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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

# Evaluation of antileishmanial activities of triglycerides isolated from roots of *Moringa stenopetala*

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**Abstract** The objective of this study was to evaluate antileishmanial activities of compounds isolated from the roots of *Moringa stenopetala.* Two compounds were isolated from the crude petroleum ether:ethyl acetate (50:50 %) extract of its roots employing column chromatographic technique using a mixture of petroleum ether and ethyl acetate mixture in different polarity (or proportions by volume). The isolated compounds were labeled as **MS-1** and **MS-2**. Their structures were determined to be triglycerides based on the observed spectroscopic ($^{1}$H-NMR, $^{13}$C-NMR, DEPT-135, and IR) data and reported data in literature. Thus, compound **MS-1** and compound **MS-2** were identified as 1,3-dilinoleoyl-2-olein and 1,3-dioleoyl-2-linolein, respectively. Evaluation of antileishmanial activities ($IC_{50}$ values) of the compounds against promatigote stage of *Leishmania aethiopica* indicated that compound MS-1 to show comparable activity with the reference compounds (amphotericin B and miltefosine) whereas compound MS-2 to be less active. The activity test results against the amastigote stage of the parasite indicated that the compounds have comparable activities when compared to each other, and relatively lower activities as compared to that of the reference compounds. The results suggested that the compounds have promising antileishmanial activities. However, further in vivo tests are recommended before drawing any conclusion about the potential of the compounds as a new antileishmanial drug candidate.

**Keywords** *Moringa stenopetala* · Triglycerides · *L. aethiopica* · Leishmaniasis

## Introduction

*Moringa stenopetala* is one of the 14 *Moringa* species that belong to the family of *Moringaceae* (Mekonnen, 2002). It is a native tree in Southern Ethiopia, Northern Kenya, and Eastern Somalia. In Ethiopia, *M. stenopetala* is found in many arid areas of the Southern Ethiopia most extensively in Arbaminch and its surrounding areas such as Konso, Negelle, and Wellayta sodo at altitude of up to 1,800 m a.s.l (Stelz and Mayer, 1990; Mekonnen and Gessesse, 1998; Yishak *et al.*, 2011). It is the major vegetable crop in the region (Lindtjorn, 1983; Mekonnen and Gessesse, 1998; Demeulenaere, 2001). It can also be used as bee forage (Tessema *et al.*, 1993) and animal fodder (Jahn, 1991) as well as water purification purposes (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 hypertensionis reported by Bennett *et al.* (2003) and Mekoya (2007), stomach pains, expulsion of retained placenta during birth, asthma, and diabetes (Mekonnen and Gessesse, 1998; Mekonnen, 1999, 2002; Mussa *et al.*, 2008; Yishak *et al.*, 2011). People inhabiting around Lake Turkana also use its leaves for treatment of Leprosy (Jahn, 1991). These observations from traditional medicinal uses of the plant has led several research teams to carry out

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experimental investigations in order to find out bioactive constituents that are responsible for the observed various medicinal uses of the plant, and with an ultimate goal of discovering drugs against different diseases.

The majority of the research teams carry out biological activity tests using crude extracts obtained from different morphological 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 *M. stenopetala* was assessed in non-diabetic rabbits using blood glucose analysis. The extract was found to lower blood glucose and cholestrol in mice models (Mekonnen et al. 1997; Mekoya, 2007; Ghebreselassie *et al.*, 2011; Toma *et al.*, 2012). These findings justified the traditional use of leaves of *M. stenopetala* as antihypertensive agent by some localities of Southern Ethiopia. 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 out on guinea-pig ileum, mouse duodenum and uterus strips using ethanol extract of its leaves revealed an antispasmodic property and some oxytocic activity on uterus strips of guinea-pigs and mice. These observations were used by different authors to justify the traditional use of the leaves of *M. stenopetala* for relieving stomach pain and to expel retained placenta by women during giving birth (Mekonnen, 1999). Other experimental results also indicated antitrypanosomal, anti-fertility, and antimicrobial properties of the seed extracts. Extracts from leaves and roots of *M. stenopetala* were tested in vitro against trypomastigotes of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *L. donovani* amastigotes. The fresh root ethanol extract and the dried leaves acetone extract were found to be active against *T. brucei* whereas the other extracts were found to be inactive against the above tested parasite forms (Mekonnen *et al.*, 1999; Nibret and Wink, 2010). Reports also revealed that leaf, root, and seed extracts show low toxicity as demonstrated by the results of toxicity tests against HEPG2 cells (Mekonnen *et al.*, 2005). The results were also claimed to be consistent with the use of the leaves of the plant as vegetable by people.

