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Evaluation of Antibacterial Potential of Crude Extract of *Moringa oleifera* seed on Orthopaedics Wound Isolates and Characterization of Phenylmethanamine and Benzyl Isothiocyanate Derivatives

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ABSTRACT

The antibacterial activity of crude aqueous and methanolic extracts of *Moringa oleifera* seed on some orthopaedic wounds isolates which include *Klebsiella pneumoniae, Proteus vulgaris, Providencia stuartii, Escherichia coli, Streptococcus* sp., *Pseudomonas fluorescens, Acinetobacter baumannii, Burkholderia cepacia, Yersinia enterocolitica, Proteus mirabilis, Serratia rubidae, Salmonella pullorum* and *Klebsiella oxycota* was investigated. Both the crude aqueous and methanolic extracts of the seed demonstrated an appreciable inhibitory effects on the isolates with zone of growth inhibition ranging from 15 to 30.5 mm with aqueous extract and 9 to 20 mm with methanolic extract. Both extracts compared favourably with the reference standard antibiotics used. Minimum inhibitory concentration of the seed ranged from 0.875 to 5.0 μg mL^{-1} in aqueous extract and 0.875 to 2.5 μg mL^{-1} in methanolic extract. Phytochemical investigation of the methanolic extract of the seed led to the isolation and identification of new benzyl isothiocyanate and phenylmethanamine derivatives named 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxyl)-benzyl isothiocyanate (4) and 4-O-α-L-rhamnopyranosyloxy-N-glucopyranosyl-1→2-fructopyranosyloxy phenylmethanamine (5) along with three known compounds, 4-hydroxyphenyl acetic acid (1), O-methyl-4-(4'-O-acetyl-α-L-rhamnosyloxy) benzyl thiocarbamate (2) and 4-(α-L-rhamnopyranosyloxyl)-benzyl isothiocyanate (3). The structures were elucidated by extensive spectroscopic analyses which include Infra Red, Ultra Violet, Mass Spectrophotometer, 1D and 2D Nuclear Magnetic Resonance spectra as well as by comparison with literature data. Both the crude aqueous and methanolic extracts displayed broad spectrum of activity as they inhibited both the Gram negative and Gram positive bacteria tested.

Key words: *Moringa*, antibacterial activity, phenylmethanamine, benzyl isothiocyanate derivative, seed

INTRODUCTION

Moringa oleifera is an ancient tree that is historically known to posses numerous medicinal qualities (Posmontier, 2011) and it's a native to the sub-Himalayan parts of India, Pakistan, Bangladesh and Afghanistan. This rapidly-growing drumstick tree was utilized by the ancient Romans, Greeks and Egyptians and has become widely cultivated and naturalized in many locations in the tropics and sub tropics, West, East and South Africa, Latin America, the Caribbean, Florida and the Pacific Islands. Previous studies have reported the antimicrobial properties of the various parts of *Moringa* roots, flowers, bark and stem including seeds (Lockett *et al*., 2000; Ghebremichael *et al*., 2005; Anwar and Rashid, 2007; Walter *et al*., 2011). *Moringa* seeds are also known for its coagulation properties for treatment of water and waste water (Katayon *et al*., 2005; Oluduro and Aderiye, 2007). The seeds are used as a sexual virility drug for treating erectile dysfunction in men and also in women for prolonging sexual activity.

Traditional herbalists in Nigeria use variety of herbal preparations to treat different kinds of ailments including microbial infections (Oluduro and Omoboye, 2010). In folklore medicine, the preparation of medicinal plants in the treatment of microbial infection (both topical and systemic) have attracted a lot of the attention of scientists as readily available alternatives to the existing drugs to which many infectious microorganism have been resistant. *Moringa oleifera* preparations had been reported to possess antibiotic, antitripanosomal, hypotensive, antispasmodic, antiulcer, anti-inflammatory, hypoglycemic activities, as well as considerable efficacy in antibiosis and even reduction of *Schistosome cercariae* titre (Fahey, 2005; Posmontier, 2011). Its larvicidal and repellent potential has also been reported recently (Prabhu *et al*., 2011).

The seed kernel of *M. oleifera* seed has been employed in the treatment of bronchial asthma and found to show an appreciable decrease in severity of symptoms of asthma and also simultaneous improvement in respiratory functions (Fahey *et al*., 2001).

In particular, this plant family is rich in compounds containing the simple sugar, rhamnose and it is rich in a fairly unique group of compounds such as glucosinolates and isothiocyanates (Fahey *et al*., 2001; Bennett *et al*., 2003). For example, specific components of *Moringa* preparations that have been reported to have hypo-tensive, anticancer and antibacterial activity include 4-(4'-O-acetyl-α-L-rhamnopyranosyloxy)benzyl isothiocyanate [1], 4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate [2, 21], niazimicin [3], pterygospermin [4], benzyl isothiocyanate [5] and 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxyl)-benzyl thiocarboxamide [21]. While these compounds are relatively unique to the *Moringa* family, it is also rich in a number of vitamins and minerals as well as other more commonly recognized phytochemicals such as the carotenoids (including β-carotene or pro-vitamin A). These attributes are all discussed extensively by Fuglier (1999).

