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Isolation of Compounds from Acetone Extract of Root Wood of *Moringa stenopetala* and Evaluation of their Antibacterial Activities

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ABSTRACT

The main objective of this study was to isolate compounds from root wood of *Moringa stenopetala* and evaluate antibacterial activities of the isolated compounds. Crude gradient extracts were obtained from five solvents (petroleum ether, chloroform, acetone, methanol and water) with increasing solvent polarity using cold maceration technique. The *in vitro* antibacterial activity evaluation of gradient extracts and isolated compounds was done on four different pathogenic bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella* Typhimurium) using agar disc diffusion technique. Among the five crude extracts evaluated, the acetone extract was found to be the most active against the tested strains. Thus, this extract was subsequently subjected to column chromatographic separation which led to isolation of four compounds (MS-1, MS-2, MS-3 and MS-4). The chemical structures of the compounds were found to be cholest-5-en-3-ol, palmitic acid, n-octacosane and oleic acid, respectively, based on physical properties and spectroscopic (IR and NMR) data as well as literature reports. Three of the compounds namely cholest-5-en-3-ol, palmitic acid and oleic acid showed highest activity against *E. coli*. The observed antibacterial activities of the crude extract and the isolated compounds could justify the traditional use of the plant for the treatment of different bacterial infections. Thus, further test is recommended on large number of bacterial strains to decide their potential as candidates in development of antibacterial drugs.

Key words: *Moringa stenopetala*, antibacterial activity, crude extraction, IR-NMR, isolation, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium

INTRODUCTION

Moringa stenopetala is one of the 14 *Moringa* species that belong to the family of Moringaceae (Mekonnen, 2002; Abuye *et al*., 2003). It is a tree with 6-10 m tall and its trunk is about 60 cm in diameter at breast height. The plant is native to the horn of Africa particularly in Southern Ethiopia, North Kenya and Eastern Somalia. In Ethiopia, *Moringa stenopetala* is found in many arid zones of the Southern Ethiopia most extensively between Arba Minch and surrounding area, Konso, Negelle and Wellayta Sodo at altitude of up to 1800 m.a.s.l. (Stelz and Mayer, 1990; Mekonnen and Gessesse, 1998; Abuye *et al*., 2003; Yisehak *et al*., 2011). It is also a major vegetable crop in the region (Lindtjorn, 1983; Mekonnen and Gessesse, 1998; Demeulenaere, 2001) and can also be used as bee forage (Tessema *et al*., 1993) and animal fodder (Jahn, 1991) as well as for

purification of water (Eilert *et al*., 1981; Gottsch, 1984; Berger *et al*., 1984; Hundie and Abebe, 1991; Sajidu *et al*., 2006; Sahilu, 2010). The plant has several medicinal uses in areas where it is native. Local people use the plant parts to treat malaria, leishmaniasis and hypertension (Bennett *et al*., 2003; Mekoya, 2007), stomach pain, expulsion of retained placenta during birth, asthma, epilepsy, diarrhea, diabetes and wound healing (Mekonnen and Gessesse, 1998; Mekonnen, 1999; Mekonnen, 2002; Mussa *et al*., 2008; Yisehak *et al*., 2011) and Leprosy (Jahn, 1991).

The diverse medicinal use of the plant by local people has led several research teams to carry out experimental investigations to find out the bioactive constituents (crude or pure) that are responsible for various medicinal uses of the plant with an ultimate goal of justifying the traditional use of the plant species or discovering drugs against different diseases. Some research teams carried out biological activity tests using crude extracts obtained from different parts of the plant whereas very few of them used pure compounds isolated from different parts of the plant. For instance, hypoglycemic effect of leaf extract of Moringa stenopetala was assessed in non-diabetic rabbits using blood glucose analysis. The plant extract was found to lower blood glucose and cholesterol in mice models (Mekonnen *et al*., 1997; Mekoya, 2007; Ghebreselassie *et al*., 2011; Toma *et al*., 2012). The crude water and n-hexane extracts of its seeds have been reported to show antibacterial activities (Asres, 1995; Sahilu, 2010; Walter *et al*., 2011). Another experiment carried on guinea-pig ileum and mouse duodenum and uterus strips using ethanol extract of leaves also revealed antispasmodic property with some cytotoxic activity on uterus strips of guinea-pigs and mice. These results were claimed to be consistent with the traditional use of the leaves of Moringa stenopetala for relieving stomach pain and to expel retained placenta by women during giving birth (Mekonnen, 1999). Other experimental results also indicated antitrypanosomal, antileishmanial, anti-fertility and the antimicrobial properties of crude extracts from the seed, leaves and roots of Moringa stenopetala (Mekonnen *et al*., 1999; Nibret and Wink, 2010; Biffa, 2005). Reports also revealed low toxicity of leaves, root and seed extracts as demonstrated by cytotoxicity tests on HEPG2 cells (Mekonnen *et al*., 2005).

