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Anticancer activity of Lovage (*Levisticum officinale* Koch) extracts

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List of Abbreviations

Column chromatography	CC
Correlation spectroscopy	COSY
Deoxyribonucleic acid	DNA
Deuterated chloroform	CDCl3
Dimethyl sulphoxide	DMSO
Distortionless Enhancement by Polarization Transfer	DEPT
Doublet	*d*
Doublet of a doublet	*dd*
Dulbecco's Modified Eagle's medium	DMEM
Ethyl acetate	EtOAc
Foetal calf serum	FCS
Hertz	Hz
Heteronuclear Multiple Bond Coherence	HMBC
Heteronuclear Multiple Quantum Coherence	HMQC
Medium Pressure Liquid Chromatography	MPLC
Megahertz	MHz
Methanol	MeOH
Multiplet	*m*
Nuclear Magnetic Resonance	NMR
Part per million	ppm
Phosphate buffered saline	PBS
Retardation factor	R*f*
Roswell Park Memorial Institute	RPMI
Singlet	*s*
Thin layer chromatography	TLC
Ultraviolet	UV
Volume per volume	v/v
Weight per volume	w/v

Abstract

Plants are considered a major source of active compounds used in remedies to treat and alleviate many diseases and disorders. The aim of this study was to prepare solvent extracts from the root of *Levisticum officinale* Koch (Lovage), and to assess whether they had any anticancer properties. Two human cancer cell lines were used in the anticancer screening assays (THP-1, a leukaemia cell line and MCF7, epithelial breast cancer cells) and a modified normal cell line (PNT2A, prostate epithelial cells). The n-hexane, ethyl acetate, methanol and methanol/water crude extracts of Lovage roots were prepared and tested for cytotoxicity using a resazurin metabolic indicator assay. The most cytotoxic extract was the hexane, followed by the ethyl acetate, while the methanol and the methanol/water extracts demonstrated no cytotoxicity. Further fractionation of the n-hexane and the ethyl acetate extracts and compound structure elucidation by NMR spectroscopic analysis led to selection of one fraction from the ethyl acetate (LoE18-19) and two from the n-hexane fractions (LoH26-36 and LoH49-60). Fractions LoH26-36 and LoH49-60 showed high toxicity (IC_{50} ≤0.1 mg/ml) against THP-1 cells after 24 hours exposure (IC_{50} value of 0.001 mg/ml) and lower activity against MCF-7 cells (IC_{50} values of 0.466 mg/ml and 0.635 mg/ml for LoH26-36 and LoH49-60, respectively) and PNT2A cells (IC_{50} values of 0.145 mg/ml and 0.572 mg/ml for LoH26-36 and LoH49-60, respectively). The ethyl acetate fraction LoE18-19 demonstrated high toxicity towards the MCF-7 cells (IC_{50} value of 0.022 mg/ml) and a moderate toxicity (IC_{50} ≥0.1-≤0.5 mg/ml) against THP-1 cells (IC_{50} value of 0.287 mg/ml), but no toxic effect towards PNT2A cells (IC_{50} value of 1.833 mg/ml). NMR spectroscopic analysis showed that the chemicals in these fractions were major compounds with impurities. The fraction from the ethyl acetate (LoE18-19) was

tentatively identified as 3-methoxy-4-hydroxycinnamic acid (ferulic acid). The *n*-hexane fraction (LoH26-36) was tentatively identified as 1,2,3-propanetriol trilinoleate (trilinolein) mixed with a steroid ester and the hexane fraction (LoH49-60) was a mixture of 1,9-heptadecadien-4,6-diyne-3,8-diol (falcarindiol) with other minor compounds. The toxicity against cell lines observed in this study may be attributed to the anticancer and the anti-tumour properties of these compounds.

Chapter one: Introduction

1.1 Medicinal plants

Human beings have been exploiting plants for centuries as medicines or food additives with varying success to cure and prevent diseases. Plants have probably been used as medicines due to the need for curing diseases and to survive. Ancient medicinal practices originated as early as 4000 – 6000 BC and medicine has its roots in all parts of the world from Asia through the Middle East to Africa and Europe [1].

Written reports by Sumerians dated over 5000 years describe the potential of medicinal plants [2]. Egyptian medicine dates back to about 2900 BC, but the first formal record (the Ebers Papyrus) was written about 1500 BC; it describes around 800 prescriptions from a compilation of about 700 drugs mostly of plant and animal origin [3]. These prescriptions were administered in a variety of forms such as gargles, snuffs, poultices and infusions alone or mixed with honey, beer, wine or milk [4]. The famous Persian physician Aviennica contributed much to the sciences of Pharmacy and Medicine by his compilation of the Canon of *Medicinae*. Interestingly, it was the Arabs who opened the first drug stores in the eighth century [4]. Today, herbs like fenugreek (*Trigonella foenum-graecum*) and caraway (*Carum carvi*) are still sold in Arab countries and around the world as remedies of many symptoms in a variety of local traditional medicines.

According to the World Health Organization (WHO), around 80% of the population in developing countries is dependent on herbal medicine for basic healthcare needs [5]. The demand for natural products is not limited to developing countries; the use of herbal medicine in developed countries is also increasing. For example, one in

four of the population in the UK consumes herbal medicines frequently with an overall yearly spending of US$230 million [6, 7].

Natural products or their derivatives were included in 14 of the top 35 drugs in 2000 based on worldwide sales [8]. During the 70s of the previous century, 25% of all drugs dispensed in the USA contained compounds derived from flowering plants. Table 1 lists major drugs derived from plants adopted from [9].

Table 1: List of major drugs derived from plants [9].

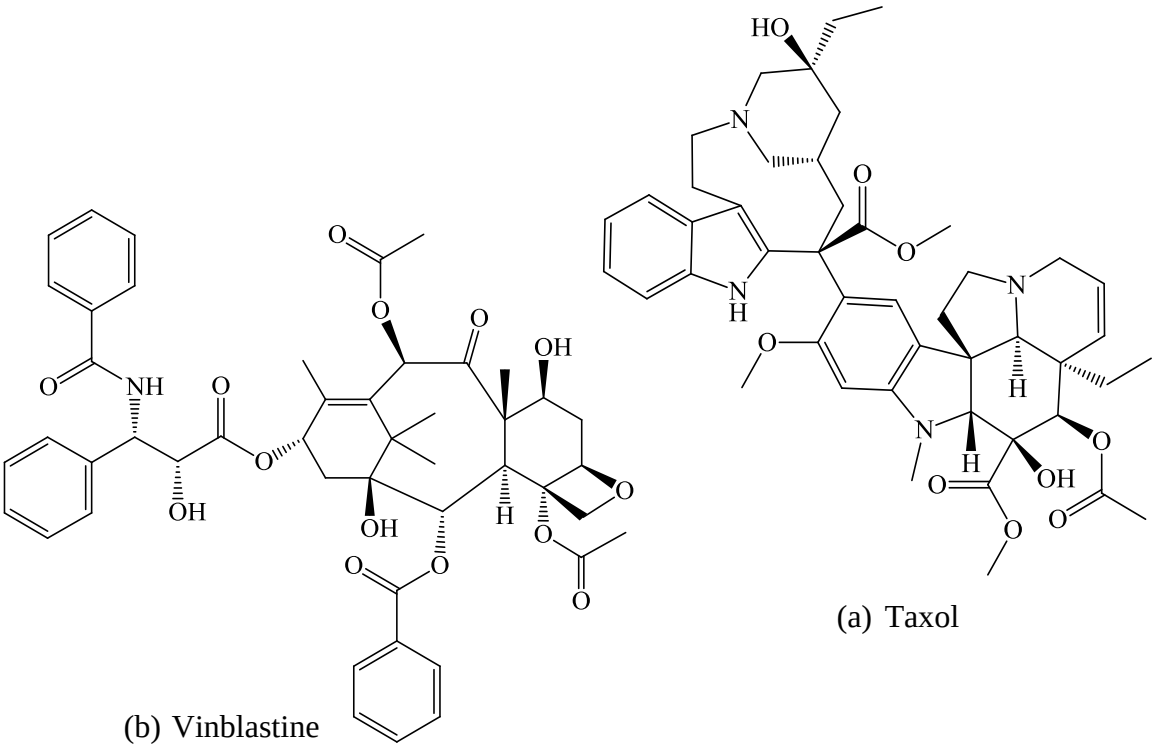
Drug	**Source**	**Pharmacological Action**	**Chemical Family**
Artemisinin	*Artemisia annua*	Antimalarial	Sesquiterpene, lactone
Codeine, morphine	*Papaver somniferum*	Analgesics	Opiate alkaloids
Cocaine	*Erythroxylum coca*	Local anaesthetic	Cocaine alkaloid
Digoxin	*Digitalis pupurea*	Cardiotoinc	Steroidal glycoside
Galanthamine	*Leucojum aestivum*	Cholinesterase inhibitor	Isoquinoline alkaloid
Quinine	*Cinchona ledgeriana*	Antimalarial	Quinoline alkaloid
Taxol	*Taxus brevifolia*	Antineoplastic	Diterpene
Vincrystine, vinblastine	*Catharanthus roseus*	Antineoplastic	Bis-indole alkaloids

1.1.1 Medicinal plants in trade

Raw plant material sold in the trade consists chiefly of seeds, wood, leaves, flowers, roots or sometimes the whole plant. During the 1990s, the overall import trade accounted on average of 400,000 tons of raw plant materials with a value of more than 1 billion USD. The international trade is limited to a few countries; 12 countries unequally share about 80-82% of the overall world importation and exportation [10]. Asia and Europe dominates the domestic import at 42% and 33%, respectively. China and India are the most important export countries. On the other hand, three countries, Japan, USA and China are important import and trade centres for pharmaceutical plants [11]. Recent advances in drug development have allowed the introduction of new medicines derived from natural products. In cancer treatment, two plant-derived natural products; paclitaxel and camptothecin were estimated to account for almost 30% of the worldwide anticancer market or about $3 billion of $9 billion per year [12, 13].

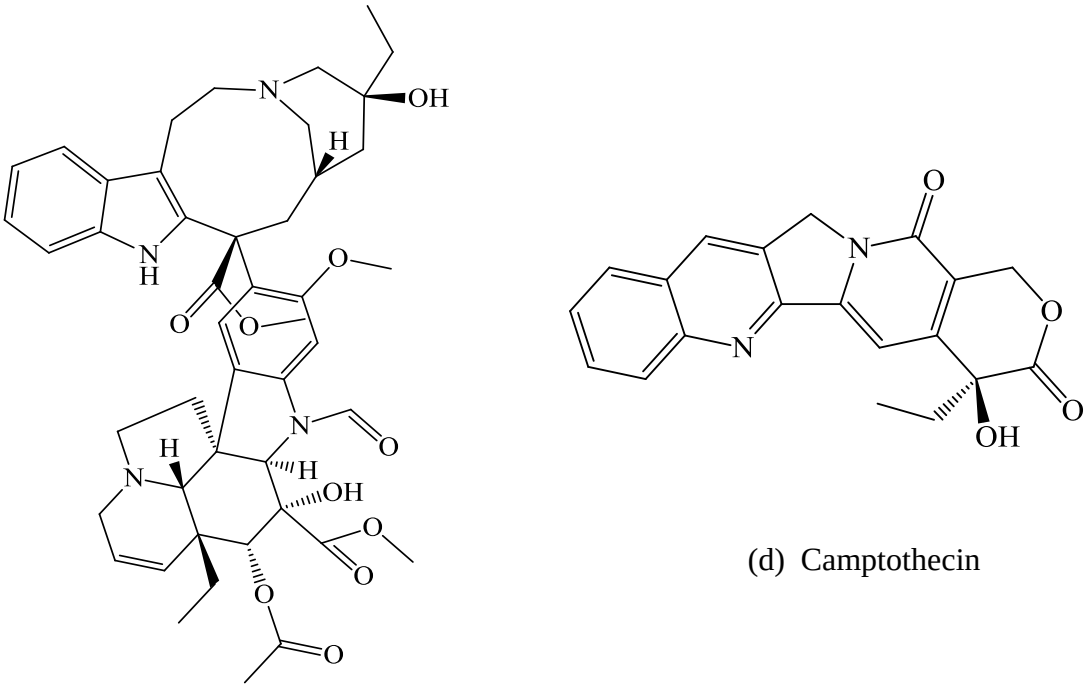
1.1.2 Natural products used in anticancer therapy

Plants have long been used in the treatment of cancer [14]. About 60% of the 92 anticancer drugs commercially available in the US and worldwide-approved anticancer drugs are of natural origin [15, 16]. Numerous types of bioactive compounds have been isolated from plant sources. Several of them are currently in clinical trials or preclinical trials or undergoing further investigation. For instance, plant-derived antitumor drugs such as taxol (Figure 1a), vinblastine (Figure 1b), vincristine (Figure 1c) and camptothecin (Figure 1d) have considerably enhanced the efficacy of chemotherapy against some cancers [17-20]. Table 2 lists some plants used in the prevention and treatment of cancers.



