SUPPLEMENTARY MATERIAL

Antimicrobial Compounds from Root, Stem Bark and Seeds of *Melia volkensii*

Rahab W. Kamau¹; Benard F. Juma^{1*} and Lilechi D. Baraza¹

¹ Department of Pure and Applied Chemistry, Masinde Muliro University of Science and Technology, P.O. Box 190-50100, Kakamega; Kenya

Abstract

Three compounds; toosendanin (**1**), kulactone (**2**) and scopoletin (**3**) were isolated from either the root-bark and/or the stem bark of *Melia volkensii*. Their structures were determined on the basis of spectroscopic data generated and by comparison with data from literature. **1** and **2**, isolated for the first time from *M. volkensii*, exhibited significant ($p < 0.05$) activity against *Escherichia coli* with Minimum Inhibitory Concentration of 12.5 $\mu\text{g/mL}$, close to that of neomycin (6.25 $\mu\text{g/mL}$). The compounds also exhibited high activity against *Aspergillus niger* (MIC 6.25 $\mu\text{g/mL}$ compared to 2.5 $\mu\text{g/mL}$ for clotrimazole). Dichloromethane and methanol seed, hexane stem-bark and methanol root-bark extracts exhibited activities towards *E. coli*, *Staphylococcus aureus*, *A. niger* and *Plasmodium falciparum*, respectively. Antimicrobial activity of the plant towards *A. niger*, *P. falciparum* and *S. aureus* is reported for the first time in the current work.

Keywords: *Melia volkensii*, antibacterial; antimalarial; *Plasmodium falciparum*

Table S1: ^1H - and ^{13}C - NMR (600 and 150 MHz in CD_3OD & 300 and 75 MHz in CDCl_3 data for compounds **1** and **2**, respectively

Compound 1		Compound 2		
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1	4.28 <i>d</i> (4.8)	70.9	1.84 <i>q</i> (3.0,5.4)	38.3
2	2.74 <i>td</i> (4.8, 15.6)	37.2	2.15 <i>dt</i> (3.6,13.8)	34.8
	1.82 <i>d</i> (14.2)	37.2		
3	5.20 <i>d</i> (4.2)	74.8	-	216.3
4	-	41.5	-	47.8
5	2.81 <i>dd</i> (4.2, 10.2)	29.6	1.79 <i>m</i>	52.6
6	1.72 <i>td</i> (3.6, 14.4)	26.2	2.17 <i>q</i> (10.2,13.2)	24.4
	2.55 <i>dt</i> (1.8,14.4)			
7	3.58 <i>s</i>	70.7	5.40 <i>dd</i> (3.0,6.6)	118.5
8	-	43.6	-	143.5
9	3.35 <i>s</i>	50.1	2.48 <i>m</i>	47.9
10	-	42.9	-	39.6
11	-	209.1	1.80 <i>q</i> (3.0,5.4)	16.9
12	5.34 <i>s</i>	79.7	1.50 <i>m</i>	29.3
13	-	46.9	-	35.5
14	-	73.6	-	55.2
15	3.80 <i>d</i> (2.4)	59.9	1.78 <i>t</i> (6.9)	35.7
16	2.03 <i>t</i> (3.0)	34.8	4.30 <i>q</i> (3.0,8.2)	82.5
	2.12 <i>dd</i> (6.6,7.2)			
17	2.88 <i>dd</i> (6.6,11.4)	39.8	2.31 <i>m</i>	58.2
18	1.38 <i>s</i>	15.8	1.00 <i>s</i>	21.6
19	4.32 <i>d</i> (12.6)	65.4	1.07 <i>s</i>	12.5
	4.25 <i>t</i> (6.6)			
20	-	124.2	2.79 <i>td</i> (5.4,14.4)	45.5
21	7.20 <i>s</i>	142.0	-	180.6
22	6.16 <i>d</i> (0.6)	113.3	1.98 <i>q</i> (7.8,14.4)	29.5
23	7.40 <i>t</i> (1.8)	143.7	2.03 <i>m</i>	26.1
24	-	172.2	5.15 <i>t</i> (1.8)	123.5
25	1.96 <i>s</i> (OAc)	21.0	-	132.6
26	-	172.8	1.73 <i>s</i>	25.7
27	2.07 <i>s</i> (OAc)	21.4	1.67 <i>s</i>	17.9
28	4.83 <i>s</i>	97.3	1.10 <i>s</i>	24.5
29	0.83 <i>s</i>	20.0	1.16 <i>s</i>	21.5
30	1.19 <i>s</i>	23.1	1.29 <i>s</i>	32.3