There are many reports describing attempts on isolation of compounds from different parts of *Moringa stenopetala* and also evaluation of their biological activities. Eilert *et al*. (1981) reported 4-(α-L-Rhamnosyloxy) benzyl isothiocyanate as an active antimicrobial (antibacterial and antifungal) agent from seeds of *Moringa stenopetala*. Mekonen and Gebreyesus (2000) isolated three glycosides namely 4-(4'-O-acetyl-L-rhamnosyloxy)-benzyl isothiocyanate, 4-(4'-O-acetyl-L-rhamnosyloxy)-benzaldehyde and rutin from ethanolic extract of leaves. Isolation of 5,5-dimethyloxazolidine-2-thione and O-(rhamnopyranosyloxy)benzyl glucosinolate was also reported from seed extracts of *Moringa stenopetala* (Mekonnen and Drager, 2003). Various unsaturated and saturated fatty acids such as oleic acid, behenic acid and palmitic acid as well as high levels of β-sitosterol, stigmasterol and campesterol were also reported from n-hexane and chloroform:methanol (1:1) extracts of seed oils of the plant. Defatted and shell-free seeds of *Moringa stenopetala* were also reported to contain the glucosinolates such as 4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate and glucoconringiin (2-hydroxy-2-methylpropyl glucosinolate) (Lalas *et al*., 2003). Bennett *et al*. (2003) reported 4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate (from seeds) and benzyl glucosinolate (from roots), 4-(α-l-rhamnopyranosyloxy) -benzyl glucosinolate and quercetin 3-O-rhamnoglucoside, rutin and 5-caffeoylquinic acid (from leaves). Glucosinolates such as benzyl isothiocyanate, isobutyl isothiocyanate and benzene-1-isocyano-2-methyl, cyclopropane pentyl, methyl 9-octadecenoate,

methyl palmitate, nonanoic acid and [Delta]-cadinene were the major components (from oil of seeds) were reported recently (Nibret and Wink, 2010). The bioactivity test of those compounds showed high antitrypanosomal activity. Glucosinolates from seeds, leaves and roots of *Moringa stenopetala*, and their activity as antihypertension were also reported previously (Bennett *et al*., 2003; Mekonnen and Drager, 2003). Recently we have characterized two triglycerides namely; 1,3-dilinoleoyl-2-olein and 1,3-dioleoyl-2-linoleic from root extract of *Moringa stenopetala* that showed good activity on *L. aethiopica* intracellular amastigotes (Bekele, 2011). As discussed above, the *in vivo* and *in vitro* tests using crude extracts of roots of *Moringa stenopetala* showed promising biological activities including antimicrobial activities. The aim of this research was then to isolate compounds from crude extracts of root wood that would show relatively better antibacterial activities and also to evaluate antibacterial activities of the isolated compounds.

MATERIALS AND METHODS

Chemicals and apparatus: General laboratory grade solvents such as petroleum ether, chloroform, ethyl acetate, acetone and methanol (Purchased from supplied by Sigma Aldrich Chemicals Co. Ltd.) and distilled water for extraction and column elution. Silica gel (60-120 mm mesh size) and TLC (silica gel, UV-254) pre-coated on aluminum sheets were used for chromatographic analysis. Compound spots on TLC plates were detected using UV (uvitec chamber) and iodine vapor. Evaporation of solvent was carried out using a rotary evaporator (Heidolph, UK) and HY-5A Manoeuvre style vibrator (Rotary shaker) were used for extraction. A standard antibiotic disc (ciprofloxacin, 5 μg) and culture medium (Mueller Hinton agar, nutrient broth) were used for the antibacterial activity test. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 were recorded using Bruker Advance 400 MHz spectrometer. $CDCl_3$ was used as a solvent in all spectroscopic analysis. For Infrared (IR) spectra were obtained from Perkin-Elmer BX infrared spectrometer (400-4000 cm^{-1}) using $CDCl_3$ solution. Melting point apparatus (Griffin) was used for melting point determination.

Plant materials and extraction: The root wood of *Moringa stenopetala* was collected, in November 2011, from Arba Minch, Southern Ethiopia, and identified by botanist at the Department of Biology, Jimma University where a voucher number (MT/201) was given and a specimen was deposited. The collected plant material was cut into small pieces and air dried in laboratory at room temperature for forty five days under shade. The dried material was then powdered manually using grind mill. The powdered plant material (1250 g) was sequentially extracted with petroleum ether, chloroform, acetone, methanol and distilled water (7.5 L each) using maceration technique twice for 72 h with constant continuous shaking. The extracted matter was filtered first using fresh cotton plug and then using filter paper (Whatman No.1). The filtrate were concentrated using a rotary evaporator. The resulting crude extract of each solvent was weighed and stored in refrigerator below 4°C (Kohler *et al*., 2002), until used for microbial assay, TLC fractionation and chromatographic isolation of compounds.

Evaluation of antibacterial activity

Preparation of test solutions and bacterial strains for preliminary activity: Test solutions were prepared by dissolving 100 mg of each of the crude extracts in 1 mL of dimethyl sulfoxide (DMSO) to achieve final stock concentration of 100 mg mL^{-1} solution of test sample. Microorganisms used for evaluation of antibacterial activities of the crude extracts and fractions were

Staphylococcus aureus ATCC 25903, *Escherichia coli* ATCC 25722, *Pseudomonas aeruginosa* DSMZ 1117 and *Salmonella* Typhimurium ATCC 13311. These strains were donated by the Department of Biology, Jimma University. The same procedures were repeated for the antibacterial activity tests of the isolated compounds but the solutions were prepared by dissolving 30 mg of each compound in 1 mL DMSO.

