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Original Research Article

Isolation and Characterization of Pyropheophorbide-a from *Moringa oleifera* Lam

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ABSTRACT

Moringa oleifera is a plant rich in pharmacologically active compounds. This study investigated the phytochemical constituents *of Moringa oleifera* leaf extracts. Dried *M. oleifera* leaves were ground and extracted successively with hexane, ethyl acetate and methanol using a Soxhlet apparatus. Different chromatographic techniques were used to fractionate the ethyl acetate extract. Thin layer chromatography was used to pool similar fractions together. Fractions obtained were purified using sephadex glass column. The structures of isolated compounds were identified using nuclear magnetic resonance (NMR), electrospray ionization mass spectrometry (ESI-MS) and comparison with published data. Phytochemical screening revealed the presence of flavonoids, terpenoids, carbohydrates and phenols. Ethyl acetate extract which was subjected to column chromatography resulted to the isolation of two compounds: mono acetyl glycerol and pyropheophorbide-a, from the leaves *of M. oleifera.* This is the first report on the isolation of pyropheophorbide-a from the leaves *of M. oleifera*.

Keywords: *Moringa oleifera*, Isolation, Pyropheophorbide-a, Monoacetyl glycerol.

Introduction

Man has always used plants for the treatment and prevention of various disease conditions due to the presence of varied active ingredients in different parts of plants. According to the World Health Organization (WHO), 75% of people rely on plant based traditional medicines for primary health care globally; and about 85% of traditional medicines are based on the use of plant extracts. [1] The demand for natural products from plants is due to special attributes of natural products not present in synthetic drugs. They are said to be "safe" or at least safer than conventional medicine with little or no side effects, eco-friendly. Other attributes include having drug-like properties, low cost and increased evidence for an association between high consumption of fruits /vegetables and reduced risk of diseases. [2] Historically, plants are the major sources of some of the most important drugs based on their use in traditional medicine. For example, Morphine, from opium poppy (*Papaver somniferum),* which became the first pure substance of natural origin to be commercialized as a drug used as analgesic, [3] Digoxin and other digitalis glycosides, from foxglove (*Digitalis spp.*), used to treat cardiac failure, [4] Taxol, from the Pacific yew (*Taxus brevifolia*), and its semisynthetic derivative docetaxel used as anticancer treatment [5] and Quinine, from *Cinchona* bark (*Cinchona spp.*), used in the treatment of malaria. [4]

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Moringa oleifera Lam. (Syn. *Moringa pterygosperma* Gaerth) belongs to a monogeneric family of Moringaceae, [6, 7] and is widely distributed in many tropical and sub-tropical countries. [8] The plant is commonly known as drumstick or horseradish tree. In Nigeria, it is known by various local names such as: Ewe ile (Yoruba), Zogali, or Zogalla-gandi (Hausa) and *Okochiegbu* (Ibo). *Moringa oleifera* is a highly valuable plant, with nearly all parts used for medicinal and nutritive purposes. Its medicinal use has long been recognized in the *Ayurvedic* and *Unani* systems of medicine.[9] Some of the medicinal effects are antitumor, [10, 11] antipyretic, anti-inflammatory, antiulcer,[12,13] antispasmodic, [14, 15] diuretic, [14, 16] antihypertensive, [17, 18]cholesterol lowering,[19] antioxidant, anti-diabetic, hepato-protective, antibacterial and antifungal activities.[20] The potential of *M. oleifera* in the treatment of typhoid fever in Cameroon [21] and HIV/AIDS in Uganda has also been reported. [22] In Nigeria, *M. oleifera* is used to treat various diseases such as inflammation, asthma, fever, cough, pains, liver and pancreatic disorders, venereal infections, diarrhea and malaria. [23, 24] In addition, Igbo et al 2015 reported that ethyl acetate extract of *M. oleifera* leaves exhibited antitrypanosomal activity with minimum inhibitory concentration (MIC) valve of 25 µg/mL. [25] Other uses of *M. oleifera* include animal forage (leaves and treated seed cake), biogas (leaves), domestic cleaning agent (leaves), blue dye (wood), fencing (living trees), fertilizer (seed cake), green manure (leaves), gum (tree trunks) sugar cane, juice-clarifier (powdered seeds), ornamental plantings, bio-pesticide, pulp (wood) and water purification (powdered seeds). [26]*Moringa* species contain a wide range of fairly unique compounds called glucosinolates and isothiocyanates.[27, 28] Also, the plant family is particularly rich in rhamnose glycosides. Some pharmacologically active compounds such as 4-(4'-*O*-acetyl-α-L-rhamnopyranosyloxy)benzyl isothiocyanate, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate**,** niazimicin, pterygospermin**,** benzyl isothiocyanate**,** and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate; also, thiocarbamates, carbamates, and nitrile glycosides have been isolated from the fresh