The assignments were made from COSY, HMQC and HMBC spectra. *J* values (in Hz) are as shown in brackets

Table S2: ^1H - and ^{13}C - (600 MHz in CDCl_3) NMR data for compound **3**

C. No.	H	C	COSY	HMBC
1	-	-	-	-
2	-	161.5	-	-
3	6.30, <i>d</i> , (9.6)	113.4	C-4	C-3, C-2, C-10
4	7.62, <i>d</i> , (9.6)	143.3	C-3	C-9, C-2, C-5, C-4
5	6.95 <i>s</i>	107.5	-	C-6, C-7, C-9, C-10
6	-	143.9	-	-
7-OH	6.17	149.7	-	C-7, C-6, C-8
8	6.87 <i>s</i>	103.2	-	C-7, C-9, C-4, C-5
9	-	150.2	-	-
10	-	111.5	-	-
ArOCH₃	3.98	56.4	-	-

J values (Hz) are as shown in brackets.

Table S3. Zones of inhibition of crude extracts against growth of microbes

Plant part	Crude extract	Zone of inhibition (mm) at various concentrations			
		1000 µg/mL	500 µg/mL	250 µg/mL	100 µg/mL
<i>Escherichia coli</i>					
Stem-bark	Dichloro-methane	8.7±0.58	8.2±0.25	-	-
Seeds	Hexane	9.0±1.00	-		
	Dichloro-methane	9.8±0.25	8.0±0.15	6.3±0.06	-
	Methanol	11.7±1.53	-	-	-
<i>Staphylococcus aureus</i>					
Root-bark	Methanol	19.4±0.40	16.0±0.20	10.9±0.36	8.8±0.25
Stem-bark	Dichloro-methane	18.2±0.25	9.8±0.25	-	-
	Methanol	12.0±0.15	9.8±0.20	8.0±0.10	-
Seeds	Methanol	17.1±0.36	18.2±0.13	15.0±0.20	11.2±0.25
<i>Aspergillus niger</i>					
Root-bark	Hexane	10.0±1.00	3.5±0. 20	-	-
	Dichloro-methane	18.3±1.53	14.1±0.36	10.2±1.04	-
	Methanol	20.7±4.16	15.0±0. 50	12.9±0.10	12.3±0.26
Stem-bark	Hexane	26.7±1.53	17.0±1.00	11.2±0.29	8.8±0.25
	Dichloro-methane	13.0±1.00	10.2±0.76	9.3±0.76	8.6±0.40
	Methanol	19.8±0.29	13.2±1.26	13.0±0.15	11.8±0.76
Seed	Hexane	10.3±0.58	-	-	-
	Dichloro-methane	23.3±1.53	16.1±0.66	11.0±1.00	-
	Methanol	19.2±1.04	15.0±0.20	-	-

Figure S1;- Percentage growth inhibition of *Plasmodium falciparum* by 0.01g/mL crude extracts from *Melia volkensii*

Experimental

1.1 General

TLC analysis was done using analytical silica gel 60 PF₂₅₄₊₃₆₆ pre-coated alumina plates (Merck, 0.25 mm thick). The resulting spots on TLC plates were visualized under UV light (254 nm and 365nm) and located using H₂SO₄/vanillin spray or by exposure to iodine vapour. Column chromatography was carried out on Silica Gel (Loba Chemie, 60-120 mesh) or (CDH, 60-120 mesh). Gel filtration for compounds' purification was achieved using Sephadex LH-20. The ¹H and ¹³C-NMR spectra were recorded in CDCl₃ or CD₃OD at 300 or 600 MHz and 75 or 150 MHz, respectively, on a Bruker Avance DPX 300 and DRX 600 Spectrometers. The full scan HR-ESI were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, USA) equipped with an infinity cell, a 7.0 Tesla superconducting magnet (Bruker, Karlsruhe, Germany).

1.2 Plant Material

Fresh root-bark and stem bark of *Melia volkensii* were collected from Kibwezi, Eastern Kenya, and its voucher specimen (MA/BIO/00289) was preserved in the department of Biological sciences, Faculty of Science, Masinde Muliro University of Science and Technology.

