

Antioxidant, Proteinase Inhibitory and Membrane Stabilization Potentials of *Moringa oleifera* Seed Oil

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SUMMARY

The chemical composition, antioxidant and anti-inflammatory activity of the widely consumed *Moringa oleifera* seed used for management of stress and a wide variety of diseases in Northern Nigeria is here investigated. The seed oil, dichloromethane and ethanol extracts were obtained by solvent extraction. Fatty acid profile was validated by producing the methyl esters from the *Moringa oleifera* seed using conventional and direct extraction methods. For the direct method, the reaction was performed in a one necked glass reactor equipped with a reflux condenser. The esterification reaction was confirmed using fourier transform infrared (FT-IR) spectroscopy and the chemical composition of the fatty acid methyl esters (FAMES) were determined using gas chromatography mass spectrometry GC-FID/GC-MS. The *in vitro* free radical scavenging potential was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) and hydrogen peroxide assays while the anti-inflammatory activity was determined using the *in vitro* proteinase inhibitory and membrane-stabilization assays. While the GC-MS results of the two extraction methods were similar and comparable, the direct extraction method of the FAMES saves time, minimizes use of solvents and affords better gas chromatography elution of fatty acids than the conventional multistage method. The oil obtained was found to contain high levels of oleic acid (up to 72.08 %) followed by palmitic, stearic, docosanoic, anachidic and lignoceric acid up to levels of 12.94, 9.64, 3.15, 1.60, and 0.56 % respectively. The seed oil and extracts showed higher DPPH scavenging activity (74.98 %), high ABTS scavenging activity (83.44 %) and moderate hydrogen peroxide scavenging activity (59.58 %) compared to the standard, ascorbic acid. No positive proteinase inhibitory activity was observed for the oil and extracts which showed low membrane stabilization responses at the tested concentrations (5 – 250 µg/mL). The positive health impact derived from the seed may be due to its antioxidant effect while the anti-inflammatory activity of the seed may not be through proteinase inhibition mechanism. It is suggested that the consumption of *M. oleifera* seed, seed oil and its allied products due regulated due to its low proteinase inhibitory and membrane stabilization activities.

Key Words: *Moringa oleifera*, fatty acid, antioxidants, anti-inflammatory, membrane stabilization.

***Moringa oleifera* Tohum Yağının Antioksidan, Proteinaz İnhibitörü ve Membran Stabilizasyon Potansiyelleri**

ÖZET

Kuzey Nijerya'da stres yönetimi ve çok çeşitli hastalıkların tedavisinde kullanılan *Moringa oleifera* tohumunun; kimyasal bileşimi, antioksidan ve anti-inflamatuar aktivitesi araştırılmıştır. Tohumun yağı, diklorometan ve etanol ekstraktları, solvan ekstraksiyonu ile elde edilmiştir. *Moringa oleifera* tohumundan klasik ve doğrudan ekstraksiyon yöntemleri kullanılarak metil esterlerin üretilmesiyle yağ asidi profili valide edilmiştir. Reaksiyon; doğrudan yöntem için, bir geri akış kondansatörü ile donatılmış bir boyunlu cam reaktörde gerçekleştirilmiştir. Esterleştirme reaksiyonu, fourier transform kızılötesi (FT-IR) spektroskopisi kullanılarak doğrulanmış olup yağ asidi metil esterlerinin (FAME) kimyasal bileşimi, gaz kromatografisi kütle spektrometresi GC-FID / GC-MS kullanılarak belirlenmiştir. *In vitro* serbest radikal süpürücü potansiyeli 1,1-difenil-2-pikrilhidrazil (DPPH), 2,2'-azinobis-3-etilbenzotiazolin-6-sülfonat (ABTS) ve hidrojen peroksit analizleri kullanılarak değerlendirilken anti-inflamatuar aktivite *in vitro* proteinaz inhibitörü ve membran stabilizasyon analizleri kullanılarak belirlenmiştir. İki ekstraksiyon yönteminin GC-MS sonuçları benzer ve karşılaştırılabilir olsa da, FAMES'in doğrudan ekstraksiyon yöntemi zaman tasarrufu sağlamış, solventlerin kullanımını en aza indirmiş ve klasik çok aşamalı yöntemle göre gaz kromatografisi ile yağ asitlerinin daha iyi elüsyonunu sağlamıştır. Elde edilen yağın, yüksek seviyelerde oleik asit (% 72.08'e kadar), ardından sırasıyla 12.94, 9.64, 3.15, 1.60 ve % 0.56 seviyelerinde palmitik, stearik, dokosanoik, araşidik ve lignoserik asit içerdiği bulunmuştur. Tohum yağı ve ekstraktları, daha yüksek DPPH süpürücü aktivitesi (% 74.98), yüksek ABTS süpürücü aktivitesi (% 83.44) ve orta düzeyde hidrojen peroksit süpürücü aktivitesi (% 59.58); standart askorbik asitle karşılaştırıldığında daha yüksek olmuştur. Test edilen konsantrasyonlarda (5 - 250 µg / mL) düşük membran stabilizasyon cevapları gösteren yağ ve ekstraktlar için herhangi bir pozitif proteinaz inhibitör aktivitesi gözlenmemiştir. Tohumdan elde edilen sağlık üzerine pozitif etkisi, antioksidan etkisine bağlı olabilirken, tohumun anti-inflamatuar aktivitesi proteinaz inhibisyon mekanizması yoluyla olmayabilir. *M. oleifera* tohumu, tohum yağı ve benzer ürünlerinin tüketiminin düzenlenmesi, düşük proteinaz inhibitörü ve membran stabilizasyon aktiviteleri nedeniyle önerilmektedir.

