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Isoprenoids and Fatty Acids Derivatives from the Chloroform Fraction of the Antimycobacterial Methanol Extract *Ximeniaamericana* Lam. (Olacaceae) Stem Bark

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ABSTRACT

This study investigated the triterpenoids and fatty acid derivatives, and the *in vitro growth inhibitory effect against clinical strains of Mycobacteria tuberculosis* of the stem bark of *Ximenia Americanaa plant widely used in* ethno-medicine for the treatment of bacterial and skin infections, poison, post-partum hemorrhage, anaemia, and dysentery. The macerated methanol extract (XAM) of the stem bark was evaluated for anti-tuberculosis activity using the Lowensten Jensen method against de-contaminated clinical strains of *Mycobacterium tuberculosis*. The XAM was fractionated by open column chromatography on a normal phase silica gel column with a 25 % stepwise gradient of chloroform-methanol as mobile phase. The constituents of the non-polar column fractions eluted with 100% chloroform were characterized using Gas Chromatography-Mass spectroscopic (GC-MS) techniques and by comparison with reference NIST library compound. The XAM (5 mg/mL) inhibited the growth of the *Mycobacterium tuberculosis*. GC-MS analysis of the non-polar column fractions afforded Two lupane-type triterpenoids: Lup-20-(29)-en-3-one (15) and lupeol (16), three phytosteroids: campesterol (11), stigmasterol (12) and gamma-sitosterol (14), one fridelane-type triterpenoid: Friedelan-3-one (8), one oleanane-type triterpenoid: 12-oleanen-3-one (13), and the fatty acids: Palmitic acid methyl ester (1), Palmitic acid (2), 11-octadecenoic acid methyl ester (3), Octadecanoic acid methyl ester (4), Cis-13-Octadecenoic acid (5), 10,13-octadecadiynoic acid methyl ester (6), Docosanoic acid (7), Tetracosanoic acid (9), and Hexacosanoic acid methyl ester (10). The presence of these bioactive triterpenoids and fatty acids could offer an explanation for the ethno-medicinal uses of this plant. Further work is on-going to isolate in pure form, and characterized the bioactive constituents in the XAM with the view of discovery lead compounds for the treatment of tuberculosis and associated opportunistic bacterial infections.

Key words: *Ximeniaamericana*, isorenoids, fatty acids, tuberculosis, drug discovery

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INTRODUCTION

The worrisome global health challenge of drug resistant tuberculosis infections is making attention to be shifted to the use of the rich forest bio-diversities to combat this disease. Plant derived isoprenoids commonly called terpenoids and fatty acids are not only useful as chemosytemic markers, several of them are increasingly being reported to have potential as leads in the development of newer drugs for the treatment of diseases. Several plant derived isoprenoids with anti-tuberculosis activity have been documented (Cantrell *et al* 2001; Higuchi *et al* 2008; Akihisa*et al* 2005; Mann *et al* 2011). The Olacaceae family of plants of which *Ximenia Americana* is a specie, is known to contain bioactive triterpenoids and fatty acids derivatives. *Ximeniaamericana* commonly called tallow wood, yellow plum or sea lemon, is a small sprawling tree of woodlands widely distributed in the tropical region of Africa and America (Pott and Pott, 1994; Uchôa*et al* 2016).The lemon-yellow or orange-red fruits have a pleasant plum-like flavor. In Asia, the young leaves are cooked as a vegetable though with high cyanide as anti-nutrients. Virtually all its morphological parts are used in ethno-medicine (James*et al* 2008; Braga 1960). Preparations from the roots are

