ANALYSIS OF THE HUSK AND KERNEL OF THE SEEDS OF *MORINGA STENOPETALA*

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ABSTRACT. The ethanol extract of the kernel of the endemic plant *Moringa stenopetala* after silica gel column chromatography led to the isolation of 4-(α-L-rhamnopyranosyloxy)benzyl glucosinolate (**1**) and sucrose. The oil obtained by Soxhlet extraction with petrol was trans-esterified with BF_3-MeOH and analyzed by GC-MS. The results showed the presence of diverse fatty acids namely palmitic (11%), palmitoleic (1.2%), stearic (11%), oleic (63%), linoleic (1.2%), arachidic (6%) and the rare behenic acids (6%). The white glossy seed husk which comprises 25% of the whole seed yielded two compounds namely 4-(σ-L-rhamnosyloxy)benzyl alcohol (**2**) and allantoin (**3**). To the best of our knowledge this is the first report of compound **2** as a natural product. Allantoin is reported here for the first time from the genus *Moringa.*

INTRODUCTION

Moringaceae is a unique family of only one genus namely *Moringa* [1], comprising 14 species found in Asia and Africa. The dominant Asian species, *M. Oleifera* Lam. is widely distributed in the Indian subcontinent and also recently introduced in different parts of tropical Africa. *M. stenopetala* (Baker f.) Cufod. is endemic to East Africa mainly southern Ethiopia and northern Kenya. This species is known by different local names such as *Shiferaw* (Amharic), *Aleko* (Gamo Gofa), *Shalkayda* (Konso), *Haleko* (Burji), and *Halakwa* (Wollayta). Its common descriptive English name is cabbage tree (English),

M. stenopetala is a highly valued plant cultivated in home gardens in southern Ethiopia where the leaves are eaten as vegetables [2] and traditionally used against malaria [3], hypertension, asthma, and diabetes. It is also used to heal stomach-ache and expel retaining placenta in women [4]. Pharmacological reports showed that the leaves of *M. stenopetala* showed anti-leishmanial [5], antispasmodic [4], anti-hyperglycemic [6], antibacterial [7], and blood pressure lowering properties [8]. The major secondary metabolite in the leaves is rutin, 4-(4'-O-acetyl-σ-L-rhamnosyloxy) benzylisothiocyanate, and4-(4'-O-acetyl-σ-L-rhamnosyloxy) benzadehyde [9].

Moringa seeds are obtained from the pods of the *Moringa* tree. The seeds are covered by white fluffy husk which encapsulates the kernel (Figure 1). Removal of the husk by de-hulling yields the soft edible kernel, which when pressed yields oil. The seed cake, which is a byproduct obtained after pressing, is used for water treatment [10]. 4-(α-L-rhamnopyranosyloxy) benzylglucosinolate (**1**) was reported before from the seeds [3]. Two compounds from the husk and one additional compound from the kernel are reported here.

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Figure 1. Picture of *Moringa* seed before and after removal of husk.

EXPERIMENTAL

Plant material

Seeds of *M. stenopetala* were collected from Arba Minch. The plant was identified by Mr. Melaku Wondafrash; voucher specimen (YM002/2015) deposited at the National Herbarium of Addis Ababa University, Ethiopia.

Chemicals and instruments

All solvents used were analytical grade. Melting points were determined in Digital Melting Point Apparatus. Analytical TLC was run on a 0.25 mm thick layer of silica gel GF_{254} (Merck) on aluminum plate. Spots were detected by observation under UV light (254 nm) followed by spraying with vanillin in H_2SO_4. Column chromatography was performed using silica gel (230-400 mesh) Merck. Solvent was freed using Rotavapor BÜCHI, RE 121. NMR spectra were measured on Bruker Avance 400 NMR Spectrometer. UV-Vis spectra were recorded on T60 UV-Visible Spectrophotometer. Optical rotations were measured using Autopol®IV Automatic Polarimeter. The IR spectra of compounds were recorded using a Perkin-Elmer BX Spectrometer (400-4000 cm^{-1}) as KBr pellets.

GC-MS analysis were performed using Agilent Technologies 7820A gas chromatograph system equipped with HP-5 capillary column (30 m x 0.25 m; coating thickness, 0.25 µm) and Agilent technologies 5977 E mass spectroscopy ion trap detector. Analytical conditions were as follows: Injector and transfer line temperature are 220 and 260 ^{o}C, respectively; oven temperature programmed from 60 ^{o}C to 240 ^{o}C at 3 ^{o}C/min; carrier gas, helium at 1 mL/min; injection 5 µL; split ratio, 1:30. Identification of the constituents was based on search through mass\hunter\library\NIST11.L and mass\hunter\library\W9N11.L.

