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Evaluation of the Phytochemical, Nutritive, Antimicrobial, Antioxidant and GC-MS analysis of *Plukenetia Conophora* Mull. Arg. (Euphorbiaceae) Husk

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ABSTRACT

Background: Nigerian walnut or African walnut as it is popularly called belongs to the family Euphorbiaceae. It has been used in traditional medicine as an antimicrobial, antioxidant, male fertility agent, treatment of skin conditions, prolong and constant hiccup alongside CVD. This is the very first study of the husk plant part of the Nigerian walnut, usually considered as waste. The study aimed at evaluating of the phytochemical, antimicrobial, antioxidant activities, chemical characterization and proximate analysis of *Plukenetia conophora* (Euphorbiaceae) husk.

Methods: The husk extraction was carried out by maceration in dichloromethane-methanol 1:1 ratio for 72 hr, followed by concentration on a rotary evaporator at a temperature of 40^{o}C and subsequent drying in a desiccator. The phytochemical screening and proximate analysis were undertaken. The antimicrobial activities were determined by the agar well diffusion method, while for antioxidant activities, DPPH assay was employed. The bioactive compounds were identified by means of the GCMS.

Results: The phytochemical screening result showed alkaloids, flavonoids, saponins, phenols and steroids were present. The antimicrobial result demonstrated activity against the range of bacteria investigated and some of the fungi tested. The antioxidant result was shown to be active. The GCMS analysis revealed that Linolenic acid, Oleic acid and methyl stearate were in abundance. The proximate analysis showed it's a good source of fibre, which supports gut health.

Conclusion: The findings from this study showed that Nigerian walnut husks can pave the way for the development of novel pharmaceuticals through the waste to wealth approach.

Keywords: Antimicrobial, Antioxidant, *Plukenitia conophora*

1. INTRODUCTION

Antimicrobial resistance (AMR) and oxidative stress attributed diseases are the major cause of public and global health challenges, WHO has stated that AMR is a top global development and health threat, in 2019 it accounted directly for 1.27 million deaths. In addition to death and disabilities AMR has a significant economic cost, the World Bank estimates an additional 1 trillion dollars additional economic cost by 2050. Antimicrobials are used in treating bacterial and fungal diseases, it is one of the most therapeutic discoveries of the 20th century. However, there has been an emergence of antimicrobial resistance, this is because of the emergence of drug resistant pathogens that is the effect of indiscriminate use, incessant and misuse of antimicrobials [1, 2]. Therefore, there's a need for research on novel antimicrobials and it is well known that plants produce various secondary metabolites for protecting themselves against various pathogens. Hence numerous researchers have paid great attention to plant based traditional medicine

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practices and biologically active compounds and phytochemicals isolated from plant species used in herbal medicine with satisfactory therapeutic index for the development of novel natural antimicrobials [3, 4]. The walnut (*Tetracarpidium conophorum*) oil has been proven to have antimicrobial and antifungal activities, the walnut oil demonstrated broad spectrum activity on bacterial and fungal clinical isolates [5]. As previously mentioned *T conophorum* has been used traditionally for treating prostate cancer and has been used as an antioxidant. Antioxidants (reduction) and oxidation balance is believed to be a very critical concept to biologically maintain a healthy system. However, under condition of oxidative stress, ROS (Reactive Oxygen Species) like superoxide, peroxyl radicals and hydroxy are generated. These ROS plays a major role in the pathophysiology and etiology of human aging and of course diseases such as coronary heart disease, cancer, Alzheimer's disease (a depression disorder), inflammation, cataracts and other neurodegenerative disorders. Plant medicine has been used over the years by people due to its variety of natural products that are closely compatible with human organisms and pose reduced side effects alongside pharmacological activities, which has led plant medicine to be incorporated into western or modern medicine. But the major challenge with herbal medicine is not having scientific evidence, therefore there's a need to back up the people's herbal medicine beliefs and practices, by making it evidence based. Amongst these plant medicines is Nigerian walnut popularly known as African Walnut; *Plukenetia conophora* mull. (Arg), family Euphorbiaceae. This African walnut botanical name was formerly *Tetracarpidium conophorum*. This Nigerian walnut or Afrcan walnut as it is popularly called is native to the Western and Southern Africa, majorly in Nigeria, Congo and Cameroon. This Nigerian walnut is a climber with fruit that is edible often as snacks when husked (hulled), roasted or boiled and shelled. The fruits are green with four to three round seeds in each. The bark, leaves seeds and oils of *Tetracarpidium conophorum* are used medicinally which most has been proven scientifically, their uses include antihelminthic, male fertility agent, antibacterial especially gram positive, treatment of skin conditions (eczema, skin rash, pruritus, skin irritations, psoriasis and other skin infections caused by bacteria and fungi), reduce toothache, treat prostate cancer, antioxidants, treat depression, antipyretic and common cold. Walnut husk or hull is the greenish part of the fruit that encapsulates the seeds, it's often discarded as waste during walnut processing, however recent studies on the English walnut (*Juglans regia*) husk and the American Black walnut (*Juglans nigra*) husk [6] has proved to be rich in health benefits through research having scientific backing so much so it's tincture has become popularly sold as health products. This has sparked the interest to question if the Nigerian walnut (*Tetracarpidium conophorum*) husk can also be scientifically proven to be an antioxidant and an antimicrobial as the natives claim it to be medicinally useful.
The antioxidant activity of the leaves of *T. conophorum* has been established [7]. Lastly the proximate composition of *T. conophorum* has been revealed to be protein rich (29.14%), fat rich (54.14%), carbohydrate rich (4.17%), ash rich (3.32%) and rich in several vitamins [8]. There's a need to ascertain these properties for the husk part. To the best of our knowledge, this is the very first study of the husk plant part of the Nigerian walnut (*T. Conophorum*) and has exhaustively revealed compounds that could be responsible for its preventive alongside therapeutic action such as antimicrobial, antioxidants and being used as animal food.