used to treat rheumatism (Mevy*et al* 2006), fever, jaundice, headaches, irregular menstrual flow, gastric disorders and as antiseptic (Uchôa*et al* 2016) while that from the leaves are used as laxative and in the treatment of measles (Omer and Elnima, 2003). The flowers infusion is used to reduce bloody diarrhea (Braga 1960). Scientific validation of its anticancer and antineoplastic (Voss *et al* 2006), antimicrobial (Omer and Elnima, 2003; Geyid*et al* 2005; Kon*éet al* 2004; Kawo*et al* 2011; Da Silva*et al* 2015), antipyretic (Soro*et al* 2009), radical scavenging (Maikai*et al* 2010; Le *et al* 2012) and pesticidal (Fatope*et al* 2000) have been documented. Literature on the phytochemistry of the roots (Fatope*et al* 2000), leaves and stem (Uchôa*et al* 2016) are documented. The root also contains the fatty acids: tariric acid and 10Z, 14E, 16E-octadeca-10, 14, 16-triene-12-ynoic acid (Fatope*et al* 2000). This present study reports on the anti-mycobacterial effects and the Gas Chromatography-Mass spectroscopy (GC-MS) characterisation of isoprenoids and fatty acids from the stem bark of *Ximeniaamericana.*

MATERIALS AND METHODS

The *Ximeniaamericana*stem bark were collected from the farmlands in Chaza Village, Suleja, Niger State Nigeria and authenticated at the Herbarium of the National Institute for Pharmaceutical Research and Development, Idu, Abuja, Nigeria. A voucher specimen (NIPRD/H/6417) has been deposited at the herbarium of the same institute.

Preparation of the crude methanol extract

The cold maceration extraction technique was used. The powdered dried plant material was macerated in absolute methanol for 72 hours and filtered to obtain the methanol filtrate. The residue was then repeatedly extracted with more portions of the methanol until a colorless extract was obtained. This is to achieve exhaustive extraction. The methanol filtrates were pooled together and concentrated by evaporation to dryness using a rotary evaporator. The weights of the dried crude methanol extract (XAM) was noted

Antimycobacterial susceptibility test

The agar dilution method using the egg-enriched Lowenstein-Jensen (LJ) medium (Jensen 1955) was used. Briefly, the 500 mg of the dried methanol extract of *Ximenia Americana* stem bark was dissolved in 4 ml dimethyl sulphoxide and diluted to 100 ml with the egg-enriched Lowenstein-Jansen (LJ) medium to give a final test concentration of 5 mg/ml. Isoniazid (10 ml of 200 μg/ml re-constituted to 100ml with the egg-enriched LJ medium), and dihydrostreptomycin (10 ml of 800 μg/ml re-constituted in the egg-enriched LJ medium) were used as standard control drugs at respective final concentrations of 0.2, and 0, 8.0μg/ml. 20 ml of the LJ medium on which the test sample/standard drug have been incorporated were poured into separate slant bottles which have been previously sterilized to form slants. De-contaminated clinical *Mycobacterium tuberculosis* isolate (positive to NO_3^- reduction, negative catalyst labile test and shows the presence of serpentinous cords on zinc smear) diluted in sterile water to 10^{-2} and 10^{-4}; corresponding to 1.0 and 0.5 McFarland respectively was used for inoculation. A 10.0 μlaliquot of each inoculants concentration (10^{-2} and 10^{-4}) was inoculated, in triplicates, into separate standard drugs, XAM and negative control LJ media slants and incubated for six weeks at 37 ºC. Inoculated media were checked after three days for non-*Mycobacterium. Tuberculosis* contamination and subsequently monitored weekly for growth. Colony counting was done following the International Union against Tuberculosis guideline.

Preparation of the isoprenoid and fatty acid rich extract

The methanol extract (XAM) obtained was then fractionated by eluting with chloroform using column chromatography packed with normal phase silica gel (Mesh 60-120) as adsorbent. The pooled fraction eluted with the chloroform was dried to obtained the triterpenoid and fatty acid rich extract XAC

Phytochemical screening

Confirmatory phytochemical tests for C-30 Isoprenoids (triterpenoids and steroids) were carried out on the XAC using the standard Lieberman-Buchard and Salkwoski phytochemical tests (Harborne 1998; Houghton and Raman 1999).