(a) Taxol

(b) Vinblastine



(c) Vincristine

(d) Camptothecin

Figure 1: Plant-derived anticancer agents in clinical use [21].

Table 2: List of some plants used in the treatment or prevention of cancer

Plant Name	Use as anticancer	References
Allium sativum	Active against erythroleukaemia as well as breast and prostate cancer	[22]
Annona triloba	Acetogenins from this herb have been found to be active against certain lung and breast cancers	[23]
Aronia melanocarpa	Active against colorectal and colon cancer	[24]
Asparagus officinalis	Possess antioxidant and antitumor activities against HeLa and BEL-7404 cells	[25]
Asparagus racemosus	Significant anticancer activity using MCF-7, HT-29 and A-498 cell lines	[26]
Brassica oleracea	Prevents lung, stomach, colorectal, breast, and other cancers	[27]
Camellia sinensis	Used to prevent prostate, colon, and gastric cancers. It is also used to prevent skin cancer or damage from ultraviolet radiation	[28]
Catharanthus roseus	Vinblastine and vincristine, largely used in combination with other cancer chemotherapeutic agents for the treatment of leukaemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma	[14]
Chrysanthemum morifolum	May reverse precancerous gastrointestinal lesions	[29]
Plant Name	**Use as anticancer**	**References**

Combretum caffrum	Contains combretastatins, the most potent of which is combretastatin CA-4, which is active against colon, lung and leukaemia cancers	[30, 31]
Kaempferia rotunda	Active against human HT-29 colon cancer and MCF-7 breast cancer cells	[32]
Larrea divaricata	Has an antiproliferative activity on T lymphoma (BW 5147) cells *in vitro* and *in vivo* antitumor activity.	[33]
Nigella sativa	Inhibits stomach tumours, carcinoma, and Ehrlich ascites carcinoma.	[34]
Olea europaea	Active against breast cancer and leukaemia.	[35, 36]
Oxcycocus macrocarpos	Plant juice reduces the number of breast cancer tumours, delay tumour development, and slows metastases of cancer to lungs and lymph nodes	[37, 38]
Polygonum tinctorium	Protection and prevention of leukaemia	[39, 40]
Raphanus sativus	Synthetic agent roscovitine, derived from the natural product olomucine, originally isolated from *Raphanus sativus L.* in Phase I clinical trials in Europe.	[41]
Taxus brevifolia	Taxol used in treatment of breast and ovarian cancer	[42, 43]

Table 2: continued.

1.2 The Apiaceae family

This project focuses on Lovage, a plant that belongs to the Apiaceae family. This is a large plant family with over 3000 species. It also includes plants like angelica, carrots, celery, and parsley, which are mostly aromatic with hollow stems. The plants of the Apiaceae family (also known as the Umbelliferae family) are known to contain bioactive components of benefit to human health making their phytomedical prospective a highly promising field of study [44]. Carrots *(Daucus carota)* belong to this family and the purple carrot has a compound called 2-pentanone, a common phytochemical, which may prevent colon carcinogenesis in cells of the colonic mucosa, by inhibiting Cyclooxygenase-2 (Prostaglandin Synthase-2) protein expression and prostaglandin production [45]. Carrots contain more beta-carotene, a pro-vitamin A and anti-cancer compound, than other common fruit and vegetables [46]. When carrots are consumed, the liver converts beta-carotene into Vitamin A, which benefits day and night vision, healthy skin and immunity. Falcarinol is found in carrots, celery and parsley, and is shown to have anti-cancer properties in breast cancer and anti-tumour properties in rat models [47, 48]. Celery (*Apium graveolens*) seed oil is rich in monoterpene hydrocarbons (74.6% of d-limonene) and has anti-inflammatory, anti-viral, anti-tumour, anti-microbial, anti-hypoglycaemic, anti-carcinogenic benefits [49-51]. Luteolin is found in celery, as well as carrots and other foods, and has anti-cancer, anti-inflammatory, anti-microbial and anti-oxidant properties as well as, inducing apoptosis, inhibiting angiogenesis, preventing carcinogenesis and a role as a cancer chemopreventative as it can reduce tumour growth and sensitise tumour cells to anti-cancer drugs [52, 53].

1.2.1 Lovage: plant taxonomy and distribution

Lovage (*Levisticum officinale* Koch, Figure 2a) is an herb that grows wild in temperate zones around the world. It is also sometimes called European Lovage, garden Lovage, Lavose, Love parsley and Sea parsley. It is classified under the class Dicotyledons, subclass Rosidae, order Apiales, family Apiaceae, genus *Levisticum* and species *L. officinale* W.D.J. Koch. Lovage can be found growing wild across Europe and Asia, parts of Britain, as well as the Eastern US, although it is believed to originate in Afghanistan and Iran. The Scottish or Scots Lovage (*Ligusticum scoticum*, Figure 2b) is a long-lived perennial in the same family as *L. officinale*. It belongs to the genus *Ligusticum* and species *L. scoticum*. It is common on cliffs and rocky shores of Scotland. Considering its spice properties, Lovage is now widely farmed all over Europe and the US.

1.2.2 Characteristics of Lovage

The herb bears dark green leaves and greenish yellow flowers. The plants, including the roots, have a strong celery-like odour as a result of essential oils as well as oleoresins that are produced from the leaves, seeds, and roots [54]. The terms Lovage oil and Lovage extract are used interchangeably to identify the steam distillation product derived from Lovage plant roots. Lovage and its extraction products have a long history and widespread use as flavouring substances, fragrance materials and herbal medicines [55]. Lovage extract has been recognised as GRAS (Generally Recognised As Safe) for use as a flavour ingredient by the US Food and Drug Administration and the Flavour and Extract Manufacturers Association (FEMA No. 2650) [56]. A limited toxicological database exists for Lovage extract. Lovage is

relatively non-toxic following acute exposure by oral and dermal routes. With the exception of occluded application to rabbit skin, Lovage extract is historically considered to be generally non-irritating and non-sensitising in animal studies and on human volunteers [55]. Lovage essential oil has been shown to be anti-bacterial against 20 out of 25 genera of bacteria [57] and inhibits human head and neck squamous carcinoma cell (HNSCC) growth [58]. Elbadawy (2007) demonstrated that solvent crude extracts of Lovage leaves - hexane and methanol: water (1:1), possessed anti-inflammatory activity, while MacAskill (2009) showed that hexane, chloroform and methanol extracts significantly inhibited TNF-α production by THP-1 cells stimulated with bacterial lipopolysaccharide [59].

1.2.3 Traditional uses of Lovage

Lovage has several uses, including medicinal and culinary. Research has shown that Lovage is an extremely beneficial herb for the digestive and respiratory systems. It has warming and stimulating therapeutic effects on both body systems [60, 61]. In addition, Lovage is effective in healing ailments such as poor appetite, indigestion, bronchitis, gas and colic [61]. Lovage also possesses considerable diuretic and antimicrobial properties and hence it is normally administered for healing urinary tract problems [62]. Apart from these features, Lovage promotes menstruation and lessens accompanied pain and may also help in improving blood circulation. However, skin photosensitivity and dermatitis during harvesting have been reported; this may be due to compounds such as furocoumarins [63].

Figure 2: (a) Lovage (L. officinale Koch), and (b) Scottish Lovage (L. scoticum).

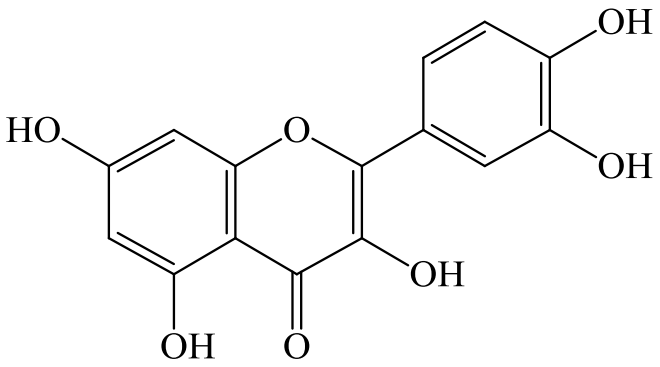
1.2.4 Constituents of Lovage

Lovage contains a volatile oil, coumarins, plant acids, beta-sitosterol, gums and resins [64]. About 70% of the constituents of the volatile oil are a chain of lactone derivatives called phthalides, while coumarins include bergapten, psoralen and umbelliferone. Lovage is considered a high source of the free-radical-scavenging flavonoid, quercetin; one kilogram of Lovage yields about 1700 mg of quercetin [65].

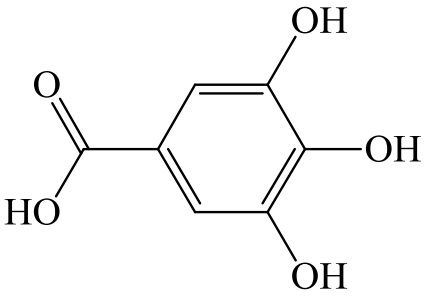
Quercetin (Figure 3a) has been shown *in vitro* to inhibit growth of colorectal cancer cells [66-68], possibly by up-regulation in the expression of tumour suppressor genes [66] and modulation of cell-cycle related and apoptosis genes [68]. It has also been shown to have potential activity against prostate cancer. It can attenuate the function of the androgen receptor (AR), inhibiting AR-mediated expression of prostate-specific antigen (PSA) [69], up-regulating tumour suppressor genes while down-regulating oncogenes and cell cycle genes [70], and inhibit other receptors involved in growth and metastasis of prostate cancer [71]. Quercetin has also been shown *in vitro* to have activity against leukaemia [72] and pancreatic tumour cells [73]. Other preliminary studies suggest that quercetin could have inhibitory effects on other cancer types, including breast, ovary, endometrial, non-small-cell lung, gastric and squamous cell [74].

Gallic acid is another bioactive compound found in Lovage (Figure 3b). Gallic acid has anti-fungal and antiviral properties. It also acts as an antioxidant and helps to protect cells against oxidative damage. Gallic acid was found to show cytotoxicity against cancer cells by inducing apoptosis [75]. Yoshioka *et al.* (2000) studied the

apoptotic activity of gallic acid in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines. The study showed that several derivatives of gallic acid including ethylgallate 2,3,4-trihydroxybenzoic acid and ellagic acid induce apoptotic cell death in these cancer cell lines [76].



(a) Quercetin



(b) Gallic acid

Figure 3: Chemical structure of two important compounds found in Lovage extracts, (a) Quercetin and (b) Gallic acid.

1.3 Aims and objectives of this research

The aim of this project was to investigate the anticancer properties of Lovage root extracts. The existence of bioactive flavonoids such as quercetin and gallic acid raise the proposition that anticancer activity may occur through the induction of apoptosis pathways [77, 78]. Cytotoxicity of the solvent extracts from the roots of *L. officinale* Koch were to be tested on different cancerous and normal cell lines and crude extracts exhibiting anticancer activity selected for further characterisation using thin layer chromatography (TLC) and purification using column chromatography. Identification of compounds in fractions is possible through Nuclear Magnetic Resonance (NMR) spectroscopy and anticancer activity through apoptosis detected using a fragmentation assay using different human cancer cell lines. Therefore, the experimental objectives were to:

1) Prepare crude solvent extracts using four solvents in increasing order of polarity; n-hexane, ethyl acetate, methanol and methanol/water (50:50).

2) Determine the cytotoxicity of the crude solvent extracts on human cancer cell lines (THP-1 and MCF7) in comparison with PNT2A (considered as a normal cell line).

3) Fractionate crude extracts by chromatography (column and vacuum liquid).

4) Re-test cytotoxicity of the isolated compounds.

5) Perform NMR to identify the active compounds in fractions with anti-proliferation activity.

