Isolation and Characterization of Bioactive Compounds from *M. Stenopetala* Root Extracts for Medicinal Values

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ABSTRACT

Introduction: The worldwide investigation of plant species for the treatment of various diseases and infections is still valuable. In developing countries, such as Ethiopia, where health care coverage is less, traditional medicine has played a significant role in treating health problems. *Moringa stenopetala* is among plant species widely used in traditional medicine for treatments of human health cases.

Aims: This study was conducted to isolate bioactive compounds from *Moringa stenopetala* root for medicinal purposes.

Methodology: The dried root of *Moringa stenopetala* was extracted with different solvents: n-hexane, dichloromethane/methanol (1:1) and methanol using maceration.

Results: Phytochemical analysis confirmed the presence of alkaloids, saponins, flavonoids, phenols, steroids, glycosides, terpenoids and tannins. Compounds were isolated and the compounds were characterized using results from spectroscopic techniques (IR, ¹H-NMR, ¹³C-NMR and DEPT-135). The results signify that isolated and characterized compounds are (5E, 7Z, 10E)-4,5,6,7-tetramethylnonadeca-5,7,10-trienoic acid and (4E,6E,8E)-pentadeca-4,6,8-trienoic acid with a structure that nearly look like simple fatty acids.

Conclusion: Fatty acids are known to possess antimicrobial activities of different strains and the result of this work supports the indigenous knowledge of the community of using this tree for antimalarial and anti-pain treatments.

Key Words: Crude extract, Characterization, *M. Stenopetala*, NMR, IR, Isolation

INTRODUCTION

Medicinal plants are a rich source of compounds that can be used to develop drug synthesis. Parts or the whole plant of medicinal plants that may be used for medical purposes. The active compounds in most parts of the medicinal plants have direct or indirect therapeutic effects and are used as medicinal agents.¹ Africa is a rich source of medicinal plants. About 800 species of the medicinal plants grown in Ethiopia are used for treating different medical conditions and, hence, Ethiopia is considered as the home of some of the most diverse plant species in Africa.²

Moringa belongs to the flowering plant family *Moringaceae*. It comprises about 13 species that range from tiny herbs to massive trees in size.³ The most widely known species is *Moringa oleifera*, a multipurpose tree native to

India and cultivated throughout the tropics. An overview study of Alessandro et al.⁴ saw the cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* from its leaves constituents. *M. stenopetala*, an African species, is also widely grown, but to a much lesser extent than *M. oleifera*. *M. stenopetala* is an important indigenous vegetable in southwestern Ethiopia where it is cultivated as a food crop⁵ in southern parts of Ethiopia like Konso and Darashe.⁶ The fresh leaves of the plant are cooked and consumed by the local communities. Its root parts are often used to treat malaria and other health problems. Various reports also show the antileishmanial, antitrypanosomal, antifertility, and antidiabetic activities of *M. stenopetala*. People with high blood pressure boil the leaves and drink the water to get relief from their ailment.^{6,7}

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For the investigation of constituents of the traditional medicinal plants, several researchers reported biological activities of crude extracts from different parts of *Moringa* species as well as isolation of pure compounds. Of the common bioactive compounds alkaloids, flavonoids, phenols, glycosides are capable of inhibiting the growth of various kinds of bacteria,⁸ terpenoids show significant anti-viral, anti-bacterial,⁹ anti-malarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer activities,⁸ tannins known for their anti-tumour activities,¹⁰ saponins and steroids are effective in anti-inflammatory actions.¹¹ Gopinath et al.¹² conducted research in India for isolation and identification of therapeutic compounds from *Moringa oleifera* and its antimicrobial activity. Isolation, characterization and derivatization of some bioactive components in *Moringa oleifera* leaves are identified by a study conducted in Nigeria.¹³

According to a study conducted in Arba Minch, Ethiopia, isolation of compounds from acetone extract of the root of *Moringa stenopetala* and evaluation of their antibacterial activities were done.¹⁴ Irrespective of some previous works on the phytochemical investigation from *Moringa stenopetala*, more intensive advanced scientific output awaits to know the constituents of this species with regards to the indigenous knowledge of the society. Thus, in this research phytochemical investigation was carried out to identify secondary metabolites and isolation of compounds from the root part of this plant.