Anahtar Kelimeler: *Moringa oleifera*, yağ asidi, antioksidanlar, antiinflamatuar, membran stabilizasyonu.

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INTRODUCTION

Moringa oleifera Lam., the most widely cultivated of the family Moringaceae, is a multipurpose medicinal plant well known for its numerous pharmacological activities in Africa and Asia folkloric medicine (Leone et al., 2015). It is widely grown in tropical and subtropical countries for food, medicine and economic purpose (Stohs and Hartman, 2015). Almost all parts of the plant are reported to be of significant medicinal applications in the tropical and subtropical countries where they are indigenous (Oliveira et al., 1999). The leaves of *Moringa oleifera* are eaten in both raw and cooked form in African countries, such as Ghana, Ethiopia, Nigeria and Malawi (Oluduro, 2012; Olson, 2002). The seed or extracted seed oil is consumed raw in Nigeria where it is traditionally believed to relieve stress and associated health conditions such as fatigue. These effects may be due to the quality and composition of the fatty acids in the seed. Fatty acids are known to be essential components of the human diet and it is one of the most effective sources of storage energy. It functions as insulators of delicate internal organs and plays important role in cellular activities within the membranes (Singh et al., 2002).

Various parts of the plant contain essential nutrients, minerals, proteins, vitamins, β - carotene, amino acids and phenolic acids (Nambiar and Seshadri, 2001; Ross, 1999; Farooq et al, 2007; Basuny and Al-Marzouq, 2016). The leaf is found to have hypocholesterolaemic activity (Ghasi et al., 2000), *in vivo* antihyperglycemic activity (Jaiswal et al., 2009), antihepatocarcinomic activity (Khalafalla et al., 2010), wound healing potential (Rathi et al., 2006), antioxidant (Bajpai et al., 2005; Sreelatha and Padma, 2009), *in vivo* anti-diabetic (Oriabi, 2016) and antimicrobial activities (Cáceres et al., 1991, Manikandan et al., 2016). The fruit is reported for hypocholesterolaemic activity (Mehta et al., 2003) while the root extract possesses antiinflammatory activity (Ezeamuzle et al., 1996) and antifertility activity (Shukla et al., 1988). The leaves, fruit, flowers, roots, seed, bark and pods have been reported to possess analgesic, antitumour, cardiac and circulatory stimulatory activities (Makonnen et al, 1997; Sutar et al., 2008), antipyretic, antiepileptic, anti-inflammatory and antiulcer (Pal et al, 1995) activities. Phytochemicals identified in the plant includes nitrile and saponin from the pod (Faizi et al., 1994; Sharma and Paliwal, 2013), flavonoid pigments such as kaempferol, rhamnetin, isoquercitrin and kaempferitrin from the leaves (Nair et al., 1962), moringyne and amino acids from the seed (Ram et

al., 2004), vanillin, 4-hydroxy mellein, β -sitosterol and β -sitosterone from the stem bark (Ram et al., 2006) and kaempferol-3-rutinoside from the flowers (Ram et al., 2004). The toxicological effect of the seed has been evaluated in wistar rat. It was suggested that the seed and leave extracts have hepato-protective effect (Olayemi et al., 2016).

Many concepts and traditional believes exist on the potencies of the plant to treat many illnesses. However, one of the most speculative concept about the plant is that it is a potent medicinal plants which is nontoxic and without risks to human health. The extent of the toxicity, safety, antioxidant and anti-inflammatory potentials, especially the membrane stabilization activity of the seed has not been fully explored. Also, there have been some differences about the reported chemical composition and bioactivity of the seed of *Moringa oleifera* which may be apparently due to the geographical origin of the plant. Hence, this study was set to investigate the fatty acid profile, antioxidant, anti-inflammatory and membrane stabilization potentials of the seed of *M. oleifera* of Nigerian origin.

MATERIALS AND METHODS

Plant Material

Moringa oleifera seeds were obtained during winter from the northern part of Nigeria (Kaduna) and authenticated by a professional Taxonomist at the herbarium of the department of Plant Biology of the University of Ilorin, Ilorin, Nigeria. A voucher specimen number UILH/002/1008 was obtained. The seed were dried, de-shielded, pulverized and stored in a cool place for further work.

Solvents and Reagents

Solvents and chemicals used which includes hexane, benzene, methanol, aluminium chloride, sulphuric acid, sodium sulphate, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), and ascorbic acid were of analytical grade, and where necessary, solvents were redistilled before use. Other antioxidant and anti-inflammatory assay reagents were obtained from Santa Cruz Biotechnology, US.