There are many reports describing attempts that have made on isolation of compounds from different parts of *M. stenopetala.* Alemayehu and Tarekegn (2003) isolated three glycosides namely rutin, 4-(4′-*O*-acetyl-L-rhamnosyloxy) benzylisothiocyanate, and 4-(4′-*O*-acetyl-L-rhamnosyloxy) benzaldehyde from leaf extracts. Eilert *et al.* (1981) reported 4-(alpha-L-rhamnosyloxy) benzyl isothiocyanate as an active antimicrobial agent from seeds of *M. stenopetala.* Mekonnen and Drager (2003) identified 5,5-dimethyloxazolidine-2-thione and *O*-(rhamnopyranosyloxy) benzyl glucosinolate from seed extracts of *M. stenopetala*. Different unsaturated fatty acids (e.g., oleic acid) and saturated acids (e.g., behenic acid and palmitic acid as well as high levels of β-sitosterol, stigmasterol, and campesterol have also been reported from n-hexane and chloroform:methanol (1:1) extracts of seed oils of the plant (Lalas *et al.*, 2003). Nibret and Wink (2010) have reported isolation of several compounds, which belong to glucosinates, from seeds of *M. stenopetala*. Bioactivity tests of these compounds were found to show potent trypanocidal and low cytotoxic activities against HL-60 cells. Glucosinolates from seeds, leaves, and roots of *M. stenopetala*, and their activity as antihypertensive were also reported previously (Bennett *et al.*, 2003 and Mekonnen and Drager, 2003). As discussed above, the in vivo and in vitro tests using crude extracts of roots of *M. stenopetala* showed promising biological activities including antileishmanial activities. However, our literature survey revealed that no work has been done on the evaluation of antileishmanial activities of compounds isolated from *M. stenopetala*. Thus, the aim of our work was to isolate compounds from roots of this plant, and evaluate their antileishmanial activities wishing to find compounds that could be used as leads in the discovery of antileishmanial drugs.

## Methods and materials

### Chemicals, apparatus, and general procedures

#### *Chemicals and apparatus*

General laboratory grade solvents (petroleum ether and ethyl acetate) for the extraction and isolation were purchased from Sigma-Aldrich Company. The solvents were subjected to distillation before using them for extraction and isolation purposes. Nuclear magnetic resonance data ($^{1}$H-NMR, $^{13}$C-NMR, and DEPT-135) were recorded using Bruker Advance 400 MHz spectrometer. $CDCl_3$ was used as solvent in all nuclear magnetic resonance (NMR) analyses. All NMR analyses were carried out at the Department of Chemistry, Addis Ababa University. Infra red (IR) data were recorded as KBR pellets using Perkin-Elmer BX spectrometer (400–4,000 $cm^{-1}$) at the Ethiopian pharmaceutical industry, Addis Ababa. Silica gel (60–120 mm mesh size) and TLC plate (silica gel, $UV_{254}$ pre-coated on aluminum sheets) were used for chromatographic analyses. Compounds on TLC plates were detected using UV and iodine chambers. Evaporation of solvents under reduced pressure was carried out using a rotary evaporator (Heidolph, UK).

#### *Collection and preparation of plant material*

The plant materials (root of *M. stenopetala*) were collected from the gardens of Arbaminch, Southern Ethiopia that is

about 500 km away from Addis Ababa, in January 2011. The collected plant materials were washed carefully and chopped into small pieces, and subsequently air-dried at room temperature without exposure to direct sunlight. The dried materials were then powdered using a grind mill into coarse powder and sieved to homogenize the sample. The powdered plant material was stored in suitable container until used for extraction.