1.3 Extraction and Isolation

The plant materials were air dried in shade before grinding. The root-bark (1kg) was first soaked in hexane before being subjected to cold extraction using dichloromethane for 24 hours, three times. Gradient elution vacuum Liquid Chromatography (hex/EtoAc) on this extract (54 g) gave fractions A (10 g) and B (38.9 g). Fraction A was not followed further. Fractional crystallisation (MeOH/CH₂Cl₂) of fraction B gave white powder B₁ (1.2 g) and yellow gum B₂ (35.7 g). Powder B₁ was subjected to gradient elution column chromatography (silica gel; Hex/CH₂Cl₂ and CH₂Cl₂/MeOH) to give combined fractions, B_{1a}, B_{1b} and B_{1c} (by TLC analysis). Compound **1** (414 mg, white rod-shaped crystals) crystallised out from fraction B_{1b} (CH₂Cl₂/MeOH). Fraction B₂ (35.7 g) was subjected gradient elution column chromatography (hex/EtoAc) to give combined fractions B_{2a}-B_{2f}. Compound **2** (57.8 mg; white needle-shaped crystals) was obtained by crystallisation from fraction B_{2c} (hex/CH₂Cl₂). Fraction B_{2f} (4.8 g) was chromatographed on silica gel (80 g) using CH₂Cl₂/MeOH mixtures to give combined fractions B_{2f1} and B_{2f2}. Fraction B_{2f1} was further purified using a sephadex column (1:1 MeOH/C₂Cl₂) to obtain compound **3** (8.2 mg; white powder). Compound **1** (50 mg) was also isolated from dichloromethane stem-bark using similar procedures. Other crude extracts were obtained in a similar manner as described above.

1.4 Bioactivity Tests

A combination of agar well diffusion and serial dilution technique with some modification (Sen & Batra, 2012) was applied to all crude extracts and two pure compounds for both antibacterial and antifungal activity. Antiplasmodial activity of root-bark and seeds extracts from *Melia volkensii* was done by parasite lactate dehydrogenase (pLDH) method with some modifications (Ali et al., 2010).

1.4.1 Preparation of Test Samples

Antibacterial and antifungal test samples were prepared and concentrations of the samples varied by serial dilutions to make 500, 250 and 100 µg/mL as well as 100, 50, 25, 12.5, 6.25 and 2.5 µg/mL for crude extracts and isolated compounds, respectively. For each of the crude extracts subjected to antiplasmodial assay, 0.01 g of the sample was dissolved in dimethyl sulfoxide and pre-diluted in 1mL of Roswell Park Memorial Institute (RPMI) 1640 medium to make 10,000 µg/mL concentration.

1.4.2 Culture and Maintenance of Microorganisms

E. coli and *S. aureus* bacteria and *As. niger* fungi, were obtained from Department of Biological Sciences, Masinde Muliro University of Science and Technology. Nutrient agar and potato dextrose agar were used to culture bacteria and fungi, respectively. Nutrient/potato dextrose agar was dissolved in distilled water at a ratio of 1:26 agar (grams) to water (mL) and the mixture was sterilized by autoclaving at 121 °C for 15 minutes and then cooled for 5 minutes.

A Dd2 clone of *P. falciparum* was obtained from cryopreserved stocks at the US Army Medical Research Unit, Kenya. The parasite cultures were maintained in complete media made up of in-complete RPMI 1640 media supplemented with 0.2 % bicarbonate, 50 µg/mL gentamicin, 0.5% heat inactivated serum, 0.2 % glucose, 1 % lipids, 0.44 % hypoxanthine and group O+ human erythrocytes diluted to 5% haematocrit to mimic the human host environment. Synchronized cultures were obtained by enriching the young ring stage trophozoites using 5 % D-sorbitol. A 6 mL asynchronous parasite culture of approximately 7 % parasitaemia was centrifuged at 9.5 g for 5 minutes and the supernatant aspirated. The remaining packed cells (~300 µL) were re-suspended in 6 mL 5% D-sorbitol and incubated for 10 minutes. Thereafter, the cells were washed twice in RPMI 1640 medium and the supernatant containing the lysed mature parasites removed.