Preparation of fresh inoculums for bioactivity test of crude extracts using disk diffusion method: Active cultures were prepared using Muller Hinton agar culture media to grow organisms. The culture media was boiled in distilled water to dissolve media and autoclaved at 121°C for 20 min and poured to sterile Petri dish. When the medium solidified, standard strains were uniformly inoculated on it. Five well-isolated colonies of the same morphological type were selected from an agar plate culture and the top of each colony was touched with a loop and the growth was transferred into a tube containing 4.5 mL of a suitable nutrient broth medium. The broth culture was incubated at 37°C until it achieves or exceeds the turbidity of the 0.5 McFarland standards for 8 h. The turbidity of the actively growing broth culture was adjusted with sterile saline solution to obtain turbidity optically comparable to that of the 0.5 McFarland standards which was resulted in a suspension containing approximately 1-2×10^8 CFU mL^{-1} for different strains (CLSI, 2009). Inoculums containing 1 to 2×10^8 CFU mL^{-1} of bacteria were spread on the solid plates with a sterile swab moistened with the bacterial suspension and 100 mg mL^{-1} concentration of 50 μL of the working suspension/solution of the same concentration of the sample plant gradient extracts and same volume of extraction solvent and DMSO for negative control was impregnated using No. 1 Whatman filter paper disc (diameter 6 mm) with the help of micropipette. Simultaneously ciprofloxacin disc (5 μg) was also used as positive control. Plates were left for 5 min till the extract diffuse in the medium with the lid closed and incubated at 37°C for 24 h. After overnight incubation, the plates were observed for the zone of inhibition (ZI) and the diameter of the inhibition zone was measured using ruler and mean was recorded (Anonymous, 1996; Stephen, 2005). The same procedures were employed in antibacterial evaluations of the isolated compounds.

Isolation and structural elucidation of compounds: The crude extract that showed superior antibacterial activity was subjected to column chromatography packed with silica gel (60-120 mm mesh size) to isolate compounds. The silica gel was dried at 100°C for 1 h to activate it and a glass column of 3 cm diameter and 60 cm length was packed with the activated 100 g silica gel slurry dissolved in petroleum ether. The crude extract (12 g) of acetone extract of root wood was dissolved in minimum quantity of acetone and then adsorbed onto 24 g of silica gel. Then the solvent was allowed to evaporate and the dry sample adsorbed to the silica gel was applied to the column that was already packed with silica gel. The choice of solvent system used for isolation of compounds was made after carrying out the TLC analyses of the crude extract in various combinations solvents of different polarity. Finally, petroleum ether and ethyl acetate mixture was found to give good separation. Therefore, petroleum ether and ethyl acetate mixture in different combination with increasing polarity (in the ratio 98:2, 96:4, 94:6, 92:8, 90:10, 88:12, 86:14, 84:16) was used to elute the chromatographic column. A total of 350 fractions each with 40 mL were collected. Solvents were removed from the fractions under reduced pressure using rotary evaporator. The identity of the fraction was examined by TLC on silica gel plate. The spots developed were visualized under UV light at 254 and 365 nm and then by exposure to iodine vapor. The fractions that showed the same

TLC development profiles (color and R_f) were combined and concentrated to dryness under reduced pressure using rotary evaporator (Hemavani and Thippeswamy, 2012). Evaporation of solvents resulted in various fractions of different physical property. The structures of the compounds were elucidated based on combined spectral data which include Infra Red, Nuclear Magnetic Resonance (^{1}H-NMR, ^{13}C-NMR and DEPT-135) spectra as well as by comparison with literature data. All spectroscopic analysis were carried out at Department of chemistry, Addis Ababa University.

RESULTS AND DISCUSSION

In this study, antibacterial activities of crude extracts of root that were obtained through gradient extractions using solvents such as petroleum ether, chloroform, methanol and acetone and water.

Evaluation of antibacterial activities of crude extracts from root wood of *Moringa stenopetala*: Gradient extraction of 100 g of dried plant material in petroleum ether, chloroform, acetone, methanol and water afforded 0.65, 0.75, 0.45, 1.0 and 1.25 g crude extracts. *In vitro* test of antibacterial activities of the crude extracts against four bacterial species namely *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella* Typhimurium showed that acetone extract to be relatively more effective against all the test bacterial species followed by petroleum extract. On the other hand, the chloroform extract was found to act on none of the bacterial species (Table 1). Moreover, the antibacterial activities of methanol and aqueous extracts were not significant as demonstrated by their low bacterial inhibition zones (Table 1). The inhibition zone data of acetone extract (mm) were comparable to that of the reference drug against most of the bacterial species (Table 1). Therefore, the crude acetone extract of root wood of *Moringa stenopetala* was selected as the best candidate for chromatographic isolation of compounds.

Isolation of compounds from crude acetone extract of root wood of *Moringa stenopetala*: Based on the observed results of antibacterial activity tests of the crude extracts (Table 1), root wood was subjected to gradient extraction in bulk. The gradient extraction gave 12 g crude acetone extract from 1250 g of plant material. This amount of the acetone crude extract was then subjected to column chromatography using different combinations of petroleum and ethyl acetate as mobile phases with increasing polarity.

This led to isolation of six compounds from the crude acetone extract. Fractions 28-32 eluted by petroleum ether and ethyl acetate (98:2%) were combined based on their TLC profile similarity. Evaporation of solvent from these fractions gave 56 mg of white amorphous powder compound labeled as MS-1. Its R_f value was determined as 0.45 in petroleum-ethyl acetate (95:5). Another white crystalline solid compound (44 mg) was obtained by combining fractions 36-38 that were eluted with 4% ethyl acetate in petroleum ether. The compound was labeled as MS-2 and its Rf

Table 1: Antibacterial inhibition zones (mm) of crude extracts of root wood of *Moringa stenopetala*

						Drug	
Bacterial species	Pet. ether extract	Chloroform extract	Acetone extract	Methanol extract	Aq. extract	Cipro	DMSO
E. coli	10.0	na	15	10	na	31	na
P. aeruginosa	12.5	na	25	12	8	33	na
Salmonella Typhimurium	13.5	na	27	NA	12	30	na
S. aureus	12.0	na	23	na	7	32	na

DMSO: Dimethyl sulfoxide, Cipro: Ciprofloxacin; na: Not active

value was determined as 0.34 in petroleum-ethyl acetate mixture (95:5%). A 43 mg of third compound, white needle sharped crystalline, labeled as MS-3 was isolated from the combined fractions 43-45 that were eluted with 6% ethyl acetate in petroleum ether. Its R_f value was determined to be 0.57 in petroleum-ethyl acetate mixture (92:8). A light yellow oily compound (77 mg) of labeled as MS-4 was obtained by combining fractions 47-50 that were eluted with 8% ethyl acetate in petroleum. Its R_f value was determined to be 0.53 in petroleum ether-ethyl acetate mixture (90:10%).

Structure elucidation of the isolated compounds: The structures of the compounds (MS-1, MS-2, MS-3 and MS-4) that were isolated from acetone extract of the root wood of *Moringa stenopetala* were elucidated (proposed) based data obtained from spectroscopic (IR and NMR) analyses and comparing the data with reported data in literature as discussed below.

Structural elucidation of compound MS-1: Analysis of IR spectrum of MS-1 showed that the compound does not possess a carbonyl group which is confirmed by absence of medium and strong bands ranging 1810-1710 cm^{-1}. The bands ranging from 1100-660 cm^{-1} are characteristic bands of steroids and bands at 3022, 1530 and 928 cm^{-1} can be attributed to trisubstituted double bonds. The ^{1}H-NMR spectrum of MS-1 (Table 2) showed a single peak is at 7.2 ppm. This represents the residual peak of $CHCl_3$ from $CDCl_3$ solvent. A multiplet centered at 5.36 ppm was attributed to coupling of the H-atom (bonded to the sp^2 C-atom H-6) with the H-atoms on the adjacent CH_2.

The peak at 3.54 ppm was attributed to an H atom (H-3) bonded to -OH group. Moreover, the ^{1}H-NMR spectrums showed five methyl signals at 0.94, 0.86, 0.88, 0.84 and 0.69 ppm. Two doublets are also observed at 0.82 and 0.86 ppm, respectively. These peaks correspond to the H-atoms attached to the external CH_3 groups on the cholest-5-en-3-ol molecule. These groups exhibit diastereotopicity and therefore, act differently to generate four peaks. Another singlet is observed at 1.02 ppm, corresponding to the additional CH_3 side group. It is important to note that it is not known which singlet corresponds exactly to each of the sp^3 hybridized methyl groups. The singlets at 1.03 ppm correspond to either of the methyl groups located inside the ring system of the cholest-5-en-3-ol. Extensive couphng is observed ranging from 1.25-2.35 ppm corresponding to the remaining H-atoms found throughout the cholest-5-en-3-ol molecule. This compound shows characteristics steroid signals at 0.69 and 3.54 ppm. The observed ^{1}H-NMR spectrum of MS-1 was found to be consistent to that of cholest-5-en-3-ol reported in literature (Bajia, 2007; Yasmin, 2008; Rohlfsen, 2009). The analysis of ^{13}C-NMR and DEPT-135 spectra of compound MS-1 revealed the presence of twenty seven carbon atoms in the molecule. The spectra indicated presence of five CH_3, eleven CH_2, eight CH and three quaternary carbons. DEPT-135 spectrum also confirmed the

Table 2: ^{1}H-NMR ($CDCl_3$, 400 MHz) data of compound MS-1 along with reported data for cholest-5-en-3-ol

H	^{1}H-NMR data of MS-1	^{1}H-NMR data of cholest-5-en-3-ol*
H-6	5.36	5.34
H-3	3.54	3.52
Me-19	0.94	0.98
Me-21	0.86	0.91
Me-26	0.84	0.84
Me-27	0.88	0.87
Me-18	0.69	0.65

*(Bajia, 2007; Manea *et al*., 2006; Rohlfsen, 2009; Yasmin, 2008)

Table 3: ^{13}C-NMR and DEPT-135 ($CDCl_3$ 400 MHz) spectral data of compound MS-1 along with the reported ^{13}C-NMR data of cholest-5-en-3-ol

C. No.	^{13}C-NMR of MS-1 (ppm)	DEPT-135 data of MS-1	^{13}C-NMR of cholest-5-en-3-ol*	Nature of carbon
1	37.252	37.253	37.2	CH_2
2	31.651	31.651	31.6	CH_2
3	71.823	71.824	71.8	CH
4	45.826	45.825	45.9	CH_2
5	140.752	-	140.7	C
6	121.740	121.74	121.7	CH
7	29.719	31.923	29.2	CH_2
8	31.902	31.899	31.9	CH
9	50.122	50.125	50.2	CH
10	36.511	-	36.5	C
11	21.089	21.089	21.1	CH_2
12	39.773	39.775	39.8	CH_2
13	42.289	-	42.3	C
14	56.767	56.769	56.8	CH
15	24.313	24.312	24.3	CH_2
16	26.038	28.261	26.2	CH_2
17	56.044	56.046	56.1	CH
18	11.872	11.873	11.9	CH_3
19	19.034	19.034	19.0	CH_3
20	36.156	36.159	36.1	CH
21	18.787	18.787	18.7	CH_3
22	33.848	33.935	33.9	CH_2
23	28.260	28.261	28.2	CH_2
24	23.059	23.060	23.1	CH
25	19.413	19.413	19.4	CH_3
26	19.840	19.841	19.8	CH_2
27	11.990	11.990	11.9	CH_3