2. METHODS AND MATERIALS

2.1 Materials

2.1.1 Equipment

Analytical weighing balance, Thermometer, Gas-Chromatography Mass Spectrophotometer, UV Spectrophotometer, Rotary Evaporator, Retort Stand, Separatory funnel, Erlenmeyer flask, Desiccator, Hot air oven, Water bath, Whatman filter paper no. 1, Sterile pipette, Test tubes, Volumetric flasks, Glass funnel (pyrex) Muffle furnace, Beakers, Porcelain Crucibles, Petri dishes, Sterile pasteur's pipette, Incubator, Meter rule, Universal bottles, Stop watch, masking tape, cotton wool.

2.1.2 Solvents and Reagents

Dichloromethane, Methanol, N-Hexane, Distilled Water, Dimethyl sulfoxide, Diphenyl picryl hydralazine, Sulphuric acid.

2.2 Methods

2.2.1 Collection of Samples

The sample selected for this study is *Plukeneetia conophora*, commonly known as Nigerian walnut. The sample (Nigerian walnut) husk used was collected in June 2023. Prior to inclusion in the study, the sample was subject to identification and authentication procedures conducted by a botanist affiliated with the University of Port Harcourt.

2.2.2 Preparation of the Sample

The collected Nigerian walnut husk were washed thoroughly to remove surface impurities and contaminants. Following this, the walnut husk was subjected to a drying process. It was sun-dried as a dehydration process naturally. Upon achieving the desired dryness, the dehydrated Nigerian walnut husk was finely ground into a powder form using a grinder. The finely powdered sample was then carefully stored in airtight glass containers for subsequent utilization, ensuring protection against moisture and external contaminants.

2.2.3 Extraction

A total of 200 grams of the finely powdered Nigerian walnut husk sample was accurately weighed and subsequently transferred into a glass macerating jar. The maceration process commenced by immersing the powder in 200 liter of dichloromethane-methanol, maintaining a precise ratio of 1:1. This maceration was carried out at room temperature within the glass jar, utilizing intermittent agitation for a duration of 72 hours. Following the maceration process, the resulting dichloromethane-methanol extract underwent filtration utilizing Whatman NO. 1 filter paper to separate the solvent from the extract. The filtrate obtained was re-macerated with 100 ml methanol for 48 hours, then filtered and subsequently subjected to concentration through a rotary evaporator and the yield of the concentrated extract was recorded, encompassing both the weight and the extract's percentage yield. Upon completion of the concentration process, the extract was carefully transferred into a secure glass container and further dried within an activated desiccator for 72 hr. The extract was transferred into a fridge where it was stored until further analysis necessitated its use.

2.2.4 Partitioning of the extract

The dichloromethane-methanol extract obtained previously was used for the partitioning process. 5 grams of the extract was dissolved in 100ml of 90% aqueous methanol yielding a concentrated solution suitable for partitioning. The concentrated extract solution was combined with an 30ml of n-hexane in a separating funnel. Vigorous shaking ensured thorough mixing and partitioning of the components. The mixture was allowed to settle, resulting in the formation of two distinct layers: an aqueous methanol layer and a non-polar n-Hexane layer. The n-Hexane layer was carefully separated and set aside and the process repeated severally until all the n-hexane soluble constituents were extracted. This non polar n-hexane extract was concentrated using a rotary evaporator to obtain the n-hexane fraction.

2.2.5 Phytochemical Screening

The Phytochemical screenings were carried out as modified by Okoroafor and Isirima [9, 10].

2.2.6 Antimicrobial Susceptibility Testing by Agar Well Diffusion

2.2.6.1 Bacteria

The bacterial strains used were *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Sterile petri dishes were labelled in triplicate for each bacterial strain. Mueller-Hinton agar pour was uniformly inoculated with 0.1ml of standardized bacterial cultures and thoroughly mixed. After pouring the inoculated agar into the labelled petri dishes, it was allowed to cool and solidify on the workbench. Then, a sterile 6mm cork borer was used to aseptically create wells in the solidified agar. Preparation of 100% solutions of the extract was carried out. A few drops of the extract solutions were added into the corresponding wells on the agar plates. The plates were incubated at 37°C for 24 hours under optimal conditions. After incubation, observation for zones of inhibition around each well was conducted, with Gentamicin serving as the positive control and Dimethyl sulfoxide (DMSO) as the negative control. Accurate measurements of the diameter of these zones were taken using a calliper.