Extraction of Pulverized Seed Material

The extraction from the pulverized seed material was obtained using different extraction techniques in order to determine the chemical composition of the seed.

Soxhlet Extraction

The pulverized seed sample weighing 100 g was extracted with hexane using the soxhlet extractor at 65°C for approximately 7 hours. The extract which was

filtered and concentrated using a rotary evaporator yielded yellow oil (Atolani et al., 2012).

Cold Extraction

2 kg of the pulverized seed material was extracted with ethanol for 9 days with constant changing of the solvent at regular interval. The extracts obtained from each batch were merged, filtered and concentrated at reduced temperature using the rotary evaporator to afford (37.11 g) ethanol extract. Following the nine days of extraction with ethanol, the sample was further extracted with water for five days. Thereafter, the aqueous extract was filtered and partially concentrated using an open water-bath to yield aqueous extract which was partitioned with dichloromethane (DCM) in a separating funnel. The DCM fraction was concentrated using the rotary evaporator to yield 46.31 g DCM extract (Atolani et al., 2013).

Acid Hydrolysis/Trans-esterification

1 g of the oil obtained via soxhlet extraction was weighed into a round bottom flask and 5mL of 2M HCl in methanol was added to it. The mixture was refluxed for approximately 1 hour and the mixture allowed to cool. The mixture was then extracted with n-hexane in a separating funnels and the organic phase was washed repeatedly with hexane and concentrated. The derived oil, fatty acid methyl esters (FAMES) was put in a vial and kept in a refrigerator at 4°C until further analysis (Atolani et al., 2012).

Direct Preparation of FAMES from *M. oleifera* seed

The fatty acid methyl esters (FAMES) were also obtained from the seed of *Moringa oleifera* in single step without first extracting the oil using reported protocol with slight modification (Atolani et al., 2015). This was carried out by refluxing 20g of the seed materials with a mixture comprising of methanol: hexane: benzene: H_2SO_4 , in the ratio 41: 36: 20: 2 v/v and 1g of $AlCl_3$. The solution was refluxed for 1 hour at 80°C in a round bottom flask and then allowed to cool to room temperature. After cooling, the mixture was decanted, filtered and then extracted with more volumes of hexane in a separating funnel. After allowing the mixture to settle down, two phases were formed; the upper phase containing the FAMES and the lower aqueous layer contains majorly the reagent mixtures. The aqueous layer was collected and the organic layer was further washed severally with water to neutrality and the concentrated via distillation. Anhydrous sodium carbonate was added to remove traces of water molecules in the prepared FAMES. The success of the trans-esterification was confirmed using the Fourier Transform-Infrared Spectroscopy which

showed disappearance of the hydroxyl functional group of the fatty acids.

Infrared Spectrometer and Absorbance Measurements

The infrared spectrum was recorded on a Shimadzu (8400s) Fourier Transform-Infrared Spectroscopy (FT-IR) Spectrophotometer, Shimadzu, Japan, using potassium bromide (KBr) powder. Absorbance measurements for the assays were recorded on a Spectra Max (Plus) UV multiscan spectrophotometer, US.

GC-FID/GC-MS Analyses

The oil (FAMES) obtained from the seed of *Moringa oleifera* was analyzed using an Agilent Technology 7890A gas chromatograph equipped with a fused silica capillary column HP-5MS (30 m by 0.32, 0.5 μ m film thickness) on ultra-pure helium gas and coupled to a mass selective detector (mass spectrometer). The injection and interface were operated at 250 and 380°C respectively. The oven temperature was raised from 60 to 300°C at a heating rate of 5°C min⁻¹ and held isothermally at that temperature. The sample was analyzed in a splitless mode. Scan start time-end time; 3.77-29.31 minutes. The constituents of the fatty acids in the oil were identified on the basis of their mass-spectral fragmentation patterns compared to those of the reference compounds on the NIST database.

In vitro Antioxidant Assays (Radical Scavenging Activity Assays)

In order to evaluate the free radical scavenging activity of the oil and extracts from the seed, three antioxidant assays which includes DPPH, ABTS and hydrogen peroxide scavenging activities were adopted.

DPPH Free Radical Scavenging Activity Assay

This spectrophotometric assay uses the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) as a reagent. The assay was carried out in duplicate according to the method previously reported (Atolani et al., 2012; Atolani and Olatunji, 2016). The DPPH free radical reagent was freshly prepared at a 0.1 mM concentration in methanol and kept in a dark bottle in the refrigerator overnight. Standard solution (ascorbic acid) and extracts were prepared in triplicate at concentrations 0.02 mg/mL, 0.04 mg/mL, 0.06 mg/mL, 0.08 mg/mL and 0.1 mg/mL. 2 mL of DPPH was added to all samples which were shaken and immediately incubated in the dark for 30 minutes. The absorbance value was measured at 517 nm. Blank experiment was also carried out to determine the absorbance of DPPH before interacting with the sample. The decreasing absorbance of the DPPH solution indicated an increase in DPPH radical

scavenging activity. The activity was given as percent DPPH radical scavenging, and was calculated using the equation:

$$\% \text{ AA} = 100 \times [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})] / (\text{Abs}_{\text{control}})$$

Where: % AA means percentage antioxidant activity, Abs is the absorbance of the control and Abs is the absorbance of the sample at 517 nm. Results were expressed as mean values \pm standard deviations (SD).