*(Bajia, 2007; Manea *et al*., 2006; Yasmin, 2008)

presence of methyl carbon at 11.990 (C-27), 19.413 (C-25), 18.787 (C-21) and 11.873 (C-18) ppm, and methine carbons at 71.824 (C-3), 121.74 (C-6), 31.899 (C-8), 50.125 (C-9), 56.769 (C-14), 56.046 (C-17), 36.156 (C-20) and 23.060 (C-24) ppm. The absence of peaks at 140.752 (C-5), 36.511(C-10) and 42.289 (C-13) ppm in the DEPT-135 spectrum which were observed in the ^{13}C-NMR spectrum also confirmed the presence of quaternary carbon atoms in MS-1. The experimental ^{13}C-NMR and DEPT-135 chemical shift values on spectral data (Table 3). Are also consistent with that of cholest-5-en-3-ol reported in literature (Bajia, 2007; Manea *et al*., 2006). To the best our knowledge this is the first to report of isolation of cholest-5-en-3-ol from *Moringa stenopetala*. The observed spectral data and comparison with reports in literature that MS-1 to most likely as cholest-5-en-3-ol (Fig. 1). Moreover, the observed melting point of MS-1 (146-150°C) was comparable to the reported melting point value of cholest-5-en-3-ol (i.e., 148-150°C) (Williams *et al*., 1965).

Structural elucidation of compound MS-2: In the IR ($CDCl_3$) spectrum of MS-2, a band at 1706 cm^{-1} indicated the presence of carbonyl group of carboxylic acid. The band of medium intensity at 1212 cm^{-1} indicates C-O stretching of carbonyl carbon of carboxylic acid and absorption

at 1468 cm^{-1} indicating bending of -CH_2- in compound. MS-2. Analysis of the ^{1}H-NMR ($CDCl_3$, 400 MHz): spectrum of MS-2 showed a single peak is at 7.2 ppm. This represents the residual peak of $CHCl_3$ from $CDCl_3$ solvent. ^{1}H-NMR of MS-2 exhibited a triplet signal at 2.34 ppm for hydrogens adjacent to carbonyl group which are slightly deshellded. The spectrum also indicates multiplate signals at 1.63 for methylene (H-3) ppm, broad singlet at 1.27 ppm (chain) and intense triplet at 0.88 ppm for methyl hydrogens (H-16). The observed ^{1}H-NMR spectral data (Table 4) of compound MS-2 was found to be consistent to that of palmitic acid reported in literature (Joshi *et al*., 2009).

The analysis of ^{13}C-NMR spectrum of compound MS-2 showed signals at 179.857 (C-1) carbonyl carbon of fatty acids, 34.031 (C-2), 24.694 (C-3), 29.346 (C-4), 29.457 (C-5), 29.615 (C-6 and 7), 29.265 (C-8), 29.720 (C9-C12 of the chain), 29.078 (C-13), 31.948 (C-14), 22.718 (C-15) and 14.153 (C-16) ppm (Table 5). The DEPT-135 spectrum of the MS-2 showed a single signal at δ 14.151 indicating the presence of one methyl carbon (Table 5). The observed ^{1}H-NMR spectrum of MS-2 was found to be consistent with that of the data reported for palmitic acid (Joshi *et al*., 2009) (Fig. 1). Moreover, the observed melting point of MS-2 (61-64.5°C) was comparable to the reported melting point value of palmitic acid (i.e., 63-64°C) (Syed, 2006; Joshi *et al*., 2009).

Structural elucidation of compound MS-3: Analysis of IR ($CDCl_3$) spectrum of MS-3 indicated that it has no carbonyl group, aromatic ring and carbon-carbon multiple bonds. The bands near 3000 cm^{-1} due to C-H stretching, along with weaker peaks ranging 1475-1370 cm^{-1} from C-H and 1016 cm^{-1} from C-C bending indicates that the compound is most likely an alkane. Its ^{1}H-NMR spectrum of MS-3 exhibited a single peak at 7.2 ppm. This represents the residual peak of $CHCl_3$ from $CDCl_3$ solvent. The intense triplet peak at 0.90 ppm indicated that presence of protons of methyl (-CH_3) groups whereas the peaks at 1.27 ppm indicated that the methylene proton adjacent

Table 4: ^{1}H-NMR ($CDCl_3$, 400 MHz) data of compound MS-2 and reported ^{1}H-NMR data of palmitic acid

^{1}H	^{1}H-NMR data of MS-2	Reported ^{1}H-NMR data of palmitic acid*
H-2	2.34	2.32
H-3	1.63	1.61
H-4-H-15 (Chain)	1.27	1.20
H-16	0.88	0.86

*(Syed, 2006; Joshi *et al*., 2009)

