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ISOLATION AND CHARACTERIZATION OF COMPOUNDS FROM ROOTS OF *MORINGA STENOPETALA*, AND EVALUATION OF THEIR ANTILEISHMANIAL ACTIVITIES

BY

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JIMMA, ETHIOPIA

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Advisors: Legesse Adane (Ph.D)

Prof. Asrat Hailu

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List of Abbreviations

DCL: Diffuse Cutaneous Leishmaniasis

IC_{50}: 50% inhibitory concentration

IR: Infra Red

LCL: Localized Cutaneous Leishmaniasis

MCL: Mucocutaneous Leishmaniasis

NMR: Nuclear Magnetic Resonance

VL: Visceral Leishmaniasis

MS: *Moringa stenopetala*

Abstract

The objective of this study was to isolate compounds from the roots of *Moringa stenopetala,* and to evaluate their antileishmanial activity based on previous research results which revealed that the root extracts of this plant have promising antileishmanial activity.

Three compounds were isolated from the roots of this plant species. The compounds are **MS-1, MS-2** and **MS-3**. The structures of **MS-2** and **MS-3** were proposed to be triglycerides based on the observed spectral (^{1}HNMR, ^{13}CNMR, DEPT and IR) data and reported data in literature. Thus, **MS-2** and **MS-3** were identified as 1,3-dilinoleoyl-2-olein and 1, 3-dioleoyl-2-linolein, respectively. However, the structure of **MS-1** was not proposed since it was not possible to do so based on the available spectral data.

The bio-activity *in vitro* test was done using microculture radioisotope technique which determines the growth inhibition (IC_{50} values) of the isolated compounds. The results indicated that **MS-2** and **MS-3** were found to be more active as compared to **MS-1** in their inhibitory activities of promastigotes of the test strain *L. aethiopica*. With regard to their antileishamnial activities, the inhibitory activities of the compounds against the amastigotes of the parasite were found to be comparable to each other and to the reference drugs (Amphoteisine B and Miltefosine) (Section 3.0).

1.0 INTRODUCTION

1.1 The Use of Natural Products in Treatment of Leishmaniasis

The use of medicinal plants to treat several human diseases has long histories that goes to the time of human civilization and are still used in many parts of the world, particularly people in the developing countries. Moreover, many modern drugs that are in use are obtained from plant sources. For instance, the widely used drugs such as digoxin, reserpine, morphine, cocaine and vincristine are some of the typical examples obtained from *Digitalis* leaves, *Rauwolfia serpentine, Papaver somniferum and Erythroxzion* species respectively (Bickii *et al.*, 2007). Medicinal plants have been used for centuries for the treatment of various ailments. In Ethiopia about 95% of the population relies on traditional herbal medicine for treatment of various ailments (Goji *et al.*, 2006; Chauhun *et al.,* 2009). The Ethiopian flora is also estimated to contain between 6,500 and 7,000 species of higher plants of which about 12% are endemic (Demissew and Dagne, 2001). This suggests the need for scientific evaluation of these plants in order to identify (isolate) lead compounds for development of new drugs for treatment of different diseases.

Over 100 plants have been reported to be active against various forms of leishmanial parasites (Rocha *et al*., 2005). There are also reports on investigation of antieishmanial activities of crude extracts and/or compounds isolated from different parts of such plant species. The findings of these researches indicated the promising anileishmanial activities of such crude extracts or isolated compounds as demonstrated by *in vitro* against promastigotes and amastigotes or *in vivo* against Leishmania infected animals (Iwu *et al*., 1992; Taddese *et al*. 1993; Khalid *et al.* 2005; Luize *et al.*, 2005; Lamidi *et al.*, 2005; Fokialakis *et al.*, 2007).

Surveys conducted in different parts of Ethiopia showed the use of different medicinal plants for treatment of various infectious diseases of affecting humans (including Leishmanasis) by traditional healers (Giday *et al.,* 2003; Weldegerima *et al.,* 2008; Wirtu *et al.,* 1999; Wondimu *et al.,* 2007). For instance, the roots of *L. ocymifolia* (locally called "Ras kimir") used for treatment of Leishmaniasis (Abebe and Ayehu, 1993). Yinebe, (2008**)** also reported some Ethiopian medicinal plants showing antileishmanial actcities. *Artemesia abyssinica (Leaves),*

Croton macrostachys (berries and leaves), *Echnops kebericho* (tuber), *Erythrina brucei* (stembark), *Albizia schimperiana* (stembark and rootbark) and *Aloe* (stem) are some of the examples of the medicinal plants. The chopped roots of *M. stenopetala* are traditionally used for treating visceral leishmaniasis. The crude extracts of roots of this plant also showed a promising antileishmanial activity (Mekonnen *et al.,* 1999). These observations have encouraged us to` carry out investigations in order to evaluate antileishmanial activities of compounds isolated form root extracts of this plant hopping that the pure compounds could be more active than the crude extracts against Leishmania species.

1.2 Background Information About Leishmaniasis

Leishmaniasis is an infectious disease that is endemic to tropical and sub-tropical countries of the world. It is estimated that 350 million people are at risk, 12 million people are infected and 2 million new infections each year through out the world (Rougeron *et al.,* 2010). It is prevalent in 88 countries (72 of them developing countries) (Ioset, 2008). It is caused by obligate intramacrophage protozoa that belong to the genus *Leishmania,* and transmitted by the bite of infected female sandflies (Chappuis, 2007; Manan *et al.,* 2008). Human infection is caused by about 21 of the 30 *Leishmanial* species that infect mammals. These include the *L. donovani* complex with three species (*L. donovani, L. infantum,* and *L. chagasi*), the *L. mexicana* complex with four main species (*L. mexicana, L. amazonensis,* and *L. venezuelensis*), *L. tropica, L. major, L. aethiopica,* and the subgenus *Viannia* with four main species (*L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis,* and *L. (V.) peruviana*) (Chappuis., 2007).

As shown bellow (Figure 1), Leishmaniasis is transmitted while an infected female sandflies take blood meal from healthy people. Once the sandfly has been infected, the amastigote migrates to the alimentary canal of the insect where it attaches to local epidermal cells. The parasites mature and differentiate into motile promastigotes and move to the pharynx of the sandfly. Upon a subsequent blood meal, the promastigotes are injected into the blood stream of the victim. Then the promasigotes get phagocytized by macrophage, and reside in the parasitophorous vacuole where they transform into amastigote. Amastigotes then replicate and

released back into blood stream to infect macrophages within the skin, viscera, and blood tissues (Anonymous, 2006).

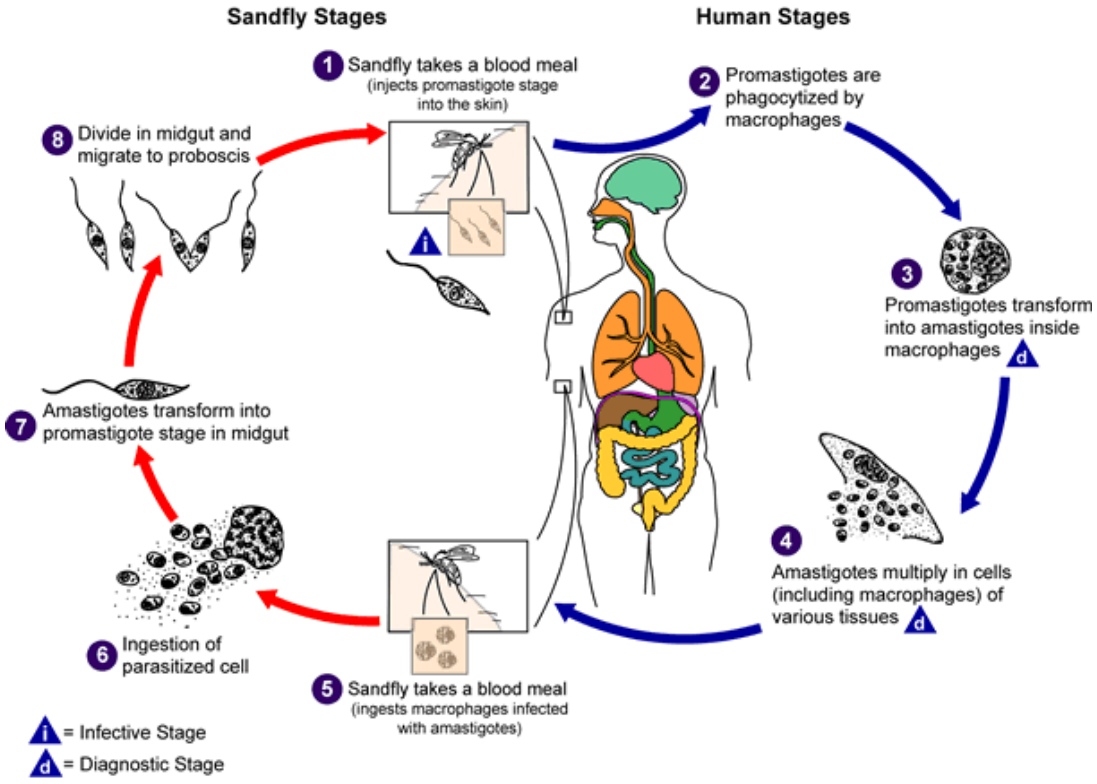

Figure 1. Life cycle of the Leishmania parasite

(Source: http://aapredbook.aappublications.org/content/images/large/2006/1/071_07.jpeg**)**

There are four main clinical fourms of Leishmaniasis, These are (i) Localized cutaneous leishmaniasis (LCL); a disease that causes lesions (or ulcers) on the face, nose, forehead and lower limbs, and usually heals on its own leaving a scar. LCL is mainly caused by *L. tropica*, *L. major* and also due to *L. aethiopica* , *L. mexicana, L. infantum*, *L. chagasi* and *L. Donovan* (Davies *et al*., 2003); (ii) Diffuse cutaneous leishmaniasis (DCL) causes disseminated nodules, plates or lumps, on the face, arms and legs. It never heals spontaneously and relapses after treatment. DCL occur due to *L. aethopica*, *L. mexicana* and *L. amazonensis* cause (Davies *et al.*, 2003). The cutaneous forms account for 50-75% of all new cases with 1-1.5 million annual incidence where as half a million cases (25%) of visceral leishmaniasis occur each year (Donald,

2003); (iii) Mucocutaneous leishmaniasis (MCL) causes extensive disfiguring destruction of mucosa and cartilage of the mouth, nose, ear and pharynx leading to a severe mutilation of the face. MCL occur mainly due to *L. aethiopica, L. brasiliensis* and also due to *L. donovani, L. major* and *L. infantum* (Paredes *et al*., 2003); (iv) Visceral leishmaniasis (VL) is characterized by irregular fever, weight loss, enlargement of spleen and liver, anemia, leukopenia, skin pigmentation and weakness associated with parasite invasion of spleen, liver, bone-marrow, lungs, oral mucosa, larynx, oesophagus, stomach, small intestine, skin and sex cells. VL is generally caused by *L. donovani* and *L. infantum*, and responsible for ~500, 000 new cases and 60, 000 deaths worldwide each year (Chappuis, 2007).

Recent reports revealed that all forms of leishmaniasis occur in Ethiopia (Hailu *et al.*, 2006). LCL case was first reported in Ethiopia in 1913. It is known to be caused by two spcies, *L. aethiopica* and *L. major.* The Ethio-Kenya and Ethio-Sudan border were suffered of VL in 1938-42. DCL and MCL are found in areas where other CL types occur and VL is distributed throughout the lowlands and semi desert areas (Ashford, 1973).

1.3 Treatment of Leishmaniasis

There are few chemotherapeutic agents to treat Leishmanisasis. These agents (drugs) can be categorized into first- and second-line drugs. The first-line drugs in the treatment of leishmaniasis are the pentavalent antimonials such as sodium stibogluconate (**1**), sodium antimony gluconate (**2**) and meglumine antimonite (**3**) whereas drugs such as pentamidine (**4**), amphotericin B (**5**), miltefosine (**6**), paromomycin (**7**), sitamaquine (**8**) and imiquimode (**9**) are examples of second-line drugs for treatment of Leishmaniasis (Figure 2). However, there are some limitations associated with these drugs. The parasite developed resistance to most of them, due to their prolonged use in certain regions of the world, and also they cause unpleasant side effects such as pain at the site of injection, gastrointestinal problems, stiff joints, cardio toxicity, and in some cases, hepatic and renal insufficiency (Davies *et al.*, 2003; Donald, 2003;). Most of the drugs are also expensive, and are not affordable by many patients living in areas where the disease is endemic Croft *et al*., 2006). Moreover, the emergence of leishmania-HIV co-infections has worsened the situation. Co-infection, especially when combined with malnutrition, contributes to an increased death rate (Haile and Anderson, 2006). The high rate of HIV infection

in population has an impact on treatment outcomes due to the development complexity in the nature of leishmania-HIV co-infections and severity of the disease due to low immunity (Paredes *et al.*, 2003). All these factors have necessitated for a continued search for other effective, safe and cheap antileishmanial agents.

