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ANTIBACTERIAL EFFECT OF AFRICAN WALNUT OIL ON PLANKTONIC AND BIOFILM CELLS OF *Staphylococcus aureus*

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Abstract
Screw press a mechanical method of extraction was used to extract oil from African walnut and fatty acid profile was carried out using gas-chromatography-mass spectrometry (GC-MS). Antibacterial activity was determined using minimum inhibitory concentration, minimum biofilm inhibitory concentration and minimum biofilm eradication concentration. Extraction's yield was 12.53 %. African walnut oil shows that linoleic acid (C18:2, 13.05%), linolenic acid (C18:3, 80.59%), stearic acid (C18, 4.41%) and palmitic acid (C16, 1.53%) were the dominant fatty acids present in it. Antibacterial activity was detected against planktonic and biofilm cells of reference bacteria and clinical isolate of Staphylococcus aureus. The results show that African walnut is a promising oil nut crop with high level of polyunsaturated fatty acids and antibacterial potential.

Key Words: *African walnut, Antibacterial activity, Biofilm, Fatty acid, Planktonic*

Introduction

Staphylococcus aureus is a gram positive bacterium that resides permanently on the surface of human skin and in the nose of healthy individuals across the globe causing a range of human infections ranging from mild to chronic infections. *S. aureus* is known to cause mild epidermal skin lesions, chronic infections including endocarditis, osteomyelitis, pneumonia, meningitis, septicemia, bacteremia and toxic shock syndrome (Qiu *et al*., 2011). In addition, *S. aureus* is known to have two growth phases: a unicellular growth phase, in which the cells are free floating in liquid (planktonic phase) and a multicellular growth phase in which the cells are sessile and live in a biofilm (Davey and O'Toole, 2000*).* In nature, *S. aureus* has been found to form biofilm on both biotic and surfaces, including indwelling hospital devices (for example bone and joint prostheses, heart valves, implanted catheters), human tissues, food and food processing facilities (Chamdit and Siripermpool, 2011). *S. aureus* biofilm are encased in an extracellular matrix known as EPS produced by them that acts as slime (Flemming and Wingender, 2010).

Biofilms formed by *S. aureus* are resistant to antibiotics thereby hindering the effectiveness of antimicrobial drugs, leading to both persistent and severe infections which are of global concerns (Atshan *et al*., 2013).

African walnut (*Tetracapidium conophorum*) is a genus in the family *Euphorbiaceae*. *T. conophorum* is cultivated in Cameroon and Nigeria (Ayoola *et al*., 2011). It is commonly called African walnut. African walnut oil is widely used in medicine for the treatment of fungal infections, skin infections, respiratory diseases and ulcers. African walnut oil has been reported to possess antimicrobial, antioxidant, antimicrobial, antiviral and immunosuppressive activities. Most research studies are based on the antimicrobial activities of walnut extracts obtained from leaves, roots, and shoots of African walnut and as such only very little information is available on the nut oil and its effect on planktonic and biofilm cells of *S. aureus*. In view of the above, the main aim of the study was to determine if African walnut oil could be a good antimicrobial agent.

Materials and Methods

Collection, Identification and Processing of African Walnut

Fresh African 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 identified and authenticated by a plant scientist in the Department of Biological Sciences, Olabisi Onabanjo University, Ogun State, Nigeria. After identification, African walnuts were sorted, washed in cold water to remove dirt adhering to the surface and manually separated to remove the black husk. The nuts were then sundried in shade for 14 days and milled using Marlex Excella grinding machine (Amazon, UK). Milled nut samples were packed in air tight containers and stored for about 3-5 days at room temperature before use.

Extraction of African walnut oil and Oil Yield Determination (%)

The step wise method described by Jokic *et al*. (2014) for screw press was used. The extraction of oil from walnut was performed using a screw expeller (Model SPU 20, Senta, Serbia). African walnut oil was obtained by pressing 200g of the nuts. The minimum and maximum nozzle size used was 7 and 12mm, 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 pressing, the screw pressed oil was centrifuged and measured using an analytical balance. The average and percentage yield of the oil was calculated. In addition, the colour and other related physical characteristics of the extracted oil were assessed by visual observation. The extracted oil was then stored in white bottles and kept in the refrigerator at 4°C until analyzed. Extraction was done repeatedly for about three times.

