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Physical properties, Chemical Composition and Antimicrobial Activity of Oil and Extracts from *Tetracarpidium conophorum (*African Walnut)

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Abstract. The oil of the powdered nut of African walnut (*Tetracarpidium conophorum)* was extracted sequentially using the expeller press (mechanical) method and solvent extraction (chemical) method by soxhlet extractor. The physical appearance of the oil reveals that African walnut oil was pale yellow in colour, clear, light without impurities. The colour of the oil extract reveals that the petroleum ether extract was golden yellow, n-hexane extract was golden brown and chloroform extract was golden/dark brown. 200g of African walnut extracted by expeller press yielded 12.53 % of oil, 30 g of the sample extracted by hexane, petroleum ether and chloroform yielded 18.06 %, 12.5 % and 11.97 %. The chemical properties of the expeller pressed oil showed the saponification values, unsaponifiable matter content, acid value, peroxide values, free fatty acids (FFA), iodine, refractive index (RI) and specific gravity (SG) of the oil was 170.1 mg KOH g^{-1}, 4.1 g/kg^{-1}, 0.92 mg KOH g^{-1}, 1.02 meq O_2 kg^{-1}, 0.56 %, 91.3 Wij's, 1.445 and 0.94. The n-hexane extract had 174.1 mg KOH g^{-1}, 3.9 g/kg^{-1}, 0.72 mg KOH g^{-1}, 1.01 meq O_2 kg^{-1}, 0.46 %, 90.1 Wij's, 1.442 and 0.88. Petroleum ether extract had 165.1 mg KOH g^{-1}, 2.5 g/kg^{-1}, 0.82 mg KOH g^{-1}, 1.00 meq O_2 kg^{-1}, 0.65 %, 87.6 Wij's, 1.440 and 0.94. Chloroform extract had 160.1 mg KOH g^{-1}, 3.2 g/kg^{-1}, 0.62 mg KOH g^{-1}, 0.94 meq O_2 kg^{-1}, 0.82 %, 89.9 Wij's, 1.440 and 0.94. The oil could be exploited for use in vegetable oil and cosmetic industries. In this study, the *in vitro* antimicrobial activities of African walnut oil and extracts were evaluated using agar-well diffusion method against clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *Cutibacterium acne*, *Malassezia furfur* and *Candida albicans* which were isolated from human skin infections. The results of the investigation revealed that African walnut oil had inhibitory effect on the growth of all the test microorganisms. On the bacterial species, *S. aureus* was highly inhibited by African walnut oil as confirmed by the zones of inhibition measured. *C. acnes* was moderately inhibited, while the growth of *E. coli* was observed to be mildly inhibited. With the fungal species, 100 % of African walnut oil proved really effective inhibiting the growth of *M. furfur* and *C. albicans.* Petroleum ether concentration (80 %) inhibited the growth of *E. coli* while *C. acnes* and *M. furfur* were inhibited at 20 % petroleum ether concentration; *C. albicans* was inhibited at 10 % concentration. The minimum inhibitory concentration of African walnut oil and petroleum ether extract revealed that 500 and 250 mg/mL were the least concentrations that prevented the growth of bacteria after 24 h incubation.

Keyword: African walnut oil, *Tetracarpidium conophorum*, physical properties, chemical composition, antimicrobial activity, skin infections, agar well diffusion method, extracts, MIC

1. Introduction

The use of plant and its products have a long history of use in folk medicine and through the years have been incorporated into modern or western medicine and in certain systems of traditional medicine (Sen and Batra, 2012). Since ancient times, many plants species have been reported to have pharmacological properties as they posses various secondary metabolites like terpenoids, saponins,

flavonoids, steroids, tannins and alkaloids which is utilized to combat pathogenic microorganisms (Chijioke *et al*., 2015; Igara *et al*., 2017).

With the development of technology along with the advancement in science, remarkable progress has been made in the field of medicine with the discoveries of many natural and synthetic drugs (Sen and Batra, 2012). Antibiotic is one of the most significant therapeutic discoveries of the 20th century that had effectiveness against serious microbial infections. However, only very few of the well-known human diseases have been treated from these synthetic drugs (Sen and Batra, 2012). This is because of the emergence of drug resistant pathogens that is the effect of indiscriminate use, incessant and misuse of antibiotics (Bello *et al*., 2013; Igara *et al*., 2017). Antibiotic resistance has increased recently and is posing a serious therapeutic problem. Plants produce a variety of compounds to protect themselves against a variety of pathogens. It is anticipated that plant extracts having different target sites other than the antibiotics sites will be active against drug resistant pathogens (Sen and Batra, 2012). Over many decades, medicinal plants have been used as traditional treatments for numerous human diseases world-wide. Hence, numerous researchers have paid great attention to plant based traditional medicine practices and biologically active compounds or phytochemicals isolated from plant species used in herbal medicines with satisfactory therapeutic index for the development of novel natural antimicrobials (Sen and Batra, 2012; Nwaoguikpe *et al*., 2012; Chijioke *et al*., 2015).

The African walnut (*T. conophorum*) belongs to the family Euphorbiaceae (Edem *et al*., 2009). However, Ayodeji and Aliyu (2018) stated that some walnut species are found in the family Olacaceae. African walnut is similar to *Juglans regia* (L.), known as the English walnut and belonging to the family Juglandaceae (Raja *et al*., 2012; Lamichhane *et al.,* 2016). African Walnut is a perennial creeping shrub that grows in temperate areas in Africa such as Cameroon, Gabon and Liberia including Nigeria (Ayoola *et al*., 2011). The immature fruits are usually green in colour but turn dark brown to black as they reach maturity (Ayodeji and Aliyu 2018). Walnuts are dry nuts which are encased in green pods. As walnut matures, the outer covering dries and falls off leaving the segment tough black shell and the white seed. The white seed nut is the edible nut (Ekwe and Ihemeje, 2013). Its range in Nigeria includes Uyo, Akamkpa, Akpabuyo, Lagos, Akure, Kogi, Ajaawa, Ogbomosho, Ibadan (Obianime and Uche, 2010; Ayodeji and Aliyu, 2018), Ife, Ekiti and Ijeshaland. it is abundant in all cocoa-producing states in Nigeria and in the southern part of Nigeria (Udedi *et al*., 2014; Nwaichi *et al*., 2017; Ayodeji and Aliyu 2018). It is commonly called African walnut because of its West African origin. *T. conophorum* is often called by different names such as awusa (Yoruba), Ukpa in Igbo, kaso or ngak in Cameroon. Walnut tree is about 40m in length and are usually harvested between July to December (Ayoola *et al*., 2011).