6) Investigate the cause of cell death using a DNA ladder assay.

Chapter two: Materials and methods

2.1 List of materials and chemicals

■ 0.22 μm filter (Millipore, UK)

■ 96-well plates (TPP, Switzerland)

■ 75 cm^2 cell culture flasks (Corning Incorporated, USA)

■ Accutase (PAA laboratories, Austria)

■ Anisaldehyde (FSA laboratory, UK)

■ Apoptotic DNA Ladder Detection Kit (APT151, Chemicon International, USA)

■ Avance DRX500 MHz NMR (Bruker, UK)

■ Chloroform-d (Sigma Aldrich, UK)

■ Dimethyl sulphoxide (Sigma Aldrich, UK)

■ Ethyl acetate (Sigma Aldrich, UK)

■ Foetal calf serum (Sigma, UK)

■ Freeze drier (Edwards Modulyo, UK)

■ L-glutamine (Sigma, UK)

■ MCF7 (European Collection Animal Cell Culture, UK)

■ Methanol (Sigma, UK)

■ Microcentrifuge (Centaur, SANYO, Japan)

■ Microscope (Olympus, Japan)

■ *n*-Hexane (Sigma Aldrich, UK)

■ Neubauer-Improved Haemocytometer (Marienfeld, Germany)

■ NMR tubes (Sigma Aldrich, UK)

■ Resazurin sodium salt (Sigma Aldrich, Germany)

■ TLC silica gel 60 F254 aluminium sheets (Merck, Germany)

■ Rotary evaporator (Büchi, Switzerland)

■ RPMI 1640 medium (Sigma, UK)

■ PNT2A (European Collection Animal Cell Culture, UK)

■ Safety Cabinet (Walker Safety Cabinets Ltd, UK)

■ Silica gel 60H for thin layer chromatography (Merck, Germany)

■ Sintered glass Buchner filter funnel (Schott Duran, Germany)

■ Soxhlet apparatus (Quickfit, UK)

■ SpectraMax M5 Microplate Reader (Molecular Devices Corporation, USA)

■ Streptomycin/ Penicillin (Cambrex, UK)

■ THP-1 cells (European Collection Animal Cell Culture, UK)

■ UV-detector 254 nm and 364 nm UVGL-58 (UVP, USA)

2.2 Solvent extraction of Lovage root

Lovage plant material that had been harvested in August 2009 from Dr Ferro's garden, in Shotts Lanarkshire, was air-dried and the roots were separated, cut into small pieces and stored for about 8 months. The stored root pieces were ground using a grinder. About 450 g of root powder was added to a filter paper sleeve. This was topped with cotton wool and inserted into a glass Soxhlet apparatus, which was attached to a condenser and a pre-weighed round-bottomed flask which contained solvent. Solvent extraction was carried out starting with the least to more polar solvents (*n*-hexane, ethyl acetate, methanol, and methanol/water). The apparatus was placed on an electromantle, at medium heat (60-80°C) and left to extract for 8–10 h or until the solvent ran clear (about 20-30 cycles) [79-82]. The extracts were evaporated to dryness at 40˚C under vacuum using a rotary evaporator. The methanol/water (50:50) extract was further lyophilised in a freeze drier overnight. The flasks were re-weighed, allowing determination of the yield of the extract and the extracts were stored at -20˚C until assessed for further purification or used in cytotoxicity assays.

2.3 Column chromatography (CC)

The hexane extract (20 g) was dissolved in 60 ml n-hexane, adsorbed onto 50 g silica gel and dried. The silica gel was packed into a 55×3 cm glass column and fractions eluted beginning with 100% n-hexane and a gradient change to reach 100% ethyl acetate (EtOAc) then the gradient was changed to 100% methanol (MeOH) as described in Table 3. Finally, the column was washed with methanol [83]. Fractions were collected in numbered 20 ml glass bottles and analysed by thin layer

chromatography (TLC). Fractions with similar TLC profiles were grouped together and further analysed by NMR.

2.4 Separation of compounds by thin layer chromatography (TLC)

After fractionation, collected fractions were analysed by TLC in order to combine similar fractions. TLC was carried out on normal phase silica gel coated aluminum sheet plates [84]. Briefly: each sample was spotted onto the plate, 1 cm up (baseline). The spots were air-dried and the plate placed into a developing tank, containing the mobile phase until the solvent had reached 1 cm from the top of the plate (solvent front). The mobile phase consisted of ethyl acetate:*n*-hexane (0:1, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, 1:0), ethyl acetate: ethanol (7:3), 100% methanol. To facilitate mobile phase migration, blotting paper (Whatman 3mm) was allowed to soak and equilibrate for 20 to 30 minutes previous to TLC runs. The plate was left to air dry, then the spots were visualised under UV light at 254nm and 366nm; visible bands were marked using a pencil. Finally, the plate was sprayed with anisaldehyde-H_2SO_4 spray (5 ml sulphuric acid, 85 ml methanol, 10 ml glacial acetic acid and 0.5 ml anisaldehyde) and heated for one minute. The R*f* values for each band were calculated by dividing the distance the band travelled, by the distance of the solvent front. On this basis, fractions containing similar compounds could be pooled, freeze-dried and further analysed by NMR to help determine the structure of the compounds. Several solvents used were assessed for their ability to fractionate the chemical constituents in the starting material.

2.5 Medium pressure liquid chromatography (MPLC) separation

Fractionation of the ethyl acetate extract was performed using VersaFlash™ from Supelco, Sigma-Aldrich (Buchs, Switzerland) column VersaPak™ (silica, spherical 23 × 110 mm, 23 g), connected to a pump manager (Buchs, Switzerland). The separation was carried out as described by Ishahara *et al.* [85]. Briefly: five grams of crude extract dissolved in ethyl acetate was mixed with sorbent silica granules. This was then left in a fume cupboard until the granules had absorbed the plant extract and the solvent evaporated. Elution was carried out using different solvent sequences as shown in Table 4, through the column flow rate at 100 ml/m and pressure 1.6bar in 2 solvent systems. Fractions were collected in 40 ml conical flasks and combined into groups according to TLC profile before being pooled together.

Table 3: Solvent system used for CC of the crude solvent extracts (n-hexane, ethyl acetate, methanol, and methanol /water).

CC solvent system
Hexane 100%
Hexane: EtOAc 90:10%
Hexane: EtOAc 80:20%
Hexane: EtOAc 70:30%
Hexane: EtOAc 50:50%
EtOAc 100%
EtOAc: MeOH 70:20%
EtOAc: MeOH 50:50%

Table 4: Solvent systems used for MPLC fractionation of the crude solvent extracts

Solvent system I: A = *n*-hexane, B = EtOAc, Flow Rate: 100 ml/m, Pressure:1.6 bar		
Time	**%A**	**%B**
0	98	2
5	98	2
25	0	100
30	0	100
Solvent system II: A = EtOAc, B = MeOH, Flow Rate: 100 ml/m, Pressure:1.6 bar		
0	100	0
5	100	0
15	20	80

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2.6 Identification of compounds by nuclear magnetic resonance (NMR) spectroscopy

NMR is a useful technique that helps to elucidate chemical structures of compounds. The samples were dissolved in deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-d_6) depending on the solubility of the compounds and placed in NMR tubes (5 mm x 178 mm). One dimensional spectroscopy (1D-NMR) included ^{1}H for determination of the kinds of protons in the compounds, while ^{13}C provided data on the number and kinds of carbon atoms in the compounds. On the other hand, 2D-NMR included ^{1}H-^{1}H correlation spectroscopy (COSY) that helped to identify compounds that are more complicated, while heteronuclear multiple quantum coherence (HMQC) was used to identify the correlation between protons and carbons atoms in samples through the ^{1}J coupling between them. Heteronuclear multiple bond connectivity (HMBC) provided the correlation between the chemical shift of the protons in the samples and the heteronucleus ^{13}C through ^{2}J and ^{3}J coupling interaction between the nuclei. The NMR spectra and data were obtained on a Jeol Eclipse 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C and HMQC and HMBC. ChemBioDraw Ultra, Version 12.0, was used to draw compound structures, and also to predict ^{1}H-NMR spectrum.

2.7 Liquid Chromatography-Mass Spectrometry (LC-MS)

This technique is associated with high-performance liquid chromatography (HPLC) and the detection was carried out with mass spectrometry (MS). In addition, HPLC is used to remove interferences from the sample. In order to help in confirmation of the identity of hexane and ethyl acetate obtained compounds, LC/MS was performed

using an Exactive HRMS mass spectrometer coupled to an Ultimate 3000 nano LC pump. Samples were dissolved in methanol (HPLC grade) to obtain 1mg/ml solution in small glass bottle. The mobile phases A and B for separation at LC/MS consisted of 0.1% (v/v) formic acid in water and in water/acetonitrile, respectively. The gradient was 5% B (0–5 min), 5–40% B (5–30 min), 40–100% B (30–35 min), 100% B (35–36 min), and 5% B (36–40 min) at a flow-rate of 300 nL/min.

2.8 Tissue culture and crude extract / isolated fractions cytotoxicity assessment

Three human cell lines were used in this assessment: THP-1 cells (leukaemic, monocytic cell line), MCF7 cells (breast cancer cell line) and PNT2A cells (normal prostate epithelium cell line). The PNT2A and MCF7 cell lines were kindly provided by Mrs Louise Young, SIPBS, University of Strathclyde. These cell lines were widely used previously in proliferation and cytotoxicity assays in our department. All procedures related to its growth and maintaining are well established [86]. The cytotoxicity was determined using resazurin, which is a metabolic indicator dye that turns from blue to pink in the presence of viable, metabolising cells. The assessment is based on cytotoxicity of the plant extracts that were tested on the cancer cell lines. This is an important preliminary assay because, if the plant extracts are cytotoxic to the cells, it may indicate anticancer activity. The plant extracts were also tested on normal cells as a control. If the extract is toxic to the controls, it would be difficult to say whether the extract has anticancer effects or is generally cytotoxic.

2.8.1 Preparation of culture medium

All procedures were carried out in a sterile environment. Complete medium was prepared by adding appropriate amounts of supplements (Table 5) and kept at 4ºC until used. Cells were grown in an incubator at 37°C, 100% humidity and 5% CO_2 and sub-cultured every 3 days to keep the cells in log phase of growth.

Table 5: Growth media and supplements used for each cell line.

Cell Line	Growth Media	Supplements
THP-1	RPMI 1640	10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 1% (v/v) L-glutamate
PNT2A		
MCF7	DMEM	10% (v/v) FCS, 1% (v/v/) fungizone, 1% (v/v) penicillin/streptomycin and 1% (v/v) L-glutamate

2.8.2 Preparation of the crude extract for cytotoxicity assay

The plant extracts were made up as required for bioassays, using DMSO to dissolve the n-hexane and the chloroform extracts and medium for the methanol extract. For the crude extracts, 40 mg was dissolved in 0.5 ml DMSO and diluted 1:20 with complete medium to give 2 mg/ml of plant extract in 10% (v/v) DMSO. All the plant extracts were filtered using a 0.22 μm filter unit prior to use.

2.8.3 THP-1 cytotoxicity assay

A 75 cm^2 flask of THP-1 cells in log phase of growth was used, containing 50 ml complete medium and placed in an incubator. Cells were sub-cultured every 3 days, 40ml from the flask was discarded (or used in bioassays) and replaced with 40 ml complete medium. The cell numbers were counted by placing 10 µl of cell suspension into both chambers of a Neubauer-Improved haemocytometer. The number needed for the bioassays was $1x10^6$ cells/ml. If the cell number was too high, they were diluted with complete medium and if too low, they were spun down in a centrifuge for 5 minutes at 700xg, and the cell pellet resuspended in an appropriate volume of medium. The cells were then counted again to ensure they were at the correct value required for the assay. The cytotoxicity of the crude extracts or isolated compounds was determined using 0.01% (w/v) resazurin solution (5 mg in 50 ml deionised H_2O). To prepare the stock solution for the assay the following was carried out; for the crude extracts, 40 mg was dissolved in 0.5 ml DMSO and diluted 1:20 with complete medium to give 2 mg/ml of plant extract in 10% (v/v) DMSO. For freeze-dried fractions, 10 mg was dissolved in 0.5 ml DMSO and diluted 1:5 with complete medium to give 2 mg/ml of plant extract in 10% (v/v) DMSO.