1.4.3 Antibacterial and Antifungal Tests

The molten agar was dispensed into sterile plates of 100 mm diameter at a ratio of 20 mL per plate and the agar allowed to solidify. 20 µL of each sample at different concentrations were placed in wells bored through the agar, at the middle of the plate. *E. coli*, *S. aureus* and *A. niger* were then streaked by spread plate method using a sterile loop uniformly on the agar, and then the plates incubated for 24 hours at 37 °C for bacteria and 25 °C for fungi. Neomycin and clotrimazole were used as positive controls for antibacterial and antifungal tests, respectively. Solvents without plant materials were used as negative control. Zones of growth inhibition were observed and measured in millimeters. The lowest concentration of tested compounds that inhibited visible bacterial growth on the culture plates was defined as minimum inhibitory concentration (MIC). The experiments were done in triplicate.

1.4.4 Plasmodium falciparum Growth Inhibition Assay

Parasitemia of synchronized *P. falciparum* culture was determined by microscopy and ensured that the cultures had mature trophozoite and schizont stages just before growth inhibition assay set-up. A Corning flat bottom sterile 96 well tissue culture plate with lid was used (triplicate wells). Additional three wells of parasitized red blood cells (pRBCs) and three wells of normal red blood cells (nRBCs) only were also included in the experimental set up. The final hematocrit in each of the wells was 1 % and a parasitemia of 0.3%. To each of the wells 50 µL of parasite suspension was added, except the triplicate wells for the nRBCs. The samples were all reconstituted in 1 mL of RPMI and 20 µL of the diluted sample added to the respective triplicate wells. To the pRBC and nRBC wells, only plain RPMI was added. The outer wells of the plate were filled with sterile water and the plate placed in a culture chamber with moistened Kimwipes to provide humidity. The chamber was then purged with mixed gas (5 % CO₂, 5 % O₂, 90 % N₂) for 2-3 min, sealed and placed in 37 °C incubator until harvest.

1.4.5 *Plasmodium falciparum* Growth Inhibition Assay (GIA) Harvest

42 hours after the GIA assay set-up the plate was removed from the assay chamber and was placed at 4 °C. Using a 50-300 µL multichannel pipettor the contents of the plate were gently mixed by pipetting up and down 5 times. After thorough mixing, 50 µl of each assay row was transferred to a similar row of C-bottom plate, containing 250 µL/well cold Phosphate Buffered Saline. The plates were then centrifuged for 10 minutes at 1,300 rpm at 4 °C then 250 µL of supernatant was removed out of C-bottom plates without disturbing the parasite/RBC pellet. The plate was kept in freezer at -20 °C until ready for Lactate Dehydrogenase (LDH) assay. The C-bottom plate was removed from the freezer and kept at room temperature for at least thirty minutes. Complete LDH substrate was prepared by adding 50 µL of 3-Acetylpyridine Adenine Dinucleotide (APAD) stock and 200 µL of Diaphorase stock to every 10 ml of nitro blue tetrazolium solution. The complete LDH substrate was added (100 µL per well) to the wells of the plate. The plate was then spun briefly (5-15 sec) at 1,800 rpm in the centrifuge to eliminate bubbles. It was covered with foil and placed on a flat-bed shaker at room temperature for 30 minutes. The plate was then read at 650 nm on SpectraMAX ELISA Reader at 30 minutes. The percent inhibition using absorbance at 650 nm (A₆₅₀), shown by SpectraMAX ELISA Reader was determined by application of the following calculation.

$$\text{Percent Inhibition} = 100\% - \left(\frac{\text{A}_{650} \text{ test sample} - \text{A}_{650} \text{ RBC only}}{\text{A}_{650} \text{ pRBC} - \text{A}_{650} \text{ RBC only}} \times 100\% \right)$$

2.0 Method of Data Analysis

Bioactivity of the isolated compounds towards *E. coli*, *S. aureus* and *A. niger* were presented as zones of inhibition in millimeters with values expressed as mean $\pm$ S.D. Plasmodial percentage inhibition was presented as mean percentage $\pm$ S.D. Statistical significance was determined using one way Analysis of Variance (ANOVA). Significant differences in activity between the test-samples and positive control was done by comparison of means by Student-Newman-Keuls (SNK) test ($\alpha = 0.05$). Samples with values of $p < 0.05$ were considered statistically significant.

3.0 References

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Sen, A. and Batra, A. 2012; Evaluation of antimicrobial activity of different solvent extracts of medicinal plant *Melia azedarach*. *Int. J. Curr. Pharm. Res.*, **4**: 67-73.

Figure S2: ^1H -NMR spectrum for Toosendanin

RWK49A (600) in CDCL₃

Figure S7: ¹³C-NMR spectrum for Scopoletin