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

Identification of Active Component of African Walnut Oil

The active components of African walnut oil were identified using gas chromatography-mass spectrometry (GC-MS) analysis. African walnut oil was first trans-esterified into fatty acid methyl esters (FAME) by Boron trifluoride

methanolysis-BF3-MeOH following the guidelines of International Olive Oil Council (IOOC, 2006). After trans-esterification, African walnut methyl ester sample (the upper hexane layer) was pre-analysed by injecting 1µL and profiling of fatty acids were done using an Agilent 6890N Gas Chromatography device (Agilent Technologies, Wokingham, United States). Profiling and quantification of fatty acids were achieved using Restek-5MS (30m x 30mm x 0.25µm) column. Column temperature was programmed at 80°C for 3 min, and then raised to 220°C at 4°C min^{-1} and then held for 3 min. The carrier gas was helium with a column flow rate of 1.12 cm^3 and the Mass detector transfer line temp: 280 °C.

Biofilm formation assays

(i) ***Phenotypic detection of slime production by congo red agar (CRA) method***

CRA plates were prepared using 3 g of TSA adding 0.1g Congo red supplemented with 1g (w/v) glucose in 100 mL of distilled water. The strains were inoculated in streaks and incubated at 37°C for 24 h. The method described by Darwish and Asfour, (2013) was used.

(ii) ***Quantification of biofilm by microtiter plate (MTP) method***

Microtiter plate test was performed according to Asthan *et al*. (2013). Biofilms of *S. aureus* ATCC 6538, ATCC 12228 and MRSA 252 were pre-formed by adding 1 mL aliquots of prepared standardized suspension into nine wells of the 24-well tissue culture plates (Corning Costa, UK) and incubated at 37°C for 12 h, 24 h and 48 h respectively. Thereafter, the free floating suspensions were removed and each well was rinsed three times with 1 mL of deionized water (27 mL in total). The plates were dried in inverted position for 20 min and the attached bacteria were fixed for 15 min at room temperature by adding 1mL of methanol per well for 20 min before they were emptied and left to dry overnight. After drying, each well was stained with 1mL of 0.1% safranin for 15 min and washed thrice with distilled water. The plates were left to dry overnight and the next day plates were visually inspected for biofilm formation. Ethanol, 95% (200mL) was added to each well to dissolve the bound cells and optical density was measured at 595nm (OD 595). The experiment was repeated three times.

Screening of African walnut oil for antimicrobial activity against MRSA 252 and ATCC 6538 planktonic and biofilm cells

Microorganisms used for the study

The microorganism strains employed in the biological assays are ATCC 6538 and ATCC 12228 which are American Type Culture Collection (ATCC) reference bacteria as well as a clinical isolate MRSA 252.

Preparation of overnight culture and standardization of inoculums

Overnight cultures were prepared using the method described by Vitko and Richardson, (2013). Single colonies of ATCC 6538, ATCC 12228 and MRSA from the streaked plates were transferred with sterile loops into 10 mL TSB broth and 10 mL MHB and the bacterial cultures were then grown overnight for 16 – 18 h at 37°C with shaking (200 rpm) in N-BIOTEK shaking incubator. After incubation, bacterial growth was checked and the optical density was determined using a spectrophotometer. The bacterial suspensions were thereafter adjusted to 0.5 McFarland standards (equivalent to

1.5 x10^8 CFU/mL) by diluting the 18 h bacterial cultures (OD600 = > 2.5) 1:100 with respective broths and growing them back in the shaking incubator for 2-3 hours to obtain 0.08 to 0.10 OD625 corresponding to 1.5 x 10^8 CFU/mL.