The seed extracts have also been found to be effective on hepatic carcinogen metabolizing enzymes, antioxidant parameters and skin pilloma-genesis in mice and the seed ointment had similar effect to neomycin against *S. aureus pyodermia* in mice (Costa-Lotufo *et al*., 2005). However, antibacterial resistance, especially among wound contaminating bacteria, is an important issue that has created a number of problems in treatment of chronic wounds infections and necessitates the search for alternative drugs or natural antibacterial agents.

Moreover, despite many researches that have been carried out on the inhibitory property of *M. oleifera* seed against several bacterial and fungal isolates from various sources, there is paucity of information about the antibacterial property of the seed on wound isolates. The present study therefore, investigates the antibacterial activity of crude aqueous extract of *M. oleifera* seed on

some orthopaedic wound isolates and characterization of novel phenylmethanamine and benzyl isothiocyanate derivatives from the methanolic extract of the seed.

MATERIALS AND METHODS

General: Solvents used for extraction and chromatography were redistilled before use. Adsorption column chromatography was performed using reversed-phase C_{18} column. All Thin Layer Chromatography (TLC) analysis were performed at ambient temperature using analytical silica gel 60 GF254+366 pre-coated aluminium backed plates (Merck, 0.25 mm thick). The resulting spots on TLC plates were visualized under UV light (254 nm) and detected by the use of vanillin/H_2SO_4, spray reagents. The UV spectra of isolated compounds were obtained on a computerized Perkin-Elmer Lambda Ultra Violet visible spectrophotometer at 200-700 nm. The Fourier transform IR spectra were recorded on Unicom spectrophotometer (in KBr pellets).

^{1}H-NMR spectra were recorded at 400 MHz and ^{13}C-NMR spectra were recorded at 100 MHz both in $CDCl_3$ with Me_4Si as internal standard and in $(CD_3)_2SO$ (DMSO) on Bruker DPX-400 MHz spectrophotometer, chemical shifts are expressed in δ (ppm) and coupling constants (J) in Hz. The Heteronuclear Multiples Quantum Correlation (HMQC) spectra that provide direct 'H and ^{13}C heteronuclear connectivity was determined. The distortionless Enhancement by Polarization Transfer spectroscopy (DEPT) was also utilized to distinguish between CH_3, CH_2 and CH. The EI- MS and FAB-MS were recorded on an APIIII (Sciex, UK). The temperature of the ionization source was 650°C, voltage 4.7 KV, N_2 as the sheat gas. The MS was obtained at a scan rate of 122×10^3 (0.5 scan per sec, dwelling time 1 sec).

Microorganisms: Bacterial isolates used for the antibacterial assay were isolated from the wounds of some orthopaedic in-patients of the Obafemi Awolowo University Teaching Hospital, Ile-Ife, Nigeria. Wounds samples of the patients were cultured on MacConkey and nutrient agar. Obtained isolates were identified and confirmed using conventional biochemical characteristics and Analytical Profile Index (API) 20E Micro tubes system (API Ltd. Product, France) where applicable and maintained on 0.8% nutrient agar slants at 4°C until used.

Collection and identification of plant material: *Moringa oleifera* (Lam.) seeds were harvested from the tree planted around the doctors' residential quarters at the State Specialist Hospital Complex in Ado-Ekiti. A voucher specimen identified by Dr. Illoh was deposited at the herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria.

Extraction and isolation: The seeds were dehusked and air dried at room temperature. The seed was ground into a fine powder using a scientific electric blender. The 100 and 500 g of the seed powder were soaked separately in 500 mL of water (aqueous extraction) and in methanol (methanol extraction) for 72 h at room temperature. Obtained filtered extracts (aqueous and methanol) were concentrated using a rotary evaporator at 45°C and 6 and 35 g of each extract were obtained.

The methanolic extract was fractionated on preparative HPLC using reverse phase C_{18} column with the gradient of solvent system $CHCl_3$-MeOH (1:1) to 100% MeOH at a flow rate of 4 mL min^{-1} as a mobile phase Fractions of about 20 mL each were collected and TLC of various fractions carried out on silica using chloroform: methanol (24:1) as the solvent system to monitor the purity of each compound. Many peaks were detected; however, only five major peaks coded as peak MO1, MO2, MO3, MO4 and MO5 were isolated pure in fairly sufficient amount for the elucidation of chemical structure.

4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxyl)-benzyl isothiocyanate (4): Light brown solid: IR (Kbr) Vmax cm^{-1} 3100, 2900; 1670, 1610, 1500, 1300, 1100 and 1000 cm^{-1}; UV (MeOH) λ_{max} 273 and 225 nm; FAB-MS m/z (rel.int.%) 496.2 $[M+Na]^+$ (10), 325 (18), 310.0 [M-163.8+H]$^+$ (5), 163.8 [M-310+H] 146 (25), 107.02 (10).

For ^1H NMR (400 MHz, $CDCl_3$ with Me_4Si) and ^{13}C NMR (100 MHz, $CDCl_3$ with Me_4Si) spectroscopic data (Table 2).