The IC₅₀ was determined on GraphPad Prism 3 software (San Diego, USA) through a nonregression analysis. The IC₅₀ was taken as the concentration of sample that scavenged 50 % of the radicals.

ABTS Free Radical Scavenging Activity Assay

The 2,2' - azinobis - 3 - ethylbenzothiazoline - 6 - sulfonate, ABTS radical cation decolorization method based on the reduction of ABTS⁺ radicals by antioxidants component of the extracts or oil was used. The adopted assay follows the procedure of Atolani et al. (2013), with slight modifications. The reaction mechanism involves the donation of electron by the samples which results into the decolorization of the solution. ABTS reagent was first dissolved in deionized water to afford a concentration 7 mM. Then, the solution of potassium persulfate was prepared at a concentration of 2.45 mM. The two solutions were mixed at a ratio of 1:1 and kept in the dark for 24-48 h. The ABTS solution was then diluted in aqueous methanol with a ratio of 1:25. A volume of 20 μ L (diluted 1:10) of aqueous samples was added to 2 mL of ABTS⁺ solution, and the mixture was kept at a standard temperature of 30 °C. The absorbance was measured at 734 nm at 10 min after initial mixing. All solutions were used on the day of preparation, and all determinations were carried out in triplicate. The ABTS antioxidant capacity (AOC) was calculated and compared with ascorbic acid using the following equation;

$$\text{AOC} = 100 \cdot [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})] / (\text{Abs}_{\text{control}})$$

Where Abs_{control} and Abs_{sample} are the absorbances of the control and the sample tested, respectively.

Hydrogen Peroxide Inhibition Activity

The ability of the *Moringa oleifera* seed oil and extracts to scavenge hydrogen peroxide (H₂O₂) radical were assessed using standard procedure (Keser et al., 2012) with slight modifications. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). 0.6 mL of this solution was added to 1.0 mL sample (100 μ g/mL). The absorbance of the hydrogen peroxide was read at 230 nm after 10 min at 28.4°C temperature against a blank solution containing

phosphate buffer solution without hydrogen peroxide. Ascorbic acid served as positive controls. The percentage scavenging of hydrogen peroxide by the samples, as an equivalence of the ascorbic acid was calculated using the following formula:

$$\% \text{ AA} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})] / (\text{Abs}_{\text{control}}) \times 100$$

Where AA means percentage antioxidant activity. H₂O₂ means hydrogen peroxide.

In vitro Anti-inflammatory Activity

The anti-inflammatory activities of the oil and extracts from *Moringa oleifera* were investigated using standard procedures of the protein inhibitory activity and the red blood cell (RBC) membrane stabilization assays.

Protein Inhibitory Assay

The test was carried out according to standard procedure (Govindappa et al., 2011). The reaction mixture (2 mL) containing 0.06 mg trypsin, 1 mL of 20 mM Tris HCl buffer (pH 7.4) and 1 mL test samples of different concentrations (5 – 250 μ g/mL) was prepared. The reaction mixture was incubated at 37°C for 5 min and then 1ml of 0.8 % (w/v) casein was added. The reaction was inhibited for additional 20 min after which 2 mL 70 % perchloric acid was added to terminate the reaction. The cloudy suspension which was obtained was centrifuged and the absorbance of the supernatant was read at 210 nm using the buffer as blank. The experiment was performed in triplicate and the percentage inhibition of proteinase was calculated using the expression:

$$\% \text{ Inhibition} = 100 \times (1 - \text{Vt}/\text{Vc}).$$

Where Vt = absorbance of test sample;

Vc = absorbance of control

Red Blood Cell (RBC) Membrane Stabilization Assay

The membrane stabilization activities of the samples were determined using procedure from reported literatures (Anosike et al., 2012; Kardile et al., 2016). Blood sample was collected from animals that have not been given any NSAIDS for 2 weeks prior to the experiment. The blood sample was mixed with equal volume of Alsever solution comprising 2 % dextrose, 0.8 % sodium citrate, 0.5 % citric acid and 0.42 % NaCl (Gandhisan et al., 1991) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline and a 10% suspension was constituted with normal saline. 50 and 100 μ g/mL of the samples was prepared in distilled water and 1 mL phosphate buffer, 2 mL hyposaline and 0.5 mL of RBC suspension was added. The mixture was incubated at 37°C for 30 minutes and centrifuged at 3,000 rpm for 20 minutes. The hemoglobin content of the supernatant solution

was estimated spectrophotometrically at 560 nm. Diclofenac (100 µg/mL) was used as reference while a control that has none of the test sample was used to monitor the experiment. The experiment was performed in triplicates and mean values determined. The percentage of RBC membrane stabilization or protection was determined using the expression:

$$\% \text{ Protection} = 100 - (\text{Absorbance of treated sample} / \text{Absorbance of control}) \times 100.$$

Data Analysis

The data analysis was carried out using analysis of variance on Graphpad Prism 3.0 software (USA). Differences among the mean values were evaluated using the Dunnett Test. The IC₅₀ was determined on GraphPad Prism 3 software (USA) through a non-regression analysis. The IC₅₀ was taken as the concentration of sample that scavenged 50 % of the radicals. Results are presented as mean ± standard error of the mean.