Antimicrobial susceptibility testing of African walnut oil

Minimum Inhibitory Concentration (MIC)

MIC of African walnut oil was determined by the micro broth dilution assay using 96 well microtiter plates described by Mahboubi *et al*. (2017) with minor modifications. Wells A-F of each plate was reserved for negative control. 100µL of sterile TSB was added to the wells from rows B to H. After this, 100µL of the stock solution of African walnut oil sample (from 1000mg/mL) were added to the wells at rows A and B. Then both the mixture of samples and sterile broth 100µL 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 were determined. After this, 100µL of each standardized inoculum (10^8 CFU/mL) was added to each well. The final volume in each well was 200µL. Streptomycin sulphate for bacteria 20µg/mL was used as positive control to monitor the results. The plates were incubated at 37ºC for 24 h. All tests were performed separately in triplicate.

Minimum Biofilm Inhibitory Concentration (MBIC)

MBIC of African walnut oil was determined by the broth micro-dilution method described by Mahboubi *et al*., (2017). MRSA 252 and ATCC 6538 biofilms were first pre-formed by placing 100µL of the standardized inoculums 10^8 CFU/mL into rows B-H of the 96-well polystyrene tissue culture plates covered with lid and incubated for 24 h at 37ºC. The bottom of each well of the microtiter plate was visually inspected for biofilm formation (adherent cells) and free-floating cells were carefully removed by rinsing each plate with 200µL of 1X sterile PBS. Then 100µL of the freshly diluted TSB containing two–fold serial dilutions of African walnut oil sample prepared to achieve concentration ranging from 0.98-500mg/mL was placed into each well and incubated for 24 h at 37°C. After incubation, supernatant from all wells was discarded and 200µL of 0.1mol/L of HCl was added to each well containing the adherent cells and incubated again for 90 min at room temperature. After this, HCl was replaced by 0.1% safranin and the plates incubated for 35 min at room temperature. Non-bounded safranin was removed by rinsing the wells three times (3X) with distilled water, and thereafter each well of the plates were incubated with 125µL of 0.2 mol NaOH at 37°C for 1 h. After incubation, 100uL from the stained dissolved biofilm in each well was pipetted to a sterile new flat-bottom 96-well microtiter polystyrene plate and its intensity was measured at a wavelength of 600 nm using a plate reader. All tests were performed separately in triplicate.

Minimum Biofilm Eradication Concentrations (MBECs) Evaluation

The MBECs of African walnut oil against *S. aureus* strains; ATCC 6538 and MRSA 252 were determined by MBEC assay using a MBEC™ biofilm Inoculator with a 96-well plate with peg lids according to the process described by Harrison *et al*. (2005). Standardized inoculum (10^8 CFU/mL) of each *S. aureus* strains was added to all tested wells of a 96-well flat bottomed microtiter plate

(Innovotech, USA). A MBEC™ Biofilm Inoculator with 96-pegs (Innovotech, USA) was inserted into the wells and incubated together for 48 h at 37°C without shaking. After 48 h, a biofilm growth check test was performed to determine the number of colonies on each peg. After checking, the inoculator lids were transferred to an aseptically prepared challenge plate containing the serial 2-fold dilutions of the African walnut oil (0.98-500mg/mL) in MHB. The peg lids were thereafter removed from the challenge plates and submerged separately in sterile distilled water for 1 min and submerged into 150µL/well of recovery medium in a new plate. Both the plates and peg lids were sonicated for 5 mins and incubated for 6, 12, 24 and 48 h respectively at 37°C. After each growth time point, the MBEC values, were determined by visually checking for turbidity in the wells of the recovery plate and plating out 100µL of suspension per well on TSA. The OD595 nm of the wells in the recovery plates were measured and adjusted for the OD value in the negative growth control well. All tests were performed separately in triplicate.

Data Analysis

MIC and MBIC data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni post-tests. Biofilm growth quantification and MBEC data were analyzed by two-way ANOVA using Bonferroni post-tests.

Results

Physical Evaluation and Yield of African Walnut Oil

The average yield and percentage yield of the screw pressed oil were 25.06g and 12.53%. The physical appearance of the oil showed it was pale yellow in colour, transparently clear, light, without impurities and liquid at room temperature (25 °C- 30°C).