2.2.6.2 Fungi

The chosen fungal strains were *Candida albicans*, *Fusarium oxysporum*, *Aspergillus niger*, and *Penicillium chrysogenum*. Sterile petri dishes were labelled in triplicate for each fungal strain. Potato Dextrose agar pour was uniformly inoculated with 0.1ml of standardized fungal cultures and thoroughly mixed. After pouring the inoculated agar into the labelled petri dishes, it was allowed to cool and solidify on the workbench. Then, wells were aseptically created in the solidified agar using a 6mm sterile cork borer. Preparation of 100% solution the extract was performed. The extract solution was added into the wells on the agar plates. The plates were incubated at 25°C for 72 hours under optimum conditions. Following incubation, observation for zones of inhibition around each well was carried out, using

Fluconazole as the positive control and Dimethyl sulfoxide (DMSO) as the negative control. Accurate measurements of the diameter of these zones were taken with a calliper.

2.2.6.3 Antimicrobial Suseptibility Determination

Sterile petri dishes were labelled in triplicates for each bacterial strain. Mueller-Hinton agar pours were inoculated with 0.1ml of standardized bacterial cultures, ensuring thorough mixing. The inoculated agar was poured into the labelled petri dishes and left to solidify on the workbench. After solidification of the agar, five (5) discs were carefully extracted from the agar layer using a sterile cork borer. This process created five (5) wells in each agar plate. These wells were labelled to accommodate five (5) concentrations of each extract identified with activity during the preliminary study. Concentrations included 50%, 25%, 12.5%, 6.25%, and 3.125%. With a separate sterile Pasteur's pipette, 0.1ml of each extract concentration was meticulously added to the corresponding wells. The plates were left on the workbench for 15 minutes to ensure proper diffusion of the extracts. Subsequently, all plates were incubated at 37°C for 24 hours. Post-incubation, the diameter of resulting zones of inhibition was measured in millimeters (mm) using a calliper, directly through the base of the plates.

2.2.7 Antioxidant Analysis

A 50 milligrams of the powdered dichloromethane-methanol extract was weighed using a weighing balance and transferred into a 50-milliliter beaker. A volume of 5 milliliters of methanol was used to dissolve the extract, eventually achieving the 50-milliliter mark within a measuring cylinder. A glass rod stirrer facilitated stirring until a homogeneous, clear solution was attained. This resulting solution was moved into a 10-milliliter volumetric flask and further diluted to generate seven distinct concentrations, ranging from 800 micrograms per milliliter to 12.5 micrograms per milliliter. Concurrently, the preparation of a negative control sample was undertaken. Additionally, a standard solution of vitamin C underwent preparation, which was subsequently diluted into three different concentrations spanning from 16 micrograms per milliliter to 4 micrograms per milliliter. For the subsequent phase, a solution of the DPPH radical was constituted by dissolving 2.4 milligrams of DPPH in a 100-milliliter volume of methanol, allowing complete dissolution until a homogeneous solution was achieved. Further steps involved the addition of 2.5 milliliters of the methanolic DPPH solution to each of the diluted concentration samples. The mixtures, post vigorous shaking, were carefully incubated in darkness at room temperature for 30 minutes. Absorbance readings of the reaction mixtures were then spectrophotometrically measured at 517 nanometers, encompassing the absorbance of the negative control sample and the standard vitamin C solution. Subsequently, the capacity to scavenge the DPPH was calculated. A plot was generated, correlating the average percentage inhibition (y-axis) against the logarithm of the individual concentrations (x-axis). The IC_{50} value was extrapolated from the graph, elucidating the extent of the antioxidant activity exhibited by the plant sample.

2.2.8 Gas Chromatography Mass-Spectroscopy (GC-MS)

A small aliquot of the powdered dichloromethane-methanol extract was prepared, and a measured volume was injected into the GC-MS system. The system employed a gas chromatograph interfaced with a mass spectrometer to separate and identify the individual components present in the extract. The chromatographic separation was achieved by subjecting the sample to a temperature-controlled column, allowing the compounds to elute based on their differing chemical properties and interaction with the stationary phase within the column. The eluted compounds were subsequently ionized and analyzed by the mass spectrometer, which generated mass spectra unique to each compound. The resulting spectra were then compared with reference databases to identify the various chemical constituents present in the extract.

2.2.9 Proximate Analysis

2.2.9.1 Moisture content (AOAC 925.10 Method - Air oven)

One gram of the sample was accurately weighed into a clean, dried porcelain evaporating dish. The dish, containing the sample, was then positioned in an oven set precisely at 105°C for a duration of six hours. The evaporating dish, now holding the dried sample, was allowed to cool in a desiccator at room temperature. Subsequently, the dish, along with the dried sample, was re-weighed, and this new weight was recorded accurately. The moisture content was calculated using the formula:

% Moisture = [(Weight of fresh sample - Weight of dried sample) / Weight of sample used] × 100.

2.2.9.2 Lipid content (AOAC 963.15 Method – Soxhlet Extraction)
Two grams of the powdered sample were carefully placed into a filter paper, which was then inserted into a Soxhlet extractor. This extractor was positioned within a pre-weighed, dried distillation flask. Acetone was introduced into the distillation flask through the condenser attached to the Soxhlet extractor. A cooled water jet was directed into the condenser, allowing the heated solvent to reflux continuously. This refluxing action resulted in the extraction of lipids from the sample, accumulating in the solvent chamber. Upon complete extraction of lipids from the sample, the condenser and extractor were disconnected, and the solvent was evaporated to concentrate the lipid extract. The concentrated lipid extract in the flask was then dried in an air oven until a constant weight was achieved. Subsequently, the flask containing the dried lipid extract was re-weighed to accurately measure the weight of the lipid.The calculation of % Lipid was performed using the formula:

% Lipid = [(Weight of flask and extract - Weight of empty flask) / Weight of sample extracted] × 100.

2.2.9.3 Ash content (AOAC 942.05 Method – Furnace)
One gram of the dried sample was precisely weighed into a preheated and pre-weighed porcelain crucible. The crucible, now containing the sample, was carefully placed into a muffle furnace, where it was exposed to a regulated temperature of 630°C for a period of three hours. Subsequently, the crucible was allowed to cool down to room temperature before being reweighed. The calculation for % Ash was performed using the formula:

% ASH = [(Weight of crucible + Ash sample - Weight of crucible and sample after ash) / Weight of sample] × 100.

2.2.9.4 Carbohydrate content (AOAC 920.39 Method – Cleg Anthrone)
0.1g of the sample was precisely weighed and placed into a 25ml volumetric flask. To this, 1ml of distilled water and 1.3ml of 62% perchloric acid were added. The mixture was vigorously shaken for 20 minutes to ensure complete homogenization. The volumetric flask was filled up to the 25ml mark with distilled water and securely stoppered. The resultant solution was then allowed to settle for decanting purposes. From the filtrate, 1ml was transferred into a 10ml test tube and diluted to volume with distilled water. Subsequently, 1ml of the working solution was pipetted into a clean test tube, to which 5ml of Anthrone reagent was added. A mixture of 1ml distilled water and 5ml Anthrone reagent was also prepared. Both mixtures were read at a wavelength of 630nm using the prepared blank (1ml distilled water and 5ml Anthrone reagent) for calibration purposes. Additionally, a solution of glucose (0.1ml) was treated as the sample with Anthrone reagent, and its absorbance was measured. The % Carbohydrate as glucose was calculated using the formula:

% CHO as glucose = (25 x absorbance of sample) / Absorbance of standard glucose.

2.2.9.5 Crude fiber determination (AOAC 962.09 Method)
Two grams of the sample were initially extracted with petroleum ether. Subsequently, the sample was boiled under reflux for 30 minutes in the presence of 200ml of dilute hydrochloric acid. After this step, the mixture underwent filtration. The resulting residue was thoroughly washed with water until it reached an acid-free state. This residue was then transferred into a beaker and boiled for an additional 30 minutes, this time with 200ml of dilute sodium hydroxide solution. Following this, the mixture was filtered once more and the residue was collected into an ignited crucible. The residue collected in the crucible underwent a washing process: three washes with 20ml ethanol and two washes with 10ml ether. After the washing steps, the residue was dried in an oven until constant weight was achieved. This dried residue was then cooled and weighed. Subsequently, the dried residue was transferred into a furnace and subjected to ignition. After the ignition process, the residue was cooled once more and weighed.The calculation for Crude Fiber content involves the following formula:

% Crude Fiber = [(Weight of dried residue - Weight of ignited crucible with residue) \Weight of sample used] × 100

2.2.9.6 Protein Content (AOAC 984.13 Method - Kjeldahl)
0.1g of the sample was precisely weighed into a clean 250ml conical flask. To this, 3 grams of digestion catalyst and 20ml of concentrated sulphuric acid were added. The mixture was heated to initiate digestion, resulting in a colour

change from black to sky-blue. After cooling the digest to room temperature, it was diluted to a final volume of 100ml using distilled water. From this diluted digest, 20ml was measured into a distillation flask. The distillation flask was secured on an electrothermal heater or hot plate and attached to a Liebig condenser, leading to a receiver containing 10ml of 2% boric acid indicator. Through a syringe attached to the mono-arm steelhead, 40ml of Sodium hydroxide was incrementally introduced into the digest until it became strongly alkaline. The mixture was then heated to boiling, and the resultant distilled ammonia gas passed through the condenser into the boric acid indicator, causing the colour to change from purple to greenish. The distillate in the receiver was titrated back to a purple colour from the greenish hue using standard 0.1N Hydrochloric acid solution. The volume of hydrochloric acid required for this colour change was noted as the titer value. The calculation for % Organic Nitrogen (used to estimate protein content) is:

% Organic Nitrogen = Titer value × Factor × 100 × 100) / (V × W)
Where:
- Titer value is the volume of standard acid (e.g., 0.1N Hydrochloric acid) used in titration.
- Factor is a conversion factor, often 1.4, representing the conversion of nitrogen to protein.
- V is the volume of the sample used in the digestion step (in liters).
- W is the weight of the sample used in grams.

This formula allows the determination of the organic nitrogen content, which is then used to estimate the protein content in the sample.

2.3 Statistical Analysis

The means were obtained from mean calculations using three determinations. The standard error of the mean (SEM) was also determined. Statistical significance of results was determined by one-way ANOVA and paired t-test using the Graph pad PRISM version 8.

3. RESULTS

3.1 Phytochemical Screening

The Phytochemistry of the Nigerian walnut husk was studied and the results the dichloromethane-methanol extract showed the presence of major classes of phytochemicals which are most likely responsible for its activity. Alkaloids, flavonoids, steroids, saponins and phenolics were present. However, anthraquinones, phlobatanin, cardiac glycosides, and triterpenoids were found to be absent in this qualitative assay as contained in Table 1.

Table 1. Phytochemical constituents of dichloromethane-methanol extract of *Plukenitia conophora*

Screened Phytochemical test	Results	Screened Phytochemical test	Result
Alkaloids		Carbohydrate	
Dragendroff test	+	Molisch	+
Mayer's test	+	Fehlings	-
Flavonoids		Phenolic compounds	
Shinoda test	+	FeCl3 test	+
Anthraquinones		Triterpenoids	
Free anthraquinones	-	Salkwoski test	-
Combined anthraquinones	-	Libermann Buchard test	-
Saponins		Cardiac glycoside	
Frothing test	+	Keller killer test	-
Emulsion	-	Kedde's test	-
Phlobatannin		Steroid	+
Hydrochloric acid test	-		

KEY: Present (+), Absent (-)

3.2 Antimicrobial Analysis

3.2.1 Antimicrobial Suseptibility

The Dichloromethane-methanol extract was tested against four pathogenic bacterial species and four pathogenic fungal species, the positive control was Gentamicin for bacterial and Fluconazole for Fungi while the negative control was DMSO. From the result obtained, the Dichloromethane-methanol exhibited activity against the panel of bacteria

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as indicated by the measured inhibitory zone diameter – IZD (in mm) and exhibited activity against two of the fungi species as indicated by the inhibitory zone diameter. The values of the IZD are presented in the Table 2, Figure 1

Table 2: Antimicrobial assay of Nigerian walnut husk showing the Inhibition zone diameter on bacteria

Organism	Inhibitory Zone Diameter (IZD) (mm)		
	Walnut Husk Extract	Positive Control (Gentamicin)	Negative Control (DMSO)
	Mean ± SEM	Mean ± SEM	Mean ± SEM
K. pneumonia	15.667 ± 1.202*	18.333 ± 0.667	0.000 ± 0.000
P. aeroginosa	6.000 ± 0.577*	13.500 ± 0.500	0.000 ± 0.000
E. coli	6.000 ± 0.577*	17.667 ± 0.667	0.000 ± 0.000
S. aureus	9.333 ± 0.333*	18.333 ± 0.667	0.000 ± 0.000

KEY: Values are presented in mean ± Standard Error of Mean (SEM); *P* value < 0.05 are represented as * against the negative control; (n = 3)

Antimicrobial assay of Nigerian walnut husk showing the Inhibition zone diameter on fungi is shown in Figure 2.

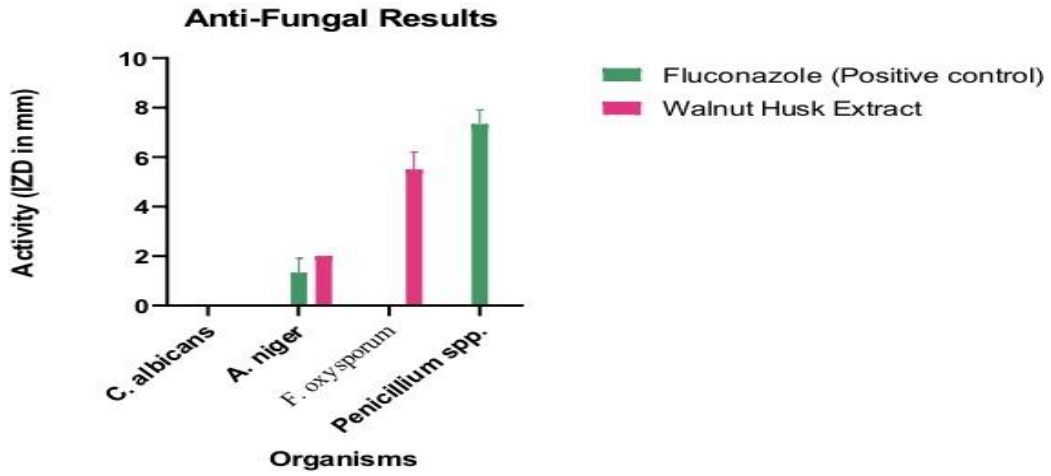

Figure 1: A chart showing the zones of inhibition of the samples and control against the test fungal organisms.

3.2.2 Minimum Inhibitory Concentration

The MIC of the Dichloromethane-methanol extract on the bacteria in which the IZD was determined, the various MIC gotten are presented in the Table 3 below.

Table 3: Minimum inhibitory concentration (MIC) of Nigerian walnut against some bacterial organisms.

Organism	Minimum Inhibitory Concentration (mg/ml)				
	50	25	12.50	6.25m	3.125
K. pneumoniae	13.00± 0.58*	9.67± 0.33*	6.33± 0.33	4.33± 0.33	0.00± 0.00
P. aeroginosa	5.33± 0.33	4.67± 0.33	3.33± 0.33	2.67± 0.33	0.00± 0.00
E. coli	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	0.00 ± 0.00	0.00 ± 0.00
S. aureus	6.33± 0.33*	4.67± 0.33*	0.00 ± 0.00	0.00 ± 0.00	0.00± 0.00

KEY; Values are presented in mean ± Standard Error of Mean (SEM) *P* value < 0.05 are represented as * (n = 3)

3.3 Antioxidant Assay

Table 4: The result of Antioxidant screening of the Dichloromethane-methanol extract of Nigerian walnut husk using DPPH

NAME	Conc (µg/ml)	Abs 1	Abs 2	$\%I_1$	$\%I_2$	$\%I_{Average}$	IC_{50}
Test Conc 1	12.500	0.188	0.178	36.913	40.268	38.591	91.51µg/ ml
Test Conc 2	25.000	0.166	0.160	44.295	46.309	45.302	
Test Conc 3	50.000	0.134	0.137	55.034	54.027	54.530	
Test Conc 4	100.000	0.109	0.103	63.423	65.436	64.430	
Test Conc 5	200.000	0.046	0.048	84.564	83.893	84.228	
Test Conc 6	400.000	0.025	0.022	91.611	92.617	92.114	
Test Conc 7	800.000	0.024	0.027	91.946	90.940	91.443	
Vit Conc 1	0.000	0.292	0.303	0.000	0.000	0.000	IC_{50} = 0.37-1.00 µg/ml
Vit Conc 2	4.000	0.139	0.154	53.356	48.322	50.839	
Vit Conc 3	8.000	0.074	0.069	75.168	76.846	76.007	
Vit Conc 4	16.000	0.000	-0.002	100.000	100.671	100.336	
Neg control	0.000	0.292	0.303	0.000	0.000	0.000	

Key; Conc – Concentration; Abs = absorbance; $\%I_1$ = percentage inhibition 1; $\%I_2$ = percentage inhibition 2 Vit = Vitamin

Calculation of percentage inhibition (%I)

% Inhibition = $((A_0 - A_1) \times 100) / A_0$; A_0 = Absorbance of Negative Control; 1 = Absorbance of Test substance

3.4 Gas Chromatography Mass Spectroscopy (GC-MS)

Table 5: Compounds found in the GC-MS of the n-hexane fraction the husk extract

S/N	Retention time (mins)	Area (%)	Mol. formular	Mol. Weight (g/mol)	Compound	Quality
1	21.277	0.22	$C_{14}H_{28}O_2$	228.3709	Dodecanoic acid, ethyl ester	81
2	25.994	0.22	$C_{13}H_{26}O_2$	214.3443	Undecanoic acid, ethyl ester	87
3	27.293	0.09	$C_{15}H_{30}O_2$	242.40	Pentadecanoic acid	89
4	28.682	0.28	$C_{17}H_{34}O_2$	270.4507	Hexadecanoic acid, methyl ester	87
5	29.045	0.33	$C_{16}H_{32}O$	254.41	9-Hexadecenoic acid(Palmitoleic acid)	87
6	29.515	6.77	$C_{16}H_{32}O$	256.4241	n-Hexadecanoic acid	99
7	30.088	0.64	$C_{10}H_{12}O_2$	164.20	Methyl 2,6-dimethyltridecanoate	92
8	32.103	0.45	$C_{19}H_{32}O_2$	292.4562	9,15-Octadecadienoic acid, methyl ester, (Z,Z)-	98
9	32.215	0.32	$C_{19}H_{36}O_2$	296.4879	9-Octadecenoic acid (Z)-, methyl ester	99
10	32.686	0.27	$C_{19}H_{38}O_2$	298.5038	Methyl stearate	91
11	33.034	24.95	$C_{19}H_{32}O_2$	294.4721	9,12-Octadecadienoic acid (Z,Z)-	99
12	33.104	23.54	$C_{19}H_{32}O_2$	294.4721	9,12-Octadecadienoic acid (Z,Z)-	99
13	33.392	5.38	$C_{19}H_{32}O_2$	294.4721	9,12-Octadecadienoic acid (Z,Z)-	99
14	33.494	9.47	$C_{18}H_{32}O$	264.4	9,17-Octadecadienal, (Z)-	94

15	33.778	3.78	$C_{18}H_{34}O_2$	282.5	Oleic Acid	95
16	33.954	6.20	$C_{18}H_{34}O_2$	282.5	cis-Vaccenic acid	74
17	34.224	3.59	$C_{18}H_{34}O_2$	282.5	Oleic Acid	90
18	34.362	2.80	$C_{15}H_{28}O_2$	240.3816	Cyclopentadecanone, 2-hydroxy-	91
19	34.523	3.12	$C_{18}H_{34}O_2$	282.4614	9-Octadecenoic acid, (E)-	92
20	35.559	2.59	$C_{20}H_{40}O$	296.5310	Octadecane, 1-(ethenyloxy)-	90
21	36.948	0.57	$C_{19}H_{36}O$	280.5	2-Methyl-Z,Z-3,13-octadecadienol	92
22	37.979	0.16	$C_{18}H_{34}O_2$	282.5	Oleic Acid	83
23	38.281	0.10	$C_{16}H_{28}O$	236.39	7,11-Hexadecadienal	90
24	38.485	0.75	$C_{17}H_{24}O_4$	292.4	2-Hydroxy-4-methoxy-7-Methyl 7,8,9,10,11,12,13,14-octahydro-6-oxabenzocyclododecen-5-one	95