To perform the cytotoxicity assay, 200 µl of prepared stock was added to the first wells of rows A-C (test cells) and G of a 96 well tissue culture plate; row G acted as a background control for coloured material. Two hundred microliter DMSO was added to the first wells of rows D and E (solvent control) and 100 µl of medium added to all the remaining wells except wells H. A 1:2 serial dilution was carried out across the plate by transferring 100 µl from one column to the next and the last 100 µl

discarded. Next, 100 µl of cells ($1x10^6$ cells/ml suspended in complete medium containing 10% (v/v) resazurin solution) was added to all the wells except row G. To row F (cells only), which acted as a positive control (cell growth), 100 µl of medium plus 10% (v/v) resazurin solution was added to each well. The medium in row H was carefully aspirated leaving behind cells, and 200 µl of sterile water plus 10% (v/v) resazurin solution was added which acted as a negative (cell death) control. The plate was then placed in an incubator overnight at 37°C, 5% CO_2 and 100% humidity. The next day, 100 µl supernatant from each well was carefully transferred to a new 96 well plate and read on a SpectraMax M5 microplate reader at 570 nm and 600 nm, to provide Day 1 readings. Any remaining medium in the wells of the original plate was carefully aspirated and replaced with 100 µl of fresh medium plus 10% (v/v) resazurin solution. The plate was then returned to the incubator overnight and was read the next day (providing Day 2 readings). The Day 2 assessment, in the absence of test agent, provided an indication of whether the cells would recover and grow. The cytotoxic effect of the treatment was determined as percentage of viability and compared to untreated cells [87]. The toxicity of compounds was determined by means of the formula:

$$\boldsymbol{Cell\ viability}(\%) = \frac{\boldsymbol{Absorbanceofsamplescells}}{\boldsymbol{Absornacesofuntreatedcells}} \times \mathbf{100}$$

2.8.4 PNT2A and MCF7 cell lines cytotoxicity assay

Both PNT2A and MCF7 cell lines adhere to the surface of the culturing flasks. Therefore, the following protocol was used to harvest the cells. Briefly, a 75 cm^2 flask of cells (75% confluence) was used for this procedure. The medium was decanted into a container of Virkon disinfectant and cells in the culture flask were treated with 5ml Accutase for 5 min at 37°C, to remove adherent cells from the surface. The cell number was counted; the number of cells needed to maintain the cells was $6x10^5$ cells/flask, while the number of cells required for the cytotoxicity bioassay was $1x10^5$ cells/ml per plate (i.e. $1x10^4$ cells in 100 µl/well).

Stock solutions of the crude extracts and isolated fractions were prepared as mentioned above. Cells (100 µl/well) were added to all rows except F and G and left for 24 hours at 37°C, 100% humidity and 5% CO_2. The following day, the medium was removed and 200 µl of stock solution was added to the first wells of rows A-D (each extract in duplicate) and 200 µl/well DMSO was added to the first well of row E (solvent control). Rows F and G contained 200 µl/well of each stock solution as the background control. Wells from column 1 to 6 in row H (cells only) acted as the positive control; and 100 µl/well of medium was added to all the remaining wells except wells 7 to 12 in rows H. A 1:2 serial dilution was carried out across the plate. After that 100 µl of complete medium containing 10% (v/v) resazurin solution was added to all rows except the wells from 7 to 12 in row H. Water plus 10% (v/v) resazurin solution (200 µl/well) was added to wells 7 to 12 in H row which acted as the negative control. Finally the plate was placed in an incubator overnight at 37°C, 5% CO_2 and 100% humidity. The readings and results were carried out as for THP-1 cells (section 2.7.3).

2.8.5 Freezing cells

Cells in log-phase of growth in a 75 cm^2 flask were transferred into 25ml universal tubes and centrifuged for 5 min at 1300xg. After removing the supernatant, the pellet was re-suspended in 5ml of complete medium and the cells counted. The cells were then centrifuged and the supernatant discarded. Freezing medium (10 ml complete medium, 10 ml FCS, 2 ml DMSO) was added to give 1×10^6 cells/ml and 1ml aliquoted into cryovials. The cells were then rapidly frozen in a -80°C freezer. Before long-term storage of the cryovials in liquid nitrogen, the cell viability was checked after one week, one vial was quickly defrosted and the contents added rapidly to 20 ml complete medium. The cells were centrifuged and the cell pellet re-suspended in 5 ml complete medium and transferred to a 25 cm^2 cell flask and incubated as mentioned above. Cell growth and replication was observed for one week with regular refreshing of growth media. The remaining cryovials were placed in liquid nitrogen storage containers after passing the cell viability check.

2.9 Detection of apoptosis using a DNA ladder assay.

For detection of the pathway involved in cell death caused by the Lovage extracts and isolated fractions, an apoptotic DNA ladder detection kit was used, and the protocol was performed according to the manufacturer's instructions [88]. Briefly, cells at 1×10^6 cells/well were incubated with 100 µl of 1 mg/ml of the stock solution, overnight at 37°C, 5% CO_2 and 100% humidity. The cells were transferred to 1.5 ml microcentrifuge tubes and spun at 500xg. Cells were washed with PBS (10 mM phosphate buffer, 137 mM sodium chloride, 2.7 mM potassium chloride) and the cells pelleted by centrifugation for 5min at 500xg. The supernatant was carefully

removed and the cells were lysed with 40 µl Tris-EDTA lysis buffer by gentle pipetting followed by addition of 5 µl of enzyme A (RNAse A) solution into crude lysates, gently mixed by pipetting and incubated at 37ºC for 10 min. A 5 µl of enzyme B (Proteinase K) solution was added into each tube, mixed gently by pipetting and incubate at 50ºC for 30 min until the lysates became clear. To the lysate 5 µl of ammonium acetate solution was added and mixed well. Then 50 µl of isopropanol was added, mixed well, and kept at -20ºC for at least 10min. The samples were centrifuged for 10min at full speed (16,000xg) to precipitate the DNA. The supernatant was removed carefully and the DNA pellet washed with 0.5 mL of ice cold 70% ethanol, and spun down. The pellet was air dried for 10 min at room temperature and then solubilised in 30 µl DNA suspension buffer (provided in the kit) and stored at -20ºC until used in gel electrophoresis. A total of 20 µl of the DNA product was loaded onto a 1% Tris acetate agarose gel containing 0.5 µg/ml ethidium bromide and electrophoresed at 5 V/cm for 1h. The gel was visualised by UV light and photographed.

Chapter Three: Results

3.1 Phytochemical screening

3.1.1 Solvent extraction and yield

The extraction of 450 g of Lovage root powder with different solvents (n-hexane, ethyl acetate, methanol and methanol/water) was carried out to obtain crude extracts. The extracts were dried by rotary evaporation under reduced pressure. The final yield of each extract was calculated as shown in Table 6. The highest yield (11.6%) was observed with the methanol extract, followed by the n-hexane and methanol/water extract (5.5% and 4.8%, respectively). The yield of the ethyl acetate extract was the lowest at (2.7%).

Table 6: Amount and yield percentage obtained from solvent extraction of Lovage root powder.

Extract	**Weight (g)**	**Yield (%)**	**Observation**
***n*-Hexane**	24.86	5.5	Oily light brown
Ethyl acetate	12.31	2.7	Oily brown
Methanol	52.32	11.6	Oily blackish brown
Methanol/water	21.47	4.8	Sticky blackish brown

3.1.2 Pooling of fractions separated by CC or MPLC, based on TLC profiles

Fractions collected from CC or MPLC were profiled by TLC and according to the similarity of the bands on the plates and the calculated R*f* values, fractions were combined. Figure 5 shows examples of TLC plates of the n-hexane fractions (Figure 4A) and ethyl acetate fractions (Figure 4B). Fractions with similar profiles were pooled together before NMR analysis. Table 7 list the pooled fractions according to TLC visualisation.

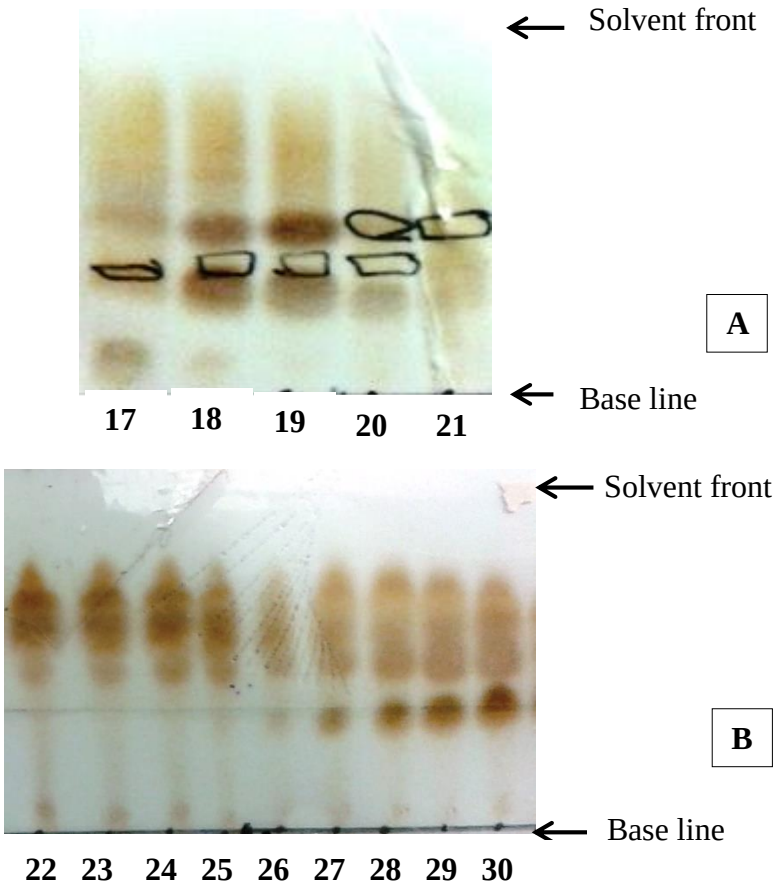


Figure 4: TLC plates for (A) ethyl acetate fractions 17-21 and (B) n-hexane fractions 22-30. Fractions with similar Rf values and band profiles were pooled together.

Table 7: List of fractions with similar Rf values and band profiles were pooled together prior to NMR. Fractions that showed aromatic compounds (in bold) were selected for further assays.

Solvent	**N-hexane**	**Ethyl acetate**
	1-12	3-5
	13-25	6-8
	26-36	9-12
	37-42	13-14
	34-44	15-17
	45-48	**18-19**
	49-60	20
	61-66	21
	67-73	22-23
	74-80	24-26
		27
		28-32
		33-38
		39-41
		42-45
		46-48
		49-50
		51
		52
		53-58

3.1.3 Chemical structure prediction of isolated fractions.

Isolated compounds were analysed by NMR spectroscopy to identify their chemical structures. The ^{1}H and ^{13}C NMR spectra of the isolated compounds were analysed by MestReNova v9 (Mestrlab Research S.L. 2014) and compared with estimated and predicted structures for compound identification.

3.1.3.1 Characterisation of LoE18-19 as 3-methoxy-4-hydroxycinnamic acid (ferulic acid) and a minor related compound.

LoE18-19 was isolated from the ethyl acetate crude extract of Lovage root using MPLC. On TLC, the fraction appeared as a purple spot after development with anisaldehyde sulphuric acid reagent and heating (Figure 5). The ^{1}H NMR (400 MHz) showed that there was a major compound with a small amount of impurity from a related compound: δ 3.80 (s, 3H, OMe), δ 6.35 (d, 1H, *J*=15.9 Hz, H-2), δ 6.80 (d, 1H, *J*=8.4 Hz, H-5′), δ 7.06 (dd, 1H, *J*=8.4,2.0 Hz, H-6′), δ 7.26 (d, 1H, *J*=2.0 Hz, H-2′), δ 7.50 (d, 1H, *J*=15.9 Hz, H-3), δ 9.58 (brs, 1H, 4′-OH), δ 12.17 (brs, 1H, 1-COOH) ppm (Figure 7). According to ^{1}H NMR data and a comparison with those given in the literature [89, 90], the structure of LoE18-19 was tentatively identified as 3-methoxy-4-hydroxy cinnamic acid (Figure 6) with the chemical folrmula: $C_{10}H_{10}O_4$ and molecular weight: 194. The major compound was detected in LC-MS (ESI, negative mode) sample at RT: 12.33 mins with mass value: 193.05 $[M-H]^-$ therefore, (M=194, $C_{10}H_{10}O_4$) The minor compound could be an isomer of ferulic acid, 3-hydroxy-4-methoxycinnamic acid, but this is only a tentative identification.