Table 5: ^{13}C-NMR and DEPT-135 ($CDCl_3$ 400 MHz) data compound MS-2 and along with the reported ^{13}C-NMR data of palmitic acid

C No.	^{13}C-NMR data of MS-2 (ppm)	DEPT-135 data of MS-2	^{13}C-NMR data palmitic acid*	Nature of carbon
1	179.857	-	179.9	Carbonyl carbon
2	34.031	34.031	34.0	CH_2
3	24.694	24.694	24.7	CH_2
4	29.346	29.390	29.3	CH_2
5	29.457	29.456	29.4	CH_2
6 and 7	29.615	29.614	29.6	CH_2
8	29.265	29.266	29.2	CH_2
9-12	29.720	29.720	29.7	CH_2
13	29.078	29.078	29.0	CH_2
14	31.948	31.949	31.9	CH_2
15	22.718	22.718	22.7	CH_2
16	14.153	14.151	14.1	CH_3

*(Syed, 2006; Joshi *et al*., 2009)

Table 6: ^{1}H-NMR ($CDCl_3$, 400 MHz) data of compound MS-3 along with the reported ^{1}H-NMR data of n-octacosane

^{1}H	^{1}H-NMR data of MS-3	Reported ^{1}H-NMR data of n-Octacosane*
H-1 and H-28	0.90	0.91
H-2 and H-27	1.63	-
H-3 – H-26	1.27	1.28

*Jian-Jan and Xi-Kui (2008), Siddiqui *et al*. (2004) and Milena *et al*. (2009)

Table 7: ^{13}C-NMR and DEPT-135 ($CDCl_3$ 400 MHz) data of compound MS-3 along with the reported ^{13}C-NMR data of n-octacosane

C. No.	^{13}C-NMR data of MS-3	DEPT-135 data of MS-3	^{13}C-NMR data of n-octacosane*
1	14.140	14.140	14.1-14.1
2	22.707	22.710	22.7-23.3
3	31.940	31.942	32.9-32.5
4	29.446	29.449	29.4-30.3
C5-C24	29.707	29.707	29.7-29.9

*Siddiqui *et al*. (2004), Milena *et al*. (2009) and Speight *et al*. (2011)

to methyl protons. A peak at 1.63 ppm indicates that protons of long chain aliphatic methylene ($-CH_2$) groups (Table 6). The ^{13}C-NMR spectrum of compound MS-3 was in the range of methyl and methylene groups.

The spectra revealed that the peaks in the range of chemical shift values 8-30 ppm indicates only saturated carbon bonds of relatively the same environment; an intense peak around 30 ppm indicate long chain saturated carbon bonds. On the other hand chemical shift values in the range of 14.0 to 34 ppm presence of methyl ($-CH_3$) and methylene ($-CH_2$) carbons (Table 7). The observed data suggested that the compound (MS-3) to be most likely a long chain alkane. The DEPT-135 data of MS-3 (Table 7) was also consistent with the above proposal. The DEPT-135 spectrum showed a single peak for presence of methyl ($-CH_3$) carbons at 14.140 ppm, methylene carbons at 22.710 to 34.0 ppm, but further analysis of ^{13}C-NMR spectra of a compound indicated that MS-3 has no indicative chemical shift values of olefinic carbons, carbonyl carbons and oxygen bonded carbon according to the spectral data. Comparing the observed ^{1}H-NMR, ^{13}C-NMR and DEPT-135 data along with literature reports enabled us to propose the chemical structure of MS-3 to be identical with that of n-octacosane (Jian-Jan and Xi-Kui, 2008; Siddiqui *et al*., 2004; Milena *et al*., 2009). Moreover, the experimental melting point of MS-3 (60-64.5°C) was found to be comparable to the reported melting point value of n-octacosane (i.e., 61-64°C) (Milana *et al*., 2009). Therefore, based on the above information, the structure of MS-3 was deduced as n-octacosane. Though, the compound was isolated from *Moringa oleifera*, this the first report of isolation of n-octacosane from *Moringa stenopetala*.

Structure elucidation of compound MS-4: In the IR spectrum the bands at 3032 and 1742 cm^{-1} indicated the C-H stretch of olefinic group and C=O stretch of carbonyl groups, respectively. Absence of a doublet band at/near 2850 and 2750 cm^{-1} indicated that the compound has no aldehydic functional group. Similarly, absence of bands around (or above) 1800 cm^{-1} also indicated that the compound is neither acid anhydride nor carboxylic acid derivative. The absence of weak bands in the range of 2000 and 1650 cm^{-1} also indicated that the compound has no aromatic functional group. The absorption band at 1742 cm^{-1} could be attributed to carbonyl group of an acid diluted with a solvent, and the intense absorption band at 1228 cm^{-1} indicates C-O stretching. Thus, the observed carbonyl group (C=O) and C-O stretching bands in the IR spectrum of

Table 8: ^{1}H- NMR ($CDCl_3$, 400 MHz) data for compound MS-4 and along with the reported ^{1}H- NMR data of oleic acid

^{1}H	^{1}H-NMR data of MS-4	^{1}H-NMR of oleic acid*
H-1	-	-
H-2	2.36	2.34
H-3	1.63	1.63
H-4	1.27	1.29
H-5	1.27	1.29
H-6	1.27	1.29
H-7	1.32	1.32
H-8	2.02	2.01
H-9	5.4	5.34
H-10	5.4	5.34
H-11	2.02	2.01
H-12	1.27	1.29
H-13	1.27	1.29
H-14	1.27	1.29
H-15	1.32	1.32
H-16	1.32	1.32
H-17	1.27	1.27
H-18	0.8	0.88