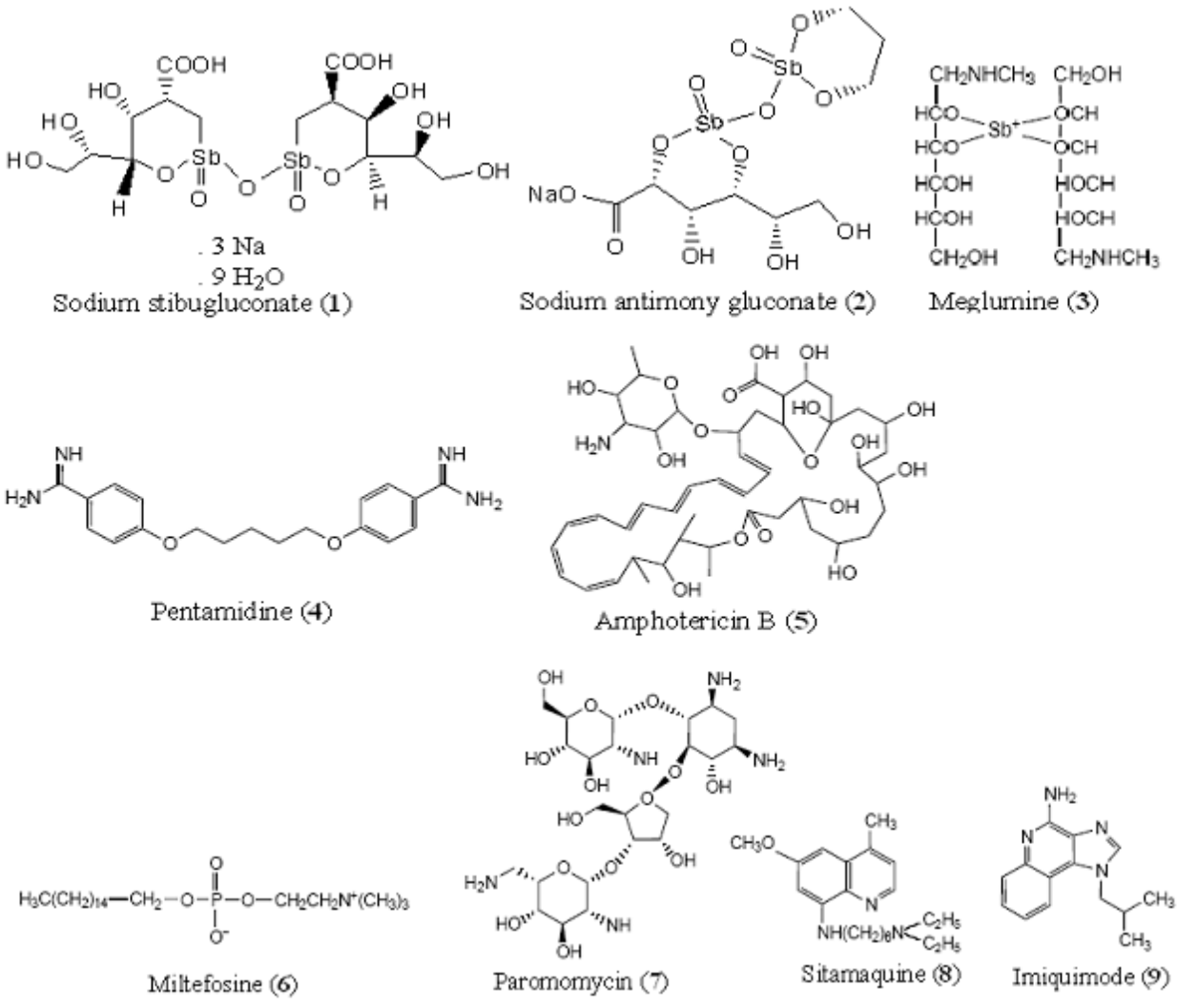

Figure 2. Structure of drugs approved for treatment of leishmaniasis (Bacab *et al*., 2001)

1.4 Medicinal Use and Phytochemical Investigations of *Moringa* species: Literature Review

There are about 14 species of *Moringa* species which belong to the family of *Moringaceae*. These are *Moringa arborea, Moringa borziana, Moringa concanensis, Moringa drouhardii, Moringa hildebrandtii, Moringa longituba, Moringa ovalifolia, Moringa peregrina, Moringa pygmaea, Moringa rivae, Moringa ruspoliana, M. stenopetala, M. oleifera* and *Moringa pterygosperma* (Ray *et al.*, 2006). Of these several Moringa species, *M. oleifera* and *M. stenopetala* are used by traditional healers to treat several diseases including leishmaniasis (Mekonnen *et al.*, 2002; Fahey *et al.*, 2005; Das *et al*., 2009; Tahany *et al*., 2010).

M. oleifera is one of the most widely cultivated species of a Monogeneric family that is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan. It was utilized by the ancient Romans, Greeks and Egyptians. Nowadays, it is now widely cultivated and has become naturalized in many locations in the tropics (Vlahof *et al.*, 2002). It is a small, fast-growing evergreen or deciduous tree that usually grows as high as 9 m. It grows at elevations from sea level to 1400 m (Ray *et al.*, 2006; Fahey *et al.*, 2005). *M. stenopetala* is native to Northern Kenya and South Western Ethiopia. It is locally called 'Shiferaw' (Amharic) and 'Aleko' (Welayita), commonly grows 6–10 m tall at altitudes between 1,000 and 1,800 m where the temperature range is 20-30 $^{\circ}$C. The trees are cultivated for their leaves that are boiled and eaten like cabbage (Mekonnen *et al.*, 1999; Mekonnen *et al.*, 2002).

1.4.1 Medicinal Uses of *Moringa* Species; *Moringa oleifera* and *Moringa stenopetala*

As described in section 1.2.2, traditional medicine and/or natural products (especially plant-based ones) have played and still playing significant roles in treatment of different kinds of livestock and human diseases. Traditionally, different parts of *M. oleifera* are used for treatment of several diseases. For instance, the stem-bark of this plant has abortifacient, antifungal and antibacterial activities. Its flowers are used as stimulants, hypoglycemic, tonic and diuretic, and also useful to increase the flow of bile. The pods of this plant species also used as antipyretic and anthelmintic. They are also used for treatment of diabetic patients. The root juice is employed in cardiac tonic, antiepileptic, nervous debility, asthma, antiparalytic, enlarged liver and spleen, deep

seated inflammation and as diuretic in calculus affection. The leaves of *M. olifera* are used in cough, given in influenza and catarrhal affections. The root-bark is also used as antiviral, anti-inflammatory and analgesic. The seeds have anti-inflammatory and antispasmodic activities, and also used in treatment of venereal diseases (Fahey *et al.*, 2005).

Many experiments have been carried out in order to confirm the traditional uses of *M. oleifera* and *M. stenopetala* claimed by traditional healers. The results of most of these experimental studies have been found to be consistent with that of the traditional medicinal uses. For instance, extracts from leaves, capsules, roots, bark and seeds of *M. oleifera* showed antimicrobial, antifungal and antibacterial activities (Alam *et al.,* 2009; Caceres *et al.,* 1991; Chen *et al.,* 2007; Doughari *et al.,* 2007; Nantachit, 2006; Renitta *et al.,* 2009; Umar, 1998). Hepatoprotective activity of *M. oleifera* roots leaves and seeds has reported by different research teams (Ghasi *et al.,* 2000; Mazumder *et al.,* 1999; Pari and Kumar, 2002). There are reports revealing anti-inflammatory activity and antioxidant activities of flowers, leaves, roots, seeds, fruit and bark of *M. oleifera* (Caceres *et al.,* 1991; Siddhuraju and Becker, 2003; Singh *et al.* 2009). Anticancer and antifertility activities of seeds, leaves and roots of *M. oleifera* have been reported by different researchers (Guevara *et al.,* 1999; Nath *et al.,* 1992; Prakash *et al.,* 1987; Shukla *et al.,* 1988).

Traditionally, the leaves and roots of *M. stenopetala* boiled in water are used to treat malaria, hypertension, stomach disorders, asthma and diabetes (Mekonnen *et al*., 1999). Aqueous extracts of its leaves also used for expulsion of retained placenta. Extracts of its roots are used to treat visceral Leshmaniasis (Mekonnen *et al.,* 2002, Mekonen *et al*., 2005). The seeds are effective for purification of water. Other experiments also demonstrated that extracts from various morphological parts of this plant showed promising biological activities. Mekonen et al. (1999, 2000) have reported hypoglycemic effect of extracts of leaves (Mekonen *et al*., 2000), and *in vitro* antitrypanosomal, antileishmanial, anti-fertility and antimicrobial properties of seeds, leaves and roots of *M. stenopetala* (Mekonnen *et al*., 1999; Mekonnen *et al.*, 2002).

1.4.2 Phytochemical Investigations of *Moringa* Species; *Moringa oleifera* and *Moringa stenopetala*

` As discussed in the previous sections, these plants have several medicinal uses. Thus, many research activities have been initiated in order to identify (isolate) specific compounds responsible for the observed biological activities or medicinal uses of these plants(Siddhuraju and Becker, 2003; Renitta *et al.,* 2009; Mekonen *et al*., 2000; Mekonen *et al*., 2005). In this section, the phytochemical studies carried out on *M. oleifera* and *M. stenopetala* are presented.

1.4.2.1 Compounds Isolated from *M. oleifera*

M. oleifera is one of the best studied plant among *Moringa* species with regard to its medicinal uses and identification compounds of potential therapeutic importance (Eilert *et al.,* 1981; Mekonnen *et al.,* 2002). Many compounds have been isolated from various parts of *M. oleifera*. Some of the compounds are presented in this section.

Several compounds have been isolated from the seeds of *M. oleifera* by different research teams. Eilert *et al.* (1981**)** reported 4(α-L-Rhamnosyloxy) benzyl isothiocyanate. Villasenor *et al.* (1989) also reported certain biosynthetically and chemically related compounds. Some of the compounds were 4-(-L-rhamnosyloxy) phenylacetonitrile, 4-hydroxyphenylacetonitrile, and 4-hydroxyphenyl-acetamide. Guevara *et al.* (1999) isolated a new compound known as O-ethyl-4-(α-L-rhamnosyloxy)benzyl carbamate along with seven known compounds such as 4(α-L-rhamnosyloxy)-benzyl isothiocyanate, niazimicin, niazirin, β-sitosterol, glycerol-1-(9-octadecanoate) and 3-O-(6'- oleoyl-β-D-glucopyranosyl)-β-sitosterol. Various sterols, tocopherols and fatty acids were also reported by Anwar and Rashid (2007) from the plant. The isolation of 4-(α-l-rhamnopyranosyloxy)- benzylglucosinolate has been reported by Bennett *et al.* (2003).

Some compounds have also been isolated from the pods of *M. oleifera*. These include O-[2'-hydroxy-3'-(2''-heptenyloxy)]-propyl undecanoate and O-ethyl-4-[(α-L-rhamnosyloxy)-benzyl] carbamate along with the known substances like methyl phydroxybenzoate and β-sitosterol (Faizi *et al.,* 1998). Similarly, Roy *et al*. (2007) reported isolation of water-soluble polysaccharide such as D-galactose, 6-O-Me-D-galactose, D-galacturonic acid, l-arabinose and l-rhamnose.

Phytoconstituents of leaves of *M. oleifera* have also been studied by some researchers Faizi *et al.* (1994) reported the isolation of two nitrile glycosides (niazirin and niazirinin) and three mustard oil glycosides (4-[(4'-O acetylalpha-L-rhamnosyloxy)benzyl]isothiocyanate, niaziminin A and niaziminin B). The authors also reported the isolation of six new and three synthetically known glycosides employing a bioassay-directed isolation method on the ethanolic extract. Most of these compounds were found to bear thiocarbamate, carbamate or nitrile groups, and were also fully acetylated glycosides which are very rare in nature (Faizi *et al.,* 1995). Murakami *et al.* (1998) isolated niaziminin B and thiocarbamate. Siddhuraju and Becker, (2003) isolated quercetin and kaempferol. Various glucosinolates and phenolic compounds that contain 4-(α-l-rhamnopyranosyloxy)-benzylglucosinolate, three monoacetyl isomers of this glucosinolate, and lower amounts of kaempferol-3-O-glucoside, kaempferol-3-O-(6"-malonyl-glucoside), 3-caffeoylquinic acid and 5-caffeoylquinic acid were also isolated from leaves of *M. oleifera* (Bennett *et al.*, 2003).

Several classes of compounds have been isolated from these parts of *M. oleifera*. Some of the compounds are aglycone of deoxy-niazimicine which was characterized as N-benzyl, S-ethyl thioformate (Nikkon *et al.*, 2003). The compound shows antibacterial and antifungal activities against *Shigella boydii, Shigella dysenteriae* and *Staphylococcus aureus.* Bennett *et al.* (2003) also reported three glucosinolates and phenolic compounds including 4-(α-l-rhamnopyranosyloxy)-benzylglucosinolate and benzyl glucosinolate. Similarly, Sashidhara *et al*. (2009) isolated and characterized aurantiamide acetate and 1, 3-dibenzylurea.

Though the parts of the plant were not specified in reports, several other compounds have also been reported from *M. oleifera*. Ndong *et al.* (2007) reported major polyphenols such as quercetin glucosides, rutin, kaempferol glycosides and chlorogenic acids. Manguro and Lemmen, (2007) reported the isolation of five flavonol glycosides that were characterised as kaempferide 3-O-(2",3"-diacetylglucoside), kaempferide 3-O-(2"-Ogalloylrhamnoside), kaempferide 3-O-(2"-O-galloylrutinoside)-7-O-α-rhamnoside, kaempferol, 3-O-[β-glucosyl-(1→2)]-[α-rhamnosyl-(1 → 6)]-β-glucoside-7-Oalpharhamnoside and kaempferol 3-O-[α-rhamnosyl-(1→2)]-[α-rhamnosyl-(1→4)]-β-glucoside-7-O-α-rhamnoside together with benzoic acid-4-O-β-glucoside, benzoic acid -4-O-α-rhamnosyl-(1→2)-β-glucoside and benzaldehyde-4-O-β-glucoside have been isolated

from the leaves. Known compounds such as kaempferol 3-O-α-rhamnoside, kaempferol, syringic acid, gallic acid and quercetin 3-O-β-glucoside were also isolated from the same extract. Yammuenart *et al*. (2008) isolated seven compounds including, linoleic sitosteroate, linoleic acid, 1,2,3-triolein, a mixture of 1,3-dilinoleoyl-2-olein,1,3-dioleoyl-2-linolein and 1,2,3-trilinolein and isothiocyanatomethylbenzene. Isolation of monoterpenoids including α-phellandrene along with p-cymene was also reported (Ogunbinu *et al*., 2009) from the plant.

1.4.2.2 Compounds Isolated from *M. stenopetala*

Though investigations on *M. stenopetala* are not extensive as compared to *M. oleifera*, some compounds are also isolated from the various parts of the plant. Three glycosides that were identified as rutin, 4-(4'-O-acetyl-L-rhamnosyloxy)-benzaldehyde and 4-(4'-O-acetyl-L-rhamnosyloxy)-benzylisothiocyanated) were reported from leaves of *M. stenopetala* (Alemayehu *et al*., 2000). Lalas *et al.* (2003) also reported compounds from seed oil of this plant. The compounds were found to contain high levels of unsaturated fatty acids, especially oleic acid and dominant saturated acids such as behenic acid and palmitic acid. High levels of β-sitosterol, campesterol and α-, β- and δ-tocopherols were also reported from the same plant part. Bennett *et al*. (2003) reported the presence of glucosinolates in *M. stenopetala* and their antihypertensive activities. The isolation of these compounds from the seeds was also reported by Mekonnen *et al.*, (2003). Nibret *et al.* (2010**)** isolated and characterized several compounds from essential oil of the seed. Some of these compounds are benzyl isocyanate, isobutyl isocyanate, benezene-1-isocyano-2-methyl, palmitic acid and oleic acid. The bioactivity tests of these compounds showed antitrypanosomal and cytotoxic activities against HL-60 cells.

As discussed above, no much work has been done on isolation and characterization of compounds and biological evaluations (*in vivo* or *in vitro*) of isolated compounds and crude extracts of different parts of this plant. Moreover, to the best of our knowledge there are no reports on the evaluation of antileishmanial activities of isolated compounds from *M. stenopetala*. Thus, this study was initiated to isolate and characterize compounds from roots of this plant and evaluate antileishmanial activities of the compounds (isolated) by *in vitro* test.

1.5 Statement of the Problem

Although some drugs have been reported for Leishmaniasis, the parasite has developed resistance to these drugs due to their prolonged use. In addition to this, unpleasant side effects of the drugs, emergence of Leishmania-HIV co-infections and expensiveness of the drugs made the disease situation more complex (Croft *et al.*, 2006; Davies *et al.*, 2003; Donald, 2003; Paredes *et al.*, 2003).

Though much effort has been done on investigation of medicinal values of *M. stenopetala* against several human diseases (including leishmaniasis), there are no reports regarding identification of specific compounds that are responsible for the antileishmanial activities of this plant. Experiments, in evaluation of antileishmanial activities, have been done using crude extracts *M. stenopetala* and also promising antileishmanial activities were reported (Mekonnen *et al.*, 1999). Thus, there is a need to identify the active constituents of the plant could be obtained for leishmaniasis drug development. In this project, an attempt was made to isolate and characterize compounds from roots of *M. stenopetala*. The isolated compounds were also subjected to *in vitro* activity test to evaluate their antileishmanial activities.

1.6 Objectives

1.6.1 General Objectives

- To investigate compounds from *M. stenopetala* and evaluate their antileishmanial activity.

1.6.2 Specific Objectives

- To extract and isolate compounds from roots of *M. stenopetala.*
- To characterize isolated compounds by using spectroscopic techniques (NMR and IR).
- To test antileishmanial activities of the isolated compounds using *in vitro* method.

2.0 MATERIALS AND METHODS

2.0 Plant Material Collection

The plant material (roots of *M. stenopetala*) was collected from Arba Minch area in southern Ethiopia, 500 km from Addis Ababa, on January 5, 2011. The collected roots were chopped into pieces to allow air drying under a shade. Voucher specimens were deposited in the Botanical Herbarium, Jimma University

2.1 Solvents and Materials

Methanol, petroleum ether and ethyl acetate were the solvents used for extraction and isolation of compounds from plant material. These solvents were general laboratory grade solvents and obtained from Aldrich Company. All solvents used in the experiment were subjected to distillation before using for extraction and isolation (chromatography). Column chromatography (CC) was carried out using silica gel (60-120 mesh size) and distilled solvents. Thin-layer chromatography (TLC) analyses were performed on pre-coated silica gel plates (GF254, Merk). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz), IR (Perkin-Elmer BX) were used for structure elucidation of the isolated compounds.

2.2 Extraction of Plant Material

The air dried plant material (500 g) was further powdered and extracted with 2 L of petroleum ether:ethyl acetate (50:50%) at room temperature for 48 hours to gain the lipophilic extracts by using maceration method (Kohlera, 2002). Filtration was carried out using Whatman filter paper. Afterwards the plant material (residue) was again air-dried and was soaked in 2 L of methanol:water (80:20%) mixture to afford the hydrophilic extracts. The solvent was then removed from the lipophilic extract using Rota Evaporator. Finally, it was weighed and stored in a refrigerator below 4ºC. The process gave 7.40 g lipophilic and 9.18 g of hydrophilic crude extracts from (petroleum ether:ethyl acetate) extract and (methanol: water) extract, respectively.

2.3 Isolation of Compounds

The crude lipophilic extract (4.2 g) was adsorbed on 4 g of silica gel and loaded on column packed with 84 g of silica gel. The solvent system used to elute the column was petroleum ether:ethyl acetate mixture. The column was then eluted sequentially with petroleum ether:ethyl acetate composition of 100:0%, 99:1%, 98:2% and 97:3% up to 90:10% respectively. 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 TLC analysis. Among the fractions collected, fractions 3-5 (labeled **MS-1**) of 100:0% petroleum ether:ethyl acetate, fractions 11-14 (labeled **MS-2**) of 98:2% petroleum ether:ethyl acetate and fractions 1-30 (labeled **MS-3**) of 97:3% petroleum ether:ethyl acetate were pure compounds and characterized to give compound **MS-1**, **MS-2** and **MS-3**, respectively. **MS-1** is brownish oil with RF value of 0.52 with 98:2% petroleum ether:chloroform, **MS-2** and **MS-3** are yellowish oil with RF value of 0.38 and 0.33 with 9:91% and 98:2% petroleum ether:ethyl acetate respectively.

2.4 Characterizations of the Isolated Compounds

The isolated compounds were characterized using different spectroscopic techniques. Nuclear magnetic resonances (^{1}H-NMR, ^{13}C-NMR and DEPT-135) were recorded using 400 MHz NMR instrument in $CDCl_3$ (at the Department of Chemistry, Addis Ababa University). Infra red (IR) spectra were recorded on IR perestinge -21 FTIR instrument (at Ethiopian pharmaceutical industry, Addis Ababa).

2.5 Evaluation of Antileishmanial Activities of the Isolated Compounds

The *in vitro* antileishmanial activity tests of the isolated compounds were carried out in Leishmaniasis Diagnostic and Research Laboratory, Faculty of Medicine, Addis Ababa University. The experiment was carried out using a test strain of *L. aethiopica* (MHOM/ET/82/117-82). RPMI-1640 (obtained Gibco, Invitrogen Co., UK), fetal calf serum (HIi-FCS), penicillin-streptomycin solution, (+)-glucose and phosphate buffer saline (PBS) (all obtained from Sigma Chemical Co., St. Louis, USA) were the materials used to prepare the culture medium (for promastigotes and amastigotes) used for the experiment. The *L. aethiopica*

strain was grown in tissue cultured flasks containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (HIFCS) and 100 IU penicillin/ml-100µg/ml streptomycin solution at 22 ^{o}C as reported in literatures (Habtemariam, 2003). The antileishmanial activities of the isolated compounds were evaluated using promastigotes and amastigote forms of the test strain (*L. aethiopica*) developed in culture medium prepared as described above. Reference drugs used were amphotericine B and meltifosine.

2.5.1 Evaluation of Antileishmanial Activities of the Isolated Compounds: Inhibition of Promastigotes

The isolation and differentiation of Marine Peritoneal Macrophage (MPM) was carried using a standard method reported in literature (Yardley, 2008). First, the MPMs were induced by injecting a 2 ml of 2% starch solution into a mouse peritoneum. 24 hrs, after injection, the test mice was killed by CO_2 suffocation, degloved and injected with cold RPMI. Then peritoneal exudates were removed using a sterile 10 ml syringe with 21G 5/8 needle. After harvesting the exudates, the cell suspension was determined by counting using a Neuebauer hemocytometer, and the cell density was adjusted to 4000. Finally, 100 µl of the cell suspension was seeded into 16-chamber tissue plane and incubated at 37 ^{o}C for 24 hrs for differentiation.

The atileishmanial activities of the compounds were evaluated by comparing their percent inhibitions of promastigotes with that of amophotericine B and meltifosine. Different concentrations of the compounds were applied to the prepared promastigotes. The concentrations of the compounds used were 1 mg and its subsequent 1/3 of the first concentration, which decreases in similar way up to fourth smallest concentration applied to determine the IC_{50} values.

2.5.2 Evaluation of Antileishmanial Activities of the Isolated Compounds: Inhibition of Amastigotes

The intracellular amastigotes forms of *L. aethiopica* were obtained using a standard method reported in literature. Briefly, promastigotes in stationary phase were used to infect differentiated MPMs 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 hrs (Habtemariam, 2003).

The test of amastigote inhibitory activities of the isolated compounds were carried out by dispensing them, in different concentrations, into wells of plates on which intracellular amastigotes were attached and the mixture was transferred to next wells for serial dilution. Then the contents were incubated at 37 $^{\circ}$C for five days in a 5% CO_2 incubator. After 5 days of exposure of the infected cells to the compounds and the reference drug, the overlay from all of the wells was removed and the plastic wells were removed from the slides. After 30 seconds exposure to 100% methanol, the rubber gasket was removed with sharp-ended forceps and the slides was stained for 10 minutes with 10 % Giemsa. After rinsing and air-drying the slides was ready for counting (x100, oil immersion). Totally, 100 macrophages were counted in each well and the % inhibition values were derived by comparing the % infection of treated to untreated cells. The same procedure was also applied for the reference compounds amphotericine B and meltifosine.

3.0 RESULTS AND DISCUSSION

Isolation of the lipophilic extract results in three pure compounds. The first compound labeled as **MS-1** was obtained by eluting the column with petroleum ether. The second compound labeled as **MS-2** was collected by eluting the column with petroleum ether:Ethyl acetate (98:2%). The third compound labeled as **MS-3** was obtained by eluting the column with petroleum ether:Ethyl acetate (97:3%). These are characterized. The structure elucidation of these compounds was done by using their ^{1}H NMR, ^{13}C NMR, DEPT and IR spectra in comparison with the reported data of these compounds in the literature.

3.0 Structure Elucidation of Compounds

3.0.1 Structure Elucidation of MS-2

Analysis of IR spectra of **MS-2** (Appendix 1) indicated that **MS-2** has no hydroxyl or carboxylic acid functional groups. This was confirmed by absence of broad bands in the range of 3650-3600 cm^{-1} (for hydroxyl) and 3400-3200 cm^{-1} (for carboxylic acids). Moreover, absence of a doublet band at/near 2850 and 2750 cm^{-1} indicated that the compound has no aldehydic functional group. Absence of bands around (or above) 1800 cm^{-1} indicated that the compound is also neither acid anhydride nor acid chloride. The absence of weak bands in the range of 2000 and 1650 cm^{-1} indicated that the compound has no aromatic functional group. Thus, the observed carbonyl group stretching band at 1745 cm^{-1} indicates that the compound is most likely an ester. The broad band at 1163 cm^{-1} also indicates the C-O stretching vibration of an ester. The strong band at 3006.80 cm^{-1} represents C-H stretch of an alkene whereas the bands at 2922 and 2852 cm^{-1} indicate C-H stretcheing of methylene and methyl groups.

In the ^{1}H-NMR spectrum of **MS-2** (Appendix 2), the peak at δ 0.90 indicated presence of protons of methyl (-CH_3) groups; the peaks at δ 1.27 and δ 1.63 indicate protons of aliphatic methylene (-CH_2) group; a peak at δ 2.02 indicates presence of protons of a methylene group that is bonded to C=C bond; the peak at δ 2.33 indicate presence of protons of methylene that is bonded to a carbonyl group; the peak at δ 2.77 indicates presence of protons of methylene group that is flanked by two C=C bonds; the peak at δ 4.17 and 4.30 indicate presence of protons of a carbon attached to oxygen whereas the peak at δ 5.38 indicates 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 (Figure 2) (Ramsewak *et al*., 2001). Thus, based on this observation the chemical structure of **MS-2** was proposed to be identical with the chemical structure of 1,3-dilinoleoyl-2-oleine (Figure 3). 1,3-dilinoleoyl-2-oleine has been isolated from of *M. oleifera* (Yammuenart *et al*., 2008). The ^{1}H-NMR data of **MS-2** and that of 1,3-dilinoleoyl-2-oleine are given in Table 1.

Table 1. ^{1}H-NMR data of **MS-2** in comparison with reported data of 1,3-dilinoleoyl-2-oleine

^{1}H	**δ ^{1}H (MS-2)**	**δ^{1}H (Reported Data)***
1a	4.14	4.12
1b	4.29	4.28
2	5.27	5.25
3a	4.14	4.12
3b	4.29	4.28
1', 1"	-	-
2', 2"	2.30, 2.35	2.28, 2.29
3', 3"	1.63	1.59
4', 4"	1.27	1.25
5', 5"	1.27	1.25
6', 6"	1.27	1.25
7', 7"	1.27	1.25
8', 8"	2.02	2.02
9', 9"	5.38	5.33
10', 10"	5.38	5.33
11', 11"	2.77, 2.02	2.75, 2.02
12'', 12"	5.38	5.33
13', 13"	5.38	5.33
14', 14"	2.02	2.02
15', 15"	1.27	1.25
16', 16"	1.27	1.25
17', 17"	1.27	1.25
18', 18"	0.88, 0.90	0.86, 0.87

* Data from Ramsewak *et al*., 2001

In the ^{13}C-NMR spectrum of **MS-2** (Appendix 3), the peaks in the range of chemical shift values 127.2- 131.9 ppm indicated C=C bonds; the three peaks at δ 173.3, 172.8 and 173.3 ppm indicate quaternary carbon atoms of (of carbonyl carbons) of ester group. On the other hand, the chemical shift values in the range of 14.10 to 68.90 ppm indicated presence of methyl (-CH_3) and oxygenated methylene (-OCH_2) carbons (Table 2). The DEPT spectrum showed peaks for the

presence of methyl (-CH_3) carbon at 14.0 and 14.1 ppm, methylene carbons at 22.6 to 62.1 ppm, oxygenated methine (-CH) carbon at 68.9 and olefinic methyne carbons at 127.1-131.9 ppm (Appendix 4). Absence of peaks at 172.8 and 173.3 ppm in DEPT spectra indicated quaternary carbon atoms (of ester carbonyl group). The peaks were observed in ^{13}C-NMR spectra but not in the DEPT spectra.

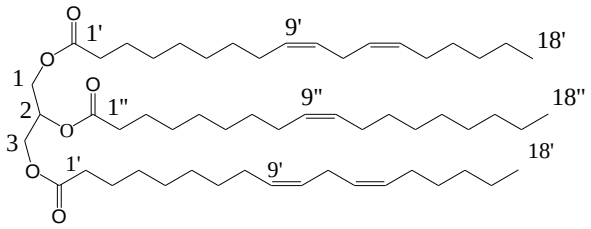

Figure 3 The proposed structure of **MS-2**

Table 2. ^{13}C-NMR and DEPT data of **MS-2** in comparison with reported data of 1,3-dilinoleoyl-2-oleine.

^{13}C	**δ ^{13}C (MS-2)**	**δ ^{13}C (Reported Data)***	**δDEPT**	**Remark**
1	62.1	62.1	62.1	-CH_2
2	68.9	68.9	68.9	-CH
3	62.1	62.1	62.1	-CH_2
1', 1"	173.3, 172.8	173.2, 172.8	-	Quaternary
2', 2"	34.0, 34.2	34.0, 34.2	34.0, 34.2	-CH_2
3', 3"	31.9, 31.5	31.9, 31.5	31.9, 31.5	-CH_2
4', 4"	22.6	22.4	22.6	-CH_2
5', 5"	22.7	22.6	22.7	-CH_2
6', 6"	24.8	24.8	24.8	-CH_2
7', 7"	24.9	24.9	24.9	-CH_2
8', 8"	27.2	27.2	27.2	-CH_2
9', 9"	127.1-131.9	127.8-130.2	127.1-131.9	-CH
10', 10"	127.1-131.9	127.8-130.2	127.1-131.9	-CH
11', 11"	25.6, 27.2	25.6, 27.2	25.6, 27.2	-CH_2
12'', 12"	127.1-131.9	127.8-130.2	127.1-131.9	-CH
13', 13"	127.1-131.9	127.8-130.2	127.1-131.9	-CH
14', 14"	27.2	27.2	27.2	-CH_2
15', 15"	29.1-29.8	29.0-29.7	29.1-29.8	-CH_2
16', 16"	29.1-29.8	29.0-29.7	29.1-29.8	-CH_2
17', 17"	29.1-29.8	29.0-29.7	29.1-29.8	-CH_2
18', 18"	14.2, 14.1	14.1, 14.0	14.1, 14.0	-CH_3

* Data from Ramsewak *et al*., 2001

3.0.2 Structure Elucidation of MS-3

Analysis of IR spectra of **MS-3** (Appendix 5) indicated that it has no alcohol or carboxylic acid functional groups like **MS-2**. This was confirmed by absence of broad bands in the range of 3650-3600 cm^{-1} (for alcohols) and 3400-3200 cm^{-1} (for carboxylic acids). Moreover, absence of a doublet band at/near 2850 and 2750 cm^{-1} indicated that the compound has no aldehydic functional group. Absence of bands around (or above) 1800 cm^{-1} indicated that the compound is also neither acid anhydride nor acid chloride. The absence of weak bands in the range of 2000 and 1650 cm^{-1} indicated that the compound has no aromatic functional group. Thus, the observed carbonyl group stretching band at 1743 cm^{-1} indicates that the compound is most likely an ester. The broad band at 1163 cm^{-1} also indicates the C-O stretching vibration of an ester. The strong band at 3006.80 cm^{-1} represents C-H stretch of an alkene whereas the bands at 2918 and 2853 cm^{-1} indicate C-H stretching of methyl groups.

In the ^{1}H-NMR spectrum of **MS-3** (Appendix 6), the peak at δ 0.88 indicated protons of methyl groups; the peaks at δ 1.27 and 1.64 indicate protons of aliphatic methylene group; a peak at δ 2.05 indicates protons of a methylene group that is bonded to C=C bond; the peak at δ 2.32 indicate protons of methylene that is bonded to a carbonyl group; the peak at δ 2.77 indicates protons of a methylene group the is flanked by two C=C bonds; the peak at δ 4.13 and 4.30 indicate the presence of protons of oxygenated carbon whereas the peak at δ 5.38 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 (Figure 3) (Ramsewak *et al.*, 2001). Thus, based on this observation the chemical structure of **MS-3** was proposed to be identical with the chemical structure of 1,3-dioleolyl-2-linolein (Figure 4). 1,3-dioleolyl-2-linolein has been isolated from *M. oleifera* (Yammuenart *et al.*, 2008). The ^{1}H-NMR data of **MS-3** and that of 1,3-dioleolyl-2-linolein are given in Table 3.

Table 3 ^{1}H-NMR data of **MS-3** in comparison with reported data of 1,3-dioleolyl-2-linolein.

^{1}H	δ^{1}H (MS-3)	δ^{1}H (reported data)*
1a	4.11	4.12
1b	4.28	4.28
2	5.28	5.25
3a	4.11	4.12
3b	4.28	4.28
1', 1"	-	-
2', 2"	2.30, 2.34	2.28, 2.29
3', 3"	1.64	1.59
4', 4"	1.27	1.25
5', 5"	1.27	1.25
6', 6"	1.27	1.25
7', 7"	1.27	1.25
8', 8"	2.01	2.02
9', 9"	5.36	5.33
10', 10"	5.36	5.33
11', 11"	2.75, 2.01	2.75, 2.02
12', 12"	5.36, 1.27	5.33
13', 13"	5.36, 1.27	5.33
14', 14"	2.01, 1.27	2.02
15', 15"	1.27	1.25
16', 16"	1.27	1.25
17', 17"	1.27	1.25
18', 18"	0.88, 0.90	0.86, 0.87

* Data from Ramsewak *et al*., 2001

The ^{13}C-NMR spectrum of **MS-3** (Appendix 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 127.8- 130.1 ppm indicated proton attached to C=C bond whereas the two peaks at δ 172.3 and 172.7 ppm quaternary carbon atoms (of the ester carbonyl group). The DEPT spectrum (Appendix 8) also confirmed the presence of methyl carbon (at 14.1 and 14.2 ppm), methylene carbon at 22.5 and 62.0 ppm and oxygenated methine carbon at 68.9 and olefinic methine carbons at127.9-130.8 ppm. Absence of peaks at 172.3 and 172.4 ppm in the DEPT spectra, which were observed in the ^{13}C-NMR spectra, also confirmed quaternary carbon atoms of ester carbonyl group.

Table 4 ^{13}C-NMR and DEPT data of **MS-3** and the reported 1,3-dioleolyl-2-linolein

^{13}C	**δ^{13}C (MS-3)**	**δ^{13}C (reported data)***	**δDEPT (MS-3)**	**Remark**
1	62.0	62.0	62.0	-CH_2
2	68.9	68.9	68.9	-CH
3	62.0	62.0	62.0	-CH_2
1', 1"	172.4, 172.8	173.2, 172.8	-	Quaternary
2', 2"	34.0, 34.1	34.0, 34.2	34.0, 34.1	-CH_2
3', 3"	31.9, 31.5	31.9, 31.5	31.9, 31.5	-CH_2
4', 4"	22.6	22.4	22.6	-CH_2
5', 5"	22.7	22.6	22.7	-CH_2
6', 6"	24.8	24.8	24.9	-CH_2
7', 7"	24.9	24.9	24.9	-CH_2
8', 8"	27.2	27.2	27.2	-CH_2
9', 9"	127.8-130.1	127.8-130.2	127.9-130.1	-CH
10', 10"	127.8-130.1	127.8-130.2	127.9-130.1	-CH
11', 11"	25.6, 27.2	25.6, 27.2	25.6, 27.2	-CH_2
12', 12"	127.8-130.1	127.8-130.2	127.9-130.1	-CH
13', 13"	127.8-130.1	127.8-130.2	127.9-130.1	-CH
14', 14"	27.2, 29.1	27.2	27.2, 29.1	-CH_2
15', 15"	29.1-29.8	29.0-29.7	29.1-29.8	-CH_2
16', 16"	29.1-29.8	29.0-29.7	29.1-29.8	-CH_2
17', 17"	29.1-29.8	29.0-29.7	29.1-29.8	-CH_2
18', 18"	14.2, 14.1	14.1, 14.0	14.2, 14.1	-CH_3

* Data from Ramsewak *et al*., 2001

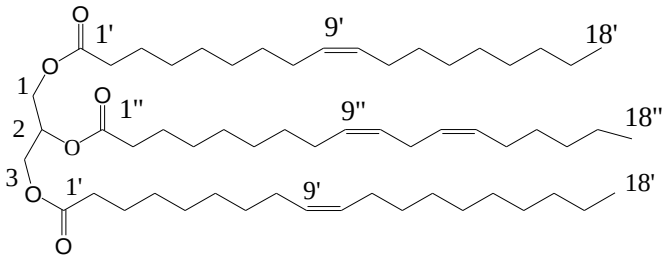

Figure 4. The proposed structure of **MS-3**

As described in section 2.2, the isolation of four triglycerides from *M. oleifera* has been reported. The four triglycerides are 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 the **MS-2** and **MS-3** 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 **MS-2** and **MS-3** 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 ^{1}H-NMR and ^{13}C-NMR data.

3.0.3 Structure Elucidation of MS-1 (Partial Characterization)

Analysis of IR spectra of **MS-1** (Appendix 9) indicated that it has no carboxylic acid, ester and aldehyde functional groups. This was confirmed by absence of carbonyl group stretching band at 1750 cm^{-1}, absence of a weak bands in the range of 2000 and 1650 cm^{-1} indicated that the compound has no aromatic functional group. The observed band at 1608 cm^{-1} represents C=C stretch of an alkene whereas the bands at 2918 and 2853 cm^{-1} indicate C-H stretches of methyl groups. The broad band in the range of 3416 and 3175 cm^{-1} represents O-H stretching of an alcohol functional group and a band at 1322 cm^{-1} indicates C-O stretching of alcohol. Thus, the observed spectrum indicates that the compound is most likely an alcohol.

