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HARUNGANA MADAGASCARIENSIS LAM EX POIR (HYPERICACEAE) FRUITS OIL EXTRACT: PHYTOCHEMISTRY AND ACUTE TOXICITY EVALUATION

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Keywords:

H. madagascariensis, Non-utilised fruits, Fatty acids derivatives, Cosmetology, Toxicity

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ABSTRACT: This study reports the phytochemistry of the oil obtained from the air-dried non-utilized fruits of *H. madagascariensis* a plant widely used in folklore medicine. The *H. madagascariensis* fruits oil (HMO) was obtained by de-fatting the chloroform extract of the air-dried fruits with n-hexane. The n-hexane extract further separated on a normal phase silica column to afford the HMO. The acute toxicity test was done on mice using the Lorke's method. Selected physico-chemical properties (specific gravity, refractive index, pH, viscosity and saponification value) were evaluated using standard procedures. Characterization of the fatty acids constituents was done using Gas Chromatography-Mass (GC-MS) and Fourier Transform Infra Red (FTIR) spectroscopic techniques. The HMO (reddish oil) had LD_{50} > 5000 mg/kg; specific gravity = 0.918; refractive index = 1.472; pH: 6.4, Viscosity: 2.252, Saponification value (mg KOH/g): 140.5. GC-MS characterization of the HMO afforded eight fatty acids derivatives (47.93% unsaturated fatty acid and 14.82% saturated fatty acids of the total peak area): palmitic acid methyl ester (7.00%), palmitic acid (2.61%), linoleyl alcohol (1.22%), linoleic acid methyl ester (26.87%), stearic acid methyl ester (4.09%), linoleic acid (12.72%), linoleic acid ethyl ester (7.12%) and behenic acid methyl ester (1.12%). FTIR spectrum showed vibrational frequencies for: OH, C=C, C=O and C-O functional groups. Aside reporting for the first time the phytochemistry of the fruit oil of *H. madagascariensis*, the presence of the unsaturated fatty acids, the promising LD_{50} and physico-chemical properties imply application of the HMO in nutraceutics and cosmetology.

INTRODUCTION: Plant derived lipids are important in nutrition, health, cosmetology and as biofuels [1, 2].

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Dietary unsaturated fatty acids are essential to human health due to their role as precursors to the eicosanoids. Unlike animal derived lipids, they are high in unsaturated fatty acids which help in the lowering of plasma cholesterol thereby reducing the risk associated with cardiovascular health. Mono unsaturated fatty acids like palmitoleic acids have been reported to increase insulin sensitivity by suppressing inflammation and inhibiting the destruction of insulin secreting pancreatic beta cells

[3]. Fatty acids derivatives of plant origin are also utilised as vehicle for drug delivery [4]. This underscores the continued search for plant derived fatty acid derivatives.

Harungana madagascariensis is a monotypic medium to tall tree species commonly called dragon's blood tree. It is used in folkloric treatment of jaundice, diarrhea, dysentery, typhoid fever, constipation, liver problems, anaemia and malaria [5]. Several scientific reports validating the folkloric use of the leaves and stem bark of this plant in the treatment of anaemia [6], diabetes [7], oxidative stress related diseases [8], bacterial infections [9-10], diarrhoea and gastro-intestinal disorders [11], and malaria [6, 12] are documented. Whereas the phytochemistry of the roots leaves and stems bark of *Harungana madagascariensis* have been investigated extensively and has several bioactive compounds like: anthracene derivatives with anti-plasmodial [12] and antibacterial flavonoids [9], reported among others, literature reports on the pharmacological activities of the fruits are few [13] with dearth of literature on the chemical constituents of this non-utilised fruits.

This present study reports the characterisation of fatty acids derivatives using Gas chromatography - Mass spectroscopic techniques as well as the evaluation of the acute toxicity and some physico-chemical properties of the fruits' oil extract from *H. madagascariensis* a plant widely used in ethno-medicine. This is a follow-up to our earlier report on the fruit of this plant [13] and it is aimed at establishing the nutritional, health and industrial benefits of this non-utilised fruits.