RESULTS AND DISCUSSION

Moringa oleifera is known to have vital nutritional value and medicinal importance. This assumption has led to the heavy consumption of the seed with less regards to what the phytochemical constituents could be. In Nigeria, especially in the northern part, the seed is consumed daily as nuts due to the stress-relieving effect that is derived from it. Compounds with antioxidant and anti-inflammatory activities are known to relieve stress (He et al., 2015). The consumption of the seed of *Moringa oleifera* seems to possess the stress relieving activity which might be due to the presence of antioxidant and anti-inflammatory phytochemicals in it. Considering that fatty acids are important antioxidant constituents of *Moringa oleifera* seed, the fatty acids profile of the seed was examined via two methods: conventional multi-steps method and the direct method, in order to fully determine the major compounds. While the conventional method involves different stages such as extraction of the lipids from pulverized seed material, hydrolysis of the lipid and esterification to obtain the fatty acid esters, the direct method combined the extraction, hydrolysis and esterification in one step by treating the pulverized seed material with specific reagents. The direct method minimizes loss due to handling at multiple stages and also saves time and resources. The chance of introduction of impurity is also minimized. From the total weight of the extract obtained, a yield of 2.3 % was obtained from the DCM extract while a yield of 1.85 % was obtained for ethanol extract.

FT-IR and GC/GC-MS results

The oil, FAMES, DCM and Ethanol extracts obtained were analyzed using FTIR spectroscopy and GC-MS spectrometry as appropriate. The FTIR spectrum of both methylated oils (conventional and direct) were quite identical as they both showed prominent peaks at 2926 and 2854 cm⁻¹ corresponding to C-H stretching of aliphatic chains of the fatty acid esters while the peak at 1747 and 1465 cm⁻¹ corresponds to carbonyl stretching and C-H bending of alkane respectively. A less intense peak corresponding to the olefinic bonds stretching was observed at 1651 cm⁻¹. The absence of prominent OH at around 3500 - 3300 cm⁻¹ suggests that the fatty acids were properly methylated.

The FTIR spectrum of the dichloromethane extract showed prominent -OH stretching vibration at 3319 cm⁻¹, C-H stretching of aliphatic hydrocarbons at 2928 and 2856 cm⁻¹ and -C=C- stretching of alkenes at 1654 cm⁻¹. The carbonyl stretching vibration was also observed at 1747 cm⁻¹. The spectrum suggests that the compounds in the DCM extract are primarily polar. The FTIR spectrum of MSO (ethanol extract) also showed prominent OH stretching vibration at 3389 cm⁻¹ and C-H of aliphatic hydrocarbons at 2926 and 2854 cm⁻¹. C=O of carbonyl stretching at 1737 and -C=C- stretching of the alkenes was indicated at 1658 cm⁻¹. The presence of aliphatic amines N-H bending vibrations were observed at 1060 and 1236 cm⁻¹ thereby suggesting the presence amines in the ethanol extracts.

The importance of food fatty acids composition on human health and nutrition cannot be overemphasized. Therefore, the determination of fatty acids composition is an essential requirement in testing herbal products (Priego-Capote et al., 2004; Williams, 2000). The fatty acid profiles obtained for FAMES using both methods are indicated in the **Table 1**. The compounds were identified on the basis of their mass spectral fragmentation patterns compared to those of the standard reference compounds stored on the spectrometer database and the NIST library. The *M. oleifera* seed oil was found to contain high concentration of oleic acid (72.08 %) followed by palmitic, stearic, docosanoic, arachidic and lignoceric acid at 12.94, 9.64, 3.15, 1.60, and 0.56 % respectively. The oil contained total of 72.08 % saturated and approximately 28 % of unsaturated fatty acid. The unsaturated fatty acid of *M. oleifera* seed oil was found to comprise of only mono-unsaturated fatty acid, precisely a mono-unsaturated omega-9 fatty acid (MUFA). The major compound, oleic acid has

also been reported to be present in large amount in sesame oil (Thomas, 2000), olive oil (Grossi et al., 2014), pecan oil (Villarreal-Lozoya et al., 2007), lime seed oil (Atolani et al., 2012), avocado and almond seed oil (Zielińska and Nowak, 2014). As a result of the high concentration of the oleic acid in olive oil, it was suggested to be the major anti-breast cancer agent in it (Win, 2005). Oleic acid is reported to synergistically enhance cancer drug effectiveness (Mendez et al. 2005). Although, the oleic acid is not classified as an essential fatty acid, it is technically essential, because the human body can only manufacture a limited amount, provided that essential EFAs are present (Rotella 2004). Fatty acid composition is one of the most relevant important

features used to determine the quality and potential health benefit of oils. The fatty acid profile obtained in this study is in agreement with literature data obtained for the seed in other region (Vlahov et al., 2002; Anwar and Bhangar, 2003; Banerji et al., 2009; Barakat and Ghazal 2016). The positive impact of the oleic acid in the seed cannot be undermined. Several studies conducted on the relative carcinogenicity and immunosuppressive activities of the various fatty acids, indicated that oleic acid was the only fatty acid with the least negative effect. Oleic acid is suspected not to raise serum cholesterol concentrations because liver enzyme cholesterol acyltransferase converts it to an inactive form (Grundy, 1994).