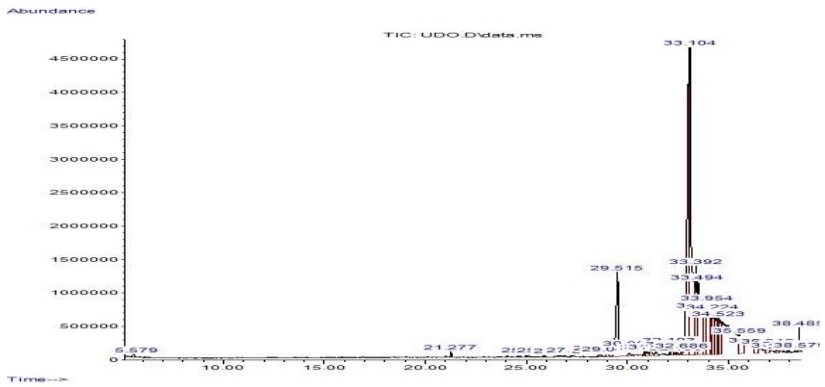

Fig 2 Gas Chromatography Mass Spectroscopy of the n-hexane fraction of the crude Dichloromethane-methanol extract of the husk of *Plukenitia conophora*

3.5 Proximate analysis

Table 6. showing the proximate analysis of the Dichloromethane-methanol extract of the Nigerian walnut husk

Parameter	Walnut Husk Sample 1 (%)	Walnut Husk Sample 2 (%)
Moisture	9.10	9.10
Ash	9.00	9.00
CHO	10.12	10.20
Protein	5.69	5.69
Lipid	3.20	3.20
Fibre	62.89	62.81

4.0 DISCUSSION

After the extraction, the crude methanol extract obtained by cold maceration was subjected to phytochemical screening. From the phyto-chemical analysis result in Table 1 above using the method of [9] as modified by Okoroafor

and Isirima, [10], the crude dichloromethane-methanol extract of the *P. conophora* contained a range of secondary metabolites from different classes. Classes of metabolites present included: Alkaloids, Flavonoids, Saponins, carbohydrates, phenolics and steroids. The plant enriched with Flavonoids and Phenolic compound which are known to contain bactericidal properties makes the plant medicinally relevant and investigative processes carried out to determined and identify the various biomolecules which are attributed to such activity [11]. Steroid present in the husk extract are secondary metabolites used pharmaceutically directly as a drug or prodrug and has antioxidants and neuroprotective activity. The saponins present in *P. conphora* acts as an effective antimicrobial agent against various bacteria, viruses, fungi, and yeasts. The saponins are secondary metabolites synthesized as defensive compounds against pathogenic microbes [12]. So the phytochemical compounds contained in plants results in the activity of that plant. The compounds contained in *Plukenitia conophora* contributes to its pharmacological effects. These compounds have inhibitory action on fungi, bacteria, and viruses. The Gas Chromatography-Mass Spectroscopy analysis of the sample was carried out as shown in Table 5 above. The Gas Chromatography spectrum indicates 23 major peak out of the original 39 peaks, However, the three most abundant compounds are 9,12-Octadecadienoicacid (Z,Z)- (Linoleic or omega 6), Oleic acid , and methyl stearate that were separated and elucidated by the Gas Chromatography Mass spectrometry. The fragmentation pattern of these compounds and their peaks were elucidated to characterize the compounds for their possible structure, name and molecular mass. All compounds elucidated by the GC-MS were found to be unsaturated hydrocarbons. The peak area is the reflection of the amount of specific analyte that is present. In this study, 9,12-Octadecadienoic acid (Z, Z)- (Linoleic or omega 6) had a retention time of 33.104 minutes and a peak area of 23.54 %. It is an unsaturated hydrocarbon with cis-double bonds at positions 9,12. It had a 99% quality match and appearing at different peaks; indicative of the abundance of this compound in the walnut husk. This compound has been reported to have antimicrobial activity [13]. Linoleic acid lowers the risk premature death and is commonly used in the skin and Cardiovascular diseases [14]. Oleic acid is an octadec-9-enoic acid in which the double bond at C-9 has Z (cis) stereochemistry. It has a role as an EC 3.1.1.1 (carboxylesterase) inhibitor, an *Escherichia coli* metabolite, a plant metabolite, an antioxidant (ChEBI) with a retention time 33.778 minutes and peak area of 3.78%. It had 95% quality match also appeared in different peaks which has been reported as a treatment for Cardiovascular diseases and antimicrobial particularly Gram positive [15]. Methyl stearate is an eighteen-chain methyl ester with a retention time of 32.686 minutes and peak area of 0.27%. It is also known as octadecanoic acid, methyl ester with 90% quality match which has been reported to have antimicrobial activity [16] alongside antioxidant and anticancer activity [17]. Further elucidation can be carried out using Liquid Chromatography-Mass Spectroscopy which would be most suitable for Polar compounds that could be thermally decomposed on the application of heat [18]. The mean proximate composition of *P. conophora* husk as shown in table 6 and figure 6 was gotten to be moisture (9.10%), ash (9.00%), carbohydrate (10.16), protein (5.69), lipid (3.20) and fibre (62.85%). Previous studies on the Nigerian walnut leaf revealed it to be protein rich (29.14%), fat rich (54.14%), carbohydrate rich (4.17%), ash rich (3.32%) and rich in several vitamins [8]. This study has shown that the walnut husk is a better source of carbohydrate, fibre and ash than the walnut leaf. The antimicrobial susceptibility test was carried out to determine the sensitivity of microbial isolates on antibiotics or to detect resistance in individual microbial isolates. The agar well diffusion method was used. The stock solution of the extracts was prepared at a concentration of 100 mg/ml. A standard antibiotic drug was used as the positive control which for antibacterial, gentamicin was gotten and fluconazole for antifungal. Upon incubation for 24 hours at 37°C, the zone of inhibition was measured as shown in Table 2. From the result, we can see that the walnut husk had potent activity on the Gram positive bacteria where for *K. pneumonia*, the walnut husk gave a mean zone of inhibition of 15mm while the positive control gave a mean zone of inhibition of 18mm which falls under the sensitive range of the CLSI (Clinical & Laboratory Standards Institute) guidelines for Gentamicin and For *S. aureus*, the walnut husk gave a mean zone of inhibition of 9 mm while the positive control gave a mean zone of inhibition of 18mm which falls under the sensitive range of the CLSI (Clinical & Laboratory Standards Institute) guidelines for Gentamicin. These values were obtained after subtraction the 6mm diameter of the bore well. According to Ajaiyeoba and Fadare [19] the methanol and ethyl acetate *P. conophora* leaf extracts have strong antibacterial properties, particularly against Gram- positive bacteria and this is in line with our husk result obtained that is most active against Gram-positive bacteria. In addition the husk had activity against two out of the four fungi worked on, For *A. niger* the walnut husk gave a mean zone of inhibition of 2 mm while the positive control gave a mean zone of inhibition of 1mm which doesn't falls under the sensitive range of the CLSI (Clinical & Laboratory Standards Institute) guidelines for fluconazole, so we can say that the clinical strain used was a little resistant while For *F. oxysporum* the walnut husk gave a mean zone of inhibition of 5.5 mm while the positive control didn't have a zone of inhibition so we can say that the clinical strain used was resistant to the positive control (fluconazole) so a comparison couldn't exactly be