*Extraction and isolation*

500 g of powdered plant material was extracted with 2 l of petroleum ether:ethyl acetate (50:50 %) at room temperature for 48 h maceration technique. The mixture was then filtered using a Whatman filter paper. Removal of solvent under reduced pressure gave a 7.40 g of crude extract. Out of 7.40 g of crude extract, 4.2 g was adsorbed onto 4 g of silica gel and was then subjected to column chromatography that was loaded with 84 g of silica gel. The column was eluted sequentially with petroleum ether:ethyl acetate mixture. The polarity of the eluting mixture was increased slowly from 100:0 to 90:10 % petroleum ether:ethyl acetate. The volume of each fractions collected was 20 ml. The purity of fractions was checked using UV visualization of the spots on TLC plates. Similar fractions were combined based on similarity of their TLC profiles. Among the collected fractions, fractions 11–14 were eluted with 98:2 % petroleum ether:ethyl acetate and fractions 1–30 of which were eluted with 97:3 % petroleum ether:ethyl acetate, and were purified and combined based on their TLC analysis. The combined fractions were concentrated under reduced pressure that resulted in two pure compounds that were labeled as **MS-1** and **MS-2**, respectively. Structures of the compounds were elucidated using spectroscopic techniques mentioned above and reported data in literature.

Evaluation of antileishmanial activities of the isolated compounds

*Test strains and source of macrophages*

The standard *Leishmania aethiopica strain* (MHOM/ET/82/117-82) (obtained from Leishmaniasis Diagnostic and Research Laboratory, Faculty of Medicine, Addis Ababa University) and murine peritoneal macrophage isolated from *BALB/c* mice were used in the in vitro assay.

*Reference drugs*

The reference drugs used were amphotericin B (Fungizone®, Bristol-Myers Squibb, Rueil-Malmaison, France) and miltefosine (Impavido®, Zantaris GmBH, Germany).

*Culture conditions*

*Promastigote culture* *Leishmania aethiopica* was grown in tissue culture flasks containing RPMI 1640 medium (Gibco, Invitrogen Co., UK), supplemented with 10 % heat-inactivated fetal calf serum (HIFCS) (Gibco, Invitrogen Co., UK), and 100 IU penicillin/ml and 100 µg/ml streptomycin solution (Sigma Chemical Co., St. Louis, USA) at 22 °C following previously described methods.

*Murine peritoneal exudate macrophages (MPEM)* The isolation and differentiation of MPEM was carried using a standard method reported in literature (Yuh-Chi *et al.*, 2012 http://ejournal.nricm.edu.tw/upload/21614/13/13-3-05.pdf). First, the MPEMs were induced by injecting a 2 ml of 2 % starch solution into a mouse peritoneum. 24 h after injection, the test mice were killed by $CO_2$ suffocation, and degloved and injected with cold RPMI. Then peritoneal exudates were removed using a sterile 10 ml syringe with 21G 5/8 needle and collected in sterile plastic tubes placed in ice bath.

*Intracellular amastigote culture* The intracellular amastigotes forms of *L. aethiopica* were obtained using a standard procedure (Yardley and Croft, 2000). Briefly, after harvesting the exudates, the cell suspension was determined by counting using a Neuebauer hemocytometer, and the cell density was adjusted to 4,000. Finally, 100 µl of the cell suspension was seeded into 16-chamber tissue plate and incubated at 37 °C for 24 h to allow differentiation of MPEMs. Promastigotes in stationary phase were used to infect differentiated MPEMs in a 16-well culture plates in the infection ratio of 7:1 (parasites to MPEM).

Biological assays

*Promastigote-based assay*

In a 96-well microliter plate, the test compound was serially diluted to twice the final test concentrations (1.00, 0.33, 0.11, 0.037, 0.012, 0.004, and 0.0013 mg) in 100 µl culture medium with each test concentration in duplicate. Then, 100 µl of suspensions containing $3.5 \times 10^6$ promastigotes/ml in a logarithmic phase were added to each well. Contents of the plates were then maintained at 22 °C in a 5 % $CO_2$ incubator. The cell density, motility, and morphology for each treatment were determined daily with an inverted microscope and the antileishmanial activity was expressed as the MIC values after 72 h of incubation. The reference drugs and medium with 1 % DMSO were included as controls.

*Antiamastigote-based assay* The intracellular amastigotes forms of *L. aethiopica* were obtained using a standard method (Yardley and Croft, 2000). *Leishmania aethiopica* promastigotes in stationary phase were used to infect differentiated MPEMs in a 16-well culture plates in the infection ratio of 7:1. Then contents of the plates were incubated at 37 °C in humidified 5 % $CO_2$ incubator for 24 h. The amastigote growth inhibitory activities of the test compound were carried by dispensing different concentrations (1.00, 0.33, 0.11, 0.037, 0.012, 0.004, and 0.0013 mg) into duplicate wells of 16-well culture plates on which intracellular amastigotes were attached. Then the contents were incubated at 37 °C for 5 days in a 5 % $CO_2$ incubator. After 5 days of exposure of the infected cells to the test compound and the reference drugs, the overlay from all of the wells was removed. After 30 s of fixing with 100 % methanol, the slides were stained with 10 % Giemsa for 10 min. After rinsing and air-drying the slides were ready for examination under oil immersion (100×). During examination, infected and uninfected macrophages were counted in each well in a total of 100 macrophages. The % inhibition values were derived by comparison with the % infection of macrophages in control wells. The same procedure was applied for the reference drugs-amphotericin B and miltefosine.