In the ^{1}H-NMR spectrum of **MS-1** (Appendix 10), the peak at 0.91 indicate the presenced of protons of methyl groups; the peaks at 1.28 and 1.59 indicated presence of protons of aliphatic methylene group; a peak at 2.05 indicates presence of protons of a methylene group that is bonded to C=C bond; the peak at 2.28 indicate presence of protons of methylene that is bonded to carbon bearing hydroxyl group; the peak at 2.76 indicated presence of protons of a methylene group that is flanked by two C=C bonds; whereas the peak at δ 5.32 indicated presence of olefinic protons.

In the ^{13}C-NMR spectrum of **MS-1** (Appendix 11), peaks observed at 127.9- 130.0 ppm indicated presence of olefinic carbons. The DEPT spectrum (Appendix 12) also confirmed the presence of methyl carbon at 14.2 ppm, methylene carbon at 22.6 and 33.8 ppm. The DEPT spectra also indicated the presence of quaternary carbon atoms at 114.2.

3.0.4 Summary of Spectral Data of the Isolated Compounds

MS-2

^{1}H-NMR (400 MHz, $CDCl_3$): 0.88 (t, 6H, H-18′ x 2), 0.90 (t, 3H, H-18"), 1.27 (m, 48H, H-(4′-7′and 15′-17′) x 2 and H-(4"-7"and 12"-17″), 1.63 (m,6H, H-3′x 2 and H-3"), 2.02 (m, 12H, H-(8′,14′)x 2 and H-(8", 11″)), 2.30 (t, 4H, H-2' x 2), 2.35 (t, 2H, H-2"), 2.77 (m, 4H, H-11" x 2),

4.14 (dd, 2H, H-la, 3a), 4.29 (dd, 2H, H-lb, 3b), 5.27 (m, 1H, H-2), 5.38 (m, 10H, H-(9′, 10′, 12', 13′) x 2 and H-(9″, 10″)) (Appendix 2); **^{13}C-NMR**: (100MHz, $CDCI_3$): 14.1 (C-18"), 14.2 (C-18' x 2), 22.5, 22.6,24.8, 24.9, 29.0-29.8 (C-(4′-7′ and 15′-17′) x 2 and C-(4"-7" and 12"-17")), 25.6 (C-11' x 2), 27.2 (C-(8′, 14′) x 2 and C-(8", 11")), 31.5 (C-3"), 31.9 (C-3′ x 2), 34.0 (C-2′ x 2), 34.2 (C-2"),62.1 (C-1), 68.9 (C-2), 127.8-131.9 (C-(9′, 10′, 12′, 13′) x 2 and C-(9", 10″)), 172.8 (C-1″), 173.3 (C-1′ x 2) (Appendix 3); **IR**: 1745 cm^{-1}, 1163 cm^{-1}, 3006.8 cm^{-1} , 2922 cm^{-1} , 2852 cm^{-1} (Appendix 1).

MS-3

^{1}H-NMR (400 MHz, $CDCl_3$): 0.88 (t, 6H, H-18′ x 2), 0.90 (t, 3H, H-18"), 1.27 (m, 48H, H-(4′-7′and 15′-17′x) 2 and H-(4"-7"and 12"-17″)),1.64 (m,6H, H-3′x 2 and H-3"),2.01(m, 12H,H-(8′,14′)x 2 and H-(8", 11″)), 2.30 (t, 4H, H-2' x 2), 2.34 (t, 2H, H-2"), 2.75 (m, 4H, H-11’ x 2), 4.11 (dd, 2H, H-la, 3a), 4.28 (dd, 2H, H-lb, 3b), 5.28 (m, 1H, H-2), 5.36 (m, 10H, H-(9′, 10′, 12', 13′) x 2 and H-(9″, 10″))(Appendix 6) ; **^{13}C- NMR**: (100 MHz, $CDCI_3$): 14.1 (C-18"), 14.2 (C-18' x 2), 22.6, 22.7, 24.8, 24.9, 29.1-29.8 (C-(4′-7′ and 15′-17′) x 2 and C-(4"-7" and 12"-17")), 25.6 (C-11' x 2), 27.2 (C-(8′, 14′) x 2 and C-(8", 11")), 31.5 (C-3"), 31.9 (C-3′ x 2), 34.0 (C-2′ x 2), 34.1 (C-2"), 62.0 (C-1), 68.9 (C-2), 127.8-130.1 (C-(9′, 10′, 12′, 13′) x 2 and C-(9", 10″)), 172.3, 172.4 (C-1′ x 2) , 172.8 (C-1″) (Appendix 7); **IR**: 1743 cm^{-1}, 1163 cm^{-1}, 3006.8 cm^{-1} , 2918 cm^{-1} , 2853 cm^{-1} (Appendix 5).

MS-1

^{1}H- NMR (400 MHz, $CDCl_3$): δ 0.91(t), 1.28 (m), 1.59 (br), 2.05 (m), 2.28 (br), 2.35 (m), 2.76 (s), 5.32 (m) (Appendix 10); **^{13}C-NMR**: (100 MHz, $CDCI_3$): δ 14.2, 22.6-33.8, 114, 127.9-130.0 (Appendix 11); **IR**: 1608 cm^{-1}, 2918 cm^{-1}, 2853 cm^{-1}, 3416-3175 cm^{-1} (Appendix 9).

3.1 *In vitro* Antileishmanial Activity Test of the Isolated Compounds

In vitro antileishmanial activity test was carried out for the isolated compounds on both stages of the parasite (promastigote and amastigote). The test strain used for the study was *L. aethiopica.* Each of the pure compounds such as; compounds labeled **MS-1, MS-2** (1, 3-dilinoleoyl-2-olein) and **MS-3** (1, 3-dioleoyl-2-linolein) were evaluated for their antileishmanial activities and found to be active against both the promastigote and amastigote forms of the parasite (Section 3.2.1 and 3.2.2).

3.2.1 Evaluation of Antileishmanial Activities of the Isolated Compounds: Inhibition of Promastigotes

The isolated compounds were subjected to antileishmanial activity tests using the promastigote stage of the parasite. The results indicated that the IC_{50} values of **MS-1**, **MS-2** and **MS-3** to be 0.24, 0.079 and 242.5 µg/ml, respectively. Among these, **MS-2** was the most active whereas **MS-3** was found to be the least active as demonstrated by its highest IC_{50} value. Comparison of these data with the observed IC_{50} values of the reference compounds (Amphoterisine B; IC_{50} = 0.004 and Miltefosine; IC_{50} =0.136 µg/ml) suggested that the two compounds (**MS-1** and **MS-2**) that showed comparable activities with the reference drugs would be used as candidates for further activity tests.

3.2.2 Evaluation of Antileishmanial Activities of the Isolated Compounds: Inhibition of Amastigote

Antileishmanial activity tests of the isolated compounds were carried out using an amastigote stage of the test strain (i.e., *L. aethopica*). The growth inhibitions (IC50) of the **MS-1**, **MS-2** and **MS-3** against the amastigotes were found to be 21.52, 40.03 and 26.79 µg/ml, respectively. The results indicated that the compounds have comparable antileishmanial activities. When compared to the corresponding activities of the reference drugs (Amphoterisine 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 the compounds are promising candidates for further antilieshmanial activity tests.

4.0 CONCLUSIONS AND FUTURE PROSPECTIVES

In the present study, three compounds (**MS-1, MS-2** and **MS-3**) were isolated from the petroleum ether:ethyl acetate extract of roots of *M. stenopetala*. Two of the compounds (**MS-2** and **MS-3**) were identified as triglycerides based on observed spectral data and comparison of the data with those data reported in literature. Thus, the structure of **MS-2** was proposed to be similar with that of a triglyceride known as 1, 3-dilinoleoyl-2-olein whereas the structure of **MS-3** was proposed to be similar to that of a triglyceride called 1, 3-dioleoyl-2-linolein. Both triglycerides have been isolated from *Moringa* species (i.e., *M. oleifera*). The third compound (**MS-1**) was partially characterized based on the IR and NMR spectral data.

The isolated compounds were subjected to *in vitro* anileishmanial activity tests using promastigote and amastigote stages of *L. eathopica*. The results indicated the compounds are less active than the reference drugs (i.e., Amphotericine B and Miltefosine) in inhibiting growth of promastigotes and amastigotes. However, the observed IC_{50} values of the compounds against amastigotes were comparable to each other and to the reference drugs (Section 3.2.2). These observations suggest that the isolated compounds are promising candidates for further antileishmanial activity tests in antileishmanial drug discovery programs.

The findings of the present study suggest more work has to be done to isolate compounds from different parts of *M. stenopetala*, and carry out antilaeishmanial activity tests. These would lead to identification of compounds which are effective in inhibiting both the wild-type and resistant Leishmanial parasites.

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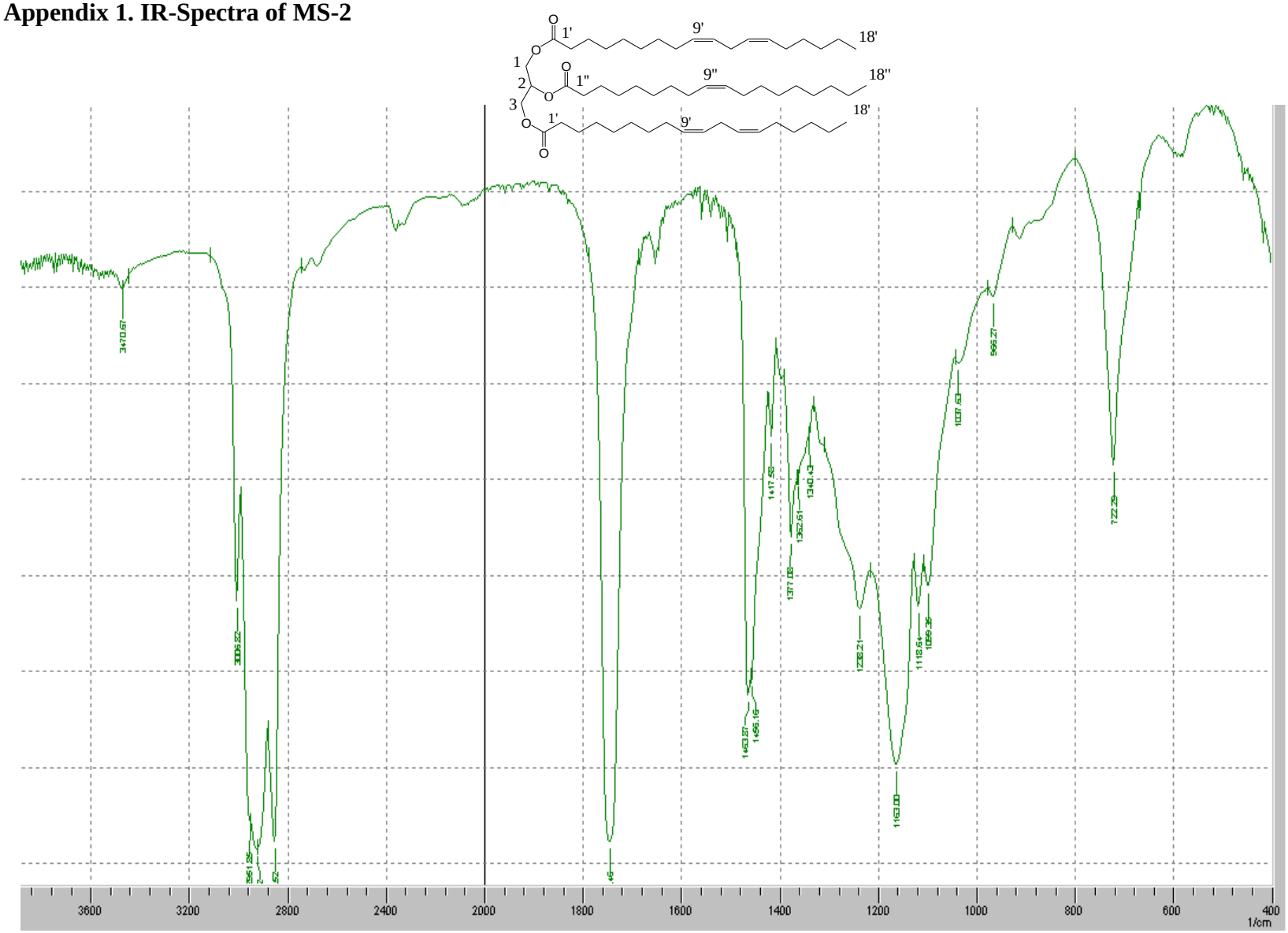
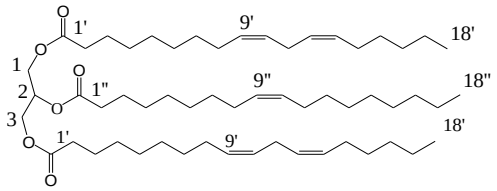
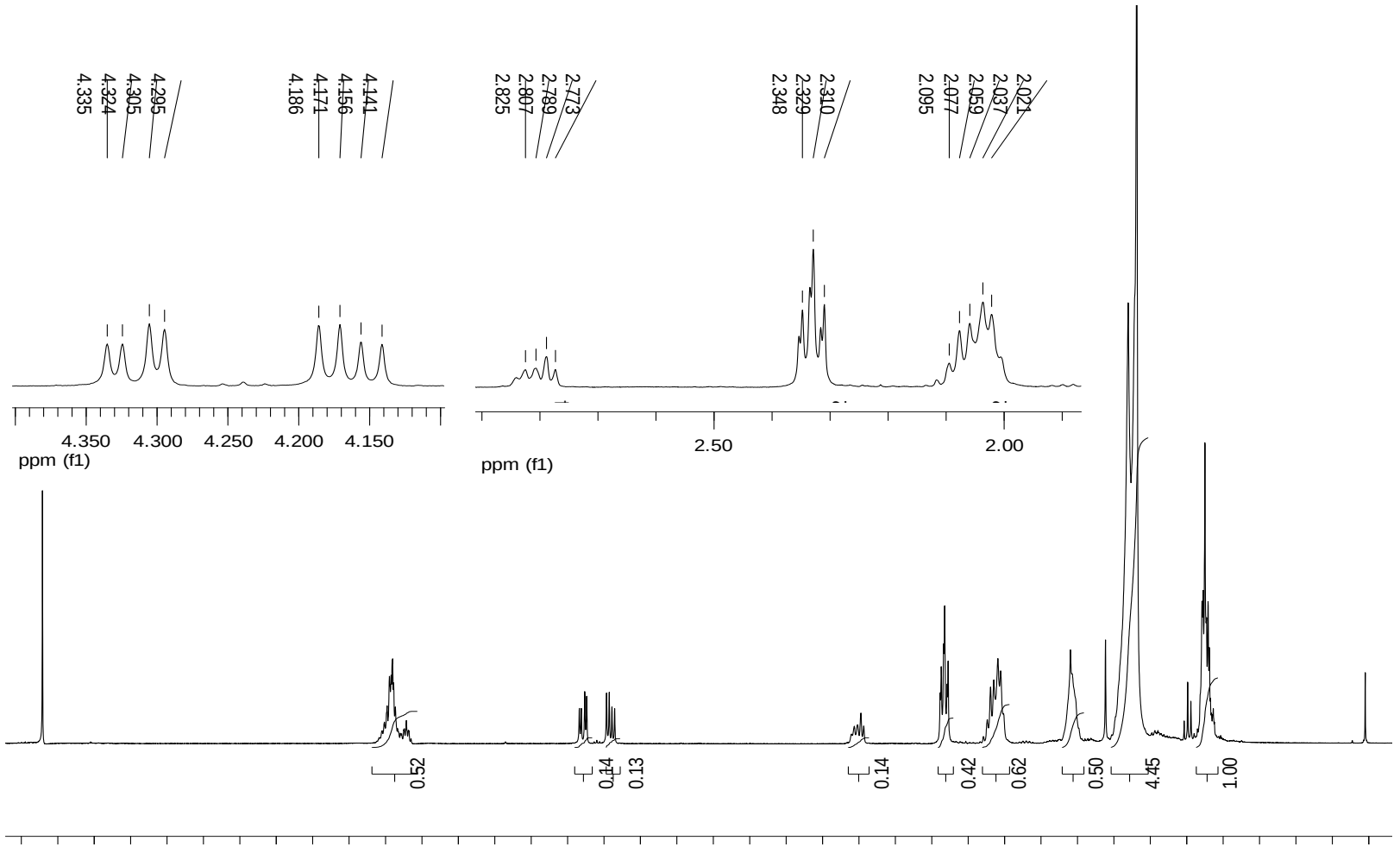
APPENDICES

Appendix 1. IR-Spectra of MS-2

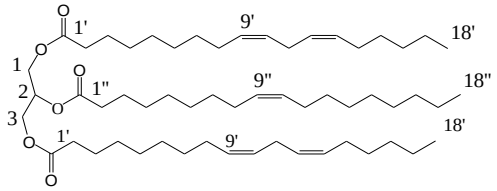


Appendix 2. ^{1}H -NMR Spectrum of MS-2 in $CDCl_3$

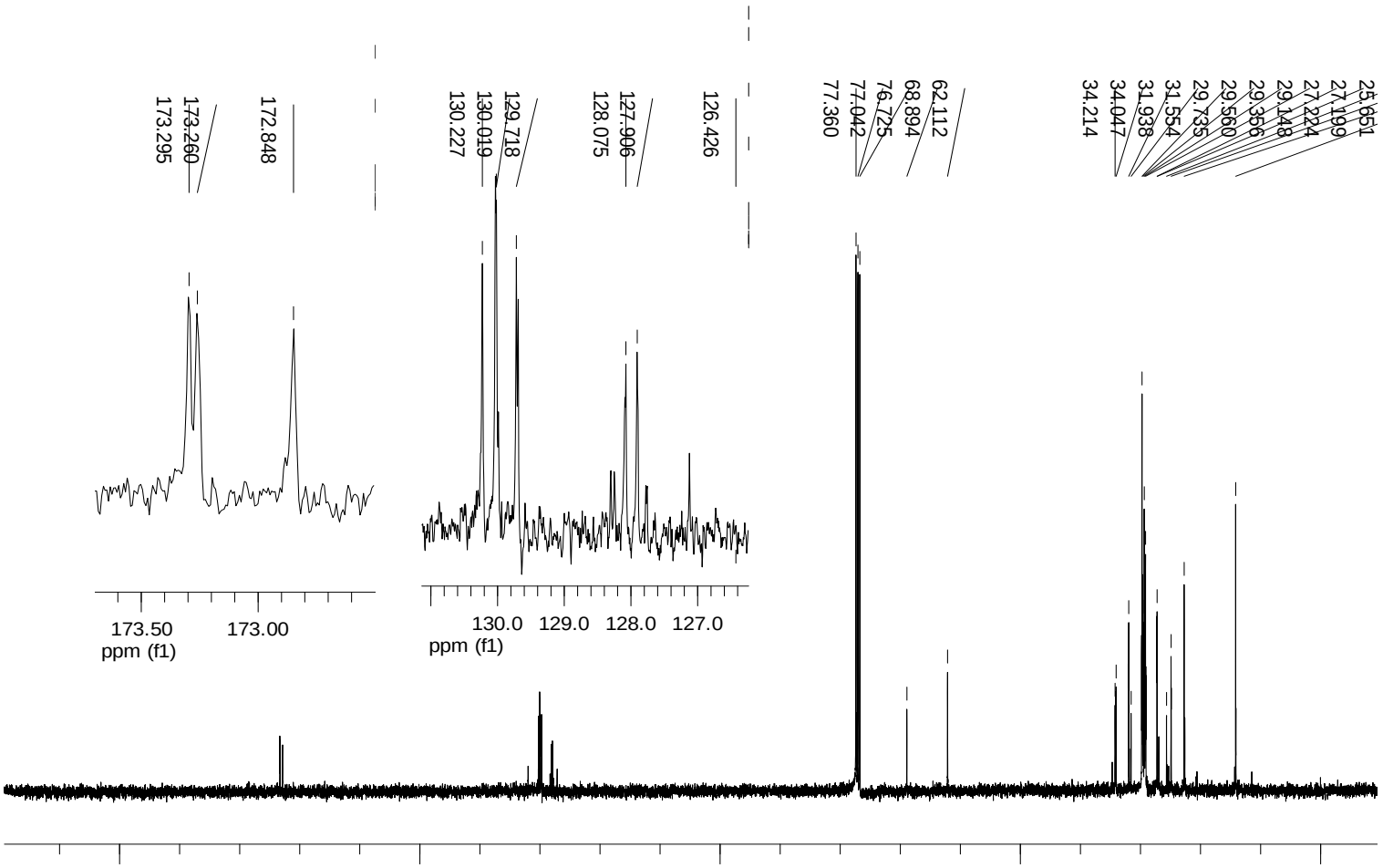




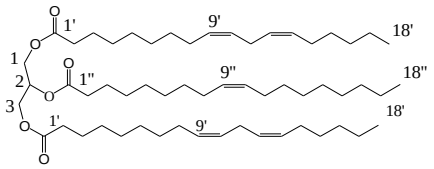
Appendix 3. ^{13}C -NMR Spectrum of MS-2 in $CDCl_3$