*Siddiqui *et al*. (2004) and Akita *et al*. (2004)

compound MS-4 indicates that the compound is most likely a carboxylic acid. The strong band at 3032 cm^{-1} represents = C-H stretch for sp^2 C-H whereas bands in the range of 938 to 672 cm^{-1} indicating =C-H bending of an unsaturated chain whereas the bands at 2934 and 2854 cm^{-1} indicate C-H stretching of methylene and methyl groups. The ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectra also confirm that the compound is a carboxylic acid with at least one C = C bond. The ^{1}H-NMR spectrum of compound MS-4 showed a single peak is at 7.28 ppm. This represents the residual peak of $CHCl_3$ from $CDCl_3$ solvent. The triplet peak at 0.8 ppm indicated presence of protons of methyl (-CH_3) groups; the peaks at 1.32 ppm and 1.63 ppm indicate protons of aliphatic methylene (-CH_2) group; a peak at 2.02 ppm (H-8 and H-11) indicates presence of protons of a methylene group that is bonded to C = C bond; the peak at 2.33 ppm indicate presence of protons of methylene that is bonded to a carbonyl group; the peak at 2.77 ppm indicates presence of protons of methylene group that is flanked by two C = C bonds whereas the peak at 5.4 ppm indicates presence of olefinic protons in the structure (Table 8). The observed IR and ^{1}H-NMR data were found to be consistent with the reported data of oleic acid (Siddiqui *et al*., 2004; Akita *et al*., 2004). Thus, based on this observation the chemical structure of compound MS-4 was proposed to be identical with the chemical structure of oleic acid (Fig. 1).

In the analysis of ^{13}C-NMR spectrum, the peaks at 130.030 and 129.734 ppm indicated the presence of C = C bond in the compound; a single peaks at 180.196 ppm indicated a quaternary carbon atom (of carbonyl carbon) of carboxylic acid. On the other hand, the chemical shift values in the range of 14.10 to 34.078 ppm indicated the presence of methyl (-CH_3) and methylene (-CH_2) carbons (Table 9). The DEPT-135 spectrum showed a single peak for the presence of methyl (-CH_3) carbon at 14.134 ppm, methylene carbons at 22.706 to 34.078 ppm and olefinic methine carbons at 130.031 and 129.737 ppm (Table 9). Absence of peaks ranging from 40-60 ppm in DEPT-135 and ^{13}C-NMR spectra indicated absence of quaternary carbon atoms in the chain of fatty acid group. Therefore, based on the above experimental results and comparing the data with literature reports,

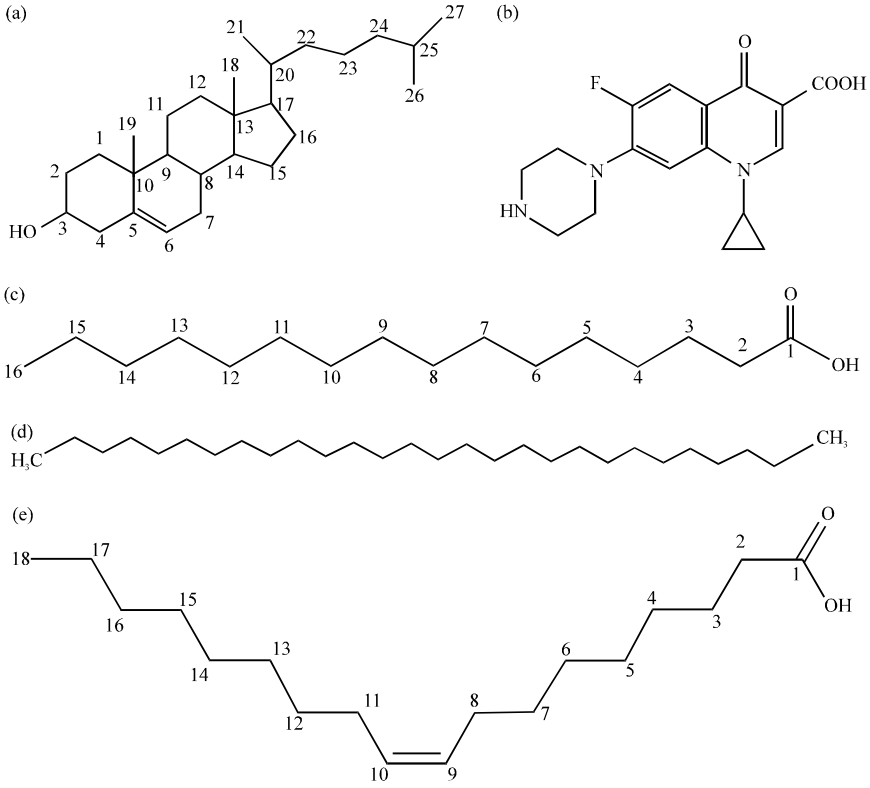

Fig. 1(a-e): The structure of the isolated compounds and the reference compound (Ciprofloxacin), (a) MS-1 (cholest-5-en-3-ol), (b) Ciprofloxacin, (c) MS-2 (palmitic acid), (d) MS-3 (n-octacosane) and (e) MS-4 (oleic acid)