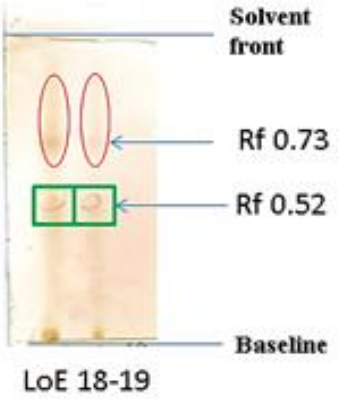


Figure 5: Image of TLC silica plate for pooled LoE18-19 fractions. The mobile phase used (n-hexane: EtOAc / 80:20).

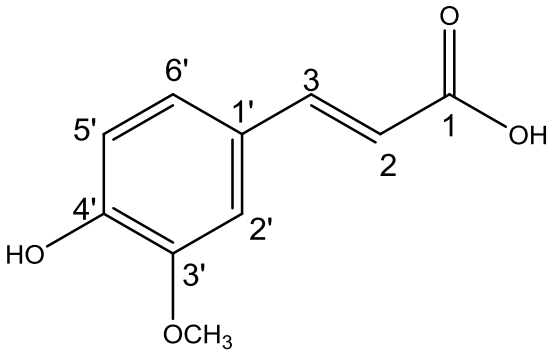


Figure 6: Chemical structure of 3-methoxy-4-hydroxycinnamic acid (ferulic acid).

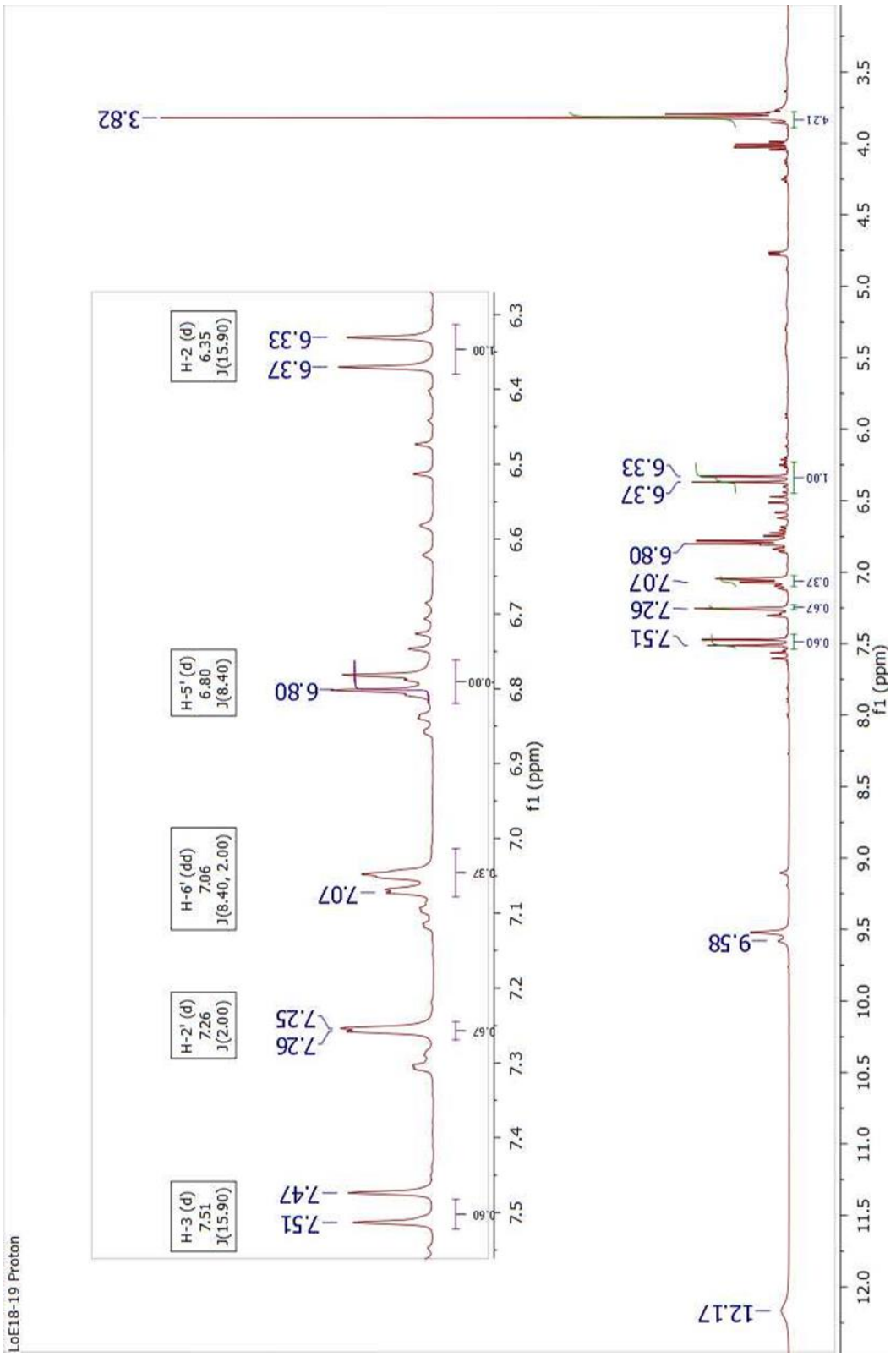


Figure 7: ^{1}H NMR spectrum of LoE18-19 processed by MestReNova software.

3.1.3.2 Characterisation of LoH26-36 as 1,2,3-propanetriol trilinoleate (trilinolein) mixed with a steroid ester.

Fractions LoH26-36 isolated from the n-hexane crude extract of Lovage root using CC. On TLC, the fraction appeared as a brownish spot after development with anisaldehyde sulphuric acid reagent and heating (Figure 8). The ^{1}H NMR (400 MHz): δ 0.72 - 1.00 (m, 9H, 18-CH_3x3), δ 1.2 - 1.23 (m, 42H), δ 1.4 - 1.6 (m, 6H), δ 1.9 - 2.1 (m, 12H), δ 2.16 - 2.24 (m, 6H, H-2x3), δ 2.65 - 2.8 (m, 6H, H-11x3), δ 4.05 - 4.30 (m, 4H, H-1/3 of glyceryl), δ 5.2 - 5.3 (m, 1H, H-2 of glyceryl), δ 5.3 – 5.4 (m. 12H, olefinics) ppm (Figure 10). The COSY spectrum is shown in Figure 11. The ^{13}C NMR (Figure 12) showed it to be a mixture of trilinolein and a steroid ester. According to ^{1}H NMR data and ^{13}C NMR with comparison with those given in the literature, the probable structure of LoH26-36 was tentatively identified as trilinolein with the chemical formula: $C_{57}H_{98}O_6$ and molecular weight: 878 (Figure 9). The major compound was detected in LC-MS (ESI, negative mode) sample with mass value: 879.38 $[M+H]^+$, therefore M=878. The steroid ester was not identified.

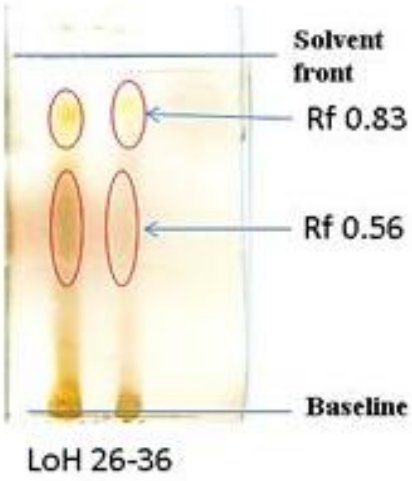


Figure 8: Image of TLC silica plate for pooled LoH 26-36 fractions. The mobile phase used (n-Hexane: EtOAc / 80:20).

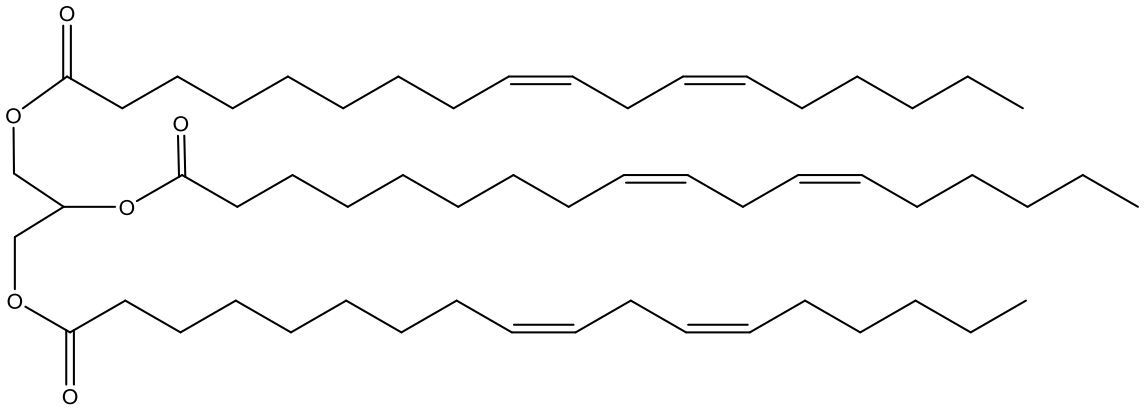


Figure 9: Chemical structure of 1,2,3-propanetriol trilinoleate (trilinolein).

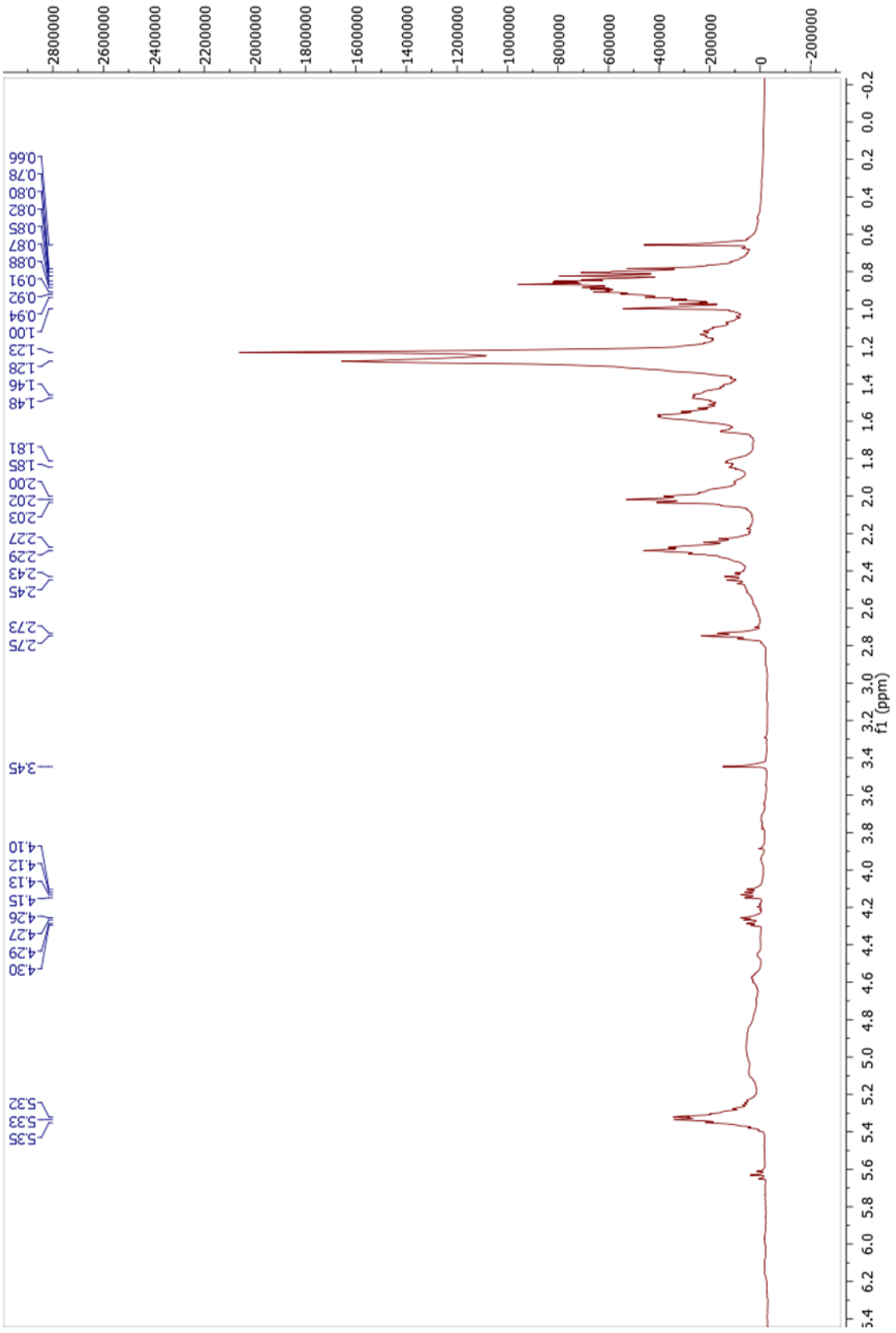


Figure 10: ^{1}H NMR spectrum of LoH26-36 processed by MestReNova software.