Currently, many of the plant species that are now cultivated world-wide for food purposes were those initially neglected and underutilized (Magbagbeola *et al.,* 2010). Some of these are potential industrial raw materials and can serve as precursor of new products. Oils derived from plant sources are obtained from plant seeds referred to as oilseeds. Oilseeds are a class of plants in which huge amount of lipids are stored in the seed tissue (Akanni *et al.,* 2005). The amount of oil present in oilseeds such as soybean and corn ranged from 10-20% and 50% in sunflower and rapeseed (Obasi *et al*., 2012). Oilseeds are among the most ancient crops domesticated by mankind. For example, there is evidence that the cultivation of linseed oil bearing varieties in the eastern part of Nigeria has been on from many decades ago. However, for many years now, globally, there has been great increase in the demand for vegetable oils with up-trend in prices (Akanni *et al.,* 2005). From the olden days, oilseed products were utilized for a variety of edible and non-edible applications. Seed oils have been extensively used for human consumption. Mostly, oils are used in food, both in cooking and as supplements. The nutritive and calorific values of oilseeds make them good sources of edible oils and fats in diets (Obasi *et al*., 2012). Besides been a vital component of human diet, oils also find importance in various industrial applications.

African walnut like many plants in Africa and other parts of the world has been proven to have numerous values including decorative, nutritive, medicinal, agricultural and industrial over the years. African walnut plant is cultivated principally for the nuts which are consumed as snacks. Many research studies have showed that walnut contain high amount of oil and is a major sources of protein, carbohydrate, fat and oils, vitamins and minerals (Ajaiyeoba and Fadare, 2006; Grace *et al*., 2016). African walnut contain uncommon fatty acids which are industrially important as they are used in the manufacture of protective coatings, dispersants, pharmaceuticals, cosmetics and a wide variety of synthetic intermediates as stabilizers in plastic formulations (Ogunwusi and Ibrahim, 2016). In addition, walnuts

have been reported as a high density food because it contains valuable bio-active phytochemical compounds such as oxalates, phytates, and tannins (Ajaiyeoba and Fadare, 2006). The ability of the nuts to reduce cholesterol levels in human seem to be the heart of their health benefits, though the nut contain many other antioxidants, which helps to supports the immune system and they also possess some anticancer properties. African walnut is used in Nigeria to increase sperm counts in men (Ajaiyeoba and Fadare, 2006; Ogunwusi and Ibrahim, 2016). Decoctions and infusions made from the green nuts and leaves of walnut have been used to treat various human infections such as candidiasis, vaginitis, conjuctivities, glomerulo nephritis, cellulitis, endocarditis and other related diseases (Grace *et al*., 2016). Furthermore, African walnuts are rich in dietary omega-3 fatty acids which play a role in the prevention of some disorders including depression dementia particularly Alzheimer's disease and the antimicrobial efficacy of the plant have been attributed to the presence of phytochemicals (Grace *et al*., 2016).

Treatment of bacterial and fungal diseases with oils and extracts from medicinal plants has been effectively carried out in Nigeria. Oil from parts of plants has been shown to exhibit antibacterial and antifungal activity against a wide range of pathogenic antibiotic resistant fungi and bacteria species (Okeke *et al*., 2001; Pawar and Thaker, 2006). Oil of plant origin has been used for various cosmetic purposes particularly in the formulation of skin and body care products. Apart from a few domestic uses of African walnut oil, local communities in Nigeria use the oil for the treatment of skin rashes and related skin infections caused by bacteria and fungi species. Although, there are no scientific justifications for this local practice, use of medicinal herbs and extracts from plant sources in the treatment of skin diseases is an age-long practice in many parts of the world (Irobi and Daramola, 1993). Treatment of skin rashes, boils, skin irritations, wounds, dermatitis and pyoderma with plant extracts is a common practice in Russia and Central Asia (Mamedov *et al*., 2005). With these claims in mind, firstly the physical screening of African walnut oil and its extracts were determined, secondly the chemical properties were determined and lastly the antibacterial and antifungal activity of the oil and extracts on some skin flora microorganisms that have been implicated in human skin infections was investigated.

2. Materials and Methods

2.1 Collection and identification of African walnut

Fresh walnuts were collected from walnut trees growing in Ogun State in September through October 2016 and a final sample of about 3 kg was randomly taken. The nuts were taken to Olabisi Onabanjo University, Ago-Iwoye, Ogun State where they were authenticated by a taxonomist. Standard methods for sample processing and preparation and analytical procedures were used.

2.2 Sample preparation and processing

After harvest and identification, African walnuts were sorted, and damaged ones discarded. The nuts were washed in cold water to remove dirt adhering to the surface. After washing, manual separation of the black husk from the nuts was done. The nuts were then sundried in shade for 14 days (two weeks). Thereafter, the nuts were milled using MarlexExcella mixer/grinding machine (Amazon, UK), packed in air tight containers and kept in the refrigerator at 4°C for further processing.