^1H NMR (400 MHz, $CDCl_3$ with Me_4Si) 7.07 (1H, d, J = 8.4), 7.36 (1H, d, J = 8.4), 4.23 (bs), α-rhamnose: δ 5.44 (1H, d, J = 3.2 Hz, H- 1'), 3.76 – 4.60 (m, H- 2', H- 3', H- 4', H- 5') , 1.26 (3H, d, J = 6.0Hz, H- 6'), Glucose: 5.18 (1H, J = 4.0 Hz, H- 1''), 3.44-3.62 (m, H- 2'', H- 3'', H- 4'', H-5'', H-6'').

^{13}C NMR (100 MHz, $CDCl_3$ with Me_4Si) 157.06 (C-1), 118.08 (C-2, C-6), 130.58 (C-3, C-5), 130.85 (C-4), 44.02 (C-7), rhamnose: 93.71 (C- 1'), 79.36 (C- 2'), 71.34 (C- 3'), 75.77 (C- 4'), 69.05 (C- 5'), 18.22 (C- 6'), Glucose: 99.87 (C- 1''), 82.53 (C- 2''), 77.98 (C- 3''), 69.45 (C- 4''), 72.81 (C- 5''), 62.02 (C- 6''), NH-C = S: 131.82.

4-O-α-L-rhamnopyranosyloxy-N-glucopyranosyl-1→2-fructopyranosyloxy phenylmethanamine (5): Light brown solid: IR (KBr) Vmax cm^{-1} 3410, 2900; 1670, 1590-1610 cm^{-1}; FAB-MS m/z (rel.int.%) 632.1 $[M+Na]^+$ (4), 463.1 [M-146+H] (4), 321 (15), 447 [M-163.8-H]$^+$ (3), 343.1 (22%), 163 [M-464.0+H]$^+$, 163.1 (M-147+146 (25), 107.02 (10).

For ^1H NMR {400 MHz, $(CD_3)_2SO$ (DMSO)} and ^{13}C NMR {(100 MHz, $(CD_3)_2SO$ (DMSO)} spectroscopic data (Table 3).

^1H NMR {400 MHz, $(CD_3)_2SO$ (DMSO)} 7.00 (1H, d, J = 8.4, H-2, H-6), 146. (23), 7.25 (1H, d, J = 8.4, H-3, H-5), 4.07 (1H, d, J = 8.0 Hz, 15.6 Hz), rhamnose : δ 5.35 (1H, bs, H- 1'), 3.44-3.78 (m, H- 2', H- 3', H- 4', H- 5') , 1.11 (3H, d, J = 6.0Hz, H- 6'), glucose: 5.17 (1H, J = 4.0 Hz, H- 1''), 3.44-3.64 (m, H- 2'', H- 3'', H- 4'', H- 5'', H- 6''), fructose: 3.81 (1H, bs, H- 1'''), 3.38 – 3.88 (m, H-3''', H- 4''', H- 5''', H- 6''').

^{13}C NMR {100 MHz, $(CD_3)_2SO$ (DMSO)} 155.01 (C-1), 116.50 (C-2, C-6), 129.10 (C-3, C-5), 129.77 (C-4), 37.02 (C-7), rhamnose: 91.71 (C- 1'), 70.40 (C- 2'), 77.00 (C- 3'), 74.25 (C- 4'), 69.71 (C- 5'), 17.89 (C- 6'), glucose: 98.46 (C- 1''), 82.53 (C- 2''), 77.98 (C- 3''), 69.45 (C- 4''), 72.81 (C- 5''), 62.02 (C- 6''), fructose: 60.92 (C- 1'''), 104.01 (C- 2'''), 81.28 (C- 3'''), 70.20 (C- 4'''), 71.60 (C- 5'''), 60.45 (C- 6''').

Antibacterial assay: The agar disc diffusion method described by Oluma *et al.* (2004) was used to determine the antibacterial activity. Two grams of concentrated seed extract was dissolved in 5% dimethyl sulfoxide (DMSO) and sterile discs (6 mm, Hi-media, India) were impregnated with 50 µL of 60 mg mL^{-1} of the seed extract. The discs were carefully and firmly placed on the Mueller-Hinton Agar (MHA) plates previously seeded with standardized bacterial suspensions (approximately 1.5×10^6 CFU mL^{-1}). Ciprofloxacin and ceftriaxone antibiotics were used as positive control and paper disc impregnated with DMSO was used as negative control. The plates were then incubated at 37°C for 24 h after which, the diameter of zones of growth inhibition around each disc was measured and recorded.

Minimum inhibitory concentration: Minimum Inhibitory Concentrations (MICs) of the crude aqueous and methanolic extracts of the seed on the wound isolates was carried out using agar

dilution technique (Andrews, 2009). The test extracts were dissolved in 5% DMSO to obtain 60 mg mL^{-1} stock solutions. Various concentrations (10.0, 5.0, 2.5, 1.25 μg mL^{-1}) were prepared from the stock solution from which 1 mL^{-1} each was added to 19 mL of sterile Mueller-Hinton Agar and poured into Petri dishes. The test organisms were then introduced into microtitre holes on the agar plates and incubated at 37°C. The least concentration of extract with no zone of growth inhibition was taken as the MIC of the extract.

RESULTS AND DISCUSSION

Antibacterial activity: All the wound isolates were sensitive to the seed extracts, with zones of inhibition varying from 6 mm in *Acinetobacter baumannii* in methanolic extract to 30.5 mm in *Streptococcus* sp. administered with 60 mg mL^{-1} of the aqueous extract of *Moringa oleifera* seed. *Streptococcus* sp. was the most sensitive to the extracts, with inhibition ranging between 20 and 30.5 mm. Meanwhile, *Acinetobacter baumannii* was the least sensitive (6-16.5 mm). *Burkholderia cepacia, Pseudomonas fluorescens* and *Yersinia enterocolitica* were resistant to 60 mg mL^{-1} of either ceftriaxone or ciprofloxacin (Table 1a). In all cases, the activity of the aqueous extract was greater when compared to the effectiveness of either the methanol extract or any of the antibiotics administered. For example, the aqueous extract recorded between 110 and 205% effectiveness on *Acinetobacter baumannii* and *Streptococcus* sp., respectively than when either of the antibiotics was used (Table 1a). Minimum inhibitory concentration of the seed ranged from 0.875 to 5.0 μg mL^{-1} in aqueous extract and 0.875 to 2.5 μg mL in methanolic extract (Table 1b). The findings concur with earlier studies. Spiliotis *et al.* (1997) reported antimicrobial activity from various varieties of *Moringa oleifera* seeds against *Bacillus cereus, Candida albicans, Streptococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Pseudomonas aeruginosa, E. coli* and *Aspergillus niger*. Jabeen *et al.* (2008) evaluated antimicrobial activity from the seeds

Table 1a: Antibacterial activity of the crude extract of *Moringa oleifera* seed on wound isolates

	Diameter of zone of growth inhibition (mm)			
			Antibiotics (30 mg mL^{-1})	
Bacterial isolates	Aqueous extract (60 mg mL^{-1})	Methanolic extract (60 mg mL^{-1})	Ceftriaxone	Ciprofloxacin
Klebsiella pneumonia	17	12	15	15
Proteus vulgaris	17	13	15	15
Providencia stuartii	17	12	R	15
Escherichia coli (ATCC2592)	22	18	15	15
Streptococcus sp.	30.5	20	15	15
Pseudomonas fluorescens	23	17	R	R
Acinetobacter baumannii	16.5	6	15	15
Burkholderia cepacia	17	14	R	R
Yersinia enterocolitica	15	11	R	R
Proteus mirabilis	16	13	6	R
Serratia rubidae	17	10	24	10
Salmonella pullorum	24	11	20	15
Klebsiella oxycota	19.5	9	15	15

R: Resistance

Table 1b: Minimum inhibitory concentration of *M. oleifera* seed

	MIC concentration (μg mL^{-1})									
	Aqueous extract					Methanolic extract				
Test organisms	10	5	2.5	1.75	0.875	10	5	2.5	1.75	0.875
Klebsiella pneumonia	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris	-	-	+	+	+	-	-	-	-	+
Providencia stuartii	+	+	+	+	+	+	+	+	+	+
Escherichia coli (ATCC2592)	-	-	-	+	+	+	+	+	+	+
Streptococcus sp.	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	-	-	+	+	+	-	-	-	+	+
Acinetobacter baumannii	-	-	+	+	+	-	-	-	-	-
Burkholderia cepacia	+	+	+	+	+	-	-	-	+	+
Yersinia enterocolitica	-	-	-	-	-	+	+	+	-	+
Proteus mirabilis	-	-	-	-	+	-	-	-	+	+
Serratia rubidae	+	+	+	+	+	+	+	+	+	+
Salmonella pullorum	+	+	+	+	+	+	+	+	+	+
Klebsiella oxycota	-	-	-	+	+	+	+	+	+	+

-: No growth; +: Growth

of *Moringa oleifera* against bacterial (*Pasteurella multocida, Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus*) and fungal (*Fusarium solani* and *Rhizopus solani*) strains. Authors reported that the zones of growth inhibition showed greater sensitivity against the bacterial strains as compared to the fungal strains and that Minimum Inhibitory Concentrations (MIC) of the extracts revealed that *Pasteurella multocida* and *Bacillus subtilis* were most sensitive strains.

The inhibitory property noticed in the seed of *M. oleifera* may be traced to the presence of various compounds present in the seed including the two newly reported benzyl isothiocyanate and phenylmethanamine derivatives in this study viz., 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxyl)-benzyl isothiocyanate (4) and 4-O- α-L-rhamnopyranosyloxy-N-glucopyranosyl-1→2-fructopyranosyloxy phenylmethanamine (5).

Structure elucidation: HPLC fractionation of the methanolic extract from the seeds of *M. oleifera* yielded compounds 1-5 (Fig. 1). Compounds 1, 2 and 3 are phenyl acetic acid derivative, benzyl isothiocyanate and thiocarbamate derivatives. Based on HMBC and comparison of its spectroscopic data (MS, ^{1}H and ^{13}C NMR) to those reported in literature, compound 1 was identified to be 4-hydroxyphenyl acetic acid (Saleem, 1995), compound 2 was identified to be O-methyl-4-(4'-O-acetyl-α-L-rhamnosyloxy) benzyl thiocarbamate commonly known as niazicin A (Faizi *et al*., 1995; Fahey, 2005) while compound 3 was identified to be 4-(α-L-rhamnopyranosyloxyl)-benzyl isothiocyanate (Faizi *et al*., 1995; Saleem, 1995; Oluduro *et al*., 2010) which has been reported isolated previously from the same plant.

The molecular formula 4 was established to be $C_{20}H_{27}NO_{10}S$ by FAB mass spectrum ($[M + Na]^+$ at m/z 496.2). The mass spectrum also gave a significant fragment ion at m/z 325.1 ($C_{12}H_{21}O_{10}$), m/z 310.1 ($C_{14}H_{16}NO_5S$), m/z 163.1 ($C_6H_{11}NO_5$), m/z 163.1 ($C_6H_{11}O_5$) and m/z 146.02 (C_8H_6NS) and m/z 107.02 (C_7H_9N) which substantiated the structure of glycoside 4. The ultraviolet/visible spectrum of compound 4 showed maximum absorption at 273 and 225 mm in MeOH. The infra-red spectrum

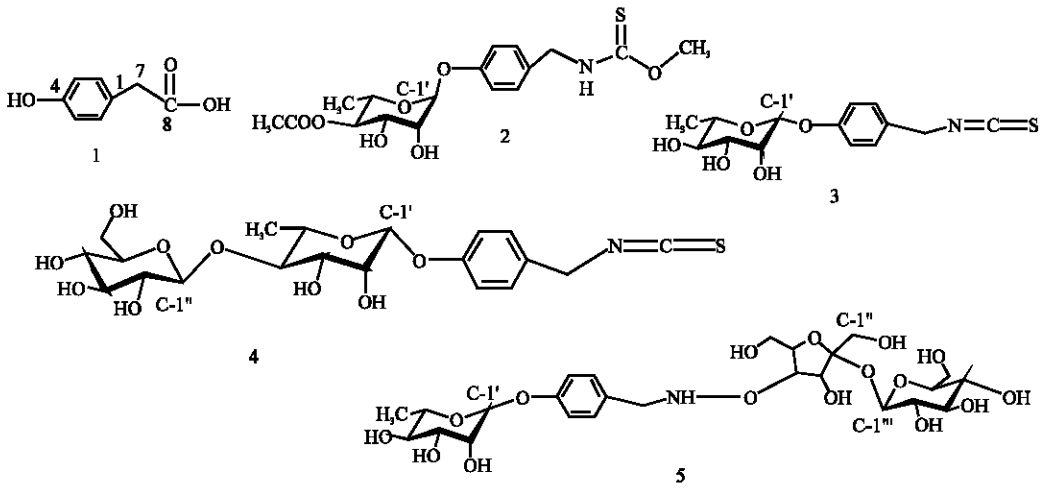

Fig. 1: HPLC fractionation of the methanolic extract from the seeds of *M. oleifera* yielded compounds 1-5

Table 2: ^{1}H- and ^{13}C-NMR data of compound 4 (400 MHz for ^{1}H-NMR, 100 MHz for ^{13}C-NMR, in $(CD_3)_2SO$ (CD_3OD)

No	^{1}H	^{13}C	HMBC
C-1		157.06	
C-2, 6	7.07 (d, 8.4 Hz)	118.08	130.58, 130.85, 157.06
C-3, 5	7.36 (d, 8.4 Hz)	130.58	118.08, 130.85,157.06
C-4		130.85	
C-7	4.23 bs	44.02	130.58, 130.85, 131.82
Rhamnose			
C-1'	5.44 (d, 3.2 Hz)	93.71	71.34, 79.36, 157.06
C-2'	4.14 (m)	79.36	71.34, 75.77, 93.71
C-3'	4.11 (m)	71.34	75.77, 79.36
C-4'	4.60 (m)	75.77	71.34, 69.05
C-5'	3.76 (m)	69.05	75.77, 71.34
C-6'	1.26 (d, 6.0 Hz)	18.22	69.71
Glucose			
C-1"	5.18 (d, 4.0 Hz)	99.87	75.77, 82.53
C-2"	3.62 (m)	82.53	77.98, 99.87
C-3"	3.48-3.50 (m)	77.98	82.53, 69.45
C-4"	3.44-3.47 (m)	69.45	77.98, 72.81
C-5"	3.56-3.60 (m)	72.81	69.45, 62.02, 77.98
C-6"	3-60-3.64 (m)	62.02	72.81, 69.45
	3.49-3.51 (m)		
NH-C = S		131.82	

Coupling patterns and coupling constants (J) in Hz are given in parentheses

also revealed several characteristic bands at the following peaks 3100, 2900; 1500, 1300,1100 and 1000 and were assigned to C-H and C-O-C (stretch asymmetric) and C-O-C (stretch symmetric) vibrations, respectively. The 1670 and 1610 bands were indicative of C = S and N-H bonds signaling the presence of thiocarbonate linkage in the molecule.

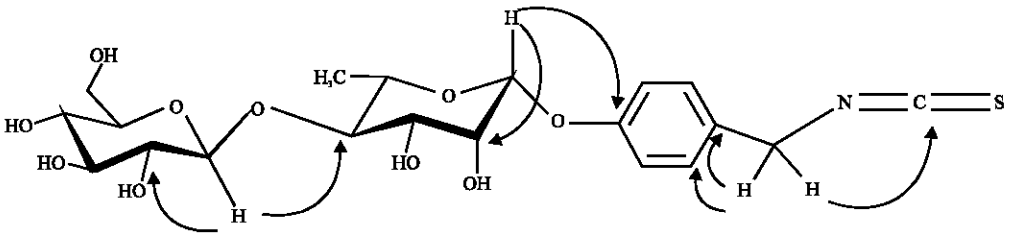

Fig. 2: Important HMBC correlations of compound 4

The ^{13}C NMR and DEPT spectra indicated that the 20 carbons of the molecule are present as three methyls (one CHMe), one methylene, one oxygenated methylene, ten methines, four aromatic CH and three quaternary carbons. The presence of two sugar moieties was evident from the structural features from ^{1}H NMR and ^{13}C NMR spectra (Table 2) which showed the presence of two anomeric protons of the sugar unit δ_H 5.44 (1 H, d, J = 3.2 Hz, H-1') attributed to C-1' (δ_C 93.71) and 5.18 (d, 4.0 Hz) attributed to C-1" (δ_C 99.87) for glucose. The anomeric proton and a methyl doublets signal at δ_H 1.26 (3H, d, J = 6.0 Hz) assignable to C - 6' also supported this assignment of the sugar as rhamnose. In the ^{13}C NMR, the four signals from δ_C 69.08 to δ_C 79.36 (Table 2) were attributable to the carbons 2, 3, 4 and 5 of the sugar moiety. It is also clear that the rhamnose is attached at C-4' (OH) because of the HMBC cross peak observed between H-1' (δ_H 5.44) and C-4 (δ_C 157.06). The glycosidic linkage between the glucose and the rhamnose was determined to be 1→4 from the observation of the long-range correlation between H-1" and C-4' in the HMBC spectrum of 4 (Fig. 2).

In the ^{1}H NMR spectrum, the two-proton mutually coupled doublets at δ 7.07 (J 8.4 Hz) and 7.36 (J 8.4 Hz) showed the presence of p-substituted benzene ring in the molecule which was supported by the correlation of theses protons at δ 7.07 (H-2, 6) and δ 7.36 (H-3, 5) with the CH carbons at 129.11 and 116.50, respectively in the HMBC and COSY spectra.

Furthermore, the 1-J coupling in the HMQC of a signal at δ_H 4.23 (H-7) with the carbon resonance at δ 44.02 in the HMQC indicated the presence of benzylic methylene. That the NH is linked with benzylic methylene carbon was evident from the diagnostic peak at m/z 107.02. The presence of isothiocyanate in 4 was very evident at the 129-131 regions of ^{13}C NMR where there are four distinct signals (three attributed to carbon at position 3, 4 and 5 of the phenyl ring while the fourth one is attributed to the C = S) (Fig. 3). These data are comparable with the values reported for 3. The difference between compounds 3 and 4 is the presence of glucose in compound 4 which is unmistakably missing in compound 3. The MS fragmentation pathways of compound 4 are shown in Fig. 3.

From the foregoing findings, compound 4 was identified as 4-(β-D-glucopyranosyl-1→4-α-L-rhamnopyranosyloxyl)-benzyl isothiocyanate which represents a new natural product described here to our knowledge, for the first time.

Compound 5 was isolated as a light brown solid. The positive- ion EI-MS exhibited a sodiated ions $[M+Na]^+$ at m/z 632.1 compatible with the molecular formula $C_{25}H_{39}NO_{16}$. The mass spectrum also gave a significant fragment ion at m/z 463.1 ($C_{19}H_{29}NO_{12}$), m/z 447.3 ($C_{19}H_{29}NO_{11}$), m/z 343.1 ($C_{12}H_{22}NO_{11}$), m/z 325.1 ($C_{12}H_{21}O_{10}$), m/z 163.1 ($C_6H_{11}O_5$) and m/z 146.0 ($C_6H_{11}O_4$) which substantiated the structure of glycoside 5. The IR spectrum showed absorption at 3410, 2900 and 1590-1610 cm^{-1} characteristic of hydroxyl group and aromatic ring nature.

