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Research Article

Chemical Constituents of *Moringa oleifera Lam.* Seeds

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ABSTRACT
Chemical studies of the ethanol extracts of the seeds of *Moringa oleifera* Lam. yielded 4-(4'-*O*-acetyl-α-L-rhamnosyloxy)benzyl isothiocyanate (1), a mixture of 1 and 4-(3'-*O*-acetyl-α-L-rhamnosyloxy)benzyl isothiocyanate (2) in a 2:1 ratio, 4-(α-L-rhamnosyloxy)benzyl isothiocyanate (3), a mixture of 3 and niazimicin (4) in a 2.5:1 ratio, triolein (5), β-sitosteryl oleate (6), and a mixture of β-sitosterol (7) and stigmasterol (8) in a 4:1 ratio from the immature seeds; 3, 5, 6, a mixture of 7 and 8 in a 3:1 ratio, oleic acid (9), and 1-octadecene (10) from the mature seeds; and 5, a mixture of 7 and 8 in a 4:1 ratio, and 9 from the brown seeds. The major constituents of the immature seeds are the isothiocyanates 1 and 3, while the major constituents of the brown seeds are the lipids 5 and 9. The structures of 1-10 were identified by NMR spectroscopy.

Keywords: *Moringa oleifera*, Moringaceae, 4-(4'-*O*-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate, 4-(3'-*O*-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate, 4-(α-L-rhamnosyloxy) benzylisothiocyanate, niazimicin, triolein, β-sitosteryl oleate, β-sitosterol, stigmasterol, oleic acid, 1-octadecene

INTRODUCTION

Moringa oleifera Lam locally known as malunggay has been used to combat malnutrition, specially among infants and nursing mothers[1]. Specific components of *M. oleifera* preparations which include the isothiocyanate compounds have been reported to exhibit hypotensive, anticancer and antibacterial activities[2]. A review of the nutritional, therapeutic and prophylactic properties of *M. oleifera* has been provided[1]. The ethanol extract of the seeds of *M. oleifera* afforded 4-(α-L-rhamnosyloxy) benzyl isothiocyanate, niazimicin and *O*-ethyl-4-(α-L-rhamnosyloxy) benzyl carbamate. The first two compounds showed very significant potential antitumor promoting activity[3]. The seeds of *Moringa oleifera* yielded 4-(α-L-rhamnosyloxy) benzyl isothiocyanate which exhibited minimum bactericidal concentration of 40 mmol/l for *Mycobacterium phlei* and 56 mmol/l for *Bacillus subtilis*[4]. The dichloromethane extract of the freeze-dried seeds of *M. oleifera* afforded 4-(4'-*O*-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate, 4-(α-L-rhamnosyloxy) benzylisothiocyanate, squalene and β-sitosterol. 4-(4'-*O*-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate and 4-(α-L-rhamnosyloxy) benzylisothiocyanate were cytotoxic against A549 with IC_{50} values of 10 and 12 mM, respectively, while Doxorubicin gave an IC_{50} value of 4 μM. These compounds were also cytotoxic against HCT 116 with IC_{50} values of 12 and 11 μM, respectively while Doxorubicin exhibited an IC_{50} value of 3 μM[5]. Another study reported the cytotoxicity of the constituents of the root bark of *M. oleifera* against doxorubicin resistant human breast cancer cell lines (Mcf-7/Adr)[6]. A recent study reported that 4-(4'-*O*-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate and 4-(α-L-rhamnosyloxy) benzylisothiocyanate significantly decreased gene expression and production of inflammatory markers in RAW macrophages. These compounds attenuated expression of *iNOS* and *IL-1*β and production of nitric oxide and TNFβ at 1 and 5 μM[7]. Another study reported the isolation of 4-(4'-*O*-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate from *M. oleifera*[8]. This study was conducted to determine the chemical constituents of *M. oelifera* seeds at different stages of maturity. We report herein the isolation of 4-(4'-*O*-acetyl-α-L-rhamnosyloxy)benzyl isothiocyanate (1), a mixture of 1 and 4-(3'-*O*-acetyl-α-L-rhamnosyloxy)benzyl isothiocyanate (2) in a 2:1 ratio, 4-(α-L-rhamnosyloxy)benzyl isothiocyanate (3), a mixture of 3 and niazimicin (4), triolein (5), β-sitosteryl oleate (6), and a mixture of β-sitosterol (7) and stigmasterol (8) in a 4:1 ratio from the immature seeds; 3-5, a mixture of 7 and 8 in a 3:1 ratio, oleic acid (9), and 1-octadecene (10) from the mature seeds; and 4, a mixture of 7 and 8 in a 4:1 ratio, and 9 from the brown seeds. The chemical structures of 1-10 are presented in Fig. 1.