MATERIALS: The fruits of *H. madagascariensis* were collected from the forest adjourning the University of Port Harcourt, Nigeria and authenticated at the Herbarium unit of the Plant Science and Biotechnology Department of the same University with Voucher Number: UPH/P/080; UPH/V/1,219. Eighteen adult mice of both sexes weighing between 23-30 g were obtained from the Animal house facility of the Department of Experimental Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Port Harcourt, Nigeria. They were kept under hygienic condition and fed with a well-balanced commercially sourced formulated diet and clean portable water. Due animal handling ethics were observed in accordance with the recommendations for proper care and use of laboratory animals (NIH Publication No. 85-23, revised, 1985). Reagents and solvents used were of analytical grade and are products of Sigma-Aldrich.

METHODS:

Preparation of the Extract: The cold maceration technique was used for the extraction of the pulverised fruits (500 g). This is to preserve the integrity of thermo-labile constituents that could be present in the fruit. Chloroform was used as the solvent for extraction. The chloroform extract obtained was then defatted by exhaustive cold maceration in n-hexane to afford the n-hexane extract (crude oil extract) after drying. The crude oil extract was further chromatographed on a normal phase silica gel column with a 5% stepwise gradient of n-hexane-chloroform (100:0 - 90:10 v//v) as mobile phase to afford a highly viscous liquid which was then treated with acetone to precipitate steroidal impurities. From the supernatant steroid-free layer was obtained the crude *H. madagascariensis* fruit oil coded HMO-c after evaporating the acetone.

Chromatographic Purification of the Crude Fruit Oil Extract: The crude oil extract (HMO-c) was further subjected to chromatography separation to remove non-fatty acids impurities. A n-hexane-chloroform 5% stepwise gradient of mobile phase on normal phase silica gel column with the elution of the reddish oily liquid at n-hexane – chloroform (100:0 – 90:10 v//v) was used for the column chromatography separation. 250mL of each gradient was used and the eluents collected in 50mL portions. Each of the reddish oily eluate was further clarified by acetone treatment to precipitate phyto-steroidal / triterpenoidal constituents with the acetone supernatant pooled to obtain the phyto-steroid free fruits oil (HMO) used for this study after removing acetone by evaporation.

Biological Evaluation of the HMO:

Acute Toxicity Test: The acute toxicity was done on mice using the Lorke's method [14]. Briefly the mice (23-30 g) were grouped into three groups (A-C) of three mice each in the first stage of the experiment. The mice in group A were given 10 mg/kg body weight (bw), while those in group B

and C got 100 mg/kg and 1000 mg/kg bw of the HMO respectively. They were observed for visible signs of toxicity for 24 hours and the number of deaths if any recorded was scored following the Lorke's method. In the second stage, due to a no death record in the first stage, the remaining nine mice were grouped into three groups (D-F) of three mice each just like in the first stage of the experiment but mice in group D were given 1600 mg/kg bw, while those in group E and F received 2900mg/kg and 5000mg/kg bw of the HMO respectively. They were observed for visible signs of toxicity for 24 hours and the number of deaths if any was recorded and scored following the Lorke's method [14].

GC-MS Characterization of the Fatty Acids Constituents: This was done on the HMO dissolved in chloroform using an Agilent gas chromatograph Model 6890, coupled to a Mass spectrometer equipped with a DB DB-1MS capillary column (30m long × 320µm nominal diameter), programmed from 120 °C (5 min) to 250 °C at 3 °C/min, with 5 min hold time. Helium was used as carrier gas (1.0ml/min) with sample injection in split mode (50:1). Injector and detector temperatures were 250 °C and 280 °C respectively. The mass spectrometer worked in electron impact mode at 70 eV with electron multiplier at 1600 V and ion source temperature at 180 °C. Mass spectra data were acquired in the scan mode in m/z range 50-550. The compounds characterised in HMO were identified by comparing their mass spectra and retention times with those of reference compounds in the NIST library [15, 16]. A quality factor > 80 used as criterion for acceptance.

Fourier Transform Infra Red (FTIR) Spectroscopic Analysis: The functional groups analysis of the fruits oil was conducted using FT-IR spectrophotometer. The HMO was examined neat and the IR spectrum was recorded.