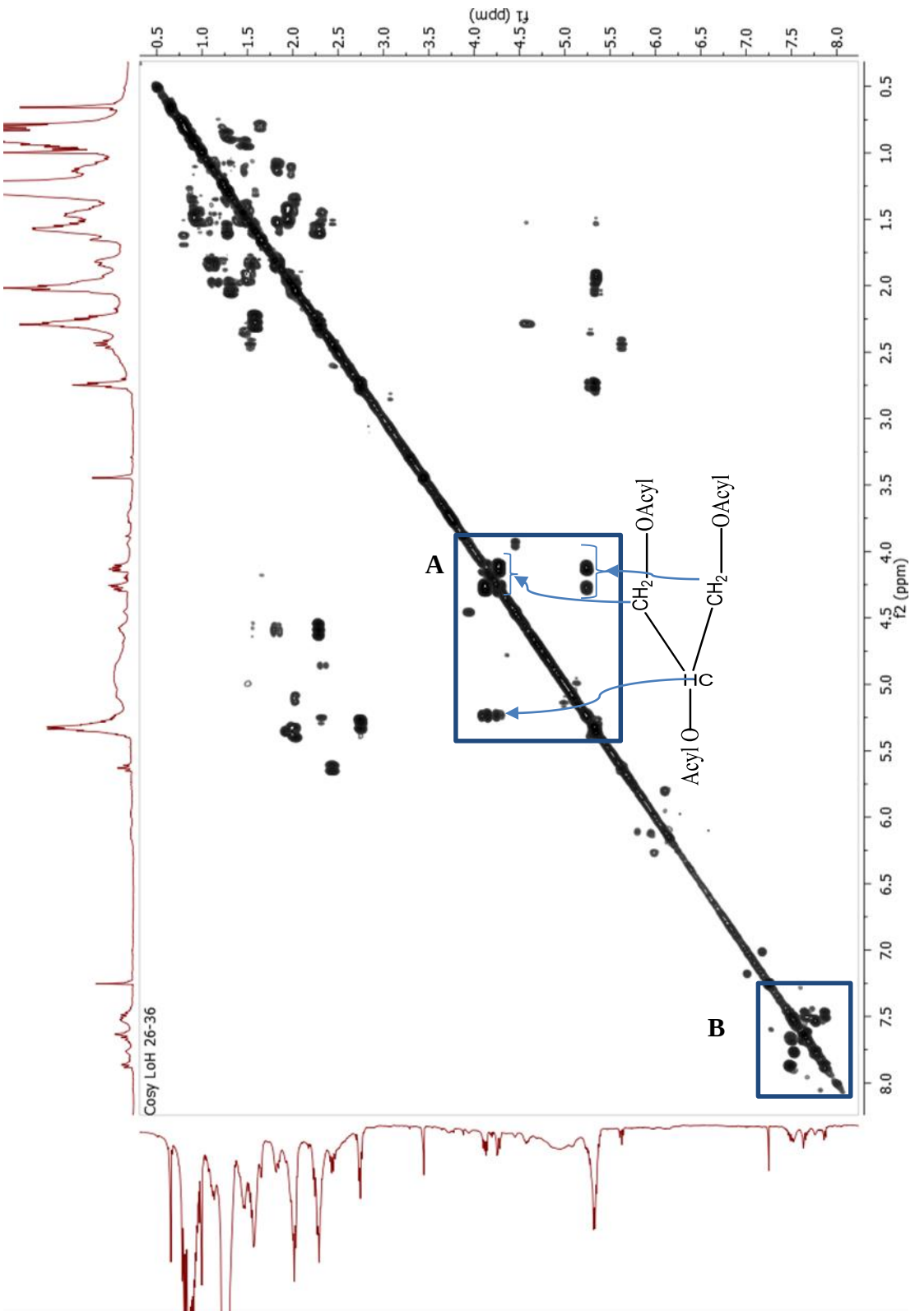


Figure 11: The COSY spectrum of LoH26-36 processed by MestReNova software.

A= region of glyceryl (acylated) protons shows CH_2 and acyl groups deshielded due to having the same J values. B= aromatic region.

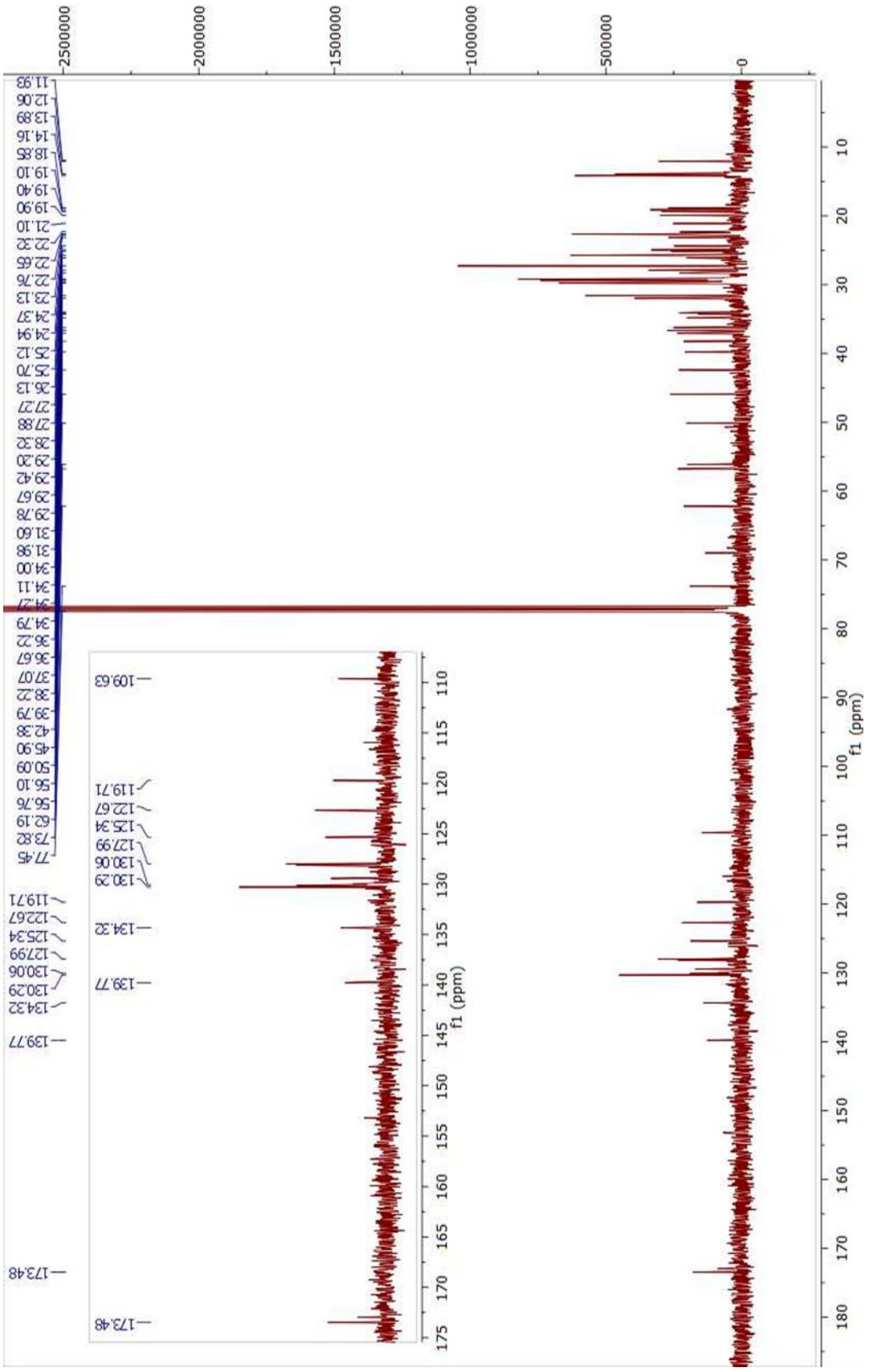


Figure 12: ^{13}C NMR spectrum of LoH26-36 processed by MestReNova software.

3.1.3.3 Characterisation of LoH49-60 as 1,9-heptadecadien-4,6-diyne-3,8-diol (falcarindiol).

Fractions LoH49-60 isolated from the n-hexane crude extract of Lovage root using CC. On TLC, the fraction appeared as a brownish dark spot after development with anisaldehyde sulphuric acid reagent and heating (Figure 13). The ^{1}H NMR (400 MHz, Chloroform-*d*) (Figure 15) δ 0.85 (t, 3H, H-17), 1.20 - 1.30 (m, 8H, H-13-16), 1.35 (m, 3H, H-12), 2.05 (m, 2H, H-11), 4.90 (brd, 1H, H-3), 5.16 (d, 1H, H-8), 5.20 (brd, 1H, H-1), 5.41 (brd, 1H), 5.48 (m, 1H, H-9), 5.51 (m, 1H, H10), 5.89 (ddd, 1H, H-2) ppm. The COSY spectrum is shown in Figure 16. The ^{13}C NMR (101 MHz, Chloroform-*d*) d 14.2 (C-17), 22.7 (C-16), 27.7 (C-11), 29.2 (C-13), 29.3 (C-12), 29.4 (C-14), 31.9 (C-15), 58.5 (C-8), 63.4 (C-3), 68.7 (C-5), 70.2 (C-10), 78.4 (C-4), 79.9 (C-7), 117.2 (C-1), 127.8 (C-9), 134.4 (C-10), 136.0 (C-2) ppm (Figure 17). The DEPT-135 (Figure 18), HMBC (Figure 19) and HMQC (Figure 20) show more details of the compound. According to ^{1}H NMR data and ^{13}C NMR with comparison with those given in the literature [91], the probable structure of LoH49-60 was tentatively identified as 1,9-heptadecadiene-4,6-diyne-3,8-diol (falcarindiol) with the chemical formula: $C_{17}H_{24}O_2$ and molecular weight: 260.37 (Figure 14).

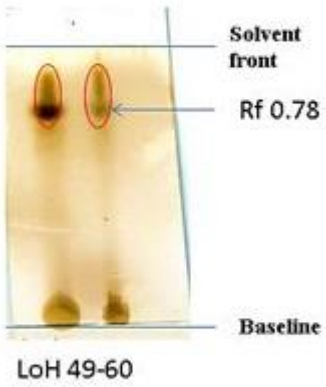


Figure 13: Image of TLC silica plate for pooled LoH 49-69 fractions. The mobile phase used (n-Hexane: EtOAc / 80:20).

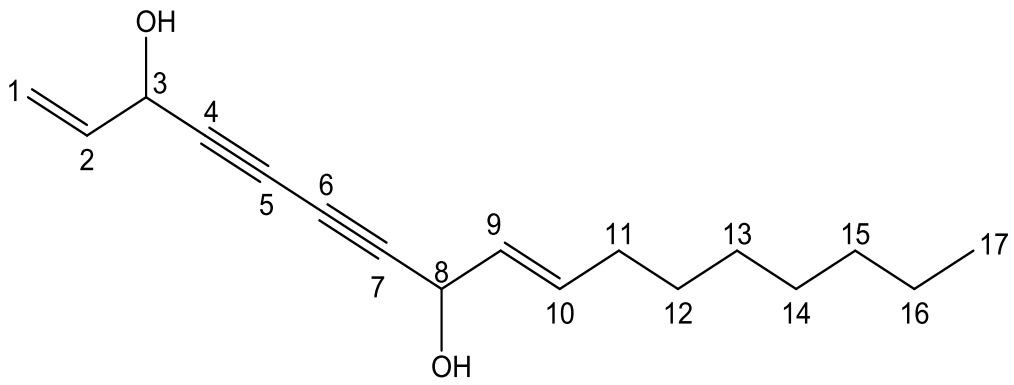


Figure 14: Chemical structure of 1,9-heptadecadiene-4,6-diyne-3,8-diol (falcarindiol).