2.3 Extraction of African walnut oil and extract

2.3.1 Oil extraction

The method of choice for the extraction of African walnut oil was expeller press (mechanical pressing) method. The step wise method described by Jokic *et al*., (2014) was used to extract oil from walnut. The extraction of oils from walnut was performed using a screw expeller (Model SPU 20, Senta, Serbia). Walnut oil was obtained by pressing 200 g of the nuts. The minimum and maximum nozzle size of the expeller used was 7 and 12 mm, respectively. The frequency had a minimum of 10 Hz and a maximum of 20 Hz. The temperature of oil presses was between 70 and 100 °C. After extraction, the physical property of the oil was recorded (Figure 1), and the amount of oil obtained was measured using an analytical balance and result obtained was used to calculate the percentage yield of oil sample.

$$\text{Percentage yield of oil} = \frac{\text{Weight of extracted oil (g)}}{\text{Weight of sample (g/nut)}} \times 100$$

2.3.2 Extracts extraction (solvent extraction)

The oil extracts were extracted by using different organic solvents such as n-hexane, petroleum ether and chloroform. Extraction was done separately with the three solvents using the solvent extraction process. Prior to extraction, the pulverized nut samples were kept in an oven at 105

^{o}C for 1 h to remove any moisture that may still be present. Thirty (30 g) of the dried nut sample was wrapped in a white muslin cloth and put into separate porous thimbles of the soxhlet extractors. Then, 200 ml of different solvents such n-hexane, petroleum ether and chloroform of HPLC grade with boiling range of 40-60 ^{o}C was separately added. The soxhlets coupled with a condenser and flask already filled with the set up were heated in a heating mantle at 65 °C to allow solvent boiling. In the process the solvent vapour travels up a distillation arm and flowed into the chamber housing the sample material. The extracts seep through the pores of the thimbles and fill the siphon tube where it flows back down into the round bottom flask. The process was allowed to continue for 8 h until clear solvents were obtained in the thimble chamber. At the end of the extraction, the resulting mixtures of the oil extracts from the three solvents were separately filtered with a 10mm Syringe-driven with a filter 0.45um to remove any impurities. The solvents were further removed completely with a rotary-evaporator (Model N-1, Eyela, Tokyo Rikakikal Co., Ltd., Japan). The oil extracts were stored in white bottles and tubes under nitrogen at 4 °C until analyzed (Figure 1). Extraction was repeated three times. The yield of extract was calculated using the formular below

Percentage yield of extracts = Weight of extracted extract (g) x 100
Weight of sample (g/nut)

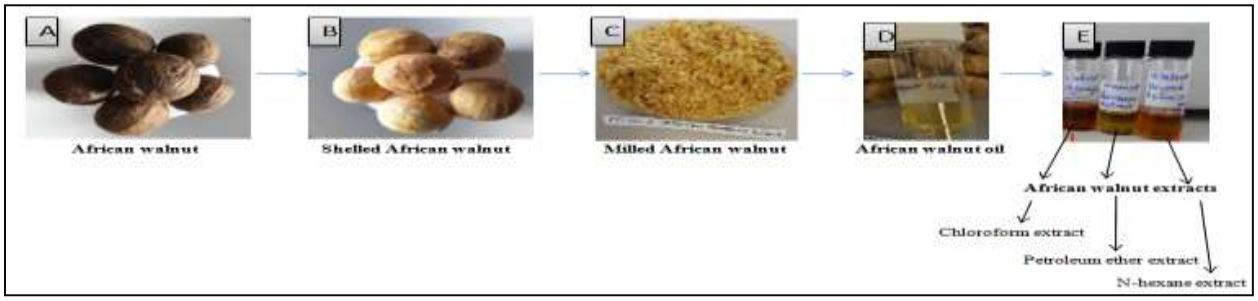

Figure 1 shows the flow chart for the extraction of oil and extracts from African walnut.

A= African walnut. B= Shelled African walnut. C= Milled African walnut. D= Expeller pressed African walnut oil E= solvent extracted extracts of African walnut.

2.4 Physical and Sensory properties

The colour, smell, taste, sedimentation and other related physical characteristics of African walnut oil and extracts were noted. A 10 man panel consisting of staff and students of Home Science and Hospitality Management, Yewa Campus, Olabisi Onabanjo University was constituted to evaluate the physical parameters of the oil. Colour measurement was done with a Lovibond colorimeter.

2.5 Chemical properties

Chemical analysis was carried out on African walnut oil to determine the Saponification values, Unsaponifiable matter content, acid value, peroxide values, free fatty acids (FFA), iodine, Refractive index (RI) and Specific gravity (SG). The procedures of Egan *et al*., (1981) and AOAC, (2000) were adopted for the estimation of Saponification values, Unsaponifiable matter content, acid value, peroxide value, free fatty acids (FFA) and iodine content, Refractive index (RI) and Specific gravity (SG) of the oil samples. The saponification value was determined by the estimation method. About 100 mg of the extracted oil was mixed with 0.5 M ethanolic potassium hydroxide solution and boiled under reflux on a water bath for 30 minutes. The solution was then titrated with 0.5 M HCl, using phenolphthalein solution as indicator. The saponification value was calculated using the titration values. For the determination of unsaponifiable matter, about 100 mg of the extracted oil was heated under reflux and saponified with 5 mL of ethanolic potassium hydroxide solution (20 % w/v) for 2 h. The unsaponifiable matter was extracted thrice with 15 mL of petroleum ether and the extracts were combined and evaporated at 40 °C under reduced pressure. The unsaponifiable residue was weighed. For peroxide determination, a known weight of African walnut oil was dissolved in a mixture of acetic acid/chloroform (3:2 v/v) and a saturated solution of KI (1 mL) was added. The liberated iodine was thereafter titrated with sodium thiosulphate solution (0.05 m) in the

presence of starch as indicator. For the free oil acidity (acid value), the titration method was used. For this determination, a known weight of African walnut oil was dissolved in a mixture of ethanol (95 %) and ether, previously neutralized with 0.1 M potassium hydroxide solution to phenolphthalein solution. The mixture was then titrated with 0.1 M potassium hydroxide solution as indicator until the solution remained faintly pink after shaking for 30 seconds. Free fatty acid (FFA) determination was by colorimetric method, while iodine value was determined using Wij's (iodine monochloride) method. In this method, a weighed amount of oil sample was dissolved in carbon tetrachloride and added to iodine monochloride. The resulting solution was kept in the dark for 30 minutes and then titrated with 0.1 M sodium thiosulphate, using starch solution as the indicator. Refractive index (RI) was measured with a Refractometer (RFM342, Bellingham + Stanley, England) while the specific gravity (SG) of the oil sample was determined gravimetrically. Both were determined using the methods of Egan *et al*., (1981). The chemical properties of African walnut oil were further compared with those of commercially available vegetable oils.