EXPERIMENTAL

General Experimental Procedure

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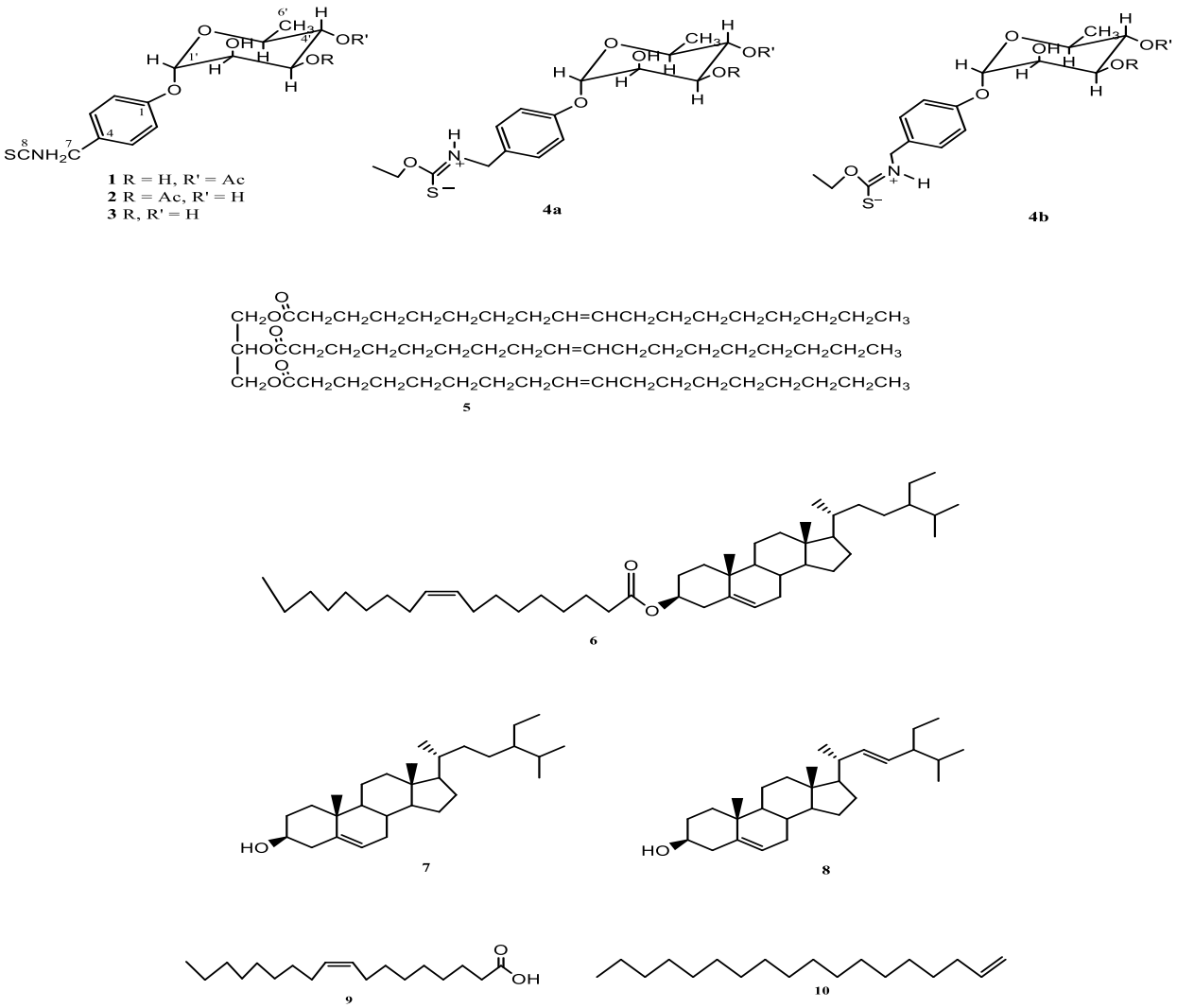

Figure 1: Chemical structures of 4-(4'-*O*-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate (1), 4-(3'-*O*-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate (2), 4-(α-L-rhamnosyloxy) benzyl isothiocyanate (3), niazimicin (4), triolein (5), β-sitosteryl oleate (6), β-sitosterol (7), stigmasterol (8), oleic acid (9), and 1-octadecene (10) from the seeds of *Moringa oleifera* Lam.

NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR spectra for all compounds except for **4** which was obtained in $(CD_3)_2SO$. Column chromatography was performed with silica gel 60 (70-230 mesh). Thin layer chromatography was performed with plastic backed plates coated with silica gel F_{254} and the plates were visualized by spraying with vanillin/H_2SO_4 solution followed by warming.

Sample Collection

Moringa oleifera Lam pods were collected from a farm in Laurel, Batangas, Philippines in April 2014.

General Isolation Procedure

A glass column 18 inches in height and 1.0inch internal diameter was used for the fractionation of the crude extracts. Ten milliliter fractions were collected. Fractions with spots of the same R_f values were combined and rechromatographed in appropriate solvent systems until TLC pure isolates were obtained. A glass column 12 inches in height and 0.5inch internal diameter was used for the rechromatography. Five milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

Isolation of Chemical Constituents of the Immature M. oleifera Seeds

The air-dried immature seeds (145 g) were ground in an osterizer. soaked in EtOH at room temperature for three days and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (19.2 g). The crude EtOH extract (17.2 g) was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment by volume. The 10% and 20% acetone in CH_2Cl_2 fractions were combined and rechromatographed (3 ×) in 15% EtOAc in petroleum ether to afford 5 (15 mg) and 6 (18 mg). The 30% acetone in CH_2Cl_2 fraction was rechromatographed in 15% EtOAc in petroleum ether to afford a mixture of 7 and 8 (22 mg) after washing with petroleum ether. The 40% to 50% acetone in CH_2Cl_2 fractions were combined and rechromatographed (3 ×)

using CH_3CN:Et_2O:CH_2Cl_2 (1.5:1.5:7 by volume ratio) to yield 1 (40 mg) and a mixture of 1 and 2 (15 mg) after trituration with petroleum ether. The 60% to 80% acetone in CH_2Cl_2 fractions were combined and rechromatographed (4 ×) using CH_3CN:Et_2O:CH_2Cl_2 (2.5:2.5:5 by volume ratio) to yield 3 (21 mg) and a mixture of 3 and 4 (55 mg) after trituration with petroleum ether.

Isolation of Chemical Constituents of the Mature M. oleifera Seeds

The air-dried mature seeds (658 g) were ground in an osterizer. soaked in EtOH at room temperature for three days and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (73.8 g). The crude EtOH extract (44.0 g) was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment by volume. The CH_2Cl_2 fraction was rechromatographed (2 ×) using petroleum ether to yield 10 (8 mg). The 10% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) in 2.5% EtOAc in petroleum ether to afford 6 (8 mg). The 20% acetone in CH_2Cl_2 fraction was rechromatographed (2 ×) in 7.5% EtOAc in petroleum ether to afford 5 (10 mg). The 30% acetone in CH_2Cl_2 fraction was rechromatographed in 15% EtOAc. The less polar fractions were combined and rechromatographed (3 ×) in the same solvent to afford a mixture of 7 and 8 (12 mg) after washing with petroleum ether. The more polar fractions were combined and rechromatographed in (3 ×) using CH_3CN:Et_2O:CH_2Cl_2 (0.5:0.5:9 by volume ratio) to yield 9 (7 mg). The 60% to 80% acetone in CH_2Cl_2 fractions were combined and rechromatographed (4 ×) using CH_3CN:Et_2O:CH_2Cl_2 (2:2:6 by volume ratio) to yield 3 (35 mg) after trituration with petroleum ether.

