/mL of African walnut oil produced smaller inhibition zones as observed in *P. fragi* ATCC 4973, *E. coli* ATCC 12806, and *L. monocytogenes* ATCC 13932.

In view of this, the results obtained in this study are in agreement with those of Abu-Zaid *et al.* (2021) who reported

that 100 mg/mL of *Nigella sativa* and *Trigonella foenum-graecum* extracts inhibited the growth of *B. cereus*, *S. aureus* and *E. coli* with inhibition zones in the range of 13-24 mm diameter. The activity of these extracts was also found to increase with the increase in concentration. The results also correlate with the findings of Mostafa *et al.* (2018) who reported that *Punica granatum* and *Syzygium aromaticum* plant extracts exhibited strong antimicrobial activity with increasing concentration against *S. aureus* at 5 mg/mL with 14.8 mm and 11.4 mm zones of inhibition. These results are in accordance with those of Hovorková *et*

*al.* (2018) who observed that plant oils containing medium chain fatty acids exhibited strong inhibitory activity against *C. perfringens*, *L. monocytogens*, and *S. aureus* at 0.14 – 4.5 mg/mL concentrations. Furthermore, these results are also related to Aseel (2020) who explored the antimicrobial activity of three plant oils, olive, sesame, and grape seed oil on both Gram positive and negative strains. In his study, he found out that olive oil and sesame seed oils inhibited the growth of Gram positive strains with increased concentration. Ajaiyeoba and Fadare (2006) who evaluated the antibacterial potential of extracts obtained from different parts of African walnut against Gram positive and Gram negative organisms using the agar cup diffusion and broth dilution techniques found that the extracts exhibited concentration-dependent antimicrobial properties on the Gram positive organisms at 10 - 100 mg/mL.

#### ***Minimum Bactericidal Concentration (MBCs) of African Walnut Oil***

The MBC of African walnut oil was confirmed by the absence of growth of Gram positive and Gram negative

bacterial strains streaked from inhibition zones obtained from the lowest MICs. African walnut oil showed potentially bactericidal activity against the tested organisms. African walnut oil exhibited bactericidal activity against the Gram positive bacteria (*B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799, and *L. monocytogenes* ATCC 13932) with MBC of 0.78 mg/mL except for Gram negative bacteria (*P. fluorescence* ATCC 13883, *P. fragi* ATCC 4973, *E. coli* ATCC 12806, *S. enterica* subsp. *enterica* serovar Typhimurium strain ATCC 14028) which were less sensitive and no growth recorded from 1.56 mg/mL.

The results obtained for MIC and MBC in this study suggested that African walnut oil can be used to control and prevent food spoilage caused by foodborne bacteria. All bacterial strains investigated in this study were chosen for their importance in food spoilage and food poisoning. For instance, the Gram positive bacterial strains (*B. cereus* ATCC 33018, *S. aureus* ATCC 25923, *C. perfringens* NCTC 8799, and *L. monocytogenes* ATCC 13932) and Gram negative bacteria (*P. fluorescence* ATCC 13883, *P. fragi* ATCC 4973, *E. coli* ATCC 12806, and *S. enterica*

subsp. enterica serovar Typhimurium strain ATCC 14028) were considered because they are among the most common source of foodborne disease in human causing several serious diseases like bacillosis, staphylococcal infections and listeriosis (Bintsis, 2017). While strains like *C. perfringes* NCTC 8799, *E. coli* ATCC 12806, *S. enterica* subsp. enterica serovar Typhimurium strain ATCC 14028, and *P. fluorescence* ATCC 13883, *P. fragi* ATCC 4973 produce an arsenal of toxins responsible for several notorious diseases and metabolites that induce gastroenteritis disease in humans (Cohen *et al.*, 2007; Mohammad *et al.*, 2022; Sun *et al.*, 2022). Results in this study are in line with those of Adewale *et al.* (2012), Mostafa *et al.* (2018), Fatokun *et al.* (2019), and Abu-Zaid *et al.* (2021) who reported that the minimum bactericidal activity of Nahar (*Mesua ferrea*), clove (*S. aromaticum*) and pomegranate (*P. granatum*), Mexican tea (*Dysphania ambrosiodes*), cumin (*Nigella sativa*) and fenugreek (*Trigonella foenum-graecum*) were exhibited at low concentrations of 2.5 and 5 mg/mL and 6 and 80 mg/mL respectively for Gram positive and Gram negative bacterial strains tested.

Numerous researchers have investigated the efficiency of plant essential oils and their effective compounds as antimicrobial compounds to prevent or control the growth of foodborne and spoilage organisms (El-Shazly *et al.*, 2017; Hovorková *et al.*, 2018; Aseel, 2020). Some researchers are also of the opinion that antimicrobial compounds of essential oils such as phytochemicals (alkaloids, terpenoid, and phenolic compounds) interact with proteins and enzymes present in the cell membranes of microorganisms causing its disruption thereby resulting in the dispersal of proton flux to the cell exterior which induces cell death or inhibition of enzymes necessary for the biosynthesis of amino acids (Gunaseena *et al.*, 2022). Other researchers have linked the inhibitory effect of essential oils to the hydrophobicity nature of the oils in disrupting bacterial cell structures (Nazzaro *et al.*, 2013). The present study suggests that African walnut oil which proves to be potentially effective against some food-borne bacterial strains can be used as natural preservatives to control food spoilage and food poisoning diseases and preserve food avoiding the

use of chemical preservatives that are harmful to the human body.