MATERIALS AND METHODS

Chemicals

Reagents of n-hexane, dichloromethane and methanol were used for gradient extraction. Additionally, ethyl acetate and n-hexane for column elution, pre-coated thin-layer chromatography (TLC) (silica gel, Uv 254) were used for chromatographic analyses. To identify phytochemicals, reagents like Dragendroff's test reagents, dilute sodium hydroxide, ferric chloride, conc. Hydrochloric acid, concentrated sulphuric acid and chloroform were used.

Materials

Rotary evaporator (Heidolph, UK) for the concentration of crude extracts, Grant (GI S400) thermostatic bath shaker for maceration of plant materials were used. Oven (model: N50L, GENLAB, WIDNES, England) for heating, analytical balance (AFP-110L) for weight measuring, UV chamber (Uvitec) for visualizing molecular segregation were used. ¹H-NMR, ¹³C-NMR and DEPT-135 spectra were recorded using Bruker (400 MHz) spectrometer while IR spectra were obtained from Perkin Elmer BX infrared spectrometer (400-4000 cm⁻¹).

Plant Materials

The root of *Moringa stenopetala* was collected in April 2019 from Konso Zone, Southern Ethiopia, and identified by Botanist at the Department of Biology, Hawassa College of Teacher Education where a voucher number (MS/0031) was given and the plant material was deposited. Then it was cut into small pieces and air-dried in the laboratory at room temperature for a month without exposure to sunlight. It was then milled to a suitable size for extraction using locally available mortar. A total of 1050 g plant powder was prepared for the further extraction procedure.

Extraction

One thousand and fifty grams of powdered *Moringa stenopetala* root was macerated by successive use of n-hexane, dichloromethane/ methanol (50%:50%) and methanol for 72 h with continuous shaking using a shaker machine one after another. Figure 4 indicates the general extraction techniques followed in this work. The resulting supernatant solution was filtered using Whatman filter paper No. 1. Residual solvent in each gradient extract was removed using Rota Vapor (Heidolph, UK) under reduced pressure. It was then dried in an oven at a temperature of 40°C to remove the solvent (dichloromethane/Methanol (1:1)) and kept in the refrigerator. The per cent yields of the extracts were calculated using

$$\text{Percentage yield} = \frac{\text{mass of the extract}}{\text{mass of plant material used for extraction}} \times 100 \quad (1)$$

Schematic flow chart of extraction techniques used in this work.

Phytochemical Screening Tests

Phytochemical examinations were carried out for all the extracts as per the standard method reported in the literature.^{15, 16} Based on these methods, identification tests for the presence of secondary metabolites namely: steroids (Liebermann

Buchard reaction), terpenoids (Salkowski test), saponins (Froth test and Foam test), flavonoids (Alkaline reagent test and Lead acetate test), tannins, alkaloids (Mayer's test, Wagner's test, Dragendroff's test and Hager's test), phenols (Ferric chloride test) and glycosides (Keller-Killani test) were carried out.

Isolation and Characterization of Compounds

Among the crude extracts carried out in this experiment, the crude extract of dichloromethane/ methanol showed the best TLC profile. Then, 5.2 g of dichloromethane/methanol extract was adsorbed onto silica gel (12 g) and subjected to column chromatographic isolation. The column (1.5 m length and 1.5 cm diameter) was then eluted using ethyl acetate and n-hexane mixture starting from 100% n-hexane (3 L) and gradually increasing polarity by adding ethyl acetate. The TLC analysis of crude extract in various combinations of solvent varying polarity was used to select the solvent system for the isolation of pure compounds. In doing so, ethyl acetate and n-hexane combinations were able to show the separation of pigments on TLC of the dichloromethane/methanol crude extracts. Hence, the mixture of ethyl acetate and n-hexane has been used in different combinations with a slight increase in non-polarity.