Isolation of Chemical Constituents of the Brown M. oleifera Seeds

The air-dried brown seeds (554 g) were ground in an osterizer. soaked in EtOH at room temperature for three days and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (26.5 g). The crude EtOH extract (20.0 g) was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment by volume. The 10% acetone in CH_2Cl_2 fraction was rechromatographed (2 ×) in 7.5% EtOAc in petroleum ether to afford **5** (45 mg). The 30% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) in 15% EtOAc in petroleum ether to afford a mixture of **7** and **8** (10 mg) after washing with petroleum ether. The 40% acetone in CH_2Cl_2 fraction was rechromatographed (2 ×) using CH_3CN:Et_2O:CH_2Cl_2 (0.5:0.5:9 by volume ratio) to yield **9** (125 mg).

4-(4'-O-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate (1)

^{1}H NMR (600 MHz, $CDCl_3$): δ 7.06 (H-2, H-6, d, *J* = 9 Hz), 7.24 (H-3, H-5, d, *J* = 9 Hz), 4.63 (H_2-7, s), 5.54 (H-1', d, *J* = 1.8 Hz), 4.13 (H-2', br s), 4.08 (H-3', d, *J* = 9.6 Hz), 4.85 (H-4', t, *J* = 9.6 Hz), 3.85 (H-5', m), 1.17 (H-6', d, *J* = 6.6 Hz), 2.12 (s, OAc), 3.19, 2.96 (2'-OH, 3'-OH, br s); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 156.0 (C-1), 116.6 (C-2, C-6), 128.4 (C-3, C-5), 128.1 (C-4), 48.2 (C-7), 132.2 (C-8), 97.3 (C-1'), 70.1 (C-2'), 70.7 (C-3'), 75.3 (C-4'), 66.5 (C-5'), 17.4 (C-6'), 172.3 and 21.0 (OAc).

4-(3'-O-acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate (2)

^{1}H NMR (600 MHz, $CDCl_3$): δ 7.06 (H-2, H-6, d, *J* = 9 Hz), 7.23 (H-3, H-5, d, *J* = 9 Hz), 4.63 (H_2-7, s), 5.48 (H-1', d, *J* = 1.8 Hz), 4.13 (H-2', br s), 4.07 (H-3', d, *J* = 9.6 Hz), 4.87 (H-4', t, *J* = 9.6 Hz), 3.84 (H-5', m), 1.29 (H-6', d, *J* = 6.0 Hz), 2.19 (s, OAc), 3.22, 3.10 (2'-OH, 4'-OH, br s); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 156.0 (C-1), 116.6 (C-2, C-6), 128.4 (C-3, C-5), 128.1 (C-4), 48.2 (C-7), 132.2 (C-8), 97.3 (C-1'), 69.5 (C-2'), 74.5 (C-3'), 71.2 (C-4'), 69.4 (C-5'), 17.5 (C-6'), 172.1 and 21.0 (OAc).

4-(α-L-Rhamnosyloxy) benzyl isothiocyanate (3)

^{1}H NMR (600 MHz, $CDCl_3$): δ 6.93 (H-2, H-6, d, *J* = 9.0 Hz), 7.11 (H-3, H-5, d, *J* = 9.6 Hz), 4.56 (H_2-7, s), 5.44 (H-1', br s), 4.13 (H-2', br s), 3.99 (H-3', dd, *J* = 3.0, 9.6 Hz), 3.57 (H-4', t, *J* = 9.6 Hz), 3.66 (H-5', m), 1.21 (H-6', d, *J* = 6 Hz), 5.14, 4.95, 4.83 (OH, br s); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 155.8 (C-1), 116.6 (C-2, C-6),128.4 (C-3, C-5), 128.2 (C-4), 48.1 (C-7), 131.9 (C-8), 97.8 (C-1'), 70.7 (C-2'), 71.2 (C-3'), 72.6 (C-4'), 69.1 (C-5'), 17.5 (C-6').