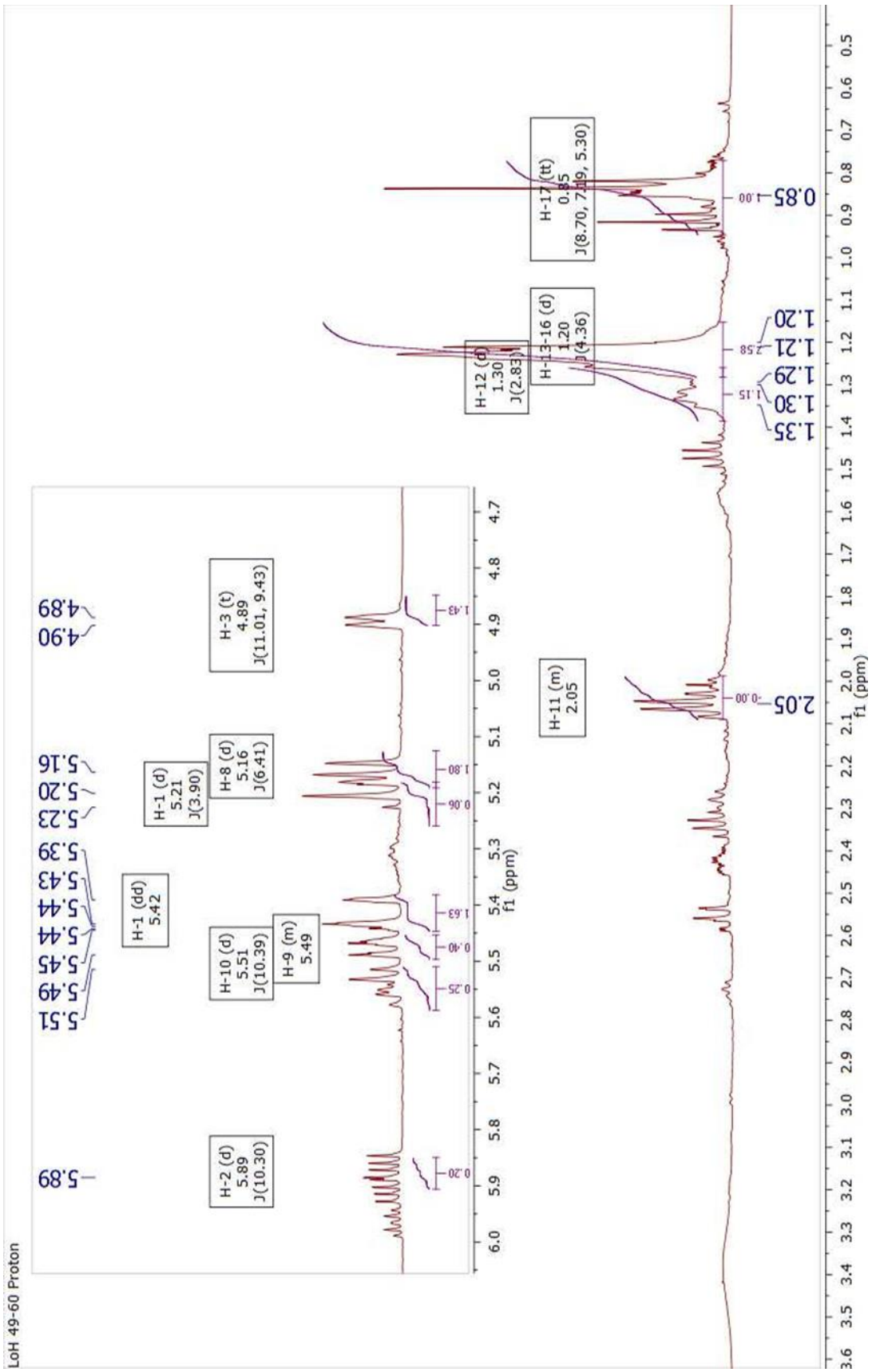


Figure 15: ^{1}H NMR spectrum of LoH49-60 processed by MestReNova software.

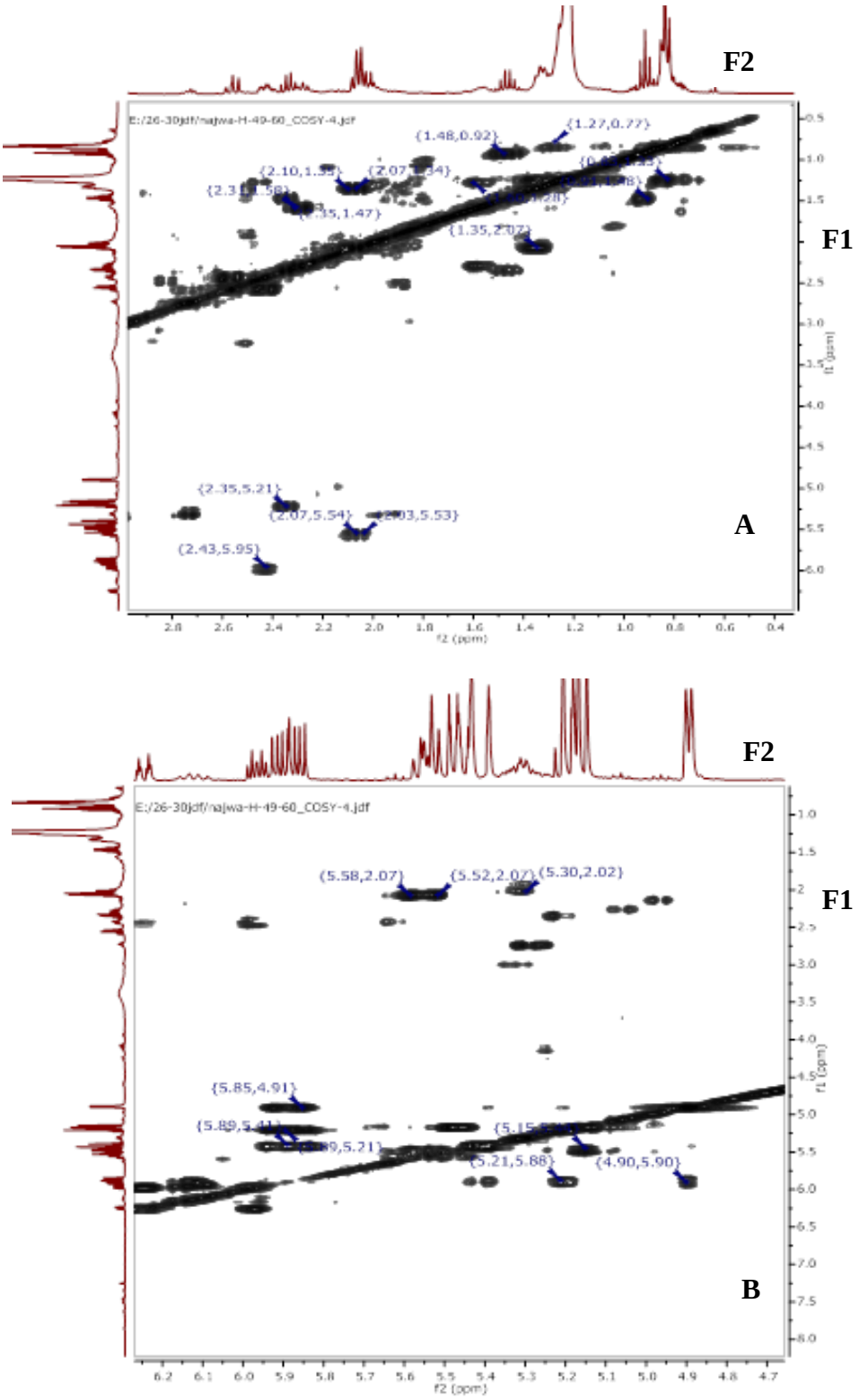


Figure 16: The COSY spectrum of LoH49-60 showing the aliphatic [A,(F2)] proton correlations and the olefinic/alcohol [B, (F2)] correlations [processed by MestReNova software].

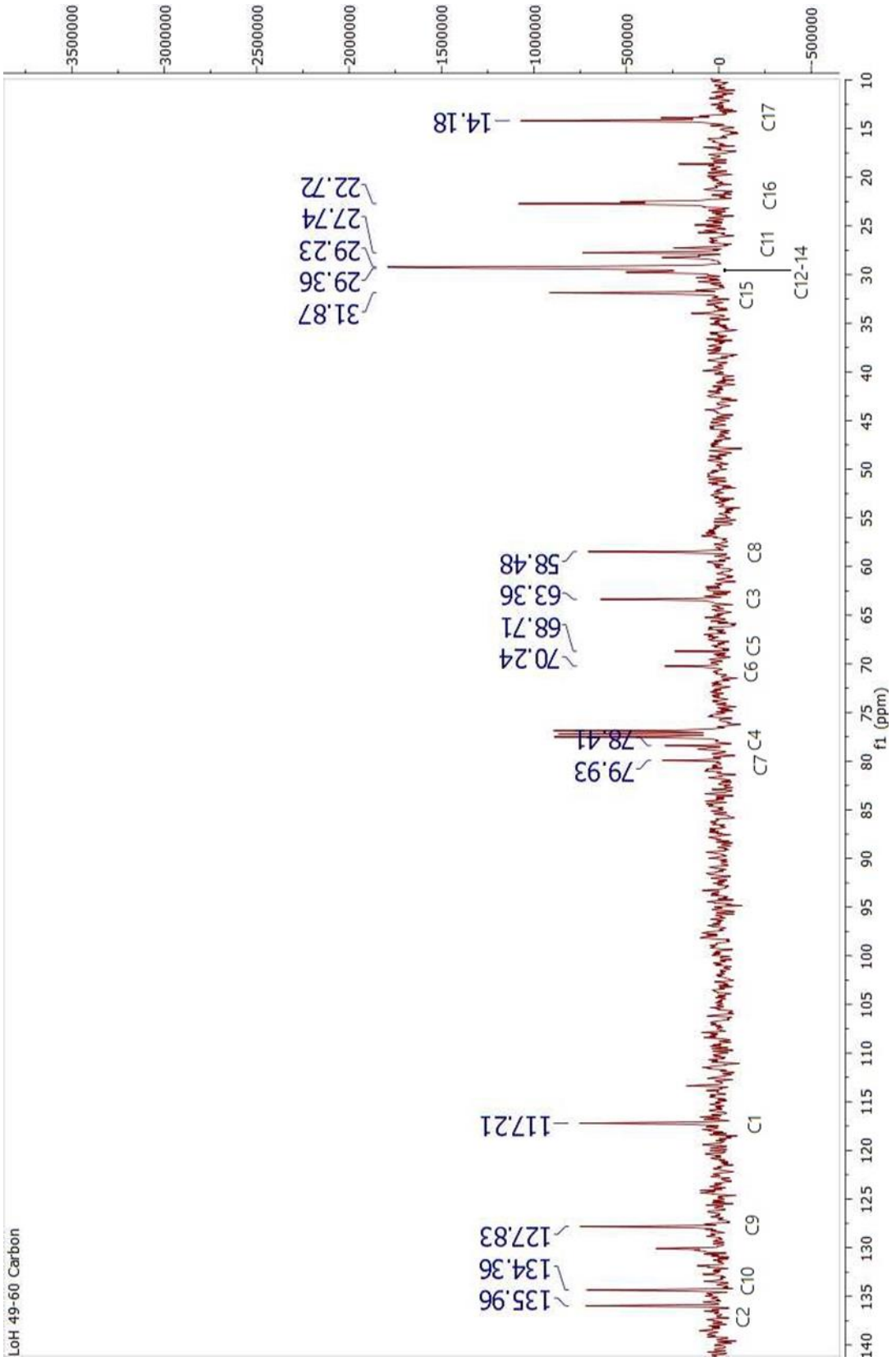


Figure 17: ^{13}C NMR spectrum of LoH49-60 processed by MestReNova software.