GC-MS Characterization of the triterpenoid and fatty acids constituents:

This was done on the XAC dissolved in chloroform using an Agilent gas chromatograph Model 6890, coupled to a Mass spectrometer equipped with a DB DB-1MS capillary column (30 m long × 320 µm 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 (1.0 ml/min) with sample injection in split mode (50:1). Injector and detector temperature were 250 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 characterized in XAC were identified by comparing their mass spectra, match factor (MF), reverse match factor (RMF), quality factor and retention times with those of reference compounds in the NIST library (Swigar and Silverstein 1981; Adams 1989). A MF or RMF of 900 or greater is an excellent match; 800–900, a good match; 700–800, a fair match. Less than 600 is a very poor match (Stein 2011). A quality factor > 80 % and MF/RMF > 800 was used as criterion for acceptance in this study.

RESULTS

Antimycobacterial susceptibility test (see Table 1): The XAM(yield 18.5 % w/w) showed a promising growth inhibition effects at the test concentration (5 mg/ml) with less than 19 colonies growth units at 1.0Mcfarlandand a no colony growth

at 0.5 Mcfarlandinnoculum concentration of the *M. tuberculosis*. The observed no colony growth at both 1.0 and 0.5 Mcfarlandinoculums concentration for the two standard drugs and the observed growth for the negative control is indicative that the *M. tuberculosis strains* used is viable and susceptible to both the standard drugs and the XAM.

Table 1: Antimycobacterial susceptibility test

Sample code	Description of sample	Final test concentration	Weekly observation report on innoculum growth												Remark
			1.0Mcfarland						0.5 Mcfarland						
			I	II	III	IV	V	VI	I	II	III	IV	V	VI	
Egg-Gly-LJ	Negative control	Not applicable	G	G	G	G	G	3+	G	G	G	G	G	1+	Viable innoculum
INH	Standard drug isoniazid	0.2 µg/ml	-	-	-	-	-	-	-	-	-	-	-	-	INH susceptible
DHS	Standard drug dihydrostreptomycin	8.0 µg/ml	-	-	-	-	-	-	-	-	-	-	-	-	DHS susceptible
XAM	*Ximenia Americana* stem bark methanol extract	5.0 mg/ml	-	-	-	-	-	+	-	-	-	-	-	-	Susceptible

Key: - =No innoculum growth observed, += 1-19 colonies growth observed, 1+=20-100 colonies growth observed, 2+= 100-200 colonies growth observed, 3+= 200-500 colonies growth observed, 4+ = > 500 confluent growth and G= observable on set of growth but were not quantified until the 6th week

GC-MSCharacterisation of chloroform fraction (XAC) of the anti-mycobacterial stem bark methanol extract of *X. americana* (See Table 2 and Figure 1): Two lupane-type triterpenoids: Lup-20-(29)-en-3-one (15) and lupeol (16), three phytosteroids: campesterol (11), stigmasterol (12) and gamma-sitosterol (14), one fridelane-type triterpenoid: Friedelan-3-one (8), one oleanane-type triterpenoid: 12-oleanen-3-one (13), and the fatty acids: Palmitic acid methyl ester (1), Palmitic acid (2), 11-octadecenoic acid methyl ester (3), Octadecanoic acid methyl ester (4), Cis-13-Octadecenoic acid (5), 10,13-octadecadiynoic acid methyl ester(6), Docosanoic acid (7), Tetracosanoic acid (9), and Hexacosanoic acid methyl ester (10). These accounted for constituents corresponding to 73.22 % of the total peak area.

Table 2: GC-MS analysis the chloroform fraction of the anti-mycobacterial stem bark methanol extract of X. americana