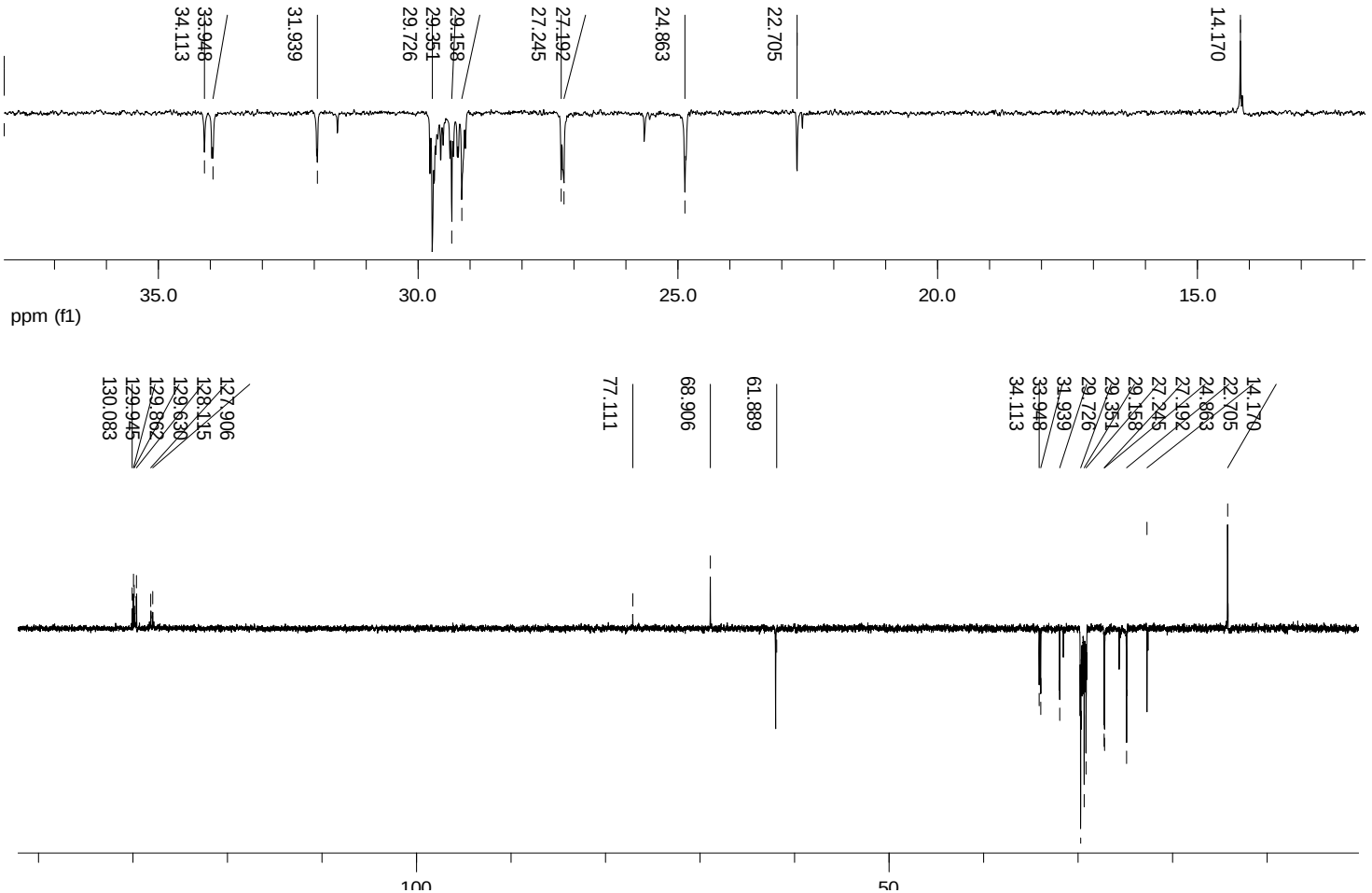
13C-NMR Spectra of compound 2



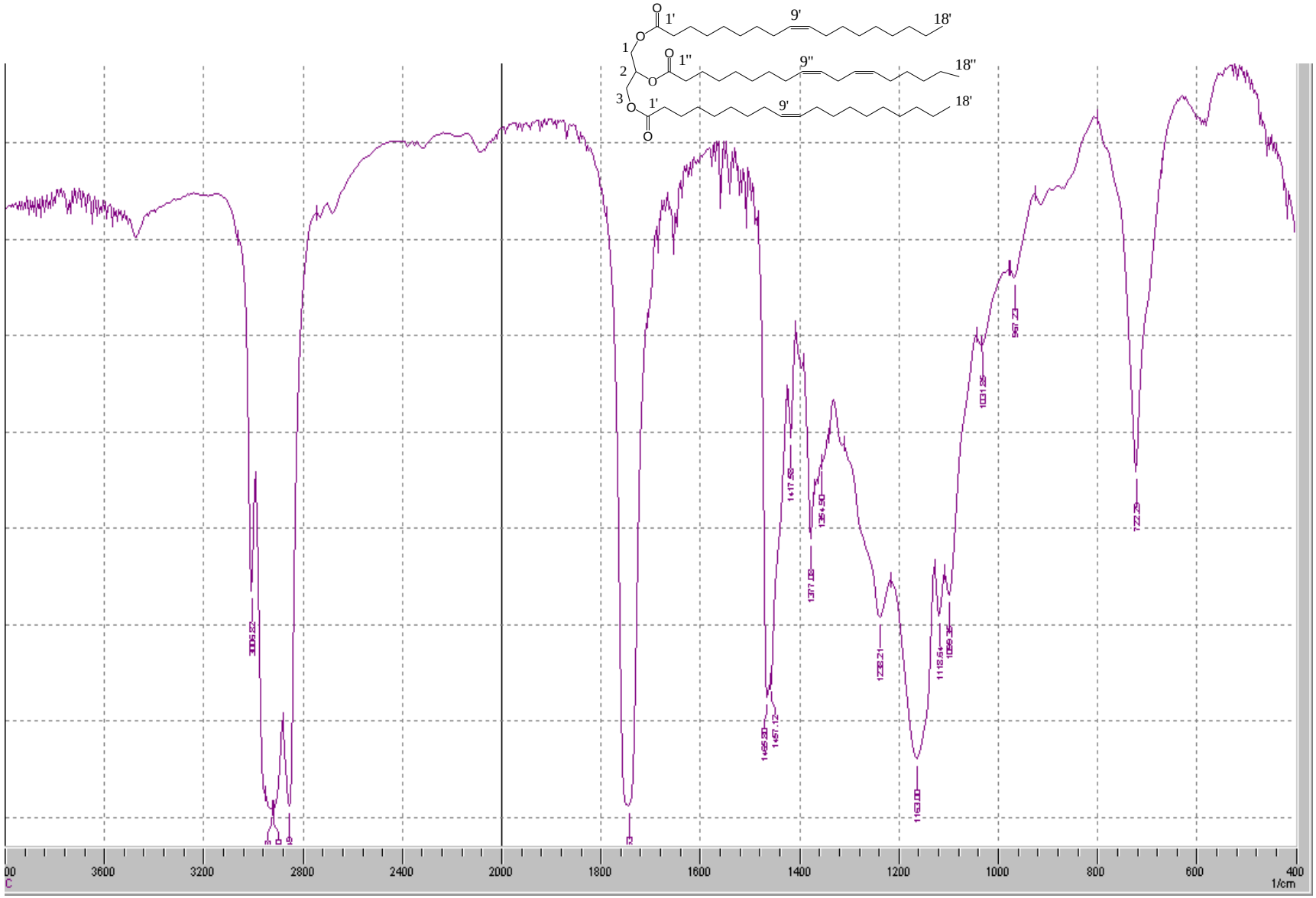
Appendix 4. DEPT Spectrum of MS-2 in $CDCl_3$



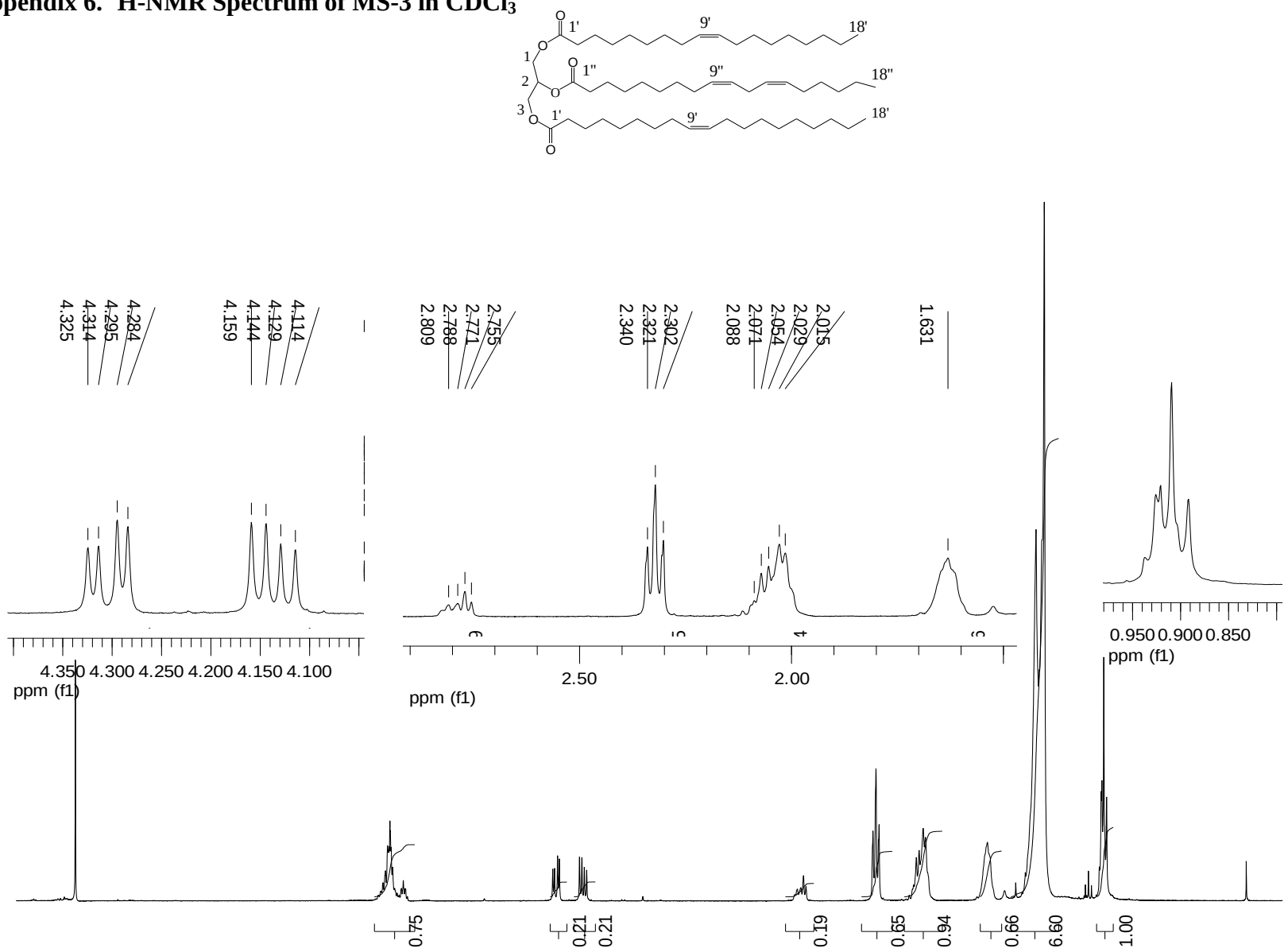
DEPT-Spectra of compound 2



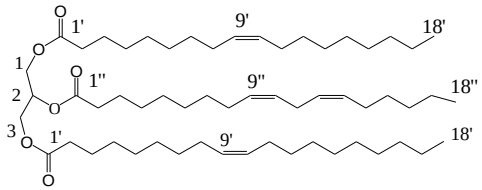
Appendix 5. IR Spectrum of MS-3



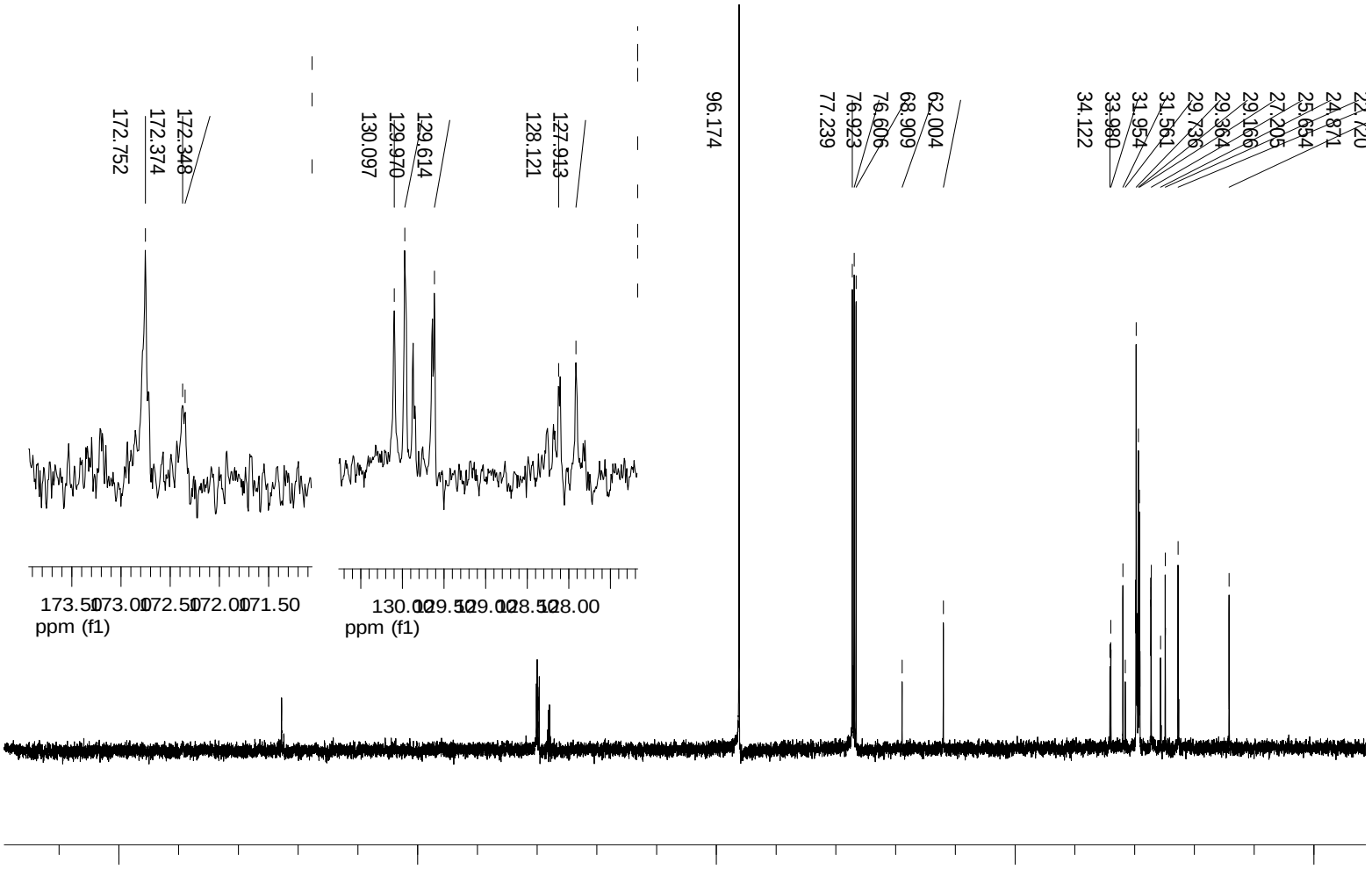
Appendix 6. ^{1}H-NMR Spectrum of MS-3 in $CDCl_3$



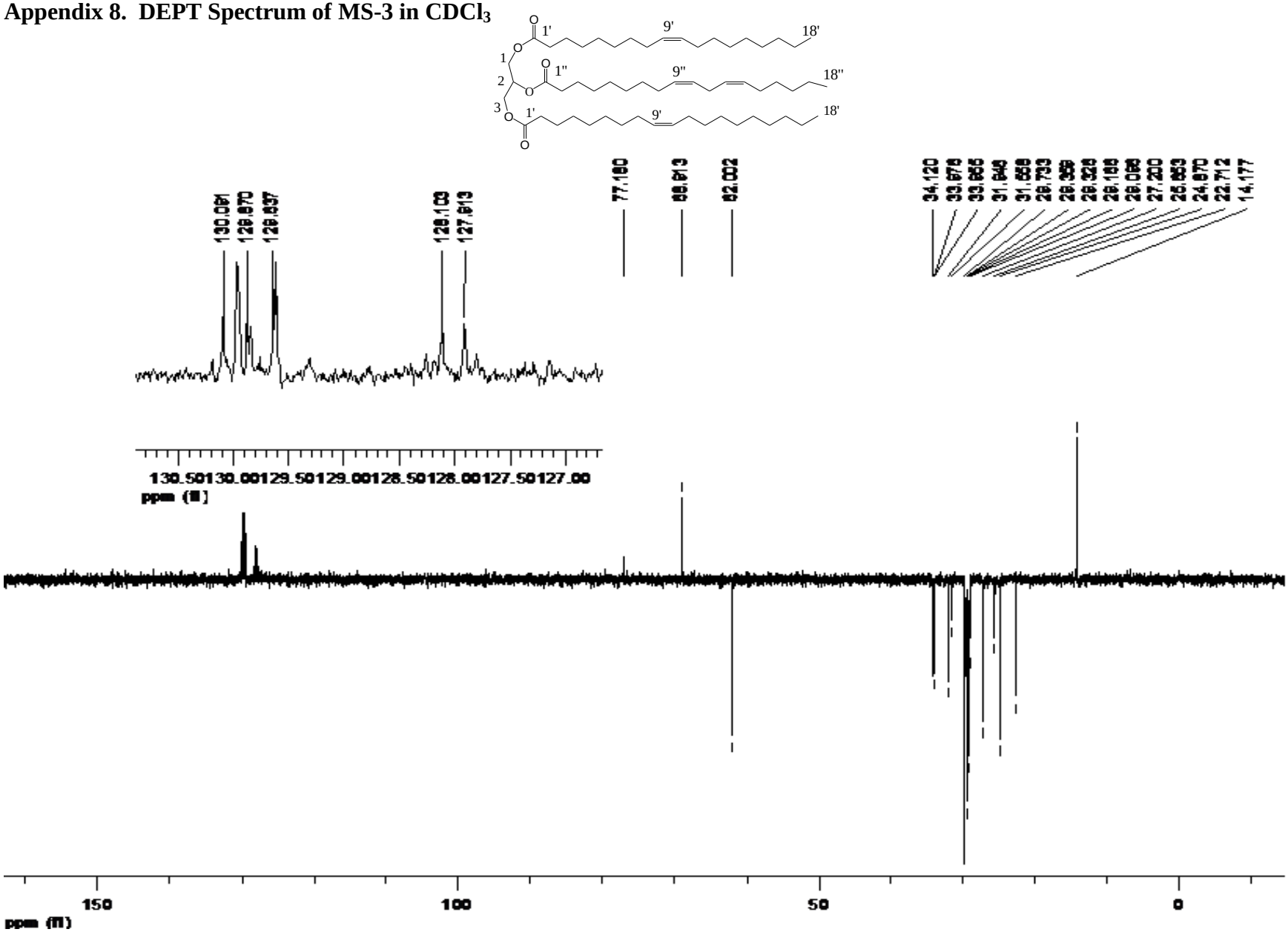
Appendix 7. ^{13}C -NMR Spectrum of MS-3 in $CDCl_3$