The collected fraction was concentrated using a Rotary evaporator. Fractions were tested using TLC. The spots on the TLC plates were visualized using UV light (at 254 nm and 365 nm) followed by iodine vapour. Pure isolates were produced in (n-hexane/ethyl acetate) fractions (%: %): 85:15 and 80:20 in a combination of n-hexane and ethyl acetate, respectively. The column chromatographic separation led to the isolation of two pure compounds. The structural elucidations of these compounds were carried out

based on data obtained from spectroscopic techniques (IR and NMR).

RESULTS AND DISCUSSIONS

Mass of Crude Extracts

Depending on the method indicated materials and methods, crude extracts were carried out and allowed to dry completely. The percentage yield of successive extracts of the root of *M. stenopetala* was determined. As presented in Table 1, the gradient extraction of the roots of the targeted plant using three different solvents systems produce different yields. The percentage of crude extract of methanol was found to be higher than the other crude extracts, due to the ranges of polarity. However, was dichloromethane/methanol (1:1) solvent was selected for further extraction as it showed an excellent TLC profile.

Table 1: The percentage yield of each gradient extract

The solvent system used for extraction	Mass of Crude extract (g)	Yield (%)
n-hexane	1.8	0.17
Dichloromethane/Methanol (1:1)	5.2	0.495
Methanol	40	3.81

Phytochemical Screening Tests

Preliminary Phytochemical screening tests were performed on eight types of bioactive compounds. As can be seen from Table 2, n-hexane, methanol and dichloromethane/methanol (1:1) crude extracts showed the presence of alkaloids, flavonoids, phenols, glycosides, terpenoids, tannins, saponins and steroids except tannins is absent in crude extract of methanol.

Table 2: Phytochemical analysis of *M. stenopetala* crude extracts

Bioactive component	Reagents used	The solvent used for crude extraction					
		n-hexane		Dichloromethane/methanol		Methanol	
		Result	Observed Colour	Result	Observed colour	Result	Observed colour
Alkaloids	Dragendroff's reagent	++	Red colour	++	Red colour	++	Red colour
Flavanoids	Alkaline reagent	++	Deep yellow colour	++	Deep yellow colour	++	Deep yellow colour
Phenols	Ferric chloride	++	Bluish black colour	++	Bluish black colour	++	Bluish black colour
Glycosides	Keller-Killani test	++	Light reddish-brown	++	Light reddish-brown	++	Light reddish-brown
Terpenoids	Salkowski test	++	Radish brown colour	++	Radish brown colour	++	Radish brown colour
Tannins	Ferric chloride	++	Brownish green	++	Brownish green	--	--
Saponins	Distilled water	++	Strong foam formation	++	Strong foam formation	++	Strong foam formation
Steroids	Liebermann Buchard reaction	++	Light blue-green	++	Light blue-green	++	Light blue-green

Key: Strong (++) Absent (--)

Structural Elucidation of Compound 1

Compound 1, **MS1**, was obtained as a white crystal appearance (38.6 mg). It was isolated from a combined 15% of ethyl acetate in n-hexane for elution of the chromatography. The absence of doublet band at/near 2850 and 2750 cm^{-1} in the IR (400-4000 cm^{-1}) spectrum (Appendix A – Fig A1) indicate that the compound has no aldehyde functional group. The absence of a strong band in the range or around 1700-1800 cm^{-1} shows that the compound has no carbonyl functional group. The absence of weak bands in the range of 2000 and 1650 cm^{-1} indicates that the compound has no aromatic functional group. On the other hand, the observed stretching broadband at 3430 cm^{-1} indicates the presence of the carboxylic acid functional group. The strong band around 2900 cm^{-1} represents the C-H stretch of alkenes whereas the bands around 2854.45 cm^{-1} indicate the C-H stretching of methyl groups. The observed data suggest that compound **MS1** could be a carboxylic acid possessing a C=C double bond in its chain.