2.6 Test organisms

All the strains of bacteria and fungi species used in this study were clinical isolates. The bacteria species were obtained from the Department of Medical Microbiology and Parasitology, University Teaching Hospital, (UCH) Ibadan, while the fungi species were obtained from the Federal Medical Centre, (FMC) Abeokuta. The bacterial species used included *Staphylococcus aureus*, *Escherichia coli*, *Cutibacterium acnes.* While the fungi species are *Candida albicans* and *Malassezia furfur*. Five strains of each species of organisms were used. Each of the strains has designated laboratory codes assigned to them; all the isolates were confirmed from source to be implicated in some kind of skin infection (Table 1). No information is made available on the specific biochemical and characteristics that distinguish the strains.

Table 1: Source of test microorganisms

Test microorganisms	Strain codes	Source of test microorganisms	Body parts from which the test organisms were isolated
Bacterial strains			
S. aureus	2341	UCH	Isolated from skin rash
	3411	UCH	Isolated from boil on the armpit
	3621	UCH	Isolated from skin rash
	3001	UCH	Isolated from boil on the buttocks
	1190	UCH	Isolated from boil on the face
E. coli	2333	UCH	Isolated from boil on the face
	1411	UCH	Isolated from skin rash
	1093	UCH	Isolated from skin rash
	1114	UCH	Isolated from boil on the neck
	1039	UCH	Isolated from infected skin (razor) rash
C. acnes	3143	UCH	Isolated from skin acne
	2030	UCH	Isolated from skin rash
	1190	UCH	Isolated from skin lesions associated with dermatomycoses
	1390	UCH	Isolated from skin acnes
	1102	UCH	Isolated from skin infection site on nails
Fungal strains			
M. furfur	2556	FMC	Isolated from skin infection (Pityriasis)
	2035	FMC	Isolated from skin infected lesion
	2441	FMC	Isolated from skin razor rash
	2099	FMC	Isolated from skin infection lesion
	2016	FMC	Isolated from skin infection lesion
C. albicans	1001	FMC	Isolated from skin rash
	1425	FMC	Isolated from infected skin (razor rash)
	1346	FMC	Isolated from dried scaly skin rash
	1922	FMC	Isolated from skin rash associated with superficial granulation
	2140	FMC	Isolated from skin infected with Tinea

UCH = University of College Hospital FMC= Federal Medical Centre

2.7 Re-identification of the organisms

Identification of all the isolates in this study was done in accordance with the technique of Cheesbrough, (2010). The identities of the isolates were however reconfirmed using standard morphological, biochemical methods and mycological diagnostic methods. All the bacterial test organisms were aseptically grown on 5 ml nutrient broth overnight at 37^{o}C and then subcultured onto MacConkey agar (MAC), nutrient agar (NA) and cystein lactose electrolyte deficient (CLED) medium plates to get pure cultures of the isolates. These plates were incubated at 37^{o}C for 24 hours. *Candida albicans, and Malassezia furfur* were cultured on Sabouraud's Dextrose Agar (SDA) plate or Columbia sheep blood agar supplemented with olive oil. Cultured plates were incubated at 25°C for 48 hours. Pure cultures of these isolates were identified biochemically using standard microbiological identification techniques described by Cheesbrough, (2010).

2.8 Microbiological screening

2.8.1 Preparation of culture media and its sterilization

Microbial culture media including nutrient agar (NA) and nutrient broth (NB) were purchased from Oxoid, UK. Mueller Hinton Broth (MHB), and Mueller Hinton agar (MHA) was obtained from Sigma Aldrich, Dorset, UK. All these media were suitable for the growth of bacteria cultures. For fungi culture, Sabouraud's Dextrose broth (SDB) Sabouraud's Dextrose Agar (SDA) was obtained from ThermoScientific, Hampshire, UK. All media used were prepared according to manufacturer's instruction. The media for both bacterial and fungal cultures were made up in large volumes as follows: NA, (28 g), NB, (13 g), MHB (23 g), MHA (38 g), SDB (13 g) and SDA (65 g) were separately made up in 1 litre (1000 ml) of deionised water and sterilized at 121^{o}C for 15 minutes in an autoclave and subsequently allowed to cool after sterilization to about 45 ^{o}C (temperature at which the agars remains molten) before pouring into Petri dishes to solidify.

2.8.2 Preparation and standardization of inoculums for antimicrobial activity test

In order to effectively investigate the antibacterial activity of African walnut oil, inoculums for agar well diffusion tests were prepared in accordance with the guidelines of Clinical Laboratory Standards Institute, CLSI (formerly the National Committee for Clinical Laboratory Standards) (CLSI 2013) and Sen and Batra, (2012). Using aseptic techniques, the bacteria strains were cultured separately in 10 ml of nutrient broth and Mueller Hinton Broth overnight or to stationary phase (OD600 = >2.5). From the overnight culture, using streak plate method, a loop full of the tested strains were streaked across the respective agar plates and incubated at 37 ºC for 24 hours. From the incubated plates, inoculums were prepared by making a direct broth suspension of four to five well isolated colonies of *Staphylococcus aureus, Escherichia coli, Cutibacterium acnes with* the same morphological type in freshly prepared 10 ml broths in separate test tubes and incubated in a shaking incubator (Camlab, UK) at 37 ºC for 18 h with 200 rpm. The bacterial suspensions were thereafter adjusted 0.5 McFarland standards (equivalent to 1.5 x10^{8} CFU/ml). This was done by diluting the 18 h bacterial cultures 1:100 with respective sterile broths before growing them back in the shaking incubator for approximately 2-3 hours (mid-log phase) to obtain 0.08 to 0.10 OD625 corresponding to 1.5 x 10^{8} CFU/ml. The correct density of the turbidity standard was verified using a spectrophotometer with a 1-cm light path and matched cuvette (Star Labs, UK) to determine the absorbance at 625nm. Blank of MHB alone was used to calibrate the spectrophotometer before measuring the samples. To ensure conformity of the suspension's turbidity with McFarland standard, both the suspensions and the prepared McFarland standard were also compared visually. Furthermore, the inoculums suspension was used within 30 minutes of standardization, which is a very important factor to avoid any change of the size of inoculums or loss of their viability.