Active Components Identified in African Walnut Oil

Five (5) main fatty acids methyl esters were identified in African walnut oil with retention times ranging from 18.68 to 32.39 min (Table 1).

Table 1: Fatty acid methyl esters identified in 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

Phenotypic Characteristics of S. aureus Strains

Phenotypic result shows that MRSA 252 and ATCC 6538 are positive biofilm formers producing black colonies on Congo red agar while ATCC 12228 was considered a negative biofilm producer (Fig. 1).

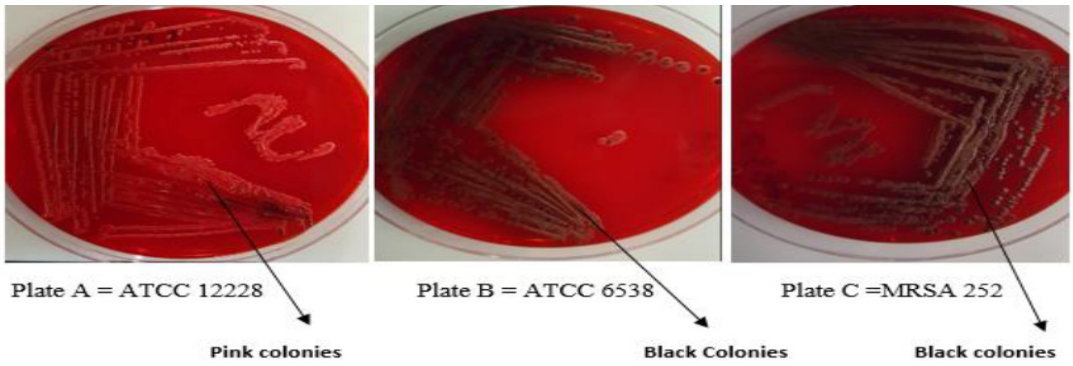

Fig. 1: Biofilm formations by ATCC 12228, ATCC 6538 and MRSA 252 on CRA plates
Plate A. Red colonies, non-biofilm former (negative biofilm producer); Plate B. black colonies of ATCC 6538, positive biofilm former; Plate C. black colonies of MRSA 252 positive biofilm former.

Quantification of biofilms formed by S. aureus strains

After 12 h, 24 h and 48 hours at OD 595 nm, biofilm formation of the strains progressively increased with incubation time. At 12 hours, optical densities of MRSA 252 and ATCC 6538 were low as 1.96 and 1.8. At 24 hours, OD of both MRSA 252 and ATCC 6538 were 3.91 and 2.71. Surprisingly at 48 hours an increased OD of 5.5 and 3.87 was measured (Fig. 2).