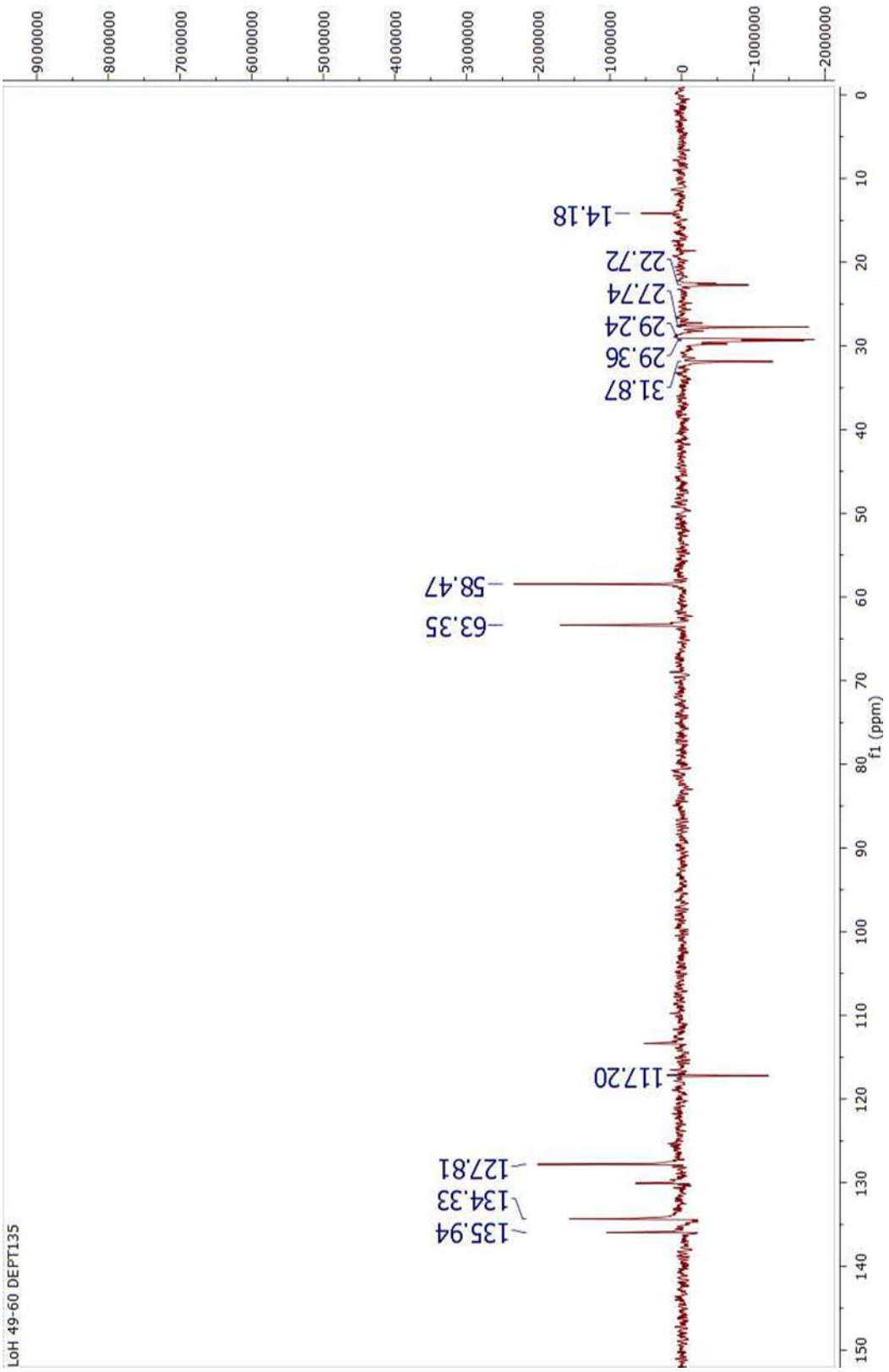


Figure 18: The DEPT135 NMR spectrum of LoH49-60 processed by MestReNova software.

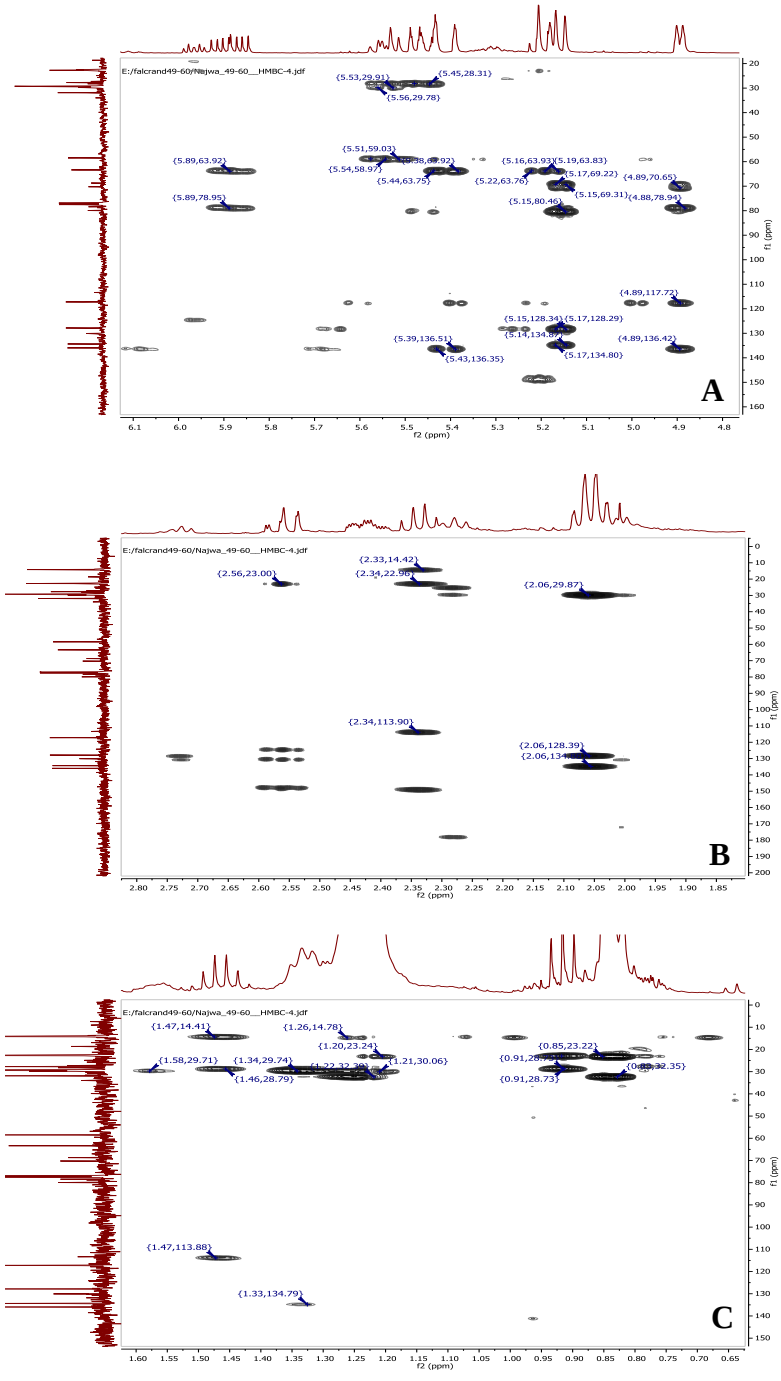


Figure 19: The HMBC spectrum of LoH49-60 processed by MestReNova software, (A): the olefinic/alcohol proton regions, (B): the allylic proton region and (C): the aliphatic proton region all with the full carbon spectral regions.

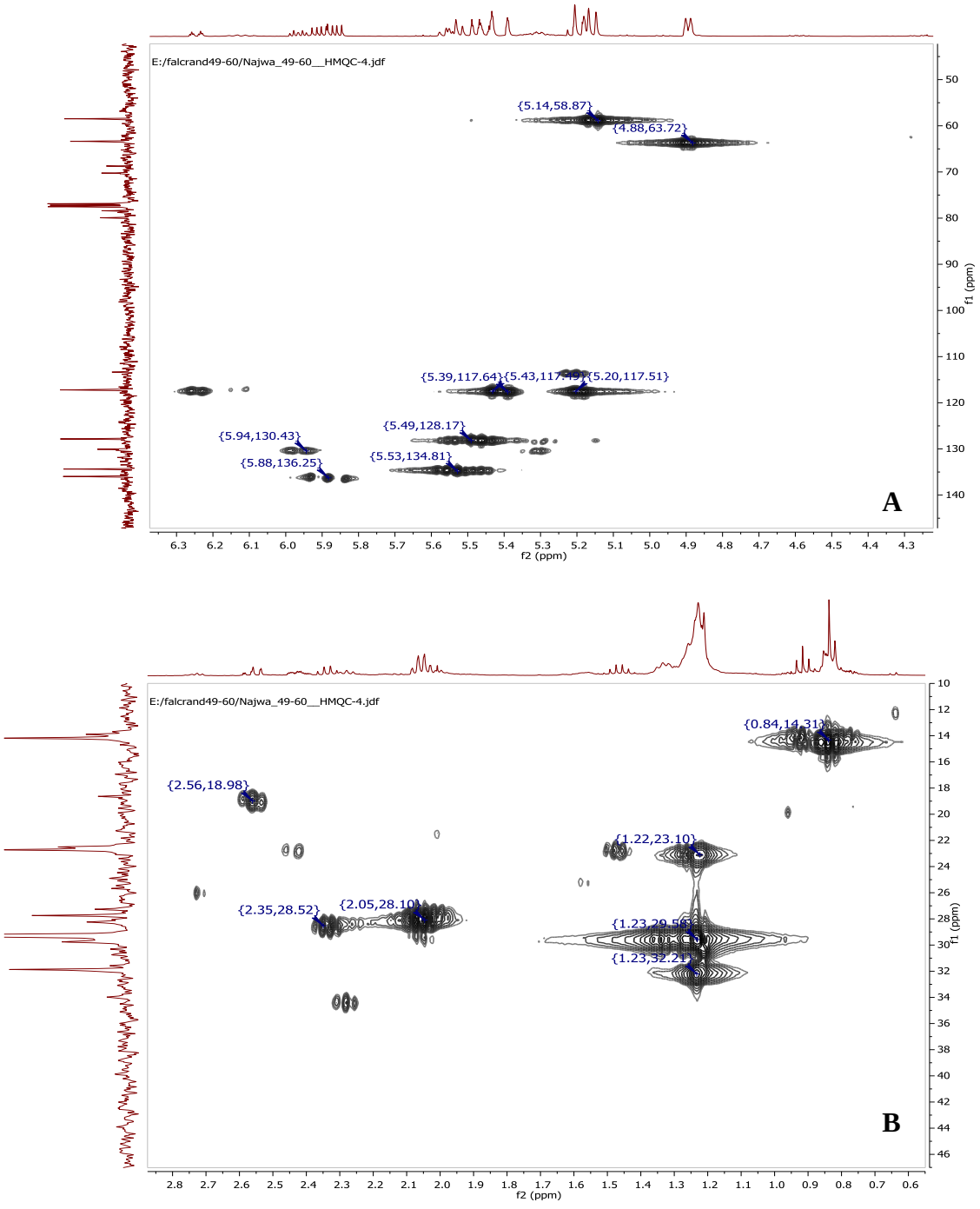


Figure 20: The HMQC spectrum of LoH49-60 processed by MestReNova software, (A): the olefinic/alcohol proton regions with the full carbon spectrum and (B): the aliphatic proton region with aliphatic carbon regions expanded.

3.2 Biological activity

3.2.1 Cytotoxicity of crude extracts on THP-1, MCF7 and PNT2A cells

Cytotoxicity assessment was carried out against THP-1 (leukaemic monocytes), MCF7 (epithelial breast cancer) and PNT2A cells (prostate epithelial normal) using crude solvent extracts of Lovage root. Cytotoxicity was defined as ≥ 50% of treated cell growth compared with untreated controls. The assay protocol involved replacing the media and extract (test agