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Full Length Research Paper

Phytosterols from *Spondias mombin* Linn with Antimycobacterial Activities

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ABSTRACT

The growing problems of tuberculosis have led to the search for new anti-Mtb agents from higher plants. The stem bark of *Spondias mombin* was evaluated for its *in vitro* activity against *Mycobacterium tuberculosis* (H37Rv strain). Bioassay-guided fractionation of the methanol extract was carried out by Vacuum Liquid Chromatography (VLC) on Silica gel (230-400 mesh) and purification was done using HPLC and TLC. *In vitro* antimycobacterial susceptibility was performed by a fluorometric microplate alamar blue assay (MABA) and percentage mycobacterial inhibition was calculated. The structures of the isolated compounds were established by spectroscopic analysis. The active VLC fraction exhibited 91% inhibition against *M. tuberculosis* H37Rv at a concentration of 40 µg/mL. The HPLC fraction SMi-15 containing compounds **1** and **2** showed 92.8% inhibition against *M. tuberculosis*. Two new antimycobacterial phytosterols were isolated from the stem bark of *S. mombin* and the structureswere identified as mombintane I (**1**) and mombintane II (**2**). The stem bark extractives of *S. mombin* contain antitubercular principles of the class phytosterol and support an important potential of triterpenoids.

Keywords: *Spondias mombin*, *Mycobacterium tuberculosis*, antimycobacterial, mombintane I, mombintane II

INTRODUCTION

Spondias mombin is widely cultivated and naturalized in tropical Africa. A bark-slash exudes a clear sticky gum. Generally, each part of the plant has medicinal uses. A tea of the flowers and leaves is taken to relieve various inflammatory conditions and stomachache. The tea is also reputed to have wound healing potential (Burkill, 1995; Villegas *et al*., 1997). The stem bark of *S. mombin* is used traditionally in West Africa for the treatment of cough and other respiratory disorders (Morton, 1987). The phytochemical and pharmacological investigation of the leaf of *S. mombin* has been reported previously (Corthout*et al*., 1991; Corthout*et al*., 1992; Corthout*et al*., 1994; Coates *et al*., 1994; Ayoka*et al*., 2006; Fred-Jaiyesimi *et al*,2009; Silva *et al*., 2011). We reported previously the antitubercular property of the stem bark fractions of *S. mombin* (Olugbuyiro *et al*., 2009). In the course of further study on the chemical constituents of an anti-Mtb fraction of *S. mombin*, we report herein the isolation and structural elucidation of two new active

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phytosterols- mombintane I (**1**) and mombintane II (**2**) from the stem bark.

MATERIALS AND METHODS

General

The methanol extract prepared from stem bark of *S. mombin* was analyzed by VLC (Si gel 230-400 mesh, Merck) and HPLC (Waters Prep LC system 4000). Characterization was done using TLC chromatography (Si gel 60 F_{254} plates), NMR (400 MHz, in $CDCl_3$) and Mass spectroscopy (Bruker ESI-micrOTOF). *Mycobaterium tuberculosis* (H37Rv) was provided by Dr. S. Franzblau, College of Pharmacy, University of Illinois at Chicago.

Plant material

Spondiasmombin stem bark was collected in the environs of University of Ibadan, Nigeria in August, 2007 and identified by Dr. O.A. Ugbogu of Forestry Research Institute of Nigeria, Ibadan. A voucher specimen (FHI NO. 107896) was deposited.

Extraction and isolation

Bioassay guided fractionation of the crude extract was done by Vacuum Liquid Chromatography (VLC) using normal phase conditions. VLC was performed on Si gel 230-400 mesh (Merck) with gradient elution using hexane, EtOAc, MeOH and water in the order of increasing polarity. Eleven fractions were collected. The active portions VL2-3 were pooled and subjected to RP-HPLC (Luna C_8 column 21.2 x 250 mm) using a linear gradient from 85% water-15% acetonitrile to 100% methanol with flow rate 15ml/min. A total of 22 fractions were collected and similar fractions were pooled based on their proton NMR profile and the HPLC chromatogram. Altogether, 12 fractions were obtained and submitted for anti-Mtb assay. One of the active fractions, SMi-15 (ACN/MeOH 78:22), was subjected to further purification by reversed-phase HPLC with C_8 10 x 250 mm; eluted with ACN/MeOH (90:10-100:0) at a flow rate of 3ml/min which gave rise to 5 compounds. SMi-15-4 (**1**) and SMi-15-5 (**2**) were selected for characterization based on the MS and proton NMR spectra.

MicroplateAlamar Blue Assay (MABA)

In vitro antimycobacterial activitywas evaluated against *Mycobacterium tuberculosis* H37Rv using the microplate Alamar blue assay (MABA) as previously described (Collins and Franzblau, 1997; Franzblau et al., 1998). Antimicrobial susceptibility testing was performed in black, clear-bottomed, 96-well microplates(black view plates; Packard Instrument Company, Meriden, Conn.) in order to minimize background fluorescence. Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either dimethyl sulfoxide or distilled deionized water, and subsequent twofold dilutions were performed in 0.1 ml of 7H9GC (no Tween 80) in the microplates. Inocula were initially diluted 1:2 in 7H9GC and 0.1 ml was added to wells which resulted in bacterial titers of1 x 10^6 CFU/ml in plate wells for H37Rv. Wells containing drug only were used to detect autofluorescence of compounds. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37°C. Starting at day 4 of incubation, 20 µl of 10 x alamar blue solution (Alamar Biosciences/Accumed, Westlake, Ohio) and 12.5 µl of 20% Tween 80 were added to one B well and one M well, and plates were reincubated at 37°C. Wells were observed at 12 and 24 h for a color change from blue to pink and for a reading of ≥50,000 fluorescence units (FU). Fluorescence was measured in a Cytofluor II microplate fluorometer (PerSeptiveBiosystems, Framingham, Mass.) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. If the B wells became pink by 24 h, reagent was added to the entire plate. If the well remained blue or ≤50,000 FU was measured, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C, and results were recorded at 24 h post-reagent addition. Rifampicin was used as the reference drug.

Percent inhibition was defined as 1 - (test well mean FU/mean FU of triplicate B wells) x 100. The lowest drug concentration effecting an inhibition of ≥90% was considered the MIC.

RESULTS AND DISCUSSION

The anti-Mtb active fraction purified by RP-HPLC resulted to two compounds (**1** and **2**). Compound **1** was obtained as off-white amorphous solid by reversed-phase HPLC with Luna C_8 column. It was positive to Liebermann-Burchard test for a triterpene. ^{1}H NMR spectrum of **1** showed characteristic signals (Table 1) assignable to a sterol moiety (Yayli and Baltaci; 1996; Chen *et al*., 1998; Kökdila *et al.* 2002; Yan *et al.* 2007). The DEPT NMR analysis showed six methyls, nine methylenes, and ten methines. In the down field region of the DEPT spectrum there were two peaks, at δ_C 149.4 (C) and 121.8 ppm (CH) assignable to one olefinic bond which is located at Δ^9 position.

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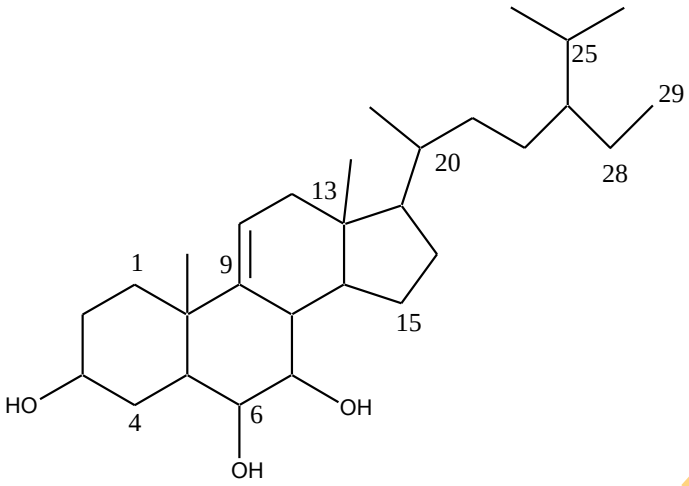

Fig. 1a.

Compound **1**- *Mombintane* I [*Stigmasta-9-en-3, 6, 7-triol*]: Isolated as off-white amorphous solid (1.8mg) R_f 0.80; ^{1}H- NMR ($CDCl_3$, 400 MHz): δ ppm 5.37 (1H, H-11), 3.68 (1H, H-7), 3.52 (1H, H-6), 3.50 (1H, H-3), 2.40 (1H, s, 3-OH), 2.35 (1H, s, 6-OH), 2.33 (1H, s, 7-OH), 2.02 (1H, H-8), 2.01(2H, H-12), 1.98 (1H H-25), 1.64 (1H, H-20), 1.59 (2H, H-2), 1.57 (2H, H-4), 1.55 (1H, H-5), 1.53 (1H, H-14), 1.47 (2H, H-15), 1.47 (2H, H-16), 1.47 (1H, H-17), 1.46 (1H), 1.40 (1H, H-1), 1.29 (2H, H-28), 1.26 (3H, H-19), 1.25 (2H, H-22), 1.25 (1H, H-23), 1.17 (3H, s, H-18), 1.26 (3H, s, H-19), 1.10 (3H, s, H-21), 0.87 (3H, H-29), 0.90 (3H, H-26), 0.90 (3H, H-27). ^{13}C- NMR ($CDCl_3$, 400 MHz): δ ppm 149.4 (C-9), 121.8 (C-11), 84.4 (C-7), 75.5 (C-6), 73.8 (C-3), 51.3 (C-17), 50.1 (C-14), 46.0 (C-24), 41.3 (C-13), 38.6 (C-12), 35.6 (C-5), 34.1 (C-10), 33.7 (C-22), 31.9 (C-8), 30.0 (C-2), 30.0 (C-25), 29.7 (C-20), 29.6 (C-23), 29.2 (C-4), 27.2 (C-1), 25.6 (C-28), 24.7 (C-15), 24.7(C-20), 22.6 (C-16), 21.0 (C-18), 21.1 (C-19), 19.3 (C-26), 19.2 (C-27), 18.2 (C-21), 14.1 (C-29). ESI-MS *m/z* 447 [M - H]$^-$, 430 [M-OH]$^-$, 413 [M-H-2H_2O]$^-$, HRESIMS *m/z* 446.3197 [M-H]$^-$, (calcd for $C_{29}H_{52}O_3$).

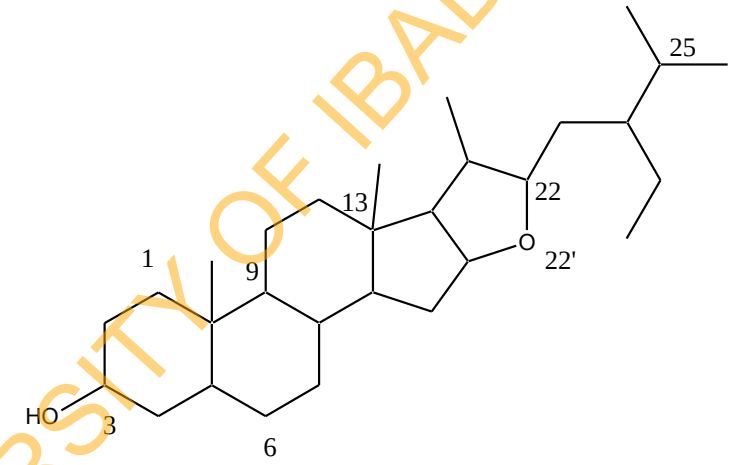

Fig. 1b.

Compound **2**- *Mombintane II* [*3-hydroxy, 22-epoxystigmastane*]: Isolated as off-white amorphous solid (1.7mg) R_f 0.86; ^{1}H- NMR ($CDCl_3$, 400 MHz): δ ppm 4.24 (1H, H-22), 4.22 (1H, H-16), 3.68 (1H, H-20), 3.50 (1H, H-3), 2.30 (1H, H-25), 2.29 (1H, H-17), 2.01 (3-OH), 1.66 (2H, H-15), 1.62 (1H, H-2), 1.54 (2H, H-4), 1.47 (1H, H-24), 1.41 (1H, H-5), 1.41 (1H, H-8), 1.40 (2H, H-6), 1.40 (2H, H-7), 1.40 (1H, H- 9), 1.40 (2H, H-11), 1.40 (1H, H-14), 1.37 (2H, H-1), 1.37 (2H, H-12), 1.29 (2H, H-28), 1.21 (3H, s, H-18), 1.21 (3H, s, H-19), 1.06 (3H, s, H-21), 0.92 (3H, s, H-26), 0.90 (3H, s, H-27), 0.88 (3H, s, H-29). ^{13}C-NMR ($CDCl_3$, 400 MHz): δ ppm 99.9 (C-22), 73.1 (C-16), 68.1 (C-3), 55.7 (C-9), 55.6 (C-17), 47.0 (C-14), 45.4 (C-12), 41.4 (C-5), 40.2 (C-24), 37.0 (C-10), 36.8 (C-4), 36.8 (C-13), 36.0 (C-8), 34.0 (C-1), 33.0 (C-23), 31.9 (C-7), 30.9 (C-25), 30.2 (C-2), 29.5 (C-6), 29.5 (C-15), 29.5 (C-20), 26.2 (C-28), 22.1 (C-11), 21.0 (C-26), 21.0 (C-27), 12.6 (C-18), 12.4 (C-19), 12.2 (C-21), 12.2 (C-29). ESI-MS *m/z* 430 [M - H]$^-$, 413 [M-OH]$^-$ HRESIMS *m/z* 430.3199 [M-H]$^-$, (calcd for $C_{29}H_{50}O_2$).