Determination of Specific Gravity, pH, Refractive Index, and Viscosity: This was done using the specific gravity bottle. A dried and tarred 25ml specific gravity bottle was filled with distilled water and weighed. The water was poured out and the bottle dried. The specific gravity bottle was then filled with the HMO and weighed. The specific gravity of the HMO was then evaluated as the ratio of the weight of 25ml of HMO to the weight of equivalent volume of distilled water. The pH, Refractive index, and Viscosity were done respectively using the pH meter, Abbe's refractometer and Viscometer at 20 °C with the instrument pre-calibrated following the manufacturers instruction.

Determination of Saponification Value of the HMO: Two grams of the HMO was dissolved in 25ml of 0.5 M alcoholic KOH in a round bottom flask with ground glass joint. The flask was then fitted with a reflux condenser and the mixture heated in a boiling water bath for 1 hr with continuous stirring. One millitre of 1% phenolphthalein was added to the reaction mixture and titrated while still hot against 0.5 M HCl. A blank titration was also carried out and the saponification value calculated as follows:

Saponification Value = [28.05(b-a)] / weight of sample

Where b and a are the respective titre values from the blank and test titration.

RESULTS:

Characterisation of HMO:

GC-MS: See **Table 1**.

FTIR Spectrum (V cm^{-1}, neat): OH (3393.2), CH (2948.3), C=C and/or C=O (1663.3), and C-O (1102.8), and deformation frequencies for: saturated aliphatic C-H (1368.7) and cis - (or Z-) =C-H (761.3).

Safety Profile: LD_{50} (in mice) > 5000mg/kg.

Physicochemical Indices: pH: 6.4, Viscosity: 2.252, Refractive index: 1.472, Specific gravity: 0.918, Saponification value (mg KOH/g): 140.5.

DISCUSSION: GC-MS characterization of the oil afforded eight fatty acids derivatives representing a total of 62.75% (47.93 % unsaturated fatty acid and 14.82% saturated fatty acids) of the total peak area (see **Table 1**). Constituents corresponding to a total peak area of 38.25 % though resolved could not be characterised due to insufficient library data and quality factor being less than 80. The major unsaturated fatty acid derivatives include: linoleic acid methyl ester (IUPAC: 9, 12-octadecadienoic

acid methyl ester; 26.87%), linoleic acid (IUPAC: 9, 12-octadecadienoic acid; 12.72%), and linoleic acid ethyl ester (7.12%) which are all derivatives of the polyunsaturated fatty acid linoleic acid. Palmitates, stearates and behenates were the major saturated fatty acids. The FTIR spectroscopic analysis of the HMO is evident of the functional groups associated with these fatty acids which include stretching vibrations in the region for OH (3393.2 cm^{-1}), CH aliphatic (2948.3 cm^{-1}) C=C and / or C=O (1663.3 cm^{-1}), and C-O (1102.8 cm^{-1}) and the deformation frequencies for: saturated aliphatic C-H (1368.7 cm^{-1}) and olefinic =C-H (761.3 cm^{-1}). Linoleic acid (9, 12-octadecadienoic acid) is an essential PUFA which is metabolised into arachidonic acid the precursor of prostaglandins, leukotrienes, thromboxane and other related eicosanoids [17].

TABLE 1: GC-MS CHARACTERISATION OF FATTY ACIDS DERIVATIVE IN *H. MADAGASCARIENSIS* FRUITS OIL (HMO)

S. no	Compound Name	Class	Rt (mins)	% Area	Quality	Molecular Formula	Molecular ion m/z (% abundance)	Base peak m/z (% abundance)	Selected diagnostic fragment m/z (% abundance) [fragment]
1	Palmitic acid methyl ester	Saturated fatty acid ester	26.30	7.00	99	$C_{17}H_{34}O_2$	270(16.4)	74(100)	239(9.6)[M-OCH_3]
2	Palmitic acid	Saturated fatty acid	26.66	2.61	89	$C_{16}H_{32}O_2$	256(73.9)	73(100)	211(1.1)[M-COOH]
3	Linoleyl alcohol	Poly-unsaturated Fatty alcohol	27.11	1.22	95	$C_{18}H_{34}O$	266(7.2)	55(100)	248(16.4)[M-H_2O]
4	Linoleic acid methyl ester	Polyunsaturated fatty acid ester	28.13	26.87	99	$C_{18}H_{34}O_2$	294(14.6)	67(100)	263(10.4)[M-OCH_3]
5	Stearic acid methyl ester	Saturated fatty acid ester	28.29	4.09	99	$C_{18}H_{38}O_2$	298(40.8)	74(100)	267(14.3)[M-OCH_3]
6	Linoleic acid	Polyunsaturated fatty acid	28.50	12.72	99	$C_{18}H_{32}O_2$	280(38.1)	67(100)	265(7.6)[M-CH_3], 235(1.9)[M-COOH]
7	Linoleic acid ethyl ester	Polyunsaturated fatty acid ester	28.68	7.12	99	$C_{20}H_{36}O_2$	308(25.0)	67(100)	279(1.1)[M-CH_2CH_3], 263(28.6)[M-OCH_2CH_3]
8	Behenic acid methyl ester	Saturated fatty acid ester	32.11	1.12	98	$C_{23}H_{48}O_2$	354(89.2)	74(100)	323(12.3)[M-OCH_3], 311(58.5)[M-$CH_2CH_2CH_3$]
	Total Identified constituents	-	-	62.75	-	-	-	-	-
	Total un-identified	-	-	38.25	-	-	-	-	-