## Results and discussion

### Structure elucidation of compounds

As discussed above, column chromatographic isolation of 4.2 g of crude extract of petroleum ether:ethyl acetate gave compounds **MS-1** and **MS-2**. The structure elucidation of these compounds was done using their $^{1}$H-NMR, $^{13}$C-NMR, DEPT-135, and IR spectra in comparison with the reported data of these compounds in the literature.

### Structure elucidation of compound MS-1

Analysis of IR spectra of **MS-1** (Supplementary material 1) indicated that the compound has no hydroxyl or carboxylic acid functional groups. This was confirmed by the absence of broad bands in the range of 3,650–3,600 $cm^{-1}$ (for hydroxyl) and 3,400–3,200 $cm^{-1}$ (for carboxylic acids). The absence of a doublet band at/near 2,850 and 2,750 $cm^{-1}$ also indicated that the compound has no aldehydic functional group. The absence of bands around (or above) 1,800 $cm^{-1}$ indicated that the compound is neither acid anhydride nor acid chloride. The absence of weak bands in the range of 2,000 and 1,650 $cm^{-1}$ indicated that the compound has no aromatic functional group. Thus, the observed carbonyl group stretching band at 1,745 $cm^{-1}$ indicated that the compound is most likely an ester. The broad band at 1,163 $cm^{-1}$ also indicates the C–O stretching vibration of an ester. The strong band at 3,006.80 $cm^{-1}$ represents C–H stretch of an alkene whereas the bands at 2,922 and 2,852 $cm^{-1}$ were attributed to C–H stretch of methylene and methyl groups.

In the $^{1}$H-NMR spectrum of compound **MS-1** (Supplementary material 2), the triplet peak at $\delta$ 0.90 indicated the presence of protons of methyl groups adjacent to methylene carbon; the peaks at $\delta$ 1.25 and $\delta$ 1.60 indicate protons of aliphatic methylene (–$CH_2$) group; a peak at $\delta$ 2.03 indicates the presence of protons of a methylene group that is bonded to C=C bond; the peak at $\delta$ 2.33 indicates the presence of protons of methylene that is bonded to a carbonyl group; the peak at $\delta$ 2.80 indicates the presence of protons of methylene group that is flanked by two C=C bonds; the peaks at $\delta$ 4.17 and 4.30 indicate the presence of protons of a carbon attached to oxygen whereas the peak at $\delta$ 5.40 ppm indicates the presence of olefinic protons in the structure. The observed IR and $^{1}$H-NMR data were found to be consistent with the reported data of 1,3-dilinoleoyl-2-oleine (Ramsewak *et al.*, 2001). Thus, based on this observation, the chemical structure of compound **MS-1** was proposed to be identical with that of 1,3-dilinoleoyl-2-oleine (Fig. 1). 1,3-Dilinoleoyl-2-oleine has been isolated from of *Moringa oleifera* (Yammuenart *et al.*, 2008). The $^{1}$H-NMR data of compound **MS-1** and that of 1,3-dilinoleoyl-2-oleine are given in Table 1.

In the $^{13}$C-NMR spectrum of **MS-1** (Supplementary material 3), the peaks in the range of chemical shift values at $\delta$ 127.19–130.23 ppm indicated C=C bonds; the peaks at $\delta$ 173.29 and 172.85 ppm indicated quaternary carbon atoms of ester carbonyl group. On the other hand, chemical shift values in the range of 14.15–68.89 ppm indicated the presence of methyl (–$CH_3$), methylene (–$CH_2$), and oxygenated methylene (–$OCH_2$) carbons (Table 2). The DEPT-135 spectrum showed a peak for the presence of methyl (–$CH_3$) carbon at $\delta$ 14.17, methylene carbons at $\delta$ 22.71–61.89, oxygenated methine (–CH) carbon at $\delta$ 68.91, and olefinic methyne carbons at $\delta$ 127.91–131.08 (Supplementary material 4). The absence of peaks at $\delta$ 172.85 and 173.30 ppm in the DEPT-135 spectrum indicated