leaves of *M. oleifera*. [17, 18, 29] These compounds are responsible for the hypotensive activity of the leaves.
Flavonoid compounds consist of glycosides, rutinosides, malonylglycosides and traces of acetylglycosides of kaempferol, quercetin and caffeoylquinic acid.[28] Moringine and moringinine have also been isolated from the plant. Plants are source of bioactive compounds which can serve a lead in drug discovery. *M. oleifera* has been widely studied and tested for its biological properties, but it is not completely exhausted in search for more bioactive compounds. Therefore, this report is on isolation of compounds from *M. oleifera* leaf ethyl acetate extract.

Materials and Methods

General Experimental Procedures
HPLC grade solvents were obtained from Avantor VWR™ and Fisher Scientific UK. NMR experiments were performed on a JEOL (JNM LA 400) 400 MHz and Bruker (Avance III) 400 MHz spectrophotometers. Samples were dissolved in 0.650 ml deuterated chloroform for NMR analyses. ESI MS of isolated compounds were obtained in positive ionization mode using a Thermo Exactive Orbitrap mass spectrometer coupled to an Ultimate 3000 LC system. Thin Layer Chromatography (TLC) was conducted using commercially available pre-coated Merck F_{254} silica gel plates. The spots were visualized under UV light at 254 nm and 366 nm, and by spraying with anisaldehyde-sulfuric acid mixture and heated to 110ºC.

Plant Material
Fresh leaves of *M. oleifera* were collected from Gusau in Zamfara State, Nigeria on February 10, 2012. The plant material was authenticated at the University of Lagos. A voucher specimen LUH 6062 was deposited at University of Lagos Herbarium. The leaves were air dried at room temperature for a period of one week. The clean dried leaves were pulverized and stored in a refrigerator until ready for use.

Extraction
Powdered *M. oleifera* leaf 650 g was extracted successively with three liters of hexane, ethyl acetate and methanol using Soxhlet apparatus for 72 h. All extracts were filtered to remove any debris and evaporated to dryness using a rotary evaporator at 40^0C under reduced pressure; their percentage yields were determined and extracts were stored in a refrigerator until further investigation.

Phytochemical Screening
Qualitative phytochemical screening of ethyl acetate and methanol extracts were carried out according to the methods described by Trease and Evans[30] and Sofowora.[31]

Isolation and Characterization of Pyropheophorbide a
A sintered glass fitted Buchner funnel with a suction outlet was packed with TLC grade silica gel (60H, Merck, Germany) under vacuum. A non-polar solvent n-Hexane was run through the Buchner funnel under vacuum in order to achieve good packing of the column. Ethyl acetate extract (15.3 g) pre-adsorbed on silica gel was added to the packed vacuum liquid chromatography (VLC) column. The sample was eluted twice each time with 300 mL solvent mixtures of increasing polarity: hexane-ethyl acetate and ethyl acetate-methanol (10% increments of ethyl acetate and methanol, respectively). A total of 28 fractions were collected. Fractions were pooled together based on similarity of TLC profiles, and further evaporated to dryness at 40ºC under vacuum using a rotary evaporator. Fractions MOEV-EtOAc-1 and MOEV-EtOAc-2 (both eluted with 100% ethyl acetate) were combined based on TLC and similarity of their proton nuclear magnetic resonance (^{1}H-NMR).
The combined fraction (850 mg) was further purified using Sephadex. A slurry of sephadex LH-20 in methanol was added to a glass column. Fractions eluted with 100% ethyl acetate was dissolved in small quantity of methanol and applied onto the sephadex column. The column was eluted with methanol; 50 fractions (4 mL each) were collected. Compounds 1 and 2 were obtained from Sephadex column fractions MOEVS 36-47 and MOEVS 11-14, respectively. Structures of the two compounds were established using NMR techniques (^{1}H, ^{13}C and 2D) and comparison with published data.