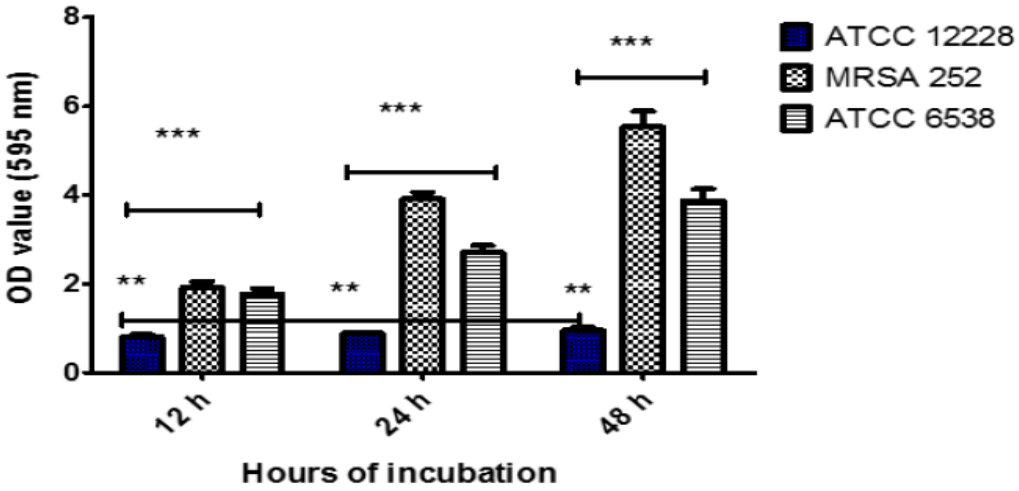

Fig. 2: Quantification of ATCC 12228, ATCC 6538 and MRSA 252 biofilms formed on polystyrene microtiter plate after 12 h, 24 h and 48 hours at OD 595 nm. Bar graphs represent the mean of biofilm formations ± SD on 24-well tissue culture plates. Significant interactions ***p<0.001; **p<0.01) were observed.

MIC and MBIC of African Walnut Oil

MRSA 252 and *S. aureus* ATCC 6538 were both susceptible to walnut oil at different concentrations tested. MIC of African walnut oil against planktonic cells of MRSA 252 was 15.6mg/mL and ATCC

6538 was 7.8mg/mL (Fig. 3a and c). MBIC values of MRSA 252 and ATCC 6538 biofilm cells were 31.25mg/mL and 15.6mg/mL. MBIC result showed that walnut oil exhibited high antibacterial activity against MRSA 252 and *S. aureus* ATCC 6538 biofilms (Fig. 3b and d). MBIC values are higher than the MIC values.

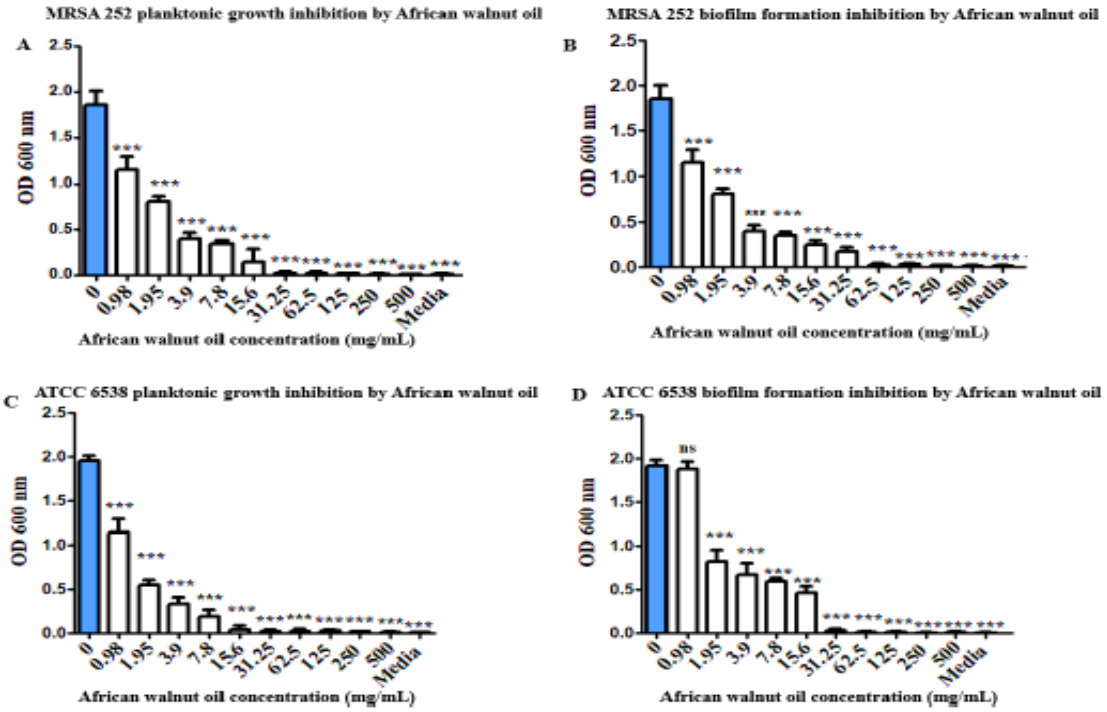

Fig. 3: Minimum inhibitory concentration and minimum biofilm inhibitory concentration of African walnut oil tested against MRSA 252 and *S. aureus* ATCC 6538 planktonic cells and preformed biofilms after 24 hours of incubation at 37°C in 96-well tissue culture polystyrene plate. Data on the graphs represent the mean OD values (Mean ± SD) of three independent experiments (n=3) and the error bars indicate standard deviations of the means. Asterisks (***) above the columns indicates significant difference of each mean with walnut oil with the corresponding growth control group ($p < 0.0001$).