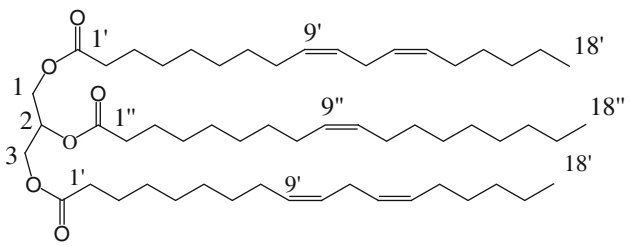


**Fig. 1** The proposed structure of compound **MS-1**

**Table 1** $^{1}$H-NMR data of compound **MS-1** in comparison with reported data of 1,3-dilinoleoyl-2-oleine (Ramsewak *et al.*, 2001)

| H no. | $\delta$ $^{1}$H (compound **MS-1**) | $\delta$ $^{1}$H (reported data) |
|---|---|---|
| 1a | 4.20 | 4.12 |
| 1b | 4.31 | 4.28 |
| 2 | 5.25 | 5.25 |
| 3a | 4.20 | 4.12 |
| 3b | 4.30 | 4.28 |
| 1′, 1″ | – | – |
| 2′, 2″ | 2.33 | 2.28, 2.29 |
| 3′, 3″ | 1.60 | 1.59 |
| 4′, 4″ | 1.25 | 1.25 |
| 5′, 5″ | 1.25 | 1.25 |
| 6′, 6″ | 1.25 | 1.25 |
| 7′, 7″ | 1.25 | 1.25 |
| 8′, 8″ | 2.03 | 2.02 |
| 9′, 9″ | 5.40 | 5.33 |
| 10′, 10″ | 5.40 | 5.33 |
| 11′, 11″ | 2.80, 2.03 | 2.75, 2.02 |
| 12′, 12″ | 5.40, 1.25 | 5.33 |
| 13′, 13″ | 5.40, 1.25 | 5.33 |
| 14′, 14″ | 2.03, 1.25 | 2.02 |
| 15′, 15″ | 1.25 | 1.25 |
| 16′, 16″ | 1.25 | 1.25 |
| 17′, 17″ | 1.25 | 1.25 |
| 18′, 18″ | 0.90 | 0.86, 0.87 |

quaternary carbon atoms (of ester carbonyl group). The peaks were observed in $^{13}$C-NMR spectra but not in the DEPT-135 spectra.

## Structure elucidation of compound MS-2

Similar to that of compound **MS-1**, IR spectrum of compound **MS-2** (Supplementary material 5) indicated that the compound has no alcohol or carboxylic acid functional groups. This was confirmed by the absence of broad bands in the range of 3,650–3,600 cm$^{-1}$ (for alcohols) and 3,400–3,200 cm$^{-1}$ (for carboxylic acids). Moreover, the absence of a doublet band at/near 2,850 and 2,750 cm$^{-1}$ indicated that the compound has no aldehydic functional group. The absence of bands around 1,800 cm$^{-1}$ indicated that the compound is neither acid anhydride nor acid chloride. The absence of weak bands in the range of 2,000 and 1,650 cm$^{-1}$ indicated that the compound has no aromatic functional group. The observed carbonyl group stretching band at 1,743 cm$^{-1}$ indicated that the compound is most likely an ester. The broad band at 1,163 cm$^{-1}$ also indicates the C–O stretching vibration of an ester. The strong band at 3,006.80 cm$^{-1}$ represents C–H stretching of an alkene whereas the bands at 2,918 and 2,853 cm$^{-1}$ indicate C–H stretching of methyl groups.

In the $^{1}$H-NMR spectrum of compound **MS-2** (Table 3; Supplementary material 6), the peak at $\delta$ 0.91 indicates protons of methyl groups; the peaks at $\delta$ 1.25 and 1.63 ppm