Results and Discussion

The percentage yield of *M. oleifera* leaf extracts produced with solvents of increasing polarity gave values for hexane 52.7 g (8.1%), ethyl acetate 22.6 g (3.5%), and methanol 81.9 g (12.6%).
Investigation of the phytochemical constituents of the ethyl acetate and methanol extracts showed the presence of flavonoids, terpenoids, carbohydrates, and phenols. Saponins, alkaloids and anthraquinones were not detected in any of the extracts (Table 1). The presence of these phytochemicals confirms the medicinal importance of *M. oleifera* leaves.
Fractionation of ethyl acetate extract gave compounds **1** (8 mg) and **2** (4.9 mg). A reddish-coloured spot detected by UV (365 nm) was observed in the TLC of fraction MOEVS 36-47. The molecular ion of compound **1** was not observed in its mass spectrum but a quasi-molecular ion at *m/z* = 546.0800 corresponding to $[M\text{-}CO_2H]^+$ was obtained suggesting a molecular formula $C_{34}H_{33}N_4O_3$. The assignments of ^{1}H-NMR and ^{13}C-NMR signals summarized in Table 2 were carried out by two-dimensional (2D) NMR: Correlation Spectroscopy (COSY), Heteronuclear single quantum coherence (HSQC) and Heteronuclear multiple bond coherence (HMBC) experiments.
The structure of compound **1** was established from ^{1}H and ^{13}C correlations observed in its 2D NMR spectra and comparison with published data.[32] The ^{1}H-NMR spectrum showed three well separated singlets (3H) due to methyl groups at δ_H 3.23, 3.41, and 3.67 ppm, an ethyl group (δ_H 1.70 (3H, t, *J* = 7.6 Hz), 3.67 (2H, q, *J* = 7.8 Hz)) and a vinyl group with protons at δ_H 7.97 (1H, dd, *J* = 11.5, 17.8 Hz), 6.29 (1H, dd, *J* = 1.3, 17.8 Hz) and 6.17 (1H, dd, *J* = 1.3, 11.5 Hz) were observed.
The ^{13}C-NMR spectrum showed 33 carbon signals and accounted for all the carbon atoms in the structure. Thus compound **1** was identified as pyropheophorbide-a (Figure 1) based on the ^{13}C and two-dimensional (2D) NMR spectra. This result compares with earlier report of pyropheophorbide-a isolated from the leaves of *Atalantia monophylla*. [32] This is the first report on the isolation of pyropheophorbide-a from *M. oleifera* leaves.
The ^{1}H-NMR spectrum of compound **2** gave signals at δ_H 2.01 (3H, s), 3.49, 3.59 (each 1H, d, *J* = 4Hz), 3.85 (1H, m), 4.04 (2H, d, *J* = *4* Hz). The ^{13}C-NMR gave a signal at 172.0 ppm indicative of a carbonyl group. Table 3 shows the ^{1}H and ^{13}C signals based on correlations observed in its 2D NMR spectrum.
The molecular ion was not observed in its mass spectrum. The peak observed at m/z = 327 is due to the loss of hydroxyl group ($[M\text{-}OH]^+$) and the mass at m/z = 313 corresponds to the loss of a CH_2 characteristic of long chain aliphatic compounds.
Compound **2** was identified as mono acetyl glycerol (Figure 2); possibly produced as a degradation product of glycerol-1(9-octadecanoate).[33]

Table 1: Qualitative phytochemical screening of *M. oleifera* leaf extracts

Class of compounds	Results
Saponins	-
Flavonoids	+
Alkaloids	-
Terpenoids	+
Anthraquinones	-
Carbohydrates	+
Phenols	+

+; indicate presence of compound
-; indicate absence of compound

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Table 2: ^{1}H and ^{13}C-NMR Chemical shifts (ppm) for compound 1 ($CDCl_3$) [a]