MBEC of African Walnut Oil

The MBEC result of MRSA 252 and ATCC 6538 shows that the challenge of MRSA 252 and *S. aureus* ATCC 6538 with African walnut oil resulted in significant decrease of viable biofilm cells in recovery medium at 6 h, 12 h, 24 h and 48 h (Table 2).

Table 2: MRSA 252 and *S. aureus* ATCC 6538 counts in growth recovery media after exposure to different concentrations of African walnut oil

African walnut oil conc.	**Viable cell count reduction in 6 h, 12 h, 24 h, and 48 h biofilms of MRSA 252 and ATCC 6538 after exposure to African walnut oil. Results expressed as log10 cfu/mL**							
	MRSA 252				***S. aureus* ATCC 6538**			
	6 h	**12 h**	**24 h**	**48 h**	**6 h**	**12 h**	**24 h**	**48 h**
Growth control	8.37 ± 0.49	8.44± 0.42	8.60 ± 0.53	8.64 ± 0.60	8.33 ± 0.30	8.43 ± 0.64	8.46 ± 0.59	8.53 ± 0.48
0.98	8.29 ± 0.56	6.59 ± 0.71	5.34 ± 0.41	3.34 ± 0.41	8.43 ± 0.43	8.16 ± 0.54	5.34 ± 0.41	3.78 ± 0.86
1.95	7.60 ± 0.78	5.99 ± 1.09	4.66 ± 1.16	2.99 ± 0.58	7.88 ± 0.70	7.82 ± 0.78	4.64 ± 1.29	3.33 ± 0.88
3.9	7.21± 0.81	6.02 ± 1.44	4.02 ± 0.72	1.85 ± 0.52	7.28 ± 0.96	6.68 ± 0.58	3.85 ± 0.51	2.52 ± 0.51
7.8	6.95 ± 0.33	5.43 ± 1.21	3.43 ± 1.21	1.69 ± 0.56	7.72 ± 0.80	5.94 ± 0.65	4.36 ± 0.59	2.03 ± 0.97
15.6	4.88 ± 1.00	3.63 ± 0.59	3.13 ± 0.71	1.54 ± 0.14	4.88 ± 0.94	4.35 ± 0.87	3.21 ± 0.66	1.52 ± 0.94
31.5	3.66 ± 1.52	3.16 ± 0.92	2.25 ± 0.16	0.27 ± 0.08	4.32 ± 0.57	2.83 ± 0.60	1.37± 0.89	1.01 ± 0.58
62.5	3.21± 0.97	2.21 ± 0.58	1.89 ± 0.42	0.29 ± 0.13	3.21 ± 0.59	2.21 ± 0.58	No counts	No counts
125	2.48 ± 0.95	1.77 ± 0.89	No counts	No counts	2.55 ± 0.49	1.76 ± 0.56	No counts	No counts
250	2.07 ± 0.48	1.41± 0.48	No counts	No counts	1.51 ± 0.58	1.41 ± 0.55	No counts	No counts
500	1.70 ± 0.43	1.24 ± 0.51	No counts	No counts	1.50 ± 0.46	1.24 ± 0.65	No counts	No counts
20mg/mL	0.91± 0.16	0.91 ± 0.16	0.91 ± 0.16	0.91 ± 0.16	0.91 ± 0.16	0.91 ± 0.16	0.91 ± 0.16	0.91 ± 0.16

Bacterial counts in both MRSA 252 and ATCC 6538 biofilms started to decrease drastically in viable count after exposure to 15.6mg/mL of African walnut oil for 6 h. However, despite the decrease at this concentration, at 24 and 48 h a better reduction in viable count was observed at 62.5mg/mL and 31.25mg/mL concentration of MRSA 252 and ATCC 6538 biofilm. MBEC was considered the lowest oil concentration able to prevent a recovery of microbial growth.