Table 9: ^{13}C-NMR and DEPT-135 data of compound MS-4 and along with the reported ^{13}C-NMR data of oleic acid

C. No.	^{13}C-NMR data of MS-4	DEPT-135 data of MS-4	^{13}C-NMR of oleic acid*		Nature of carbon
1	180.196	-	180.5	180.5	Carbonyl carbon
2	34.078	34.078	34.12	34.1	CH_2
3	24.683	24.683	24.66	24.68	CH_2
4	29.153	29.155	29.14	29.1	CH_2
5	29.073	29.073	29.07	29.0	CH_2
6	29.447	29.447	29.05	29.44	CH_2
7	29.606	29.684	29.68	29.6	CH_2
8	27.162	27.161	27.16	27.1	CH_2
9	129.734	129.737	129.7	129.7	CH
10	130.030	130.031	130.0	130.0	CH
11	27.227	27.226	27.22	27.2	CH_2
12	29.706	29.776	29.78	29.706	CH_2
13	29.338	29.337	29.33	29.338	CH_2
14	29.536	29.535	29.53	29.536	CH_2
15	29.256	29.256	29.33	29.256	CH_2
16	31.941	31.941	31.92	31.9	CH_2
17	22.707	22.706	22.68	22.7	CH_2
18	14.133	14.134	14.07	14.1	CH_3

*Pinheiro *et al*. (2009), Akita *et al*. (2004) and Jian-Jan and Xi-Kui (2008)

the compound MS-4 was identified as oleic acid that has been isolated from Moringa *oleifera* and seeds of *Moringa stenopetala* (Saleem, 1995; Jian-Jan and Xi-Kui, 2008; Nibret and Wink, 2010).

Evaluation of antibacterial activities of the isolated compounds: *In vitro* tests were carried out to evaluate antibacterial activities of the isolated compounds (MS-1 to MS-4) using four bacterial species: *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella* Typhimurium. For the sake of reproducibility, the experiments were carried out in duplicates and the average values of growth inhibition zones are used for discussions regarding the antibacterial activities of the compounds. Though the observed activities were lower than that of the reference drug (ciprofloxacin), the results showed that the compounds have comparable antibacterial activities against all bacterial species used in the experiment when compared to each other. Generally, the compounds showed superior activities against *E. coli* with compound MS-1 (cholest -5-en-3-ol) showing relatively the best antibacterial activity against *E. coli* when compared to the rest three compounds (Table 2). However, it was found to be less active than the rest three compounds against the other bacterial species (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella* Typhimurium) (Table 1). The result is consistent with previous reports that cholest-5-en-3-ol showed moderate activity against *Escherichia coli* (Gram-negative) and low activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Bajia, 2007). The compounds MS-2 (palmitic acid) and MS-4 (oleic acid) also showed superior activity against.

E. coli but relatively low activity against the rest three bacterial species (Table 10). The observed antibacterial activities of MS-2 and MS-4 were consistent with previous reports that stated their antibacterial activities (Dilika *et al*., 2000; Hinton and Ingram, 2000; Dilika *et al*., 2000; Zheng *et al*., 2005; Bazes *et al*., 2009; Lograda *et al*., 2010; Huang *et al*., 2010). A recent report by Huang *et al*. (2010) discussed extensive antibacterial activity of oleic acids against oral microorganisms including several bacterial species. The authors also suggested that oleic acid can be used as complementary ingredients in chewing gum, toothpaste, juices and milk to attack oral infections. It is important to note that MS-3 was found to show the least activity against all the test bacterial species as compared to the rest three compounds (MS-1, MS-2 and MS-4). This could be attributed to its non-polar nature. Reports indicated that the mechanism of action some polar antibacterial agents such as oleic acid and ciprofloxacin inhibiting normal function of bacterial cell growth or inhibit DNA gyrase (Sanders, 1988; Lunde *et al*., 2009). Thus, the relatively better antibacterial activities of MS-1, MS-2 and MS-4 as compared to MS-3 (n-octacosane) could be attributed to their polar nature or polar functional groups that could bind with the DNA of bacterial cells causing their death by altering the normal function of the cell membrane. To the best of our knowledge, there are no reports concerning antibacterial activity tests of n-octacosane.

Table 10: Growth inhibition zone (mm) of organisms tested for activity against isolated compounds in disc diffusion method

	Growth inhibition zone (mm)*				
Bacterial species	MS-1	MS-2	MS-3	MS-4	Ciprofloxacin
E. coli	22.75	17.5	8.25	18	31
P. aeruginosa	9	10.5	8	13.5	33
S. Typhimurium	7.5	12.5	na	10	30
S. aureus	8.5	14.5	7	15	32

*MS-1: cholest-5-en-3-ol, MS-2: palmitic acid, MS-3: n-octacosane, MS-4: oleic acid, na: not active

CONCLUSION

In conclusion, four compounds were isolated from the crude acetone extract. *In vitro* test results showed that the antibacterial activities of the isolated compounds were found to be lower than the reference compound (ciprofloxacin). When compared to each other, the antibacterial activities of the three compounds (MS-1, MS-2 and MS-4) with polar functional groups were comparable to each other and higher than that of MS-3 that is non-polar compound. The observed antibacterial activities of the crude extract and the isolated compounds could justify the traditional use of the plant for the treatment of different bacterial infections. Thus, further test is recommended on large number of bacterial strains to decide their potential as candidates in development of antibacterial drugs.

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