S/N	Compound Name	Class	Match Factor MF	Reverse Match factor RMF	Rt (mins)	% Area	Quality	Molecular Formular	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	942	942	18.89	2.96	93	$C_{17}H_{34}O_2$	270(18.4)	74(100)	239(9.6) [M-OCH_3]
2	Palmitic acid	Saturated fatty acid	917	924	21.16	6.71	96	$C_{16}H_{32}O_2$	256(50)	73(100)	227(12)[M-CH_2CH_3], 213(40)[M-$CH_2CH_2CH_3$],
3	11-octadecenoic acid methyl ester	Mono-unsaturated fatty acid ester	942	943	23.97	3.75	99	$C_{19}H_{36}O_2$	296(10)	55(100)	265(26)[M-OCH_3]
4	Octadecanoic acid, methyl ester	Saturated fatty acid ester	883	908	24.84	0.57	98	$C_{19}H_{38}O_2$	298(20)	74(100)	267(18)[M-OCH_3]
5	Cis-13-Octadecenoic acid	Unsaturated fatty acid	929	931	26.17	9.91	97	$C_{18}H_{34}O_2$	282(7)	55(100)	265(6)[M-OH]
6	10,13-octadecadiynoic acid methyl ester	Acetylenic fatty acid ester	829	833	29.17	1.78	99	$C_{19}H_{30}O_2$	290(4)	91(100)	259(10)[M-OCH_3]
7	Docosanoic acid	Saturated fatty acid	824	870	36.68	0.53	99	$C_{22}H_{44}O_2$	340(97)	57(100)	341(25)[M+1], 311(7)[M-C_2H_5], 297(40)[M-C_3H_7],
8	Friedelan-3-one	Triterpene ketone	924	928	40.63	4.55	99	$C_{30}H_{50}O$	426(38)	69(100)	411(19.6)[M-CH3]
9	Tetracosanoic acid	Saturated fatty acid	837	855	41.37	0.83	86	$C_{24}H_{48}O_2$	368(100)	368(100)	339(9)[M-C2H5], 325(9)[M-C3H7]
10	Hexacosanoic acid methyl ester	Saturated fatty acid ester	830	870	44.74	0.69	97	$C_{27}H_{54}O_2$	410(57)	74(100)	380(7)[M-OCH_3], 367(24)[M- C_2H_7]
11	Campesterol	phytosterol	836	883	49.09	1.07	96	$C_{28}H_{48}O$	400(100)	400(100) -	382(48)[M-H_2O], 273(24)[M-Aliphatic side chain], and 132(35), 120(40), 107(65) [peaks due to RDA cleavage at ring B]
12	Stigmasterol	Phytosterol	858	873	49.87	2.09	99	$C_{28}H_{48}O$	412(80)	55(100)	394(14)[M-H_2O], 273(24)[M-Aliphatic side chain], and 132(47), 120(32), 107(45) [peaks due to RDA cleavage at ring B]
13	12-olean-en-3-one	Triterpene ketone	847	871	50.58	0.93	97	$C_{30}H_{48}O$	424(12)	218(100)	409(8)[M-CH_3], and diagnostic peaks due to RDA cleavage at ring 218(100), 203(50)
14	Gamma-sitosterol	Phytosterol	878	902	52.02	26.15	96	$C_{29}H_{50}O$	414(100)	414(100)	396(50)[M-H_2O], 396(50)[M-H_2O-CH_3], 273(25)[M-Aliphatic side chain], and 32(37), 120(42), 107(52) [peaks due to RDA cleavage at ring B]
15	Lup-20(29)-en-3-one	Lupane-type triterpene ketone	814	842	52.34	4.89	93	$C_{28}H_{48}O$	424(45)	205(100)	409(50)[M-CH_3], 396(10)[M-$2CH_3$], 382(5)[M-isopropenyl side chain], 189(48)
16	Lupeol	Lupane-type triterpene alcohol	881	919	52.94	5.81	96	$C_{28}H_{50}O$	426(100)	189(100)	411(25)[M-CH_3], 382(5)[M-isopropenyl side chain], 207(90)
	% Total identified constituents					73.22					