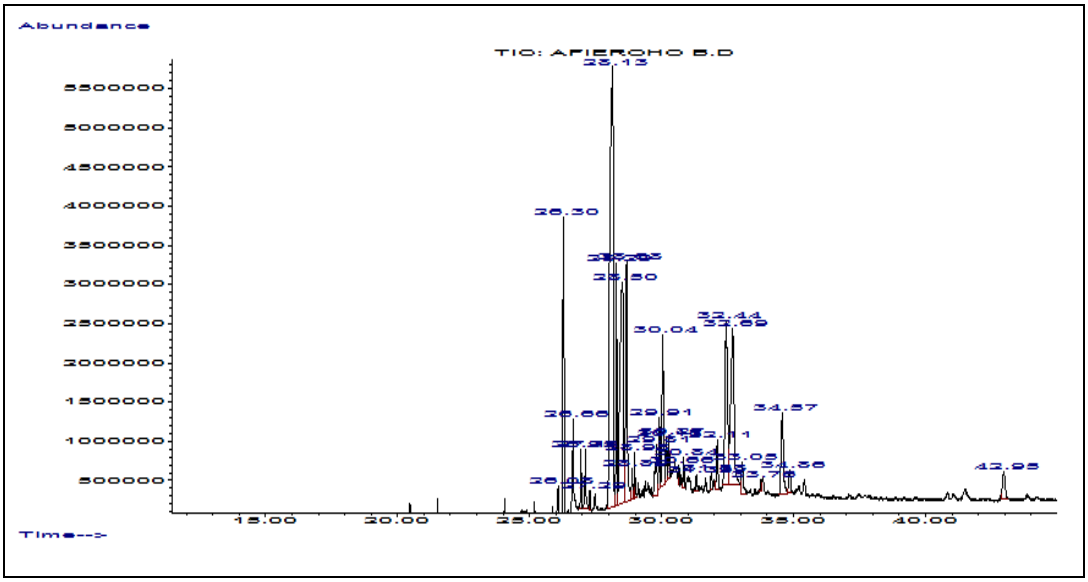

FIG. 1: TOTAL ION CHROMATOGRAM OF *H. MADAGASCARIENSIS* FRUITS OIL (HMO)

The docosanoates commonly referred to as behenates are saturated fatty acids derivatives like the stearates. Both are used as surfactants, lubricants and in cosmetology. Behenates have been reported to have hypercholesterolemic effects [18]. The trend in the Physical properties**:** pH: 6.4, Viscosity: 2.252, Refractive index: 1.472, Specific gravity: 0.918 are also indicative of vegetable oils. The very high Saponification value (140.5mg KOH/g) pointed to the potential application of the HMO in soap making. Generally, vegetable oils with high saponification values are important in the manufacture of detergents.

CONCLUSION: This work reports for the first time the phytochemistry of the fruit of *H. Madagascariensis* a plant widely used in ethnomedicine. The promising safety profile of the fruit oil extract as seen from the LD_{50} and the preponderance of unsaturated fatty acids is indicative of its nutritional and health benefits. The high saponification value as well as other promising physico-chemical properties pointed to the application of the oil extract from this non-utilised fruits in cosmetology, biodiesel and related industrial applications.

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CONFLICT OF INTEREST: There is no conflict of interest as regards the publication of this manuscript.

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