To investigate the antifungal activity of the African walnut oil and its extracts, the fungal suspensions used was prepared using three to four morphologically similar colonies of the fungal strains; *Candida albicans* and *Malassezia furfur* from a 48 h culture on Sabouraud dextrose agar. The turbidity of the fungal suspension was adjusted to 1.0 McFarland standard (equivalent to 1.5 × 10^{8} CFU/ml) with sterile normal saline (0.89% NaCl wt/vol). All experiments were performed in duplicate and repeated three times.

2.9 Determination of Antimicrobial Activity of African walnut oil and extracts

2.9.1 Agar well diffusion method

The antimicrobial activity of different concentrations of the oil was determined by modified agar well diffusion method described by Perez *et al*., (1990)

and Adeniyi *et al*., (1996). In this method, nutrient agar and Mueller Hinton agar (MHA) plates were seeded with 0.2 mL of 18 h broth cultures of each bacterial isolate. For the Fungal isolates Sabouraud's Dextrose Agar (SDA) was seeded with 0.2 mL of 24 h broth cultures of *Candida albicans, and Malassezia furfur*. The agar plates, nutrient agar, Mueller Hinton agar (MHA) and Sabouraud's Dextrose Agar (SDA) were separately seeded by spreading a small volume (0.2 mL) of the liquid inoculums (sub-cultured broth media of both bacteria and fungi isolate) by means of an L-shaped glass rod (or a "spreader") in such a way that the surface of the agar in the plates were covered with the microbes (test organisms). All test microorganisms were separately seeded into different plates. All the plates were left to dry for 1 hour. A sterile 6 mm cork-borer was used to cut two wells of equidistance in each of the plates and 0.2 mL (200 µl) of the African walnut oil was introduced into one of the two wells while the same amount of sterile oil was introduced into the second well as control (used as negative control) and all the plates were aerobically incubated at 37 ^{o}C for 24 hours for the bacteria and 48 hours for the fungi. The diameter of zones of inhibition was measured by means of linear instrument in millimeter (venier calliper) and recorded.

Antimicrobial activity of African walnut oil and solvents extracts such as petroleum ether, n-hexane and chloroform was also determined. This was determined by diluting the petroleum ether, n-hexane and chloroform extracts to get concentrations of (vol/vol) 80, 40, 20, 10 and 5 %. The different concentration of the African walnut solvent extracts were incorporated into wells on agar plates and agar plates were incubated as described above. After incubation the diameter of zones of inhibition of each concentration was measured.

2.10 Determination of minimum inhibitory concentration of African walnut oil and extracts

MIC was evaluated on African walnut oil and the extracts that showed antimicrobial activity in the agar well diffusion assay on tested isolates. Minimum Inhibitory Concentration (MIC) of walnut oil, n-hexane and petroleum ether extracts on the most inhibited bacterial strains of *S. aureus*, *E. coli* and *C. acnes* and fungal strains *M. furfur* and *Candida albicans* were determined. To effectively identify the concentration range for the experiments, initially the minimum inhibitory concentration (MIC) of walnut oil and extracts were determined by the micro broth dilution assay using 96 well microtiter plates described by Mahboubi *et al*., (2011) and Salleh *et al*., (2016). The inoculums of *S. aureus*, (2341), *E. coli (2333)* and *C. acnes (3143)* strains were prepared from 18 h overnight cultures and suspensions were standardized to 0.5 McFarland standard turbidity. Similarly, the inoculums of *M. furfur* (2556) and *Candida albicans* (1001) were prepared from 24 h overnight cultures. The standardized oils and extracts were prepared in a concentration range of 0.98 – 500 mg/ml. In setting the 96 well microtiter plate, wells A-F were reserved in each plate for negative control. Dilution of the nut oil and extracts were prepared separately in TSB and SDB for bacterial strains and fungal strains respectively. 100 µl of sterile TSB was added to the wells from rows B to H. After this, 100 µl of the stock solution of African walnut oil sample (from 1000 mg/ml) were added to the wells at rows A and B. Then both the mixture of sample and sterile broth 100 µl at row B was transferred to each well to obtain a two-fold serial dilution of the stock oil sample. Eight concentrations (500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9 mg/ml) of the oil and extracts were determined. After serially diluting the nut oil sample, 100 µl of each standardized inoculum (10^8 CFU/ml) was added to each well. The final volume in each well was 200 µl. Streptomycin sulphate for bacteria 20 µg/ml was used as positive control to monitor the results. The plates were then incubated at 37 ^{o}C for 24 hours. The minimum inhibitory concentration was the lowest concentration that prevented the growth of bacterial after 24 h incubation. This same procedure was used to determine the MICs of petroleum ether and n-hexane extracts on tested fungal strains. MICs of fungal strain were determined using SDB and plates were incubated for 48 hours.

3. Results

3.1 Physical and sensory attributes of African walnut oil and extracts

The expeller press (mechanical) method was used to extract oil from African walnut while the solvent (chemical) method was used to obtain African walnut extracts. The methods used for the extraction of oil and extracts from African walnut seem good. The recovery rate using the expeller method involving the use of mechanical extractor was however less effective compared to the use of solvent extraction. A large amount of nuts will be required for the expeller press designed for extraction of oil from African walnut. Use of expeller press to squeeze oil out of the nut is easy to use as no special skill is required, the recovery rate is slow (12.53 %). For solvent extraction, small amount of nuts are required to

obtain extracts (Table 2). Percentage yield of extracts of African walnut with solvents such as n-hexane, petroleum ether and chloroform were 18.06 %, 12.5 % and 11.97 %. The yields were obtained after three successive extractions. The results of average yield and percentage yield of oil and extract obtained from African walnut using expeller press method and solvents such as hexane, petroleum ether and chloroform is shown in table 2.