FT-MS

The high resolution positive and negative ion electrospray mass spectra were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance mass equipped with an Infinity™ cell, a 7.0 Tesla superconducting magnet, an RF-only hexapole ion guide and an external electrospray ion source. Nitrogen was used as drying gas at 150 °C. The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 µL h^{-1}. All data were

acquired with 512 k data points and zero filled to 2048 k by averaging 32 scans. The data were evaluated by the Bruker XMASS 7.0.8 software.

Extraction and isolation of kernel of M. stenopetala

Ground *M. stenopetala* kernel (230 g) were Soxhlet extracted with petrol ether (2.3 L) for 15 h, filtered and concentrated to afford 36% yellowish oil. The marc (100 g) was extracted with EtOH (500 mL) by shaking for 5 h with a mechanical shaker, filtered and concentrated to afford 3 g (3%) jelly solid. This was adsorbed and chromatographed over silica gel (230-400 mesh). Gradient elution was done using $CHCl_3$:MeOH to afford five fractions (4:1, 20 mL, 100 mg Fr1; 3:2, 30 mL, 80 mg, Fr2; 1:1, 30 mL, 50 mg, Fr3; 2:3, 40 mL, 98 mg, Fr4; 2:3, 40 mL, 300 mg, Fr 5). The fifth fraction was identified to be sucrose (300 mg). Fraction 4 (98 mg) was applied to size exclusion chromatography usingSephadex LH 20 and eluted with $CHCl_3$:MeOH (1:1) to afford two fractions (each 15 mL). The first and second fractions were identified as 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (**1**, 40 mg) and sucrose (40 mg), respectively.

Preparation of fatty acid methyl esters (FAME)

M. stenopetala oil (2 g) was placed in 25 mL round bottom flask which contained 6 mL hexane to which BF_3-MeOH (4 mL) was added. The reaction mixture was refluxed in water bath for 30 min. To the cooled mixture, 5 mL of water was added with shaking. The upper layer was separated by using separatory funnel, dried over anhydrous sodium sulphate, filtered and concentrated to afford 500 mg (25%). A small portion of the methylated fatty acids was dissolved in hexane and analyzed using GC-MS.

Extraction and isolation of compounds from the husk of M. stenopetala

The whole seed was separated first into husk (25%) and kernel. The finely powdered husk (100 g) was extracted with EtOH (600 mL), using a mechanical shaker (8 h), filtered and concentrated to afford 2 g (2%) yellowish jelly semisolid. This was adsorbed and applied on silica gel column with solvent system CH_2Cl_2:MeOH of increasing polarities as eluent to afford 27 fractions (each 40 mL). Fraction 8 (eluent CH_2Cl_2) was identified as stearic acid. Fraction 19 eluted with CH_2Cl_2:MeOH (4:1) was found to be compound **2** (20 mg) while fraction 24 eluted with 100% MeOH was identified as allantoin (20 mg).

4-(α-L-rhamnopyranosyloxy)benzylglucosinolate (1)

Yellowish jelly solid; TLC (R_f 0.5, mobile phase EtOAc:MeOH 3:2); UV λ_{max} (MeOH) nm: 245, 273; FT-MS: molecular formula $C_{20}H_{28}NO_{14}S_2^-$, *m/z* 570.0960 (calcd 570.5666 for $C_{20}H_{28}NO_{14}S_2^-$); IR ν_{max} cm^{-1}: 3400 (OH stretching), 2924 (C-H stretching), and 1613 (C=C stretching); ^{1}H-NMR (400 MHz, D_2O):δ_H 7.25 (2H, *d*, *J* = 8.40 Hz, H-3,5), 7.04 (2H, *d*, *J* = 8.40 Hz, H-2,6), δ 5.30 (1H, *d*, *J* = 1.36 Hz, H-1''), 1.13 (3H, *d*, *J* = 5.60 Hz, H-6''), 4.00 (2H, *bro. s*, H-7) and 4.00 - 3.00 (methine protons of the sugar moieties); ^{13}C-NMR (100 MHz, D_2O): δ_C162.5 (C-8), 154.6 (C-1), 129.4, (C-3, 5), 117.5 (C-2, 6), 98.8 (C-1''), 81.3 (C-1'), 60.2 (C-6'), 37.4 (C-7), 16.5 (C-6''), 79.8, 76.6, 72.0, 71.7, 70.0, 69.9, 69.4, 68.7 and 60.2 (C-6').