In the $^1\text{H-NMR}$ (400MHz, DEMSO) spectrum of compound **MS1** (Appendix A – Fig A2), the peaks at δ 0.7 and 0.9 indicate the presence of protons of two methyls ($-\text{CH}_3$) groups whereas the peak at δ 5.26, 5.40 and 5.49 indicate the presence of aliphatic protons in compound **MS1**. These $^1\text{H-NMR}$ data along with the observed IR spectral suggests that compound **MS1** be most likely fatty acid. The $^{13}\text{C-NMR}$ (400MHz) spectrum of compound **MS1** shows signals at 128.01, 128.20, 128.35, 130.17, 130.34 and 131.94 ppm which are assigned to C-5, C-7 and C-10 double bonds, respectively. $^{13}\text{C-NMR}$ (Appendix A- FigA3) and DEPT-135 (Appendix A – Fig A4) spectra show 25.67 and 29.45 ppm signals, respectively, that can be assigned to five methyls, ten methylene, four methine and four quaternary carbon atoms (Table 4). Similar to that IR data, observed NMR data of compound **MS1** were also found to be consistent with the reported data of Mulugeta et al.¹³ These indicate that the compound has a similar chemical structure with a simple fatty acid structure (Figure 1).

(5E,7Z,10E)-4,5,6,7-tetramethylnonadeca-5,7,10-trienoic acid

Figure 1: Structural elucidation of compound **MS1**.

Mulugeta et al.¹⁴ reported a compound with the structure of the fully simple aliphatic compound and quite similar with **MS1** though it has a single double bond on carbon number 9-10. Compound **MS1** has three double bonds on carbon numbers 5, 7 and 10, due to this compound **MS1** shows some spectral differences. Since **MS1** is a polyunsaturated fatty acid, it has more electron cloud density than that of the reported one because of this fact the compound is more reactive. In addition to this, **MS1** has more benefit in free radical scavenging ability as result it has a great preventing effect.

Table 3: $^{13}\text{C-NMR}$ (in ppm) and DEPT-135 (in ppm) data of compound **MS1** along with corresponding reported data of oleic acid.

Carbon atom	$^{13}\text{C-NMR}$ data MS1	$^{13}\text{C-NMR}$ data of Oleic acid [7]	DEPT-135 data of MS1	DEPT-135 data Oleic acid [7]	Nature of carbon
C-1	174.93	180.196	-	-	C
C-2	34.15	34.078	31.75	34.078	CH_2
C-3	29.46	24.683	31.41	24.683	CH_2
C-4	31.36	29.153	31.36	29.155	CH_2
C-5	137.0	29.073	-	29.073	C
C-6	128.01	29.447	-	29.447	C
C-7	131.94	29.606	-	29.684	C
C-8	130.17	27.162	130.34	27.161	CH
C-9	31.76	129.734	31.36	129.737	CH
C-10	128.01	130.030	130.17	130.031	CH_2
C-11	128.35	27.227	128.21	27.226	CH
C-12	29.46	29.706	29.46	29.776	CH_2
C-13	29.19	29.338	26.78	29.337	CH_2
C-14	29.01	29.536	22.52	29.535	CH_2
C-15	29.07	29.256	14.39	29.256	CH_3
C-16	27.07	31.941	27.07	31.941	CH_2
C-17	25.67	22.707	25.67	22.706	CH_2
C-18	22.56	14.133	22.43	14.134	CH_2
C-19	22.43	-	24.96	-	CH_3
C-20	20.49	-	40.23	-	CH_3
C-21	14.37	-	14.37	-	CH_3
C-22	14.56	-	14.37	-	CH_3
C-23	22.43	-	40.23	-	CH_3

DEPT – distortionless enforcement by polarization transfer, NMR – nuclear magnetic resonance

Structural Elucidation of Compound 2

The second compound, Compound **MS2**, was obtained as a light yellowish crystal appearance (24.46 mg). It was isolated from a combined 20% of ethyl acetate in n-hexane for elution of the chromatography. IR spectrum (Appendix B – Fig

A5) for compound **MS2** has no doublet band at/near 2850 and 2750 cm^{-1} that indicating that the compound has no aldehyde functional group. The presence of a strong band in the

range around 1700-1800 cm^{-1} also shows that the compound has a carbonyl functional group.

Table 4: ^{13}C -NMR (in ppm) and DEPT-135 (in ppm) data of compound **MS2** along with corresponding reported data of oleic acid.