**Table 2** $^{13}$C-NMR and DEPT-135 data of compound **MS-1** in comparison with reported data of 1,3-dilinoleoyl-2-oleine (Ramsewak *et al.*, 2001)

| C· no. | $\delta$ $^{13}$C (compound **MS-1**) | $\delta$ $^{13}$C (reported data) | $\delta$ DEPT-135 | Remark |
|---|---|---|---|---|
| 1 | 62.11 | 62.10 | 61.89 | –$CH_2$ |
| 2 | 68.89 | 68.90 | 68.91 | –CH |
| 3 | 62.11 | 62.11 | 61.89 | –$CH_2$ |
| 1′, 1″ | 173.30, 172.85 | 173.2, 172.8 | – | Quaternary |
| 2′, 2″ | 34.05, 34.21 | 34.0, 34.2 | 33.95, 34.11 | –$CH_2$ |
| 3′, 3″ | 31.94, 31.55 | 31.9, 31.5 | 31.94 | –$CH_2$ |
| 4′, 4″ | 22.7 | 22.4 | 22.71 | –$CH_2$ |
| 5′, 5″ | 22.7 | 22.6 | 22.71 | –$CH_2$ |
| 6′, 6″ | 24.89 | 24.8 | 24.86 | –$CH_2$ |
| 7′, 7″ | 24.89 | 24.9 | 24.86 | –$CH_2$ |
| 8′, 8″ | 27.22 | 27.2 | 27.25 | –$CH_2$ |
| 9′, 9″ | 129.72, 130.23 | 127.8–130.2 | 127.91–130.08 | –CH |
| 10′, 10″ | 127.9, 129.72 | 127.8–130.2 | 127.91–130.08 | –CH |
| 11′, 11″ | 25.65, 27.22 | 25.6, 27.2 | 24.86 | –$CH_2$ |
| 12′, 12″ | 127.91, 128.08 | 127.8–130.2 | 127.91–130.08 | –CH |
| 13′, 13″ | 131.9, 130.23 | 127.8–130.2 | 127.91–130.08 | –CH |
| 14′, 14″ | 27.22 | 27.2 | 27.22 | –$CH_2$ |
| 15′, 15″ | 29.15–29.74 | 29.0–29.7 | 29.16–29.73 | –$CH_2$ |
| 16′, 16″ | 29.15–29.74 | 29.0–29.7 | 29.16–29.73 | –$CH_2$ |
| 17′, 17″ | 29.15–29.74 | 29.0–29.7 | 29.16–29.73 | –$CH_2$ |
| 18′, 18″ | 14.15, 14.15 | 14.1, 14.0 | 14.17, 14.17 | –$CH_3$ |

**Table 3** $^{1}$H-NMR data of compound **MS-2** in comparison with reported data of 1,3-dioleolyl-2-linolein (Ramsewak *et al.*, 2001)

| H no. | $\delta$ $^{1}$H (compound **MS-2**) | $\delta$ $^{1}$H (reported data) |
|---|---|---|
| 1a | 4.12 | 4.12 |
| 1b | 4.31 | 4.28 |
| 2 | 5.25 | 5.25 |
| 3a | 4.16 | 4.12 |
| 3b | 4.29 | 4.28 |
| 1′, 1″ | – | – |
| 2′, 2″ | 2.32 | 2.28, 2.29 |
| 3′, 3″ | 1.63 | 1.59 |
| 4′, 4″ | 1.25 | 1.25 |
| 5′, 5″ | 1.25 | 1.25 |
| 6′, 6″ | 1.25 | 1.25 |
| 7′, 7″ | 1.25 | 1.25 |
| 8′, 8″ | 2.07 | 2.02 |
| 9′, 9″ | 5.40 | 5.33 |
| 10′, 10″ | 5.40 | 5.33 |
| 11′, 11″ | 2.05, 2.77 | 2.02, 2.75 |
| 12′, 12″ | 1.25, 5.40 | 5.33 |
| 13′, 13″ | 1.25, 5.40 | 5.33 |
| 14′, 14″ | 1.25, 2.03 | 2.02 |
| 15′, 15″ | 1.25 | 1.25 |
| 16′, 16″ | 1.25 | 1.25 |
| 17′, 17″ | 1.25 | 1.25 |
| 18′, 18″ | 0.91 | 0.86, 0.87 |

indicate protons of aliphatic methylene group; a peak at $\delta$ 2.03 and 2.05 ppm indicates protons of a methylene group that is bonded to C=C bond; the peak at $\delta$ 2.32 indicates protons of methylene that is bonded to a carbonyl group; the peak at $\delta$ 2.77 indicates protons of a methylene group that is flanked by two C=C bonds; the peaks at $\delta$ 4.12, 4.16, 4.29, and 4.31 indicate the presence of protons of oxygenated carbon whereas the peak at $\delta$ 5.40 ppm indicates the presence of olefinic protons. The observed IR and $^{1}$H-NMR data were found to be consistent with the reported data of 1,3-dioleolyl-2-linolein (Fig. 2) (Ramsewak *et al.*, 2001). Thus, based on this observation, the chemical structure of compound **MS-2** was proposed to be identical with the chemical structure of 1,3-dioleolyl-2-linolein. 1,3-Dioleolyl-2-linolein was previously isolated from *M. oleifera* (Yammuenart *et al.*, 2008). The $^{1}$H-NMR data of **MS-2** and that of 1,3-dioleolyl-2-linolein are given in Table 3.