Table 2: Average yield and percentage yield of African walnut oil and extracts

Mechanical method/Solvent method	Fresh weight of seeds (g)	Solvent volume (ml)	Average yield/recovery of oil/extracts after evaporation, dry weight (grams)	% yield of extracts
African walnut oil (expeller pressed oil)	200	_	25.06	12.53
N-hexane	30	200	5.42	18.06
Petroleum ether	30	200	3.75	12.5
Chloroform	30	200	3.59	11.97

Source: *Laboratory analysis, (2019).*

The physical appearance and sensory analysis of the colour, texture, taste, sedimentation properties of African walnut oil indicated that the expeller pressed oil was pale yellow in colour, clear, light, tasteless without impurities. The n-hexane extract was golden brown in colour, clear, light, without impurities, petroleum ether extract was golden yellow in colour, clear, light, without impurities and chloroform extract was golden/dark brown in colour, clear, light without impurities (Figure 1 and Table 3).

Table 3: Physical and sensory attributes of African walnut oil and extracts

	Characteristics / Properties of oil and extracts			
Attributes	**Oil/Extracts**			
	African walnut oil	**n-hexane**	**Petroleum ether**	**Chloroform**
Colour	Pale yellow, clear and transparent	golden brown, clear and transparent	golden yellow, clear and transparent	golden/dark brown, clear and transparent
Texture	Light	Light	Light	Light
Taste	Neutral, free of bitter taste, free of after tastes.	_	_	_
Smell	Neutral, free of smell coming from the plant material.	Neutral, free of smell coming from the plant material.	Neutral, free of smell coming from the plant material.	Neutral, free of smell coming from the plant material.
Sediments	Free of sediments. No impurities	Free of sediments. No impurities	Free of sediments. No impurities	Free of sediments. No impurities
State at room temperature (25°C- 30 °C)	Liquid.	Liquid.	Liquid.	Liquid.
Boiling point	-	63°C	62 °C	60 °C

Source: *Laboratory analysis, (2019).*

3.2 Chemical composition of African walnut oil and extracts

The chemical properties of oil extracted from African walnut using the expeller press method are shown in Table 4. Results for the extracts are also shown. The data (Table 4) showed the saponification values, unsaponifiable matter content, acid value, peroxide values, free fatty acids (FFA), iodine, Refractive index (RI) and Specific gravity (SG) of the oil was 170.1 mg KOH g^{-1}, 4.1 g/kg^{-1}, 0.92 mg KOH g^{-1}, 1.02 meq O_2 kg^{-1}, 0.56 %, 91.3 Wij's, 1.445 and 0.94. The n-hexane extract had 174.1 mg KOH g^{-1}, 3.9 g/kg^{-1}, 0.72 mg KOH g^{-1}, 1.01 meq O_2 kg^{-1}, 0.46 %, 90.1 Wij's, 1.442 and 0.88. Petroleum ether extract had 165.1 mg KOH g^{-1}, 2.5 g/kg^{-1}, 0.82 mg KOH g^{-1}, 1.00 meq O_2 kg^{-1}, 0.65 %, 87.6 Wij's, 1.440 and 0.94. Chloroform extract had 160.1 mg KOH g^{-1}, 3.2 g/kg^{-1}, 0.62 mg KOH g^{-1}, 0.94 meq O_2 kg^{-1}, 0.82 %, 89.9 Wij's, 1.440 and 0.94.

Table 4: Chemical properties of African walnut oil and extracts

Properties	Oil and extracts values			
	African walnut oil	n-hexane	Petroleum ether	Chloroform
Specific gravity	0.94±0.01	0.88±0.01	0.94±0.01	0.94±0.01
Refractive index	1.445±0.002	1.442±0.002	1.445±0.002	1.440±0.002
Acid value (mg KOH/g)	0.92±0.12	0.72±0.12	0.82±0.12	0.62±0.12
Free fatty acid (%)	0.56±0.10	0.46±0.10	0.65±0.10	0.82±0.10
Peroxide value (meq O_2/ kg^{-1})	1.02±0.19	1.01±0.19	1.00±0.19	0.94±0.19
Saponification number (mg KOH/g)	170.1±1.12	174.1±1.12	165.1±2.12	160.1±2.12
Unsaponifiable fraction (g/kg^{-1})	4.1±0.65	3.9±0.65	2.5±0.65	3.2±0.65
Iodine (mg of $1g^{-1}$ of oil) (Wiji's)	91.3±1.09	90.1±1.09	87.6±1.09	89.9 ±1.09

Each data is mean of three replicate readings ± Standard Deviation (SD)

Source: *Laboratory analysis, (2019).*

3.3 Microbiological screening

In the present study, oil and extracts of African walnut was evaluated for exploration of their antimicrobial activity against certain Gram negative and Gram positive bacteria and fungus which was regarded as human pathogenic microorganism. Susceptibility of the oil and extract was tested by minimum inhibitory concentration and agar well diffusion method. In this study, the results of petroleum ether and n-hexane extracts are presented because they exhibited more activity than chloroform extract.

3.4 Antimicrobial activity of African walnut oil and extracts

The result of the antibacterial and antifungal activity of African walnut oil and extracts are presented in Table 5. The result of this preliminary investigation showed that African walnut oil was active against the clinically isolated human skin pathogens like *Staphylococcus aureus, Escherichia coli, Cutibacterium acnes, Malassezia furfur* and *Candida albicans.* Results obtained in this study showed that African walnut oil exhibited inhibitory effect on the growth of clinical isolates of *Staphylococcus aureus, Escherichia coli, Cutibacterium acnes, Malassezia furfur* and *Candida albicans* tested. Significant differences ($p<0.05$) were observed in the degree of inhibition of the clinical isolates, but non-significant variations were observed inhibition among strains of the same species. On the bacterial species, *S. aureus* was highly inhibited by African walnut oil as confirmed by the zones of inhibition measured. *C. acnes* was moderately inhibited, while the growth of *E. coli* was observed to mildly inhibited. With the fungal species, 100 % of African walnut oil proved really effective inhibiting the growth of *M. furfur* and *C. albicans.* However, growth inhibition by African walnut oil was not significant between the two strains.