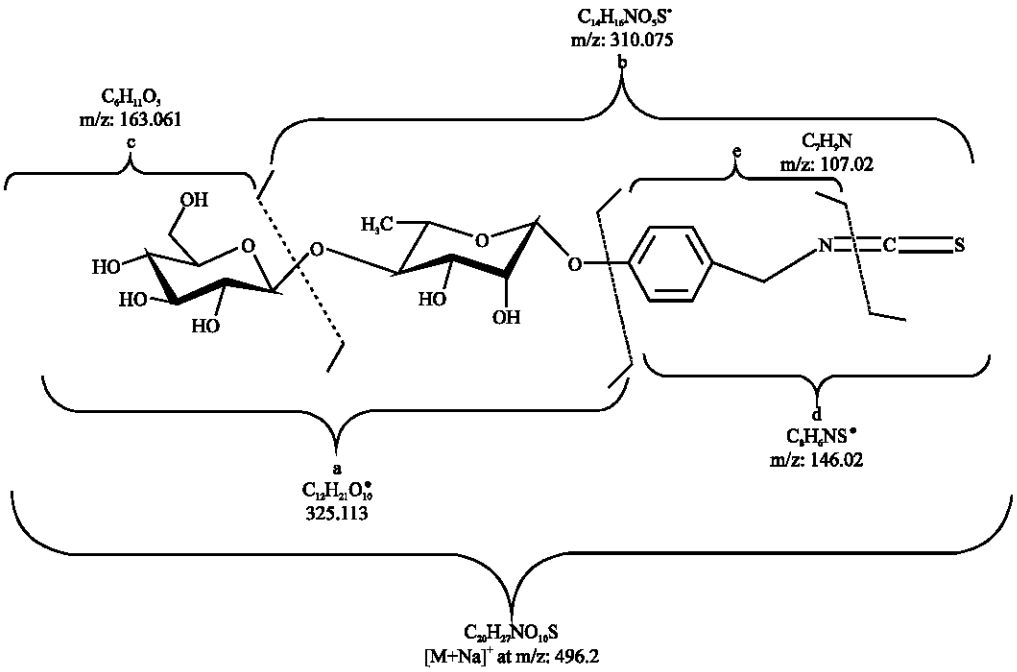

Fig. 3: MS Fragmentation pathways for compound 4

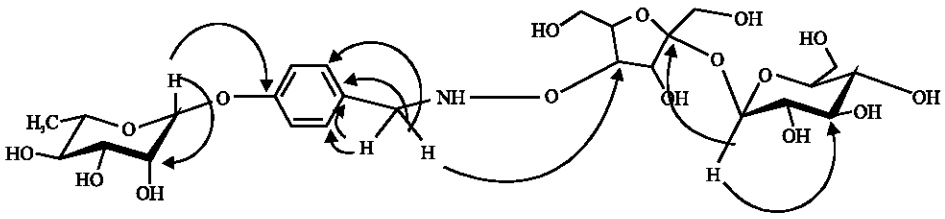

Fig. 4: Important HMBC correlations of compound 5

The ^{1}H-NMR spectrum suggested that compound 5 is a trisaccharide of phenylmethanamine on the basis of two anomeric protons (one for rhamnose and the other for glucose), at 5.35 (bs) and 5.17 (d, 4.0 Hz), a quaternary sp^{3} carbon of a fructopyranose moiety at δ_C 104.01, three oxygenated methylene at δ_C 60.45, 60.92 and 62.02 belonging to sucrose. The carbon signals at δc 17.89 and proton signal of a methyl doublets at δ_H 1.11 (3H, d, J = 6.0 Hz) assignable to C - 6' also supported this assignment of the rhamnose as part of the three sugars. The presence of 15 carbon signals in the sugar region (δ_C 60-80 ppm) together with two anomeric carbons at δ_c 98.45, 91.71, the methyl carbon of the rhamnose at δ_C 17.89 and a quaternary sp^{3} carbon of a fructose moiety at δ_C 104.01 unequivocally confirm the presence of three sugars in the phenylmethanamine aglycone.

The ^{1}H NMR spectra of 5 (Fig. 4) showed an AA'BB' (A_2B_2) system due to the protons of the phenyl ring and the assignment was further confirmed by the ^{13}C NMR spectra (δ_C 118.09 for C-2'/6' and δ_C 130.84 for C-3'/5' (Table 3). The ^{1}H-NMR spectrum indicated the presence of two anomeric protons assigned to H-1' and C-1' (δ_H 5.35, bs and δ_C 98.45) and H-2"and C-1" (δ_H 5.18, d, J = 3.64 Hz and δ_C 91.71). The coupling constants of anomeric protons at δ_H 5.18 (J = 4.00 Hz) confirmed **β**-configuration of the glucose. In the ^{13}C NMR, the fifteen signals from δ_C 60.45 to δ_C 82.53 (Table 3) were attributable to the carbons 2, 3, 4 and 5 of each of the sugar moiety.

Table 3: ^{1}H- and ^{13}C-NMR data of compound 5 (400 MHz for ^{1}H-NMR, 100 MHz for ^{13}C-NMR, in $(CD_3)_2SO$ (DMSO)