This is supported by the presence of olefinic proton signal at δ_H 5.3 (1H) ppm. The existence of molecular ion peaks at *m/z* 447, *m/z* 430 and *m/z* 413 inferred loss of two hydroxyl groups in succession from MS analysis and this established the presence of two additional hydroxyl groups on the stigmastane nucleus. The presence of proton signals at δ 2.35 (6-OH) and δ 2.33 (7-OH) also gave credence to the presence of two OH groups. The ESI-MS spectrum displayed molecular ion at *m/z* 413 [M-H]$^-$ common to stigmasterol. The molecular formula of compound **1**, $C_{29}H_{50}O_3$, was established via HRESIMS and the spectroscopic data was compared with those reported in the literature (Silverstein *et al*., 1991; Yayli and Baltaci; 1996; Chen

et al., 1998; Kökdila *et al*., 2002 and Yan *et al*., 2007). Compound **1** was identified as stigmasta-9-ene-3, 6, 7-triol and the name mombintane I is proposed for compound **1,**

Table 1.
^{1}H and ^{13}C NMR data for **1** (400 MHz , $CDCl_3$, J in Hertz and δ in ppm)

C/H No	δ_C	δ_H	C	δ_C	δ_H
1	27.2	1.40 (2H)	18	21.0	1.17(3H, s)
2	30.0	1.59 (2H)	19	21.1	1.26 (3H, s)
3	73.8	3.50 (1H)	20	29.7	1.64 (1H)
4	29.2	1.57 (2H)	21	18.2	1.10 (3H, s)
5	35.6	1.55 (1H)	22	33.7	1.26 (2H)
6	75.5	3.52 (1H)	23	29.6	1.26 (2H)
7	84.4	3.68 (1H)	24	46.0	1.46 (1H)
8	31.9	2.02 (1H)	25	30.0	1.98 (1H)
9	149.4	-	26	19.3	0.90 (3H, s)
10	34.1	-	27	19.2	0.90 (3H, s)
11	121.8	5.37 (1H)	28	25.6	1.29 (2H)
12	38.6	2.02 (2H)	29	14.1	0.87 (3H, s)
13	41.3	-	3-OH		2.40 (1H, s)
14	50.1	1.53 (1H)	6-OH		2.35 (1H,s)
15	24.7	1.47 (2H)	7-OH		2.33 (1H ,s)
16	22.6	1.47 (2H)			
17	51.3	1.47 (1H)			

Compound **2** was obtained off-white amorphous solid by reversed-phase HPLC with Luna C_8 column. It was also positive to Liebermann-Burchard test for a triterpene. Its molecular formula, $C_{29}H_{50}O_2$, was established by HRESIMS, ^{13}C NMR and DEPT spectroscopic data. ^{1}H NMR spectrum of **2** showed characteristic signals (Table 2) assignable to a sterol (Yayli and Baltaci; 1996; Chen *et al*., 1998; Kökdila *et al*. 2002, Yan *et al*. 2007). The DEPT NMR analysis showed six methyls, ten methylenes, eleven methines and two non-protonated carbon resonances assignable to stigmastane nucleus. In comparison with compound **1** however, there was absence of the olefinic bond in the down field region of both ^{13}C and ^{1}H spectra of **2**. The oxygenated carbon resonance at δ_C 68.1 revealed the presence of one hydroxyl group as supported by the proton NMR signals at δ_H 3.50 (H-3) while the signals at δ_C 99.9 (C-22) and 73.1 (C-16) could only be accommodated with the formation of a five membered ring E (tetrahydro furan) leading to 22-epoxycholestane of furostans class. The highly deshielded protons at δ_H 4.24-4.22 (H-16 and H-22) lent a support to the presence of an epoxide at C-22. The ESI-MS molecular ions in negative mode at *m/z* 430 $[M - H]^-$ and 413 $[M\text{-}OH]^-$ confirmed the fragment ions common to sterols (Yayli and Baltaci; 1996; Chen *et al*., 1998). Finally, high resolution mass measurement gave a molecular formula corresponding with $C_{29}H_{50}O_2$. The spectroscopic data suggested that the isolated molecule was furostan (**2**). Compound **2** was identified as *3-hydroxy-22-epoxystigmastane* by comparison of its ^{1}H- and ^{13}C -NMR and MS data with those reported in the literature (Yayli and Baltaci; 1996; Chen *et al*., 1998; Kökdila *et al*. 2002, Yan *et al*., 2007; DNP, 2011) and named *mombintane II*.