4-(σ-L-rhamnosyloxy)benzyl alcohol (2)

White solid; mp 153-155 ^{o}C; TLC (R_f 0.6 mobile phase EtOAc:MeOH, 4:1); $[\alpha]_{589}^{21}$ = -15 (0.4, MeOH); UV λ_{max} (MeOH) nm: 271; IR ν_{max} cm^{-1}: 3387 (OH stretching), 2920 (C-H stretching),

1612 (aromatic C=C), and 1064 (C-O stretching); ^{1}H-NMR (400 MHz, DMSO-d6):δ_H 7.22 (2H, *d*, *J* = 8.20 Hz, H-3), 6.96 (2H, *d*, *J* = 8.20 Hz, H-2), 5.32 (1H, *d*, *J* = 1.32 Hz, H-1'), δ 4.40 (2H, *d*, *J* = 4.73 Hz, H-7), 5.18 (1H, *d*, *J* = 4.23 Hz, OH), 5.92 (1H, *d*, *J* = 5.92 Hz, OH), and 4.81 (1H, *d*, *J* = 5.92 Hz, OH), 3.25 (1H, *m*, H-5'), 3.43 (1H, *m*, H-3'), 3.61(1H, *m*, H-2') and 3.62 (1H, *m*, H-4'), δ 1.07 (3H, *d*, *J* = 6.14 Hz, H-6'); ^{13}C-NMR (100 MHz, DMSO-d6): δ_C 155.3 (C-1), 136.2 (C-4), 128.4 (C-3), 116.6 (C-2), 98.8 (C-1'), δ 72.2 (C-4'), 70.8 (C-2'), 70.6 (C-3'), 69.7 (C-5'), 62.9 (C-7), 18.2 (C-6')

Allantoin (3)

White solid; mp 220-222 ^{o}C; TLC (R_f 0.3 mobile phase EtOAc:MeOH, 4:1); UV λ_{max} (MeOH) nm: 266; FT-MS: molecular formula $C_4H_6O_3N_4$,*m/z* 181.0332 (calcd 181.10537 for $C_4H_6O_3N_4Na$); IR ν_{max} cm^{-1}: 3438 (NH stretching), and 1715 (amide carbonyl); ^{1}H-NMR (400 MHz, DMSO-d6):δ_H 10.70 (1H, *br. s*, H-1'), 8.10 (1H, *s*,H-2'), 6.97 (1H, *d*, *J* = 8.00 Hz), 5.85 (2H, *s*, H-4'), 5.21 (1H, *d*, *J* = 8.00 Hz, H-3); ^{13}C-NMR (100 MHz, DMSO-d6): δ_C174.0 (C-1), 157.9 (C-2), 157.3 (C-4) and 62.8 (C-3).

RESULTS AND DISCUSSION

The composition of the yellowish oil obtained by Soxhlet extraction of the kernel of *M. stenopetala* with petrol ether (36%) was determined by gas chromatography-mass spectrometry (GC-MS), after converting the oil to fatty acid methyl esters using BF_3-MeOH. The fatty acids were identified by comparing the spectra of the components with the NIST Database. The GC-MS results showed that *M. stenopetala* oil contains a large proportion of double bond-containing fatty acids. The dominant fatty acid is oleic acid (63%), followed by palmitic (11%), stearic (11%), arachidic (6%), behenic (6%), palmitoleic (1.2%), linoleic (1.2%) and myristic acids (0.2%). These results are similar to that reported for oil from *M. stenopetala* growing in Kenya [11]. The oil of the seeds of *M. oleifera* is known as Ben Oil owing to the presence of 5-6% of behenic acid [12].