Discussion

African walnut oil was pale yellow in colour, transparently clear, light, without impurities and liquid at room temperature (25°C - 30°C). This shows that the screw pressed method of extraction used to extract oil from African walnut produced good quality oil used for this study. This is in conformity with the findings of Popoola and Yangomodou, (2006) who reported that the method of extraction of oil usually reflects on the oils quality, colour and texture.

Based on the phenotypic expression results, *S. aureus* 6538 and MRSA 252 was considered biofilm producers by Congo red agar method, in which MRSA 252 produced very black colonies compared to ATCC 6538 that produced black colonies. Similar findings have been reported in previous studies. A study on biofilm formation conducted by Atshan *et al*. (2012) and Yousefi *et al*. (2016) reported varying degrees of black colonies ranging from very black to black colonies in MSSA strains and MRSA clinical isolates. Growing *S. aureus* strains on Congo red agar plates were also in agreement with Hammadi *et al*. (2014) and findings of Arslan and Ozkardes, (2007).

Safranin-staining is predominantly used to detect the presence of extracellular substances and is used to measure and quantify the amount of biofilm formed by

S. aureus (Atshan *et al*., 2012). Visual observation of biofilm formation showed that MRSA 252 and ATCCC 6538 were biofilm formers. Both strains form fairly strong biofilms at 12 h while much stronger and highly adherent biofilms were formed at 24 and 48 h. For the safranin technique the optical density was measured and was observed that OD readings of MRSA 252 and *S. aureus* ATCC 6538 were low at 12 h (OD 595 = 1.96 and 1.8), at 24 h (OD 595 = 3.91 and 2.71) and surprisingly at 48 h (OD 595 = 5.5 and 3.87) an increased biofilm formation was observed. These values were far higher than the cut-off value of OD 595<0.5 set for positive biofilm formers indicating that MRSA 252 was a strong and highly adherent biofilm former while ATCC 6538 was a moderately strong adherent biofilm former. According to Yousefi *et al*. (2016) the ability of biofilm formation can be classified into 4 main categories: non-adherent, weakly, moderately and strongly adherent based on the OD 595.

To date, this is the first study to give detailed fatty acid composition of African walnut screw press oil from Nigeria. In this present study, polyunsaturated fatty acids were observed as major components with strong indication of linolenic acids (C18:3) as the major fatty acid component of African walnut oil. In a previous study on the fatty acids of African walnut oil, it was established that palmitic (C16:0), α-linolenic (C18:3), linoleic (C18:3) were the main components of the oil (Nkwonta, 2015). Tchiegang *et al*. (2001) also reported same types of saturated and polyunsaturated fatty acids in walnut cultivars grown in Cameroon. Result correlates with those of Nkwonta, 2015 and Tchiegang *et al*. (2001).

Plants edible oils have already been known to exhibit strong antimicrobial properties (Tabassum and Vidyasagar, 2014). In this study, results obtained have demonstrated that African walnut oil has an excellent antibacterial potential against MRSA 252 and ATCC 6538 planktonic and biofilm cells. At higher concentration, African walnut oil exerted an antibacterial activity.

Treatment with antibacterial drugs may kill free-floating cells but fail to eradicate bacteria embedded within biofilm, which can then act as a source responsible for human recurrent infection (Tabassum and Vidyasagar, 2014). African walnut oil was significantly efficient against *S. aureus* MRSA 252 and ATCC 6538 embedded in biofilms as it was observed to eradicate MRSA 252 and ATCC 6538 biofilms at low concentrations of 62.5 mg/mL and 31.25 after 12 h but with better eradication after 24 and 48 h giving low or no counts of viable cells in recovery media.

Conclusion

This report revealed that African walnut oil is rich in polyunsaturated fatty acid and it possesses an important antibacterial activity against ATCC strains and clinical isolate of *S. aureus*.

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