The result obtained also revealed that the inhibitory activity of the nut oil extracts decreases with a decrease in concentration of oil extracts in solvent, resulting in marked variation in the inhibitory concentration. While 5 % of African walnut oil was still able to inhibit the growth of *S. aureus*, inhibition of *E. coli* was only achieved at a much higher concentration of 80 % oil extract in solvent (petroleum ether). *C. acnes* and *M. furfur* were inhibited at 20 % petroleum ether concentration and *C. albicans* was inhibited at 10 % concentration.

The type of solvent used for the extraction of African walnut oil did not seem to have any significant ($p<0.05$) effect on growth inhibition of all the bacterial isolates tested, however for the fungi species inhibitory was at a lower concentration with n-hexane extract. As observed, a concentration of 10 % for *M. furfur* and 5 % for *C albicans* with petroleum ether compared with 20 % and 10 % respectively for *M. furfur* and *C albicans* with n-hexane as the extract with more inhibitory activity.

Table 5: Inhibitory effect of 100 % African walnut oil, petroleum ether and n-hexane extracts on growth effect microganisms

			Diameter of zones of inhibition (in mm)											
			Petroleum ether extract						n-hexane extract					
			Concentration (v/v)						Concentration (v/v)					
Microorganisms	**Strain**	**100% African walnut oil**	**80**	**40**	**20**	**10**	**5**	**Control**	**80**	**40**	**20**	**10**	**5**	**Control**
Staphylococcus aureus	2341	14.02	12.09	10.09	6.03	4.19	2.66	0.00	10.28	8.74	4.77	4.35	3.05	0.00
	3411	13.21	11.07	10.05	6.91	4.07	2.08	0.00	10.76	9.56	6.78	4.89	2.17	0.00
	3621	12.35	12.12	11.06	8.56	5.33	3.76	0.00	10.14	9.21	5.67	4.32	3.56	0.00
	3001	13.32	12.45	11.04	8.17	4.87	3.32	0.00	10.99	9.35	5.99	4.44	3.33	0.00
	1990	13.65	11.22	11.43	6.17	4.34	3.87	0.00	10.94	8.89	6.34	4.07	3.05	0.00
Escherichia coli	2333	2.89	1.87	0.76	0.00	0.00	0.00	0.00	0.87	0.00	0.00	0.00	0.00	0.00
	1411	1.87	1.09	0.88	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00
	1093	1.56	1.08	0.53	0.00	0.00	0.00	0.00	0.49	0.00	0.00	0.00	0.00	0.00
	1114	1.48	1.43	0.65	0.00	0.00	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00
	1039	1.85	1.49	0.67	0.00	0.00	0.00	0.00	0.54	0.00	0.00	0.00	0.00	0.00
Citibacterium acnes	3143	5.54	4.88	4.09	2.23	0.00	0.00	0.00	3.09	2.33	1.98	0.00	0.00	0.00
	2030	4.87	4.88	3.56	2.45	0.00	0.00	0.00	1.98	1.09	0.56	0.00	0.00	0.00
	1190	3.76	3.76	3.23	1.06	0.00	0.00	0.00	1.45	0.56	0.46	0.00	0.00	0.00
	1390	5.78	4.56	3.12	1.04	0.00	0.00	0.00	1.06	0.75	0.58	0.00	0.00	0.00
	1102	4.21	3.34	3.25	1.09	0.00	0.00	0.00	1.09	0.67	0.55	0.00	0.00	0.00
Malassezia furfur	2556	9.65	6.78	3.62	1.99	1.08	0.00	0.00	7.06	4.32	2.33	0.00	0.00	0.00
	2035	9.43	6.43	4.23	1.45	1.21	0.00	0.00	7.25	4.44	2.14	0.00	0.00	0.00
	2441	8.77	6.88	5.55	1.22	1.06	0.00	0.00	7.21	3.11	1.89	0.00	0.00	0.00
	2099	8.43	5.39	4.56	1.33	1.11	0.00	0.00	6.52	3.08	1.22	0.00	0.00	0.00
	2016	7.88	5.09	3.21	1.01	1.23	0.00	0.00	6.23	2.13	1.48	0.00	0.00	0.00
Candida albicans	1001	9.98	8.04	8.01	6.21	4.09	1.02	0.00	8.21	6.32	5.09	4.44	1.26	0.00
	1425	9.36	8.21	8.34	5.34	3.23	1.04	0.00	8.31	6.23	5.21	4.25	1.35	0.00
	1346	9.41	8.33	7.34	5.25	3.06	0.00	0.00	7.09	6.09	5.01	4.31	1.37	0.00
	1922	9.22	7.45	6.9	4.33	3.01	0.00	0.00	6.03	7.33	5.11	4.22	1.05	0.00
	2140	8.01	7.22	6.45	4.21	3.21	0.00	0.00	6.02	7.21	5.21	4.09	1.11	0.00

3.5 Minimum inhibitory concentration of African walnut oil and extracts

The result showed concentrations of 500 and 250 mg/mL of African walnut oil and petroleum ether inhibited the growth the bacterial and fungal strains. The minimum inhibitory concentration (MIC) of African walnut oil, petroleum ether and n-hexane extracts against the tested pathogens extracts was determined using the micro dilution methods. The minimum inhibitory concentration of African walnut oil and the extracts revealed that 500 and 250 mg/mL were the least concentrations that prevented the growth of bacteria and fungi after 24 h and 48 h incubation. Result obtained confirms the inhibitory activities of the nut oil and extracts against *S. aureus*, *E. coli*, *M. furfur* and *C. albicans* (Table 6).

Table 6: Minimum inhibitory concentration of African walnut oil, petroleum ether and n-hexane against test microorganisms

		Minimum inhibitory concentration (mg/mL)							
Microorganisms	**Strains**	**500**	**250**	**125**	**62.5**	**31.3**	**15.63**	**7.8**	**3.9**
Bacteria									
Staphylococcus aureus	2341	–	–	–	+	+	+	+	+
		–	–	–	–	+	+	+	+
		–	–	+	+	+	+	+	+
Escherichia coli	2333	–	–	–	+	+	+	+	+
		–	–	+	+	+	+	+	+
		–	+	+	+	+	+	+	+
Citibacterium acnes	3143	–	–	–	–	–	+	+	+
		–	+	+	+	+	+	+	+
		–	+	+	+	+	+	+	+
Control (without isolate)		–	–	–	–	–	+	–	–
		–	–	–	–	–	+	–	–
		–	–	–	–	–	+	–	–
Fungi									
Malassezia furfur	2556	–	–	+	+	+	+	+	+
		–	–	+	+	+	+	+	+
		–	–	+	+	+	+	+	+
Candida albicans	1001	–	–	+	+	+	+	+	+
		–	–	–	+	+	+	+	+
		–	+	+	+	+	+	+	+
Control (without isolate)		–	–	–	–	–	+	–	–
		–	–	–	–	–	+	–	–
		–	–	–	–	–	+	–	–

Key:
+ = indicating growth showed by turbidity of the broth.
– = no growth.