The marc after the above Soxhlet extraction was extracted with hot ethanol and chromatographed over silica gel to afford sucrose (340 mg) and compound **1** (40 mg) as amorphous solid:broad band at 3400 cm^{-1} due to hydroxyl stretching, molecular formula $C_{20}H_{29}NO_{14}S_2$ established from (-)-ESI-FT-MS, which displayed a molecular ion peak at *m/z* at 570.0960 (calcd 570.5666 for $C_{20}H_{28}NO_{14}S_2^-$).The peaks at *m/z* 570 and 97 are characteristic of 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate [13]. The ^{1}H-NMR spectrum of compound **1** displayed signals at δ 7.25 (2H, *d*, *J* = 8.40 Hz) and 7.04 (2H, *d*, *J* = 8.40 Hz) due to symmetrically placed protons on unsymmetrically *para* substituted aromatic ring. The anomeric proton signal at δ 5.30 (1H, *d*, *J* = 1.36 Hz) is characteristic of a rhamnopyranosyl group with α-configuration [14]. The presence of a rhamnosyl group was further substantiated by the appearance of terminal methyl signal at δ 1.13. The proton signals observed at δ 4.60 (1H, *d*, *J* = 3.2 Hz) and δ 4.00 (2H, *bro. s*) are due to H-1' and H-7, respectively.

The ^{13}C-NMR spectrum along with DEPT-135 displayed signals due to three quaternary carbons at δ 162.5 (C-8), 154.6 (C-1) and 130.6 (C-4); four methine carbon signals at δ 129.4 (C-3, 5), 117.5 (C-2, 6), 98.8 (C-1'') and 81.3 (C-1'); two methylene carbon signals at δ 37.4 (C-7) and 60.2 (C-6'); and one methyl resonance at δ 16.5 (C-6''). The remaining 8 carbon resonances observed in the region between δ 80.0 to 68.0 are attributed to methine carbon signals of the sugar moieties. The connectivity between C-7 and C-8 was evident from the observed HMBC correlation between the carbon signal at δ 162.6 (C-8) with the methylene proton signal at δ 4.00 (H-7). Another key Heteronuclear Multiple Bond Correlation (HMBC) observed was between the H-1'' signals and the quaternary carbon signal at δ 154.6 (C-1) which established the location of the rhamnopyranose group on C-1. On the basis of the above physical

and spectral evidence, compound **1** was identified as 4-(α-L-rhamnopyranosyloxy) benzylglucosinolate (**1**) (Figure 2).
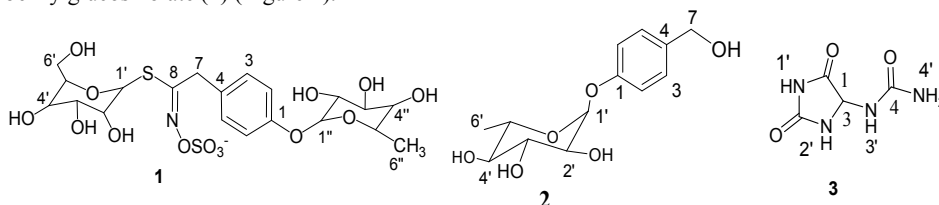

Figure 2. Constituents of *M. stenopetala* kernel (**1**) and husk (**2** and **3**).

Sucrose was isolated as a white solid and identified using NMR (^{1}H and ^{13}C), and FT-MS.Further confirmation was doneusing TLC and polarimetry against reference sucrose. Sucrose, which is reported here for the first time from *M. stenopetala*, accounts for the slight sweet taste of the seeds when chewed.

The ethanol extract of the husk of *M. stenopetala* was subjected to silica gel column chromatography which led to the isolation of two compounds **2** and **3** (Figure 2). Compound **2** was obtained as a white solid, mp 153-155 °C. It is optically active with $[\alpha]_{589}^{21}$ = -15 (c 0.4, MeOH). The UV-Vis spectrum (MeOH) of **2** displayed absorption maxima at 271 nm due to π→π* transition. The IR spectrum showed absorption band at 3387 cm^{-1} due to the presence of hydroxyl group. The proton NMR spectrum of **2** displayed signals at δ 7.22 (2H, *d*, *J* = 8.20 Hz, H-3) and δ 6.96 (2H, *d*, *J* = 8.20 Hz, H-2) due to symmetrically placed protons on unsymmetrically *para* substituted aromatic ring. The ^{1}H-NMR spectrum indicated the presence of one sugar unit. The anomeric proton signal at δ 5.32 (1H, *d*, *J* = 1.32 Hz, H-1') and the secondary methyl group resonance at δ 1.07 (3H, *d*, *J* = 6.14 Hz, H-6') suggest the sugar moiety to be a rhamnose unit. The coupling constant (*J* = 1.32 Hz) established rhamnose with α-configuration [14]. The two hydrogen doublet at δ 4.40 (2H, *d*, *J* = 4.73 Hz, H-7) is due to benzylic methylene protons on oxygenated carbon. The signals at δ 5.18 (1H, *d*, *J* = 4.23 Hz), 5.92 (1H, *d*, *J* = 5.92 Hz), and 4.81 (1H, *d*, *J* = 5.92 Hz) are attributed to protons on hydroxyl groups of sugar moiety as established by its HSQC spectrum. The series of signals at δ 3.25 (1H, *m*, H-5'), 3.43 (1H, *m*, H-3'), 3.61(1H, *m*, H-2') and 3.62 (1H, *m*, H-4') are due to methine protons of the rhamnose moiety.