### *Antifungal Activity*

The antifungal activity of African walnut oil was evaluated against three food spoilage organisms' namely *A. niger*, *P. oxalicum*, and *Trichoderma viride* using disc diffusion assay (Table 4). Results demonstrated that African walnut oil exhibited a concentration dependent activity against the three tested fungi. Overall, it was observed that as the concentration of African walnut oil increased, the activity against the fungal strains increased as represented by the diameter of the inhibition zones. The highest activity of African walnut oil was

observed in *A. niger* with a mean inhibition zone of  $27.3 \pm 0.42$  mm followed by *P. oxalicum* ( $24.8 \pm 0.21$  mm) and *T. viride* ( $22.8 \pm 0.28$  mm) at 100 mg/mL.

The mean inhibition zone of African walnut oil at concentrations of 50 mg/mL was  $24.2 \pm 0.23$  mm,  $21.3 \pm 0.19$  mm and  $19.3 \pm 0.20$  mm, 25 mg/mL was  $21.4 \pm 0.21$  mm,  $19.3 \pm 0.19$  mm, and  $17.6 \pm 0.13$  mm and 12.5 mg/mL was  $19.5 \pm 0.14$  mm,  $16.4 \pm 0.22$  mm, and  $15.3 \pm 0.12$  mm respectively. The lowest activity of African walnut oil occurred at a concentration of 1.56 mg/mL for *P. oxalicum* ( $11.8 \pm 0.06$  mm) and *T. viride* ( $9.3 \pm 0.03$  mm). Small inhibition zones were

**Table 4.** Antifungal activity of African walnut oil against fungal species causing food spoilage.

African Walnut Oil Conc. (mg/mL)	Inhibitory Zone (mm)		
	<i>A. niger</i>	<i>P. oxalicum</i>	<i>Trichoderma viride</i>
0.39	$7.8 \pm 0.04^a$	$6.3 \pm 0.04^b$	$5.5 \pm 0.01^c$
0.78	$10.5 \pm 0.09^a$	$8.2 \pm 0.07^b$	$6.7 \pm 0.02^c$
1.56	$13.2 \pm 0.06^a$	$11.8 \pm 0.06^b$	$9.3 \pm 0.03^c$
3.125	$14.2 \pm 0.06^a$	$12.7 \pm 0.07^b$	$10.3 \pm 0.03^c$
6.25	$15.1 \pm 0.09^a$	$14.2 \pm 0.15^b$	$12.2 \pm 0.09^c$
12.5	$19.5 \pm 0.14^a$	$16.4 \pm 0.22^b$	$15.3 \pm 0.12^c$
25	$21.4 \pm 0.21^a$	$19.3 \pm 0.19^b$	$17.6 \pm 0.13^c$
50	$24.2 \pm 0.23^a$	$21.3 \pm 0.19^b$	$19.3 \pm 0.20^c$
100	$27.3 \pm 0.42^a$	$24.8 \pm 0.21^b$	$22.8 \pm 0.28^c$

Data are means of three readings ( $\pm$  SD) (n=3). Values with different superscripts in the same row are significantly different ( $p < 0.05$ ).

recorded at 0.78 and 0.39 mg/mL for the three fungal strains tested. At 0.78 mg/mL,  $10.5 \pm 0.09$  mm,  $8.2 \pm 0.07$  mm, and  $6.7 \pm 0.02$  mm diameter zones were recorded, while at 0.39 mg/mL,  $7.8 \pm 0.04$  mm,  $6.3 \pm 0.04$  mm, and  $5.5 \pm 0.01$  mm diameter zones were recorded for *A. niger*, *P. oxalicum*, and *T. viride* respectively. This result correlates with that of Ajaiyeoba and Fadare (2006) who stated that solvent extracts of different parts of African walnut in the concentration range of 10 - 100 mg/mL inhibited the growth of two fungi tested.

## CONCLUSION

According to the obtained results, African walnut oil can be used as an antibacterial agent for minimizing a variety of Gram positive organisms causing foodborne diseases. The results also indicate that all tested fungi were susceptible to the oil. In addition, plant oils play significant roles in the food sector with a wide range of applications, mainly to prolong shelf-life and prevent oxidation. It is apparent from this study, that the addition of African walnut oil to food products, either by direct mixing or in edible coatings and active packaging

may help prevent food spoilage and food poisoning. In addition, the association of several compounds present in African walnut oil provides antioxidant properties, mainly due to the presence of phenolic compounds as major components (Rébufa *et al.*, 2022). These antioxidant compounds can delay or inhibit the oxidation of lipids and other molecules by inhibiting the initiation or propagation of oxidation chain reactions.

Despite the promising results, there are obvious limitations of the study which have to be pointed out. The first is that African walnut is a seasonal fruit in Nigeria that can only be collected, processed, and used for analysis when it is available. Secondly is the lack of enough previous research studies on African walnut oil activity against the microorganisms used in this research study.

## ACKNOWLEDGEMENT

The authors thank Tertiary Education Trust Fund (TETFUND), for research support fund given through Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria.

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