4. Discussion

The oil and extracts were all liquid at room temperature 30 °C. The method of extraction of oil usually reflects on the oil's quality, colour and taste. This informed the need to pay some attention to the various possible methods of extracting oil and extracts from African walnut. Although no major difference was observed in the quality of the extracted oil and extracts. Extraction of extracts with solvents seems more efficient and economical. This is in conformity with the observations of Akpuaka and Nwankwor, (2000). Use of expeller press a mechanical methods could be suitable for domestic purposes; it may be adopted for use by farmers who are familiar with a similar process which is commonly used for palm-oil extraction.

A high saponification value of 170.1 mg KOH g^{-1} indicates that the oil has low molecular weight fatty acids. This attribute is of importance in soap making as well as in shampoo making (Ajiwe, 1994; Akanni *et al*., 2005).The value obtained is the range of values (174.84 - 192.45 mg KOH g^{-1}) obtained for rapeseed oil and soyabean oil that have gained much market priority as reported by Obasi *et al*., (2012). The saponification values of 174.1, 165.1 and 160.1 mg KOH g^{-1} obtained for n-hexane, petroleum ether and chloroform extracts respectively in this study are similar to those of extracts obtained from seeds such as castor, peanut, soybean, and sunflower whose average saponification values range from 176 to 250 (Roger *et al*., 2010; Adamu *et al*., 2013).Unsaponification value was found as 4.1 g/kg^{-1}, and similar to the value of 4.7 g/kg^{-1}reported for terebinth fruit and English walnut (*Juglan regia*) by Ozcan, (2004). Obviously, this attribute of the oil can be linked with the cholesterol content it contained.

The unsaponification values 3.9, 2.5 and 3.2 g/kg^{-1}, for hexane, petroleum ether and chloroform extracts are lower than those of the oil and within range. Acid value of African walnut oil was 0.92 mg KOH g^{-1}, lower than those of soya bean (4.30 mg KOH g^{-1}) and turkey (5.61 mg KOH g^{-1}) reported by Obasi *et al*., (2012). The extracts had 0.72, 0.82 and 0.62 mg KOH g^{-1} acid values which were still much lower than that of the oil. The free fatty acid value 0.56 %, 0.46 %, 0.65 % and 0.82 % for the oil, hexane, petroleum ether and chloroform extract are low and support the value of edible oil reported by Obasi *et al*., (2012). Iodine value for the oil was 91.3 Wij's, in the range of cotton seed oil and sun flower oil iodine values (90-119 Wij's) reported by Popoola and Yangomodou, (2006). The iodine values of African walnut extracts are lower than those of sunflower (110-143), soybeans (120-143) and rubber seed (134.51) (Abayeh *et al*.,1999), but similar to those of castor oil (83.75) and Coula edulis (90-95) Adamu *et al*., (2013). This value which is in the middle range shows that the oil is semidrying and unsaturated. This further shows that the oil could be used in liquid soap formulation, just as the low acid value recorded for the oil could be of significance in paint and varnish manufacturing (Popoola and Yangomodou, 2006). Also, refractive index and specific gravity values of 1.445 and 0.94 compares favorably well with that of *Juglans regia* (1.446 and 0.97) as reported by Ozcan, (2009). Refractive index and specific gravity values obtained for the extracts correlates with those of the oil. Although, n-hexane have lower specific gravity (0.88).The specific gravity of 0.94 at 25 °C of African walnut oil and the observed melting point of 17.8 °C, which is really lower than the average tropical room temperature 25 °C, explains why the oil is liquid at room temperature. The peroxide value in the range of 0.94 - 1.02 meq O_2 kg^{-1} obtained for the extracts and oil are lower than generally recommended value for commercial edible crude vegetable oil. This is an indication that African walnut oil can resist lipolytic hydrolysis and oxidative deterioration (WHO, 2002).

The antimicrobial activity of African walnut oil and extracts especially petroleum ether as observed in this study appeared to be a broad spectrum activity as both gram positive, gram negative as well as fungal species were sensitive to the nut oil. The test microorganisms chosen for this study are known primarily as commensals or normal flora on the human skin but they can be opportunistic as they have the ability to change from a commensal or normal flora to pathogenic strains when the environment, that is the human skin supports their growth and especially when the immune system of the host is lowered or compromised. The implication of the effect of nut oil on growth inhibition is that the nut oil can help keep the pathogenic activities of these microrganisms in check. *Staphylococcus aureus* which is commonly found associated with boil and other secondary skin infections in the area of study was most inhibited by the nut oil and petroleum ether extract. The inhibition of *E.coli* by the oil was relatively low, while that of *C.acnes* was mild compared to *S. aureus.* Previous research studies on the antimicrobial activities of plant oil component indicated that lemon, cineole, citral geraniol, linalool and menthol were active against several yeast –like and filamentous fungus (Pattnaik *et al*., 1997). Previous reports have also shown that essential oils of plant sources could be used as therapeutic agent for the remedy of fungal diseases of man (Tampieri *et*

al., 2005) and plant (Soylu *et al*., 2006). Results obtained in this preliminary study gives some support to the traditional use of the nut oil for the treament of skin infections.

5. Conclusions

African walnut oil demonstrated a broad spectrum activity on bacterial and fungal clinical isolates tested. The antimicrobial activity of the nut oil and extracts shows that they can be exploited for use in traditional medicine, pharmaceutical and cosmetic industries.

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