Table 2.
^{1}H and ^{13}C NMR data for **2** (400 MHz , $CDCl_3$, J in Hertz and δ in ppm)

C/H No	δ_C	δ_H	C/H No.	δ_C	δ_H
1	34.0	1.37 (2H)	16	73.1	4.22 (1H)
2	30.2	1.62 (2H)	17	55.6	2.29 (1H)
3	68.1	3.50 (1H)	18	12.6	1.21 (3H)
4	36.8	1.54 (2H)	19	12.4	1.21 (3H)
5	41.4	1.41 (1H)	20	29.5	3.68 (1H)
6	29.5	1.40 (2H)	21	12.2	1.06 (3H)
7	31.9	1.40 (2H)	22	99.9	4.24 (1H)
8	36.0	1.41 (1H)	22’	O	--
9	55.7	1.40 (1H)	23	33.0	1.39 (2H)
10	37.0	--	24	40.2	1.47 (1H)
11	22.1	1.40 (2H)	25	30.9	2.30 (1H)
12	45.4	1.37 (2H)	26	21.0	0.92 (3H)
13	36.8	--	27	21.0	0.90 (3H)
14	47.0	1.40 (1H)	28	26.2	1.29 (2H)
15	29.5	1.66 (2H)	29	12.2	0.88 (3H)
			3-OH		2.01 (1H, s)

Antibacterial activity is one of the cited biological properties displayed by triterpenoids. *Knowltonia vesicatoria* was reported (Labuschagné *et al*., 2012) to possess antimycobacterial property. Two active triterpenoids, stigmasta-5, 23-dien-3-ol and 5-(hydroxymethyl)furan-2(5H)-one, were isolated from *K. vesicatoria* and stigmasta-5,23-dien-3-ol was found active against a drug-sensitive strain of Mtb with a MIC of 50.00 µg/mL. The triterpene, 23-hydroxy-5a-lanosta-7, 9 (11), 24-triene-3-one, has been demonstrated active against sensitive strain of *M. tuberculosis* with MIC value of 12.5 µg/mL among thirty five tested plant products (Camacho-Corona, 1998). In this study, the *in vitro* antimycobacterial susceptibility against *M.*

tuberculosis H37Rv showed that the crude extract had 27% inhibition. The crude extract potency was enhanced by purification as reflected by the most active VLC fraction (Table 3), which exhibited 91% inhibition against *M. tuberculosis*. HPLC fraction SMi-15 containing compounds **1** and **2** showed 92.8% inhibition while the reference rifampicin had 99.7% inhibition. The findings identified two new anti-Mtb compounds of phytosterol class and support an important potential of triterpenoids as previously published in literature. This suggests *S. mombin* as a potential anti-Mtb plant for generating leads that may be useful for the treatment of tuberculosis.

Table 3.
Antimycobacterial activity of the VLC column fractions of *S. mombin* stem bark

#	*Sample*	*% Inhibition*
1	VL1	2
2	VL2	91
3	VL3	69
4	VL4	32
5	VL5	26
6	VL6	27
7	VL7	27
8	VL8	13
9	VL9	24
10	VL10	8
11	VL11	22
	Drug control	
	RMP	**99**
	INH	**94**

M.TB strains: H37RV,
Inhibition of ≥ 90% was considered active,
Assays run @ 40 µg/ml
INH= isoniazid, RMP= rifampicin

Conclusion
Two new antimycobacterial phytosterols were isolated from the stem bark of *Spondias mombin*. The compounds were identified as *stigmasta-9-ene-3,6,7-triol* and *3-hydroxy-22-epoxystigmastane* by means of spectroscopic analysis. This is the first report of isolation of these phytosterols from *Spondias mombin*.

Declaration of Interest
The authors report no declarations of interest.

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