No	^{1}H	^{13}C	HMBC
C-1		155.01	
C-2, 6	7.00 (d, 8.4 Hz)	116.50	129.10, 157.06
C-3, 5	7.25 (d, 8.4 Hz)	129.10	129.77, 116.50
C-4		129.77	
C-7	4.07 (d, 15.6 Hz)	37.02	129.10, 129.77
	3.89 (d, 8.0 Hz)		
Rhamnose			
C-1'	5.35 (bs)	91.71	155.01, 70.40, 77.00
C-2'	3.78 (m)	70.40	91.71, 77.00
C-3'	3.48-3.50 (m)	77.00	70.40, 74.25, 69.71
C-4'	3.44-3.47 (m)	74.25	70.40, 69.71
C-5'	3.70 (m)	69.71	70.40, 74.25
C-6'	1.11 (d, 6.0 Hz)	17.89	
Glucose			
C-1"	5.17 (d, 4.0 Hz)	98.46	104.02, 82.53
C-2"	3.62 (m)	82.53	77.98, 98.46
C-3"	3.48-3.50 (m)	77.98	82.53, 69.45
C-4"	3.44-3.47 (m)	69.45	77.98, 72.81
C-5"	3.56-3.60 (m)	72.81	69.45, 62.02, 77.98
C-6"	3-60-3.64 (m)	62.02	72.81, 69.45
3.49-3.51 (m)			
Fructose			
C-1"'	3.81 (bs)	60.92	104.01
C-2"'		104.01	
C-3"'	3.38-3.43 (m)	81.28	70.20, 71.60
C-4"'	3.64-3.72 (m)	70.20	81.28, 71.60
C-5"'	3.76-3.88 (m)	71.60	81.28, 71.20
C-6"'	3-60-3.64 (m)	60.45	71.60, 70.20
	3.49-3.51 (m)		

Coupling patterns and coupling constants (J) in Hz are given in parentheses

From the HMBC correlations, it is also clear that the rhamnose is attached at C-1 while the glycosidic linkage in the sucrose is evident from the HMBC cross peak observed between H-1" (δ_H 5.35) and C-2"' (δ_C 104.01) of the fructose.

Furthermore, a signal at δ_H 4.07 and 3.89 (H-7) in the ^{1}H NMR spectrum (Table 3) correlated with the carbon resonance at δ 37.02 in the HMQC indicated the presence of benzylic methylene. The same signal at δ_H 4.07 and 3.89 (H-7) in HMBC cross peak with C-4 (δ_C 129.77) and C-3, 5 (δ_C 129.10). The ^{1}H NMR and $^{1}3C$ NMR data was consistent with AA'BB' (A_2B_2) system and benzylic methylene of niazicin A save the absence of thiocarbonyl carbon and presence of the sucrose in compound 5. The MS fragmentation pathways of compound 5 are shown in Fig. 5.

However, compound 5 is somewhat different from compound 4 in that it has isothiocyanate group present instead of amine unit in compound 4 and also the absence of fructopyranose unit in compound 4. In the 129-131 regions of ^{13}C NMR where there are three signals in compound 5 which are attributed to carbon at positions 3, 4 and 5, four distinct signals were evident in that same region in which case the fourth was attributed to C = S in compound 4.

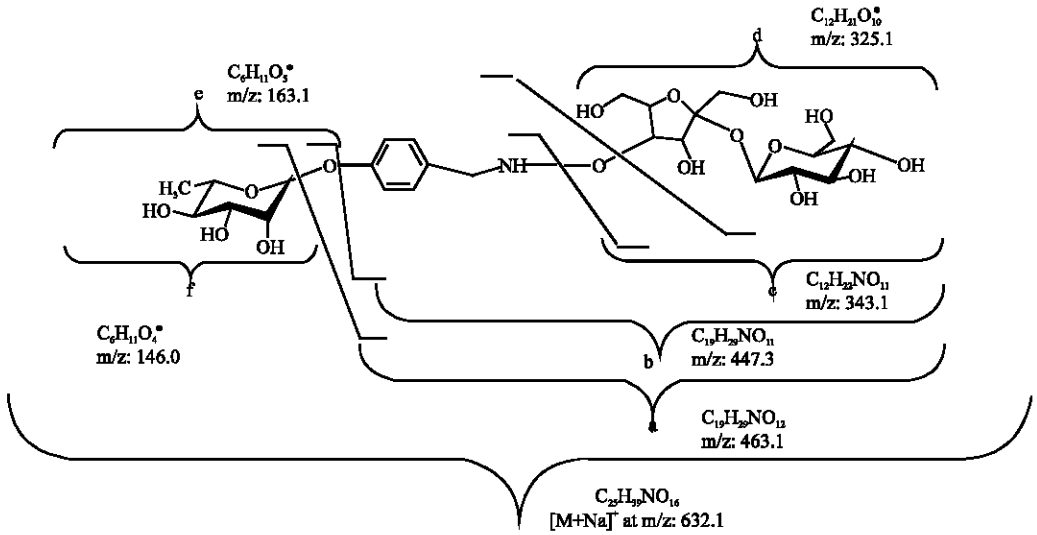

Fig. 5: MS fragmentation pathways for compound 5

From the above information compound 5 was therefore identified as 4-O-α-L-rhamnopyranosyloxy-N-glucopyranosyl-1→2-fructopyranosyloxy phenylmethanamine. This remarkable compound is isolated for the first time from *Moringa oleifera* Lam. (Moringaceae) and natural sources.

CONCLUSION

In conclusion, the aqueous seed extract showed stronger inhibitory activity on the wound isolates compared to the methanolic extract. Both the crude aqueous and methanolic extracts displayed broad spectrum of activity as they inhibit both the gram negative and gram positive bacteria used. It is suggested that the various compounds including the newly isolated compounds in this study may be responsible for the observed activity. However, further studies involving isolation of individual components (compounds) from the seed extract in sufficient quantity for activity and not just for structural elucidation alone are needed to for further clarification.

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