The $^{13}$C-NMR spectrum of **MS-2** (Supplementary material 7) was also found to be almost similar to the reported $^{13}$C-NMR spectral data of 1,3-dioleolyl-2-linolein (Table 4) (Ramsewak *et al.*, 2001). The peaks observed at $\delta$ 127.91–130.09 ppm indicated proton attached to C=C bond whereas the two peaks at $\delta$ 172.34 and 172.75 ppm

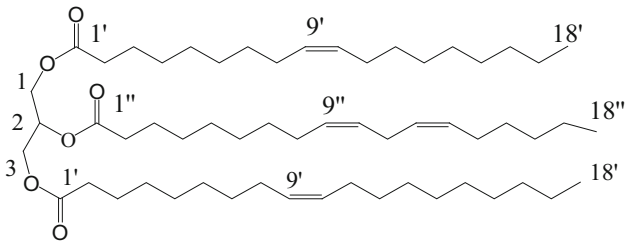


**Fig. 2** The proposed structure of compound **MS-2**

indicated quaternary carbon atoms of ester carbonyl group. The DEPT-135 spectrum (Supplementary material 8) also confirmed the presence of methyl carbon (at 14.17 ppm), methylene carbon at $\delta$ 22.71 and 62.00 ppm, and oxygenated methine carbon at 68.91, and olefinic methine carbons at $\delta$ 127.91–130.09 ppm. The absence of peaks at $\delta$ 172.75 and 172.34 ppm in the DEPT-135 spectra, which were observed in the $^{13}$C-NMR spectra, also confirmed quaternary carbon atoms of ester carbonyl group.

Isolation of four triglycerides from *M. oleifera* has been reported. The four triglycerides were 1,2,3-triolein, 1,2,3-trilinolein, 1,3-dilinoleoyl-2-olein, and 1,3-dioleoyl-2-linolein (Yammuenart *et al.*, 2008). Comparison of spectral data of compound **MS-1** and compound **MS-2** with the reported spectral data of the four triglycerides ruled out the two triglycerides (1,2,3-triolein and 1,2,3-trilinolein). Thus, the structures of compound **MS-1** and compound **MS-2** were proposed to be similar to that of triglycerides 1,3-dilinoleoyl-2-olein and 1,3-dioleoyl-2-linolein, respectively, based on the similarities of their $^{1}$H-NMR and $^{13}$C-NMR data with that of these known compounds.

## In vitro antileishmanial activity test of the isolated compounds

Compounds were subjected to antileishmanial activity tests using the promastigote and amastigote stages of *L. aethiopica*. The test against promatigotes of the parasite indicated that the $IC_{50}$ values of compound **MS-1** and compound **MS-2** to be 0.079 and 242.5 μg/ml, respectively. Compound **MS-1** was found to be relatively more active whereas compound **MS-2** was less active as demonstrated by its highest $IC_{50}$ value. Comparison of these data with the observed $IC_{50}$ values of the reference compounds (amphoterisin B; $IC_{50} = 0.004$ and miltefosine; $IC_{50} = 0.136$ μg/ml) suggested that the two compounds which showed comparable activities with the reference drugs would be used as candidates for further activity tests. Antileishmanial activity tests of the isolated compounds were also carried out using an amastigote stage of the test strain (i.e., *L. aethopica*). The growth inhibitions ($IC_{50}$) of **MS-1** and **MS-2** against the amastigotes were found to be