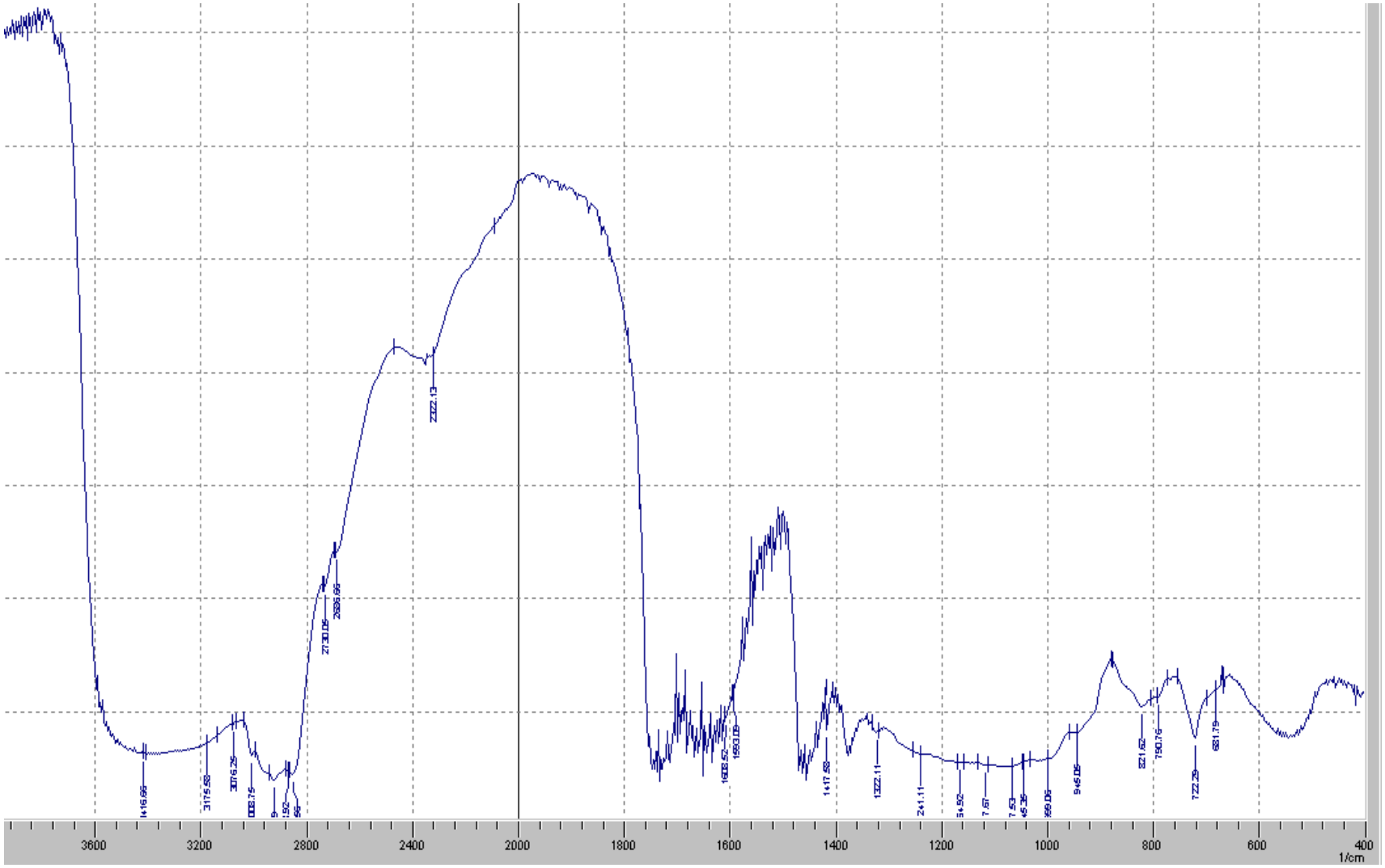
13C-NMR Spectra of compound 3



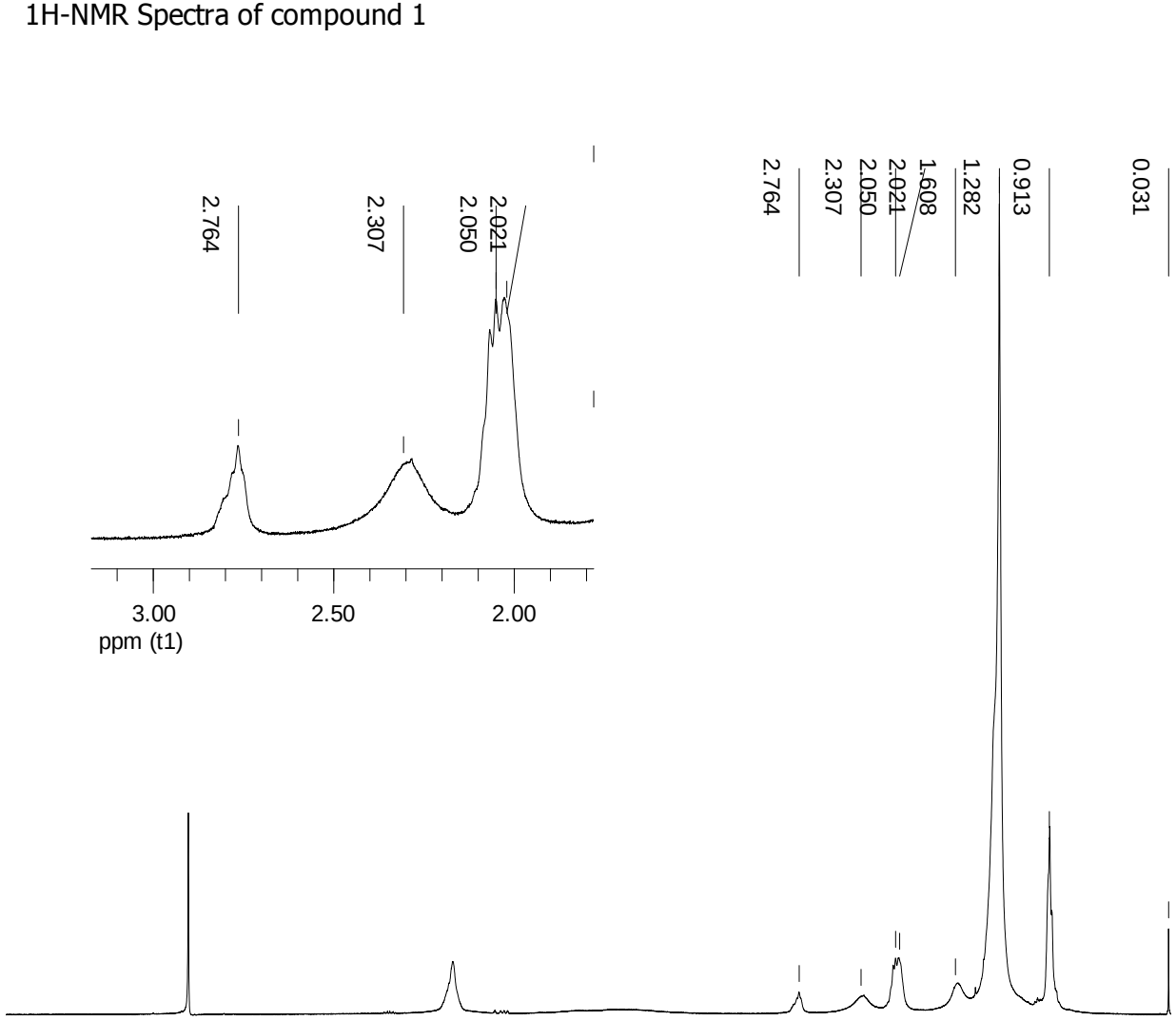
Appendix 8. DEPT Spectrum of MS-3 in $CDCl_3$



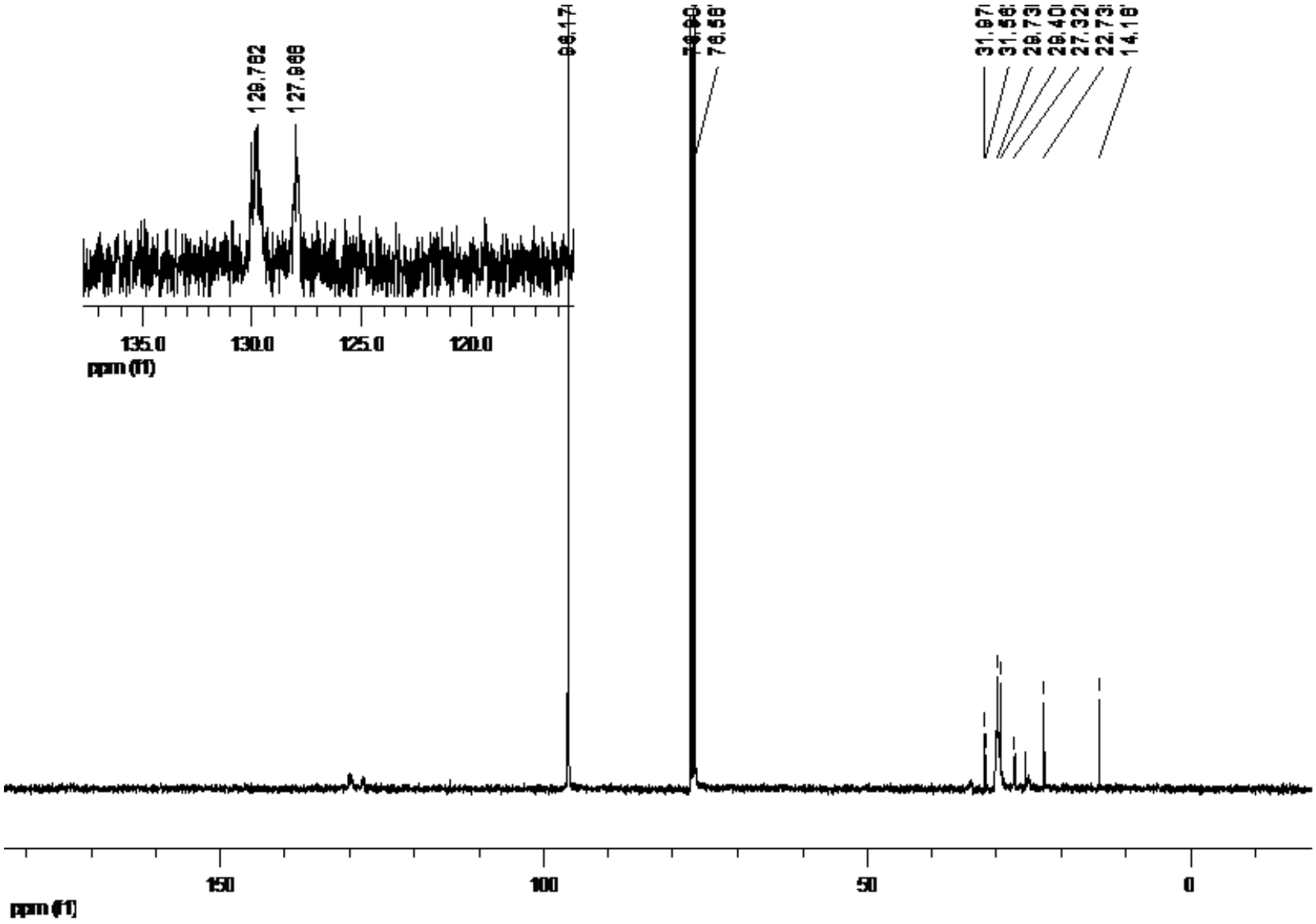
Appendix 9. IR-spectra of MS-1



Appendix 10. ^{1}H-NMR Spectrum of MS-1 in $CDCl_3$



Appendix 11. ^{13}C -NMR Spectrum of MS-1 in $CDCl_3$



Appendix 12. DEPT Spectrum of MS-1 in $CDCl_3$