made. The Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. [20]. The minimum inhibitory concentration (MIC) values of the crude extracts of *P. conophora* against the various microbial clinical isolates are shown in Table 3. These values were obtained using the agar-well diffusion method. The graphical representation of the MIC values is shown in figure 3. From the values obtained, the MIC of the bacterial strain *K. pneumonia*, were 13.000, 9.667, 6.333, 4.333 and 0.000 for 50, 25, 12.5 6.25 and 3.125 mg/ml respectively. In comparison with the MIC of *P. aeruginosa* which were 5.333, 4.667, 3.333, 2.667 and 0.000 at 50, 25, 12.5 6.25 and 3.125 mg/ml respectively and the MIC of *S. aureus* were 6.333, 4.667, 0,000, 0.000 and 0.000 mg/ml; which showed that the extract was most potent on *K. pneumonia.* Phenolic compounds and Flavonoids have anti-oxidant, anti-bacteria, cardio-protective and anti-inflammatory effect. Saponins can be used in the treatment of calciuria in humans. From the analysis carried out to evaluate the anti-oxidant potential of the crude extract Using DPPH and Ascorbic acid as the Standard. The experiment was carried out using a UV Spectrophotometer to get the Absorbance of each concentration of the crude extract that was prepared. DPPH radical scavenging assay was carried out to determine the Hydrogen atom donating ability of this plant crude Dichloromethane-methanol extract through the decolourization of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH produced violet/purple colour in methanol solution and this colour faded to different shades of yellow colour with different intensity corresponding to the various concentrations of the plant extract. The percentage scavenging activity or percentage inhibition was calculated using standard formula. From Table 4, IC_{50} of the crude Dichloromethane-methanol extract was found to be 91µg/ml, while the IC_{50} of the standard used (Ascorbic acid) was found to be ~1µg/ml. The lower the value of IC_{50}, the higher the free radical scavenging activity of the plant. The findings is tandem with Moga et al., [21] which stated that an extract of ≤ 10 µg/ml is highly active, 10-100 µg/ml active, 100-500 µg/ml moderately active and > 500µg/ml inactive. The result therefore suggest that the walnut husk extract is an active antioxidant. However, if the pure compound is isolated from the walnut extract it could yield a highly active result.

5.0 CONCLUSION

In conclusion, the phytochemical screening, proximate analysis, GCMS, antimicrobial and antioxidant activities of the Dichloromethane-methanol extract of *P. conophora* husk was investigated in this study. The results showed that the walnut husk possessed very beneficial phytoconstituents, which was correlated with the GCMS result that showed the three most abundant compounds were, 9,12-Octadecadienoic acid (Z, Z)- (Linoleic or omega 6) reported to have antimicrobial activity. Linoleic acid commonly used in skin and Cardiovascular diseases and Oleic acid; an *Escherichia coli* metabolite, a plant metabolite, an antioxidant (ChEBI) documented as a treatment for Cardiovascular diseases and antimicrobial particularly Gram positive. The third most abundant compound is Methyl stearate which has been reported to have antimicrobial activity, antioxidant and anticancer activites. The husk demonstrated good antioxidant activities and is also a good source of fiber which is highly beneficial as fiber has been shown to improve gut health. These findings, indicate that the Nigerian walnut husk has potentials as a plant-based pharmaceutical product for several diseases and thus merits further studies.

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