**Table 4** $^{13}$C-NMR and DEPT-135 data of compound **MS-2** and the reported data of 1,3-dioleolyl-2-linolein (Ramsewak *et al.*, 2001)

| C no. | $\delta$ $^{13}$C (compound **MS-2**) | $\delta$ $^{13}$C (reported data) | $\delta$ DEPT-135 (**MS-2**) | Remarks |
|---|---|---|---|---|
| 1 | 62.00 | 62.0 | 62.00 | $-CH_2$ |
| 2 | 68.91 | 68.9 | 68.91 | –CH |
| 3 | 62.00 | 62.0 | 62.00 | $-CH_2$ |
| 1′, 1″ | 172.75, 172.37 | 173.2, 172.8 | – | Quaternary |
| 2′, 2″ | 33.98, 34.12 | 34.0, 34.2 | 33.97, 34.12 | $-CH_2$ |
| 3′, 3″ | 31.95, 31.56 | 31.9, 31.5 | 31.9, 31.5 | $-CH_2$ |
| 4′, 4″ | 22.72 | 22.4 | 22.71 | $-CH_2$ |
| 5′, 5″ | 22.72 | 22.6 | 22.71 | $-CH_2$ |
| 6′, 6″ | 24.87 | 24.8 | 24.97 | $-CH_2$ |
| 7′, 7″ | 24.87 | 24.9 | 24.97 | $-CH_2$ |
| 8′, 8″ | 27.21 | 27.2 | 27.20 | $-CH_2$ |
| 9′, 9″ | 127.91–130.09 | 127.8–130.2 | 127.91–130.09 | –CH |
| 10′, 10″ | 127.91–130.09 | 127.8–130.2 | 127.91–130.09 | –CH |
| 11′, 11″ | 25.65, 27.20 | 25.6, 27.2 | 25.65, 27.20 | $-CH_2$ |
| 12′, 12″ | 27.20, 129.61 | 127.8–130.2 | 27.20, 130.09 | –CH |
| 13′, 13″ | 27.20, 130.09 | 127.8–130.2 | 27.20, 130.09 | –CH |
| 14′, 14″ | 27.20, 29.17 | 27.2 | 27.20, 31.84 | $-CH_2$ |
| 15′, 15″ | 29.17–29.74 | 29.0–29.7 | 27.20, 31.84 | $-CH_2$ |
| 16′, 16″ | 29.17–29.74 | 29.0–29.7 | 27.20, 31.84 | $-CH_2$ |
| 17′, 17″ | 29.17–29.74 | 29.0–29.7 | 27.20, 31.84 | $-CH_2$ |
| 18′, 18″ | 14.17, 14.17 | 14.1, 14.0 | 14.17, 14.17 | $-CH_3$ |

40.03 and 26.79 μg/ml, respectively. The results indicated that these compounds have comparable antileishmanial activities. When compared to the corresponding activities of the reference drugs (amphoterisin B, $IC_{50} = 0.032$ and miltefosine, $IC_{50} = 0.121$ μg/ml), they are relatively less active. However, the observed $IC_{50}$ values of the compounds indicated that they can be considered as promising candidates for further antileishmanial activity tests. There are reports that indicate antibacterial activities (Desbois and Smith, 2010) and antiviral (Williams, 1995) activities of caster oil (a triglyceride of fatty acids). Another report also indicated the use of the oil for treatment of skin infection (http://www.buzzle.com/articles/castor-oil-for-skin-care.html). All the observations suggested the compounds (**MS-1** and **MS-2**) to have promising potentials to be used as candidates for further in vivo tests in the search for safe and effective antileishmanial drugs to replace the currently available commercial drugs that face serious limitations. Some of the limitations are (i) unpleasant side effects that cause pain at the site of injection, gastrointestinal problems, stiff joints, cardio-toxicity and in some cases, hepatic and renal insufficiency (Trouiller and Torreele, 2001; Matoussi and Ameur, 2007); (ii) most of them are expensive and are not affordable by the majority of the patients (Croft and Coombs, 2003); and (iii) most of these drugs also have lost their effectiveness due to drug resistance (Mishra *et al.*, 2009; Kedzierski *et al.*, 2009).

## Conclusions

In our study, two glycerides (compounds **MS-1** and **MS-2**) were isolated from petroleum ether:ethyl acetate crude extract of roots of *M. stenopetala*. Their identities were determined to be 1,3-dilinoleoyl-2-olein and 1,3-dioleoyl-2-linolein, respectively, based on the similarities of the observed $^{1}$H-NMR and $^{13}$C-NMR data with that of reported for 1,3-dilinoleoyl-2-olein and 1,3-dioleoyl-2-linolein, respectively. The compounds were found to be active against promastigote and amastigote stages of *L. aethiopica*. The promising antileishmanial activities suggest the need for further in vivo tests to explore their potential as new antileishmanial drug candidates.

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