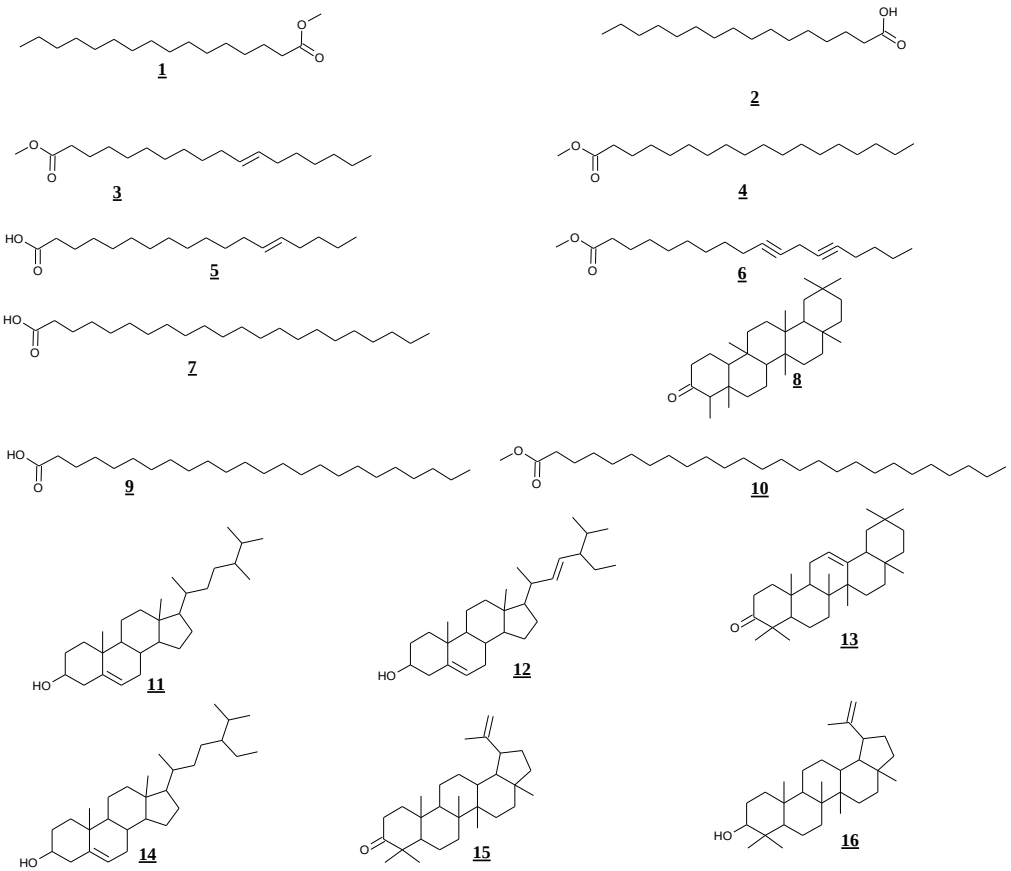

Figure 1: Fatty acids derivatives and isoprenoids characterized from the chloroform fraction of the anti-mycobacterial stem bark methanol extract of *X. americana*

DISCUSSION

Plant derived isoprenoids includes both terpenoids and steroids and are highly ubiquitous in plants with diverse biological and ecological properties. They are also used as chemosystemic markers. Several Plant derived lipids are of significance in nutrition, health, cosmetology and as biofuels. Dietary unsaturated fatty acids are essential to human health due to their role as precursors to the eicosanoids. They help in the lowering of plasma cholesterol thereby reducing the risk associated with cardiovascular health. This is a contrast to animal derived lipids. 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 (Yang *et al* 2011). Fatty acids derivatives of plant origin are also utilised as vehicle for drug delivery (Okorie *et al* 2010). 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 a hypercholesterolemic effects (Cater and Denke2001). The presence of friedelin, the lupane-type Triterpenoids : Lupeol, and Lup-20(29)-en-3-one, as well as the oleanane-type triterpenoid: 12-olean-en-3-one could explain the observed anti-mycobacterial activity of the XAM as friedelin (Akihisa*et al* 2005; Mann *et al* 2011), several lupaneand oleanane-type tritepenoids (Cantrell *et al* 2001; Higuchi *et al* 2008; Akihisa*et al* 2005) have been reported to have anti-tuberculosis activity *in vitro*. The presence of Phytosterols like campesterol, stigmasterol and sitosterol, have been reported to have cholesterol lowering effects (Rudkowska 2008; Heggen 2010).

CONCLUSION

This work reports for the first time the anti-tuberculosis effect of the stem bark of *Ximeniaamericana*a plant widely used in ethnomedicine. It also reported the characterization of bioactive saturated and unsaturated fatty acids derivatives and isoprenoids which could offer a rationale for nutritional and health benefits of this plant. Further work is on-going to isolate in the pure form the anti-mycobacterial constituents and elucidate the structure of same using spectroscopic techniques.

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