Carbon atom	^{13}C -NMR data of MS2	^{13}C -NMR data of oleic acid ¹³	DEPT-135 data of MS2	DEPT-135 data of oleic acid ¹³	Nature of carbon
C-1	174.96	180.196	-	-	C
C-2	36.7	34.078	31.75	34.078	CH_2
C-3	30.6	24.683	31.41	24.683	CH_2
C-4	132.4	29.153	132.97	29.155	CH
C-5	129.5	29.073	128.65	29.073	CH
C-6	130.4	29.447	129.71	29.447	CH
C-7	130.4	29.606	128.98	29.684	CH
C-8	128.8	27.162	127.59	27.161	CH
C-9	135.7	129.734	133.25	129.737	CH
C-10	33.8	130.030	40.17	130.031	CH_2
C-11	30.1	27.227	40.38	27.226	CH_2
C-12	29.5	29.706	29.46	29.776	CH_2
C-13	32.0	29.338	26.78	29.337	CH_2
C-14	22.8	29.536	22.52	29.535	CH_2
C-15	14.1	29.256	14.39	29.256	CH_3

The absence of weak bands in the range of 2000 and 1650 cm^{-1} indicates that the compound has no aromatic functional group. On the other hand, the observed stretching broadband at 3402.20 cm^{-1} indicates the presence of the carboxylic acid functional group. The strong band around 2923 cm^{-1} represents the C-H stretch of alkenes whereas the bands around 2854.45 cm^{-1} indicates the C-H stretching of methyl groups. The observed data suggest that compound **MS2** could be a carboxylic acid possessing a C=C double bond in its chain.

In the ^1H -NMR (400MHz, DEMISO) spectrum of compound **MS2** (Appendix Fig A6), the peaks at δ 0.8 indicate the presence of protons of one methyl ($-\text{CH}_3$) groups whereas the peak at δ 7.351, 7.629 and 7.809 indicate the presence of unsaturated methine protons in compound **MS2**. These ^1H -NMR data along with the observed IR spectral suggest that compound **MS2** to be most likely fatty acid with fifteen carbon numbers. The ^{13}C -NMR spectrum of compound **MS2** shows signals at 128.65, 128.98, 129.71, 131.29, and 133.25 ppm which are assigned to C-4, C-6 and C-8 have double bonds, respectively. ^{13}C -NMR (Appendix B – Fig A7) and DEPT-135 spectra (Appendix B- Fig A8) show that compound **MS2** has seven methylene, one methyl, six methines and one quaternary carbon (Table 5). Both IR and NMR data of compound **MS2** were also found to be consistent with the

reported data of Mulugeta et al.¹⁴ These indicate the compound has a similar chemical structure with a simple fatty acid structure (Figure 2).

Figure 2: Structural elucidation of compound **MS2**.

Previous work of Mulugeta et al.¹⁴ a compound with a structure fully simple aliphatic compound and quite similar with **MS2** elucidated in this work though it has a single double bond on carbon number 9-10. Compound **MS2** in the present work has three double bonds on carbon numbers 4, 6 and 8 and it shows some spectral difference. Since **MS2** is a polyunsaturated fatty acid, it has more electron cloud density than that of the reported one because of this fact the compound is more reactive. In addition to this **MS2** has a more benefit free radical scavenging ability effect.

CONCLUSION

Phytochemical screening of the root of *M. stenopetala* Ethiopian flora confirmed the presence of alkaloids, saponins, flavonoids, phenols, steroids, glycosides, terpenoids and tannins. Chromatographic separation dichloromethane/methanol (1:1) yielded three compounds. The two fatty acid-containing compounds (5E, 7Z, 10E)- 4,5,6,7-tetramethyl-nonadeca-5,7,10-trienoic acid and (4E,6E,8E)-pentadeca-4,6,8-trienoic acid are fully elucidated. Fatty acids are known to possess antimicrobial activities of different strains and the result of this work supports the indigenous knowledge of the community of using this tree for antimalarial and anti-pain treatments.

Supplementary Materials: The IR, NMR spectra of compounds MS1 and MS2 are included in the Supplementary Materials.

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