Niazimicin (4a/4b)

^{1}H NMR [600 MHz, $(CD_3)_2SO$]: δ 6.97 (H-2, H-6, d, *J* = 9.0 Hz), 7.20 (H-3, H-5, d, *J* = 9.0 Hz), 4.84 (H_2-7, d, *J* = 6.0 Hz), 5.32 (H-1', d, *J* = 1.2 Hz), 3.80 (H-2', m), 3.63 (H-3', m), 3.27 (H-4', m), 3.45 (H-5', m), 1.07 (H-6', d, *J* = 6 Hz), 4.38 (q, *J* = 7.2 Hz), 1.24 (t, *J* = 7.2 Hz), 9.52 (NH); ^{13}C-NMR [150 MHz, $(CD_3)_2SO$]: δ 155.1 (C-1), 116.2 (C-2, C-6),128.7 (C-3, C-5), 131.4 (C-4), 47.5 (C-7), 190.0 (C-8), 98.3 (C-1'), 70.1 (C-2'), 70.4 (C-3'), 71.7 (C-4'), 69.4 (C-5'), 17.9 (C-6'), 65.4, 14.3 (OCH_2CH_3).

Niazimicin (4a/4b)

^{1}H NMR [600 MHz, $(CD_3)_2SO$]: δ 6.97 (H-2, H-6, d, *J* = 9.0 Hz), 7.15 (H-3, H-5, d, *J* = 9.0 Hz), 4.22 (H_2-7, d, *J* = 6.0 Hz), 5.35 (H-1', d, *J* = 1.8 Hz), 3.80 (H-2', m), 3.63 (H-3', m), 3.27 (H-4', m), 3.45 (H-5', m), 1.07 (H-6', d, *J* = 6 Hz), 4.38 (q, *J* = 7.2 Hz), 1.22 (t, *J* = 7.2 Hz), 9.52 (NH); δ 155.0 (C-1), 116.3 (C-2, C-6),128.7 (C-3, C-5), 131.6 (C-4), 45.1 (C-7), 187.9 (C-8), 98.3 (C-1'), 70.1 (C-2'), 70.4 (C-3'), 71.7 (C-4'), 69.4 (C-5'), 17.6 (C-6'), 66.1, 14.0 (OCH_2CH_3).

RESULTS AND DISCUSSION

Silica gel chromatography of the EtOH extracts of *Moringa oleifera* seeds yielded 4-(4'-*O*-acetyl-α-L-rhamnosyloxy)benzyl isothiocyanate (1), a mixture of 1 and 4-(3'-*O*-acetyl-α-L-rhamnosyloxy)benzyl isothiocyanate (2) in a 2:1 ratio, 4-(α-L-rhamnosyloxy)benzyl isothiocyanate (3), a mixture of 3 and niazimicin (4) in a 2.5:1 ratio, triolein (5), β-sitosteryl oleate (6), and a mixture of β-sitosterol (7) and stigmasterol (8) in a 4:1 ratio from the immature seeds; 3, 5, 6, a mixture of 7 and 8 in a 3:1 ratio, oleic acid (9), and 1-octadecene (10) from the mature seeds; and 5, a mixture of 7 and 8 in a 4:1 ratio, and 9 from the brown seeds. The structures of 1-4 were elucidated by extensive 1D and 2D NMR spectroscopy. The structures of 1, 3, and 4 were confirmed by comparison of their ^{1}H and ^{13}C NMR data with those reported in the literature for *O*-acetyl-α-L-

rhamnosyloxy) benzyl isothiocyanate (1)[5], 4-(α-L-rhamnosyloxy) benzyl isothiocyanate (3)[5], and niazimicin (4)[9]. Niazimicin is a mixture of two rotamers (4a and 4b). An earlier study reported that 1 and 3 were the major isothiocyanates from *M. oleifera* seeds, while 2 was a minor constituent[7]. It is interesting to note that *M. oleifera* seeds contained isothiocyanates (1-3), thiocarbamate (4), lipid (5), and sterols (6-8), while the mature seeds contained isothiocyanate (3), lipids (5 and 9), and sterols (6-8) and the brown seeds contained only lipids (5, 7-9). Thus, the alkaloids (1-4) are found mainly in the immature seeds, while the brown seeds contained only lipids.

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