Table 1: Fatty acids profile of FAMES obtained by conventional and direct method

SN	Compounds	RT	Saturation	Conventional Method	Direct Method	Average (SD)
1	Palmitic acid	17.3	16:0	12.64	13.24	12.94 ± 0.42
2	Stearic acid	20.6	18:0	10.06	9.22	9.64 ± 0.59
3	Oleic Acid	21.4	18:1	71.96	72.19	72.08 ± 0.16
4	Arachidic acid	23.0	20:0	1.66	1.53	1.60 ± 0.09
5	Docosanoic acid	24.9	22:0	3.04	3.26	3.15 ± 0.16
6	Lignoceric acid	26.9	24:0	0.62	0.51	0.56 ± 0.08
% Saturated				71.96	72.19	72.08 ± 0.16
% Unsaturated				28.04	27.81	27.92 ± 0.16

RT means retention time; SD means Standard Deviation

Radical Scavenging Activities

Since single index antioxidant capacity is not considered sufficient to quantify the antioxidant potential of plant samples, three different antioxidant assays were adopted to establish the antioxidant potential of the seed oil and extracts. While it is apparent that no single method gives a comprehensive estimation of the antioxidant efficacy of plant samples, about twenty different antioxidant indices are currently in use (Luximon-Ramma et al., 2002). However, DPPH, ABTS and hydrogen peroxide antioxidant assays are widely acceptable antioxidant indices (Huang et al., 2005; Adeosun et al., 2013; Gökbulut et al., 2017; Sıpađı et al., 2017). Therefore, these three indices were used to determine the antioxidant activities of the seed oil and extracts of *Moringa oleifera* seed. The *in vitro* antioxidant

activities of the soxhlet extracted oil, DCM extract and ethanol extract were evaluated using the DPPH, ABTS and hydrogen peroxide radical scavenging assays as ascorbic acid served as standard antioxidant. The results (Table 2) of the DPPH radical scavenging assay suggests a high antioxidant potentials compared to the standard. While the DCM extract (Fig. 1) seems to have the highest activity (75.5%), the oil had the lowest IC₅₀ (1.13 ± 0.41). Ascorbic acid had a broad activity range (1.2 to 73.5%) across the tested concentrations while the oil and extracts had narrow antioxidant activity range within the upper limit. The oil had activity range 67 to 75%, DCM extract 72 to 75.5% and EtOH extract of the seed had activity in the range 70 to 74%. This study showed that *Moringa oleifera* seed oil and extracts have antioxidant activities comparable to the standard.

Table 2: DPPH radical scavenging activity of *Moringa oleifera* seed oil and extracts

Sample	%AA ± SEM* (20 µg/mL)	IC ₅₀ ± SEM* (µg/mL)	R ² Value
MS Oil	74.98 ± 0.16	1.131 ± 0.41	0.9358
DCM extract	75.54 ± 0.21	1.269 ± 0.54	0.917
EtOH extract	74.19 ± 0.20	1.167 ± 0.73	0.9512
Ascorbic acid	73.51 ± 0.31	1.141 ± 12.89	0.9961

SEM*- Standard error of mean of triplicate determinations; IC₅₀, half-maximal inhibition concentration; MS Oil-*Moringa oleifera* seed oil obtained via soxhlet extraction; DCM extract-Dichloromethane extract; EtOH extract.

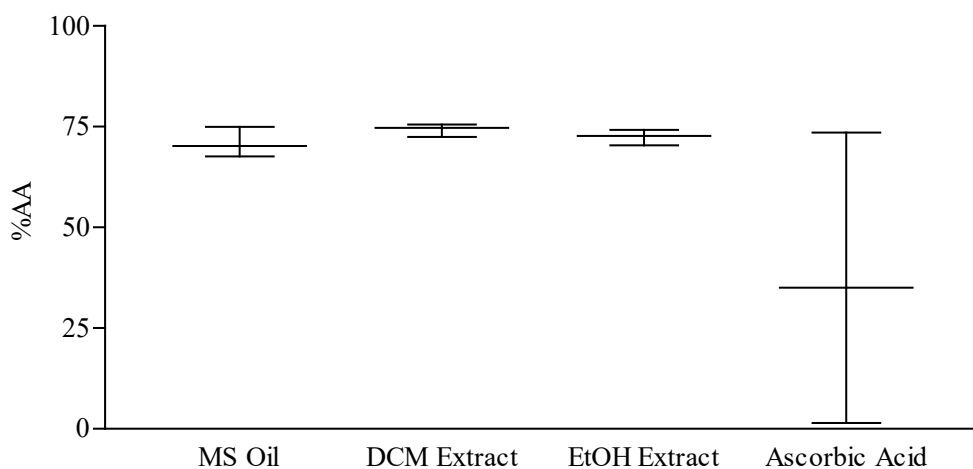


Figure 1: DPPH radical scavenging activity box and whiskers diagram indicating the spread of the percentage antioxidant capacity of MS oil, DCM extract, EtOH extract and ascorbic acid.