The proton decoupled ^{13}C-NMR spectrum with the aid of DEPT 135 of compound **2** showed the presence of two quaternary, eight methine, one methylene and one methyl carbon signals. The quaternary carbons resonating at δ 155.3 and 136.2 are due to C-1 and C-4 of the aromatic ring, respectively. The other two signals in the aromatic region at δ 128.4 and 116.6 are due to C-3, 5 and C-2, 6, respectively. The spectrum further disclosed that the sugar unit is a rhamnopyranosyl moiety from the set of the chemical shifts at δ 98.8 (C-1'), 70.8 (C-2'), 70.6 (C-3'), 72.2 (C-4'), 69.7 (C-5') and 18.3 (C-6') in agreement with the proton NMR spectrum. The benzylic methylene carbon signal was observed at δ 62.9 (C-7).

The heteronuclear multiple bond correlation (HMBC) spectrum showed correlation between methylene proton at δ 4.40 (H-7) with the carbon at 128.4 (C-3) and 136.2 (C-4) establishing the site of attachment of C-7 to C-4 of the benzene ring. Another key correlation observed was between anomeric proton signal at δ 5.32 (H-1') with the oxygenated aromatic carbon signal at δ 155.2 (C-1) which established the site of attachment of rhamnose to the aromatic ring. This was in agreement with the UV-Vis spectral analysis which showed the absence of phenolic hydroxyl as no bathochromic shift was observed on addition of sodium hydroxide to the cuvette containing methanolic solution of compound **2**. Compound **2** was identified as 4-(α-L-rhamnopyranosyloxy) benzyl alcohol, a compound that has not been reported before.

Compound **3** was identified as allantoin, white solid, mp 220-222 ^{o}C. The asymmetric and symmetric stretching of primary amide were observed in the IR spectrum at 3438 and 3343 cm^{-1}, respectively. This was substantiated by the appearance of C-N stretching of primary amide at 1385 cm^{-1}. The intense band at 1715 cm^{-1} is characteristic of five membered cyclic lactams. The FT-MS showed molecular ion at *m/z* 181.0332 (calcd 181.10537 for $C_4H_6O_3N_4Na$) compatible with the molecular composition of $C_4H_6O_3N_4.$

The ^{1}H-NMR spectrum of compound **3**, identified as allantoin, displayed signals at δ 5.21 (1H, *d*, *J* = 8.00 Hz, H-3) and 6.97 (1H, *d*, *J* = 8.00 Hz, H-3'). The latter is due to a proton on heteroatom as observed from the absence correlation to a carbon signal in its HSQC spectrum. Other signals in the proton NMR spectrum were at δ 10.70 (1H, *s*,H-1'), 8.10 (1H, *s*,H-2') and 5.85 (2H, *s*,H-4'). The downfield signal at δ 10.70 is due to H-1'. The singlet signal integrating for two protons at δ 5.85 is assigned to H-4'. The proton decoupled ^{13}C-NMR with the aid of DEPT-135 spectrum of compound **3** showed the presence of well resolved resonances of four carbon atoms, three of which turned out to be quaternary (δ 174.0 (C-1), 157.9 (C-2) and 157.3 (C-4)) and one was a methine carbon signal (δ 62.8 (C-3)). Compound **3**is known to possess anti-inflammatory, anti-asthmatic and wound healing properties [15, 16].

In conclusion, the ethanol extract of the kernel of *M. Stenopetala* gave two compounds namely 4-(α-L-rhamnopyranosyloxy)benzylglucosinolate (**1**) and sucrose. The kernel was also found to be rich in fixed oil (36%) with diverse fatty acids including myristic (0.2%), palmitic (11%), palmitoleic (1.2%), stearic (11%), oleic (63%), linoleic (1.2%), arachidic (6%), and behenic acid (6%). Furthermore from the husk 4-(σ-L-rhamnosyloxy)benzyl alcohol (**2**)andallantoin (**3**) were isolated. To the best of our knowledgecompound **2**has not been reported before as a natural product.

ACKNOWLEDGMENTS

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