Position	ppm ^{1}H, (*J*/Hz)	^{13}C ppm
1	-	141.5 (C)
2	-	131.4 (C)
2^{1}	3.41 (s)	12.1 (CH_3)
3	-	135.9 (C)
3^{1}	7.97 (1H, dd, *J* = 11.5, 17.8)	129.2 (CH)
3^{11}	6.17 (1H, dd, *J* = 1.3, 11.5), 6.29 (d, *J* =1.3,17.8)	122.6 (CH_2)
4	-	136.3 (C)
5	9.34 (s)	97.3 (CH)
6	-	155.4 (C)
7	-	136.1 (C)
7^{1}	3.23 (s)	11.2 (CH_3)
8	-	145.0 (C)
8^{1}	3.679 (q)	19.5 (CH_2)
8^{11}	1.70 (t)	17.4 (CH_3)
9	-	150.8 (C)
10	9.46 (s)	104.3 (CH)
11	-	137.9 (C)
12	-	128.3 (CH_3)
12^{1}	3.65 (s)	12.0 (CH_3)
13	-	130.3 (C)
13^{1}	-	196.6 (C)
13^{11}	5.12, 5.27	48.0 (CH_2)
14	-	149.0 (C)
15	-	106.2 (C)
16	-	160.4 (C)
17	4.50 (m)	50.1 (CH)
17^{1}	2.29 (m), 2.73 (m)	29.7 (CH_2)
17^{11}	2.38 (m), 2.65 (m)	30.7 (CH_2)
17^{111}	-	176.8 (C)
18	4.32 (q, *J* = 9.3)	51.6 (CH)
18^{1}	1.83 (d, *J* = 7.3)	23.2 (CH_3)
19	-	171.4 (C)
20	8.55 (s)	93.1 (CH)

a-Deuterated chloroform

Table 3: ^{1}H and ^{13}C-NMR Chemical Shifts (ppm) for Compound 2 ($CDCl_3$)

Position	ppm ^{1}H, (*J*/Hz)	^{13}C ppm
1	4.04 (2H, d, *J* = 4.0)	65.6 (CH_2OH)
2	3.85 (1H, m)	70.6 (CHOH)
3	3.49, 3.59 (1H each, d, *J* = 4.0Hz),	63.8 (CH_2OH)
1^{1}	2.02 (3H s)	20.9 (CH_3)
	-	172.0 (C = O)

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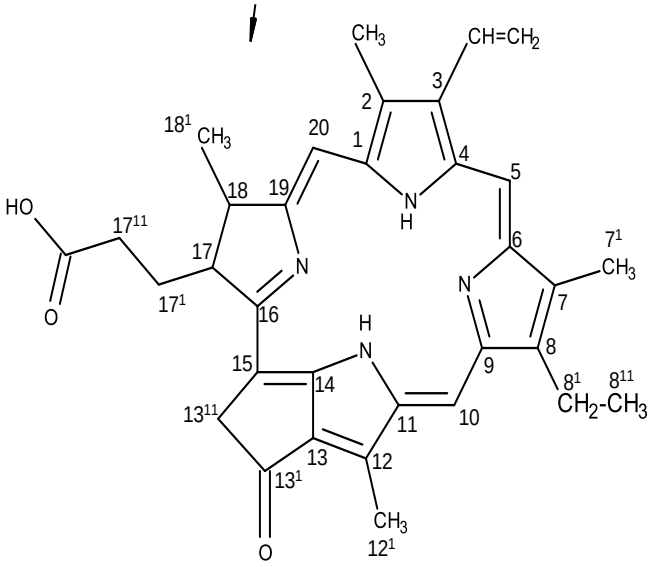

Figure 1: Structure of pyropheophorbide-a

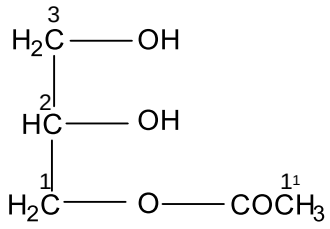

Figure 2: Structure of mono acetyl glycerol

Conclusion

The result of phytochemical screening revealed the presence of flavonoids, terpenoids, carbohydrates and phenols in ethyl acetate and methanol extracts. Fractionation of *M. oleifera* leaves ethyl acetate extract led to the isolation of pyropheophorbide-a (compound 1) an antioxidant and a photosensitizer used in photo dynamic therapy for the management of cancers. Also isolated was mono acetyl glycerol a compound with wide applications in pharmaceuticals, food and cosmetics industries. The result of this study provides scientific evidence to support the ethnopharmacological use of *M. oleifera* leaves.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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