MS Oil - *Moringa oleifera* seed oil obtained via soxhlet extraction; DCM extract-Dichloromethane extract; EtOH extract.

The ABTS radical scavenging activity results (Table 3) indicated that the *M. oleifera* seed oil (83.41 %), DCM extract (84.25 %) and EtOH extract (84.33 %) had high activities which are comparable to the standard, ascorbic acid (85.12 %). The hydrogen peroxide radical scavenging activity assays also showed good activity compared to ascorbic acid used as standard. However, the MS oil had only a moderate activity (59.58 %), while DCM had highest activity (62.17 %) and EtOH, lowest activity (55.24 %) in scavenging hydrogen peroxide radicals (Table 4).

A lot of attention is currently drawn to the antioxidant activity of foods, herbs and other natural substances consumed due to their wide recognition for the protection against cellular oxidation caused by the over secretion of reactive oxygen species (ROS). This uncontrolled condition (ROS) is known

to cause cell death, degradation of lipid membrane, inflammatory disorders, premature aging, cancer, heart diseases, diabetes and breakdown of cellular respiration. Therefore, the prompt scavenging of free radicals is essential (Azizuddin et al., 2010).

Fatty acids are important components of seeds that acts as antioxidant which when consumed as herb or in diet, could help reduce effect of oxidative stress. Cellular processes that generate pro-oxidant are reportedly managed by the consumption of diet rich in exogenous antioxidants (Gupta et al., 2014). Some fatty acids are known for their involvement in the reaction that catalyzes the oxidative decarboxylation of alpha-keto acids, such as pyruvate and alpha-ketoglutarate, in the Krebs cycle. They reportedly quench free radicals in both lipid and aqueous domains and also chelates pro-oxidant metals. Fatty acid such as lipoic acid is suggested to have some effect on other antioxidants (Packer and Witt, 1995). The strong free radical scavenging effect observed in the *M. oleifera* seed oil in this study could be associated with the overall stress and fatigue relieve effect of the seed.

Table 3: ABTS scavenging activity of the extracts of *M. oleifera* seed at 50 µg/mL

Extracts	%Inhibition ± SEM
MS Oil	83.41 ± 1.61
DCM extract	84.25 ± 0.99
EtOH extract	84.33 ± 1.60
Ascorbic acid	85.12 ± 0.34

SEM* - Standard error of mean of triplicate determinations; MS Oil- *Moringa oleifera* seed oil obtained via soxhlet extraction; DCM extract-Dichloromethane extract; EtOH extract.

Table 4: Hydrogen peroxide scavenging activity of the extracts of *M. oleifera* seed at 100 µg/mL

Samples	%Inhibition ± SEM
MS Oil	59.58 ± 0.67
DCM extract	62.17 ± 0.51
EtOH extract	55.24 ± 0.83

MSO- *M. oleifera* seed oil; DCM-Dichloromethane; EtOH-Ethanol; Values obtained are average of triplicate determinations.

Anti-inflammatory Activities

The unesterified *M. oleifera* seed oil and extracts were evaluated for their anti-inflammatory activities using the protein inhibitory and red blood cell (RBC) membrane stabilization assays. The *in vitro* protein inhibitory assay carried out as previously outlined did not show any positive response for the concentration range (5 – 250 µg/mL) tested. There was no observable difference in the absorbance of control and sample at the varying concentrations, which suggested the absence of proteinase inhibitory activity in the tested samples. Increasing the concentration of the test samples beyond the recommended threshold could improve response but with probable higher debilitating impact to other biochemical parameters. The result from this study suggested that the seed oil and extract of *Moringa oleifera* did not possess sufficient capacity to inhibit proteinase activity.

For the red blood cell (RBC) membrane stabilization assay, ETOH extract at 100 µg/mL had

the highest activity (64.99 %) which is superior to the standard drug, diclofenac with an activity of 62.15% at the same concentration (Table 5). The MS oil apparently had the lowest protecting capacity thereby indicating a note of caution to indiscriminate consumers of the oil. The membrane stabilization potential of the MS oil and DCM extract were not concentration dependent as higher activities were recorded at lower concentrations. While it is presumed that the activity threshold have been exceeded at the compared concentrations, the observation could also be due to the effect of some present phytochemicals which expresses their antagonistic potentials at higher concentrations. However, the observed phenomenon may not be absolutely deciphered until comprehensive chemical composition of all their constituents are fully established. It is suggested that there are other potential membrane stabilising agents that are present in the DCM and EtOH extracts which had a relatively higher stabilization power. Lower concentration of the oil may possess a higher stabilization capacity.

Table 5: Red blood cell (RBC) membrane stabilization activity

Samples	%Activity ± SEM
MS Oil (50 µg/mL)	35.76 ± 4.35
MS Oil (100 µg/mL)	30.84 ± 3.09
DCM extract (50 µg/mL)	41.6 ± 1.27
DCM extract (100 µg/mL)	37.86 ± 2.48
ETOH extract (50 µg/mL)	53.81 ± 4.48
ETOH extract (100 µg/mL)	64.99 ± 0.78
Diclofenac (100 µg/mL)	62.15 ± 0.02

MSO- *M. oleifera* seed oil; DCM-Dichloromethane; EtOH-Ethanol; Values obtained are average of triplicate determinations.

Methods which include inhibition of protein denaturation, inhibition of oxidative phosphorylation, lysosome membrane stabilisation, platelet aggregation and erythrocyte membrane stabilization have been employed in screening drugs, compounds, herbal preparations and extracts for anti-inflammatory activities (Oyedapo et al., 1999). The proteinase inhibitory and erythrocyte membrane stabilization activity was adopted in this study. The ability of the membrane to be stabilised required a process in which the integrity of the erythrocyte membrane and lysosomal membrane was probed by anti-inflammatory drugs. The stabilizing effect of the anti-inflammatory drugs on erythrocyte membrane was reported to be due to a stabilizing effect of the drugs on certain proteins in the membranes involved (Mizushima et al., 1970). It has also been suggested that lysosomes contained several enzymes which are involved in the process of inflammation. Lysosomal enzymes are released into the cytosol, causing damage to the surrounding tissues during inflammation. Many NSAIDs are known to stabilize lysosomal membrane leading to inhibition of inflammatory processes by restricting the release of lysosomal enzymes (Anosike et al. 2012; Chaitanya et al., 2011; Oyedapo et al., 1999; Oyedapo et al., 2010). The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Injury to red cell membrane renders the cell more susceptible to secondary damage through free radical induced lipid peroxidation. Membrane stabilization leads to the prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators (Chaitanya et al., 2011; Mounnissamy et al., 2008).

While the membrane stabilizing activity petroleum ether extract of *M.oleifera* leaf of Asian origin has been evaluated in animal models (Kumbhare et al., 2014) and *in vivo* anti-inflammatory activity by carrageenan induced rat paw edema investigated (Kumbhare and Sivakumar, 2011), search of the literature shows fewer reports on the seed. Also, while some research studies indicated that the aqueous, alcohol and chloroform extracts of *Moringa oleifera* seed of Indian origin has anti-inflammatory activity in an *in vivo* model (Minaiyan et al., 2014), other corroborative results from both *in vitro* and *in vivo* anti-inflammatory activity experiment of the aqueous seed extract indicated that the activity was due in part, to the regulation of NO production. The aqueous seed extract reduced the production of NO with greater than 90 % inhibition when compared to the controls. The results revealed that while the diluted seed extract could be relatively safe, the concentrated aqueous seed extract, or preparations with higher protein content may be potentially harmful. This supports

previous cautions against the indiscriminate increase of the concentration of the extract in the treatment of portable water (Araújo et al., 2013; Kavitha et al., 2012). It has been reported that prolonged consumption of water treated with *Moringa oleifera* seed may cause liver damage (Oluduro and Aderiye, 2009).

One of the resultant effects of the uncontrolled free radical generation in the system is inflammation. Inflammation, in the first instance, is a self-defense reaction that occurs under specific condition which develops into a chronic state and become a causative factor in the aetiology of a broad range of pathologies if not promptly managed (Ajmone-Cat et al., 2010). This study suggests that the positive health impact derived from the seed may be due to its antioxidant effect while the anti-inflammatory activity of the seed may not be through proteinase inhibition mechanism. A previous report of the antioxidant and hepatoprotective activities of the leave of the plant in animals suggest that the reported anti-inflammatory effect was probably due the presence of the antioxidants such as phenolics and flavonoids with radical scavenging activities (Singh et al., 2014).

CONCLUSION

The screening of antioxidant and anti-inflammatory potential of *M.oleifera* seed traditionally consumed as herbs indicated that the seed is endowed with potentially exploitable free radical scavenging and anti-inflammatory constituents. The data reported herein is the first report detailing the fatty acid profile, antioxidant and anti-inflammatory activities of *M.oleifera* collected from northern Nigeria where the seed is heavily consumed. The oil and extracts of *M.oleifera* seed exhibited prominent free radical scavenging activities. However, since no positive proteinase inhibitory and low membrane stabilization responses were observed for the oil and extracts, it is suggested that the anti-inflammatory activity of the seed of *Moringa oleifera* may be via other anti-inflammatory mechanisms. These findings would be of immense use to nutraceuticals scientists and nutritionists formulating antioxidant-rich therapeutic diets. The result would also be a source of valuable information to consumers as it also adds to the pool of knowledge on the antioxidant potential of the commonly consumed seed. The entire protein profile, safety profile and *in vivo* toxicity studies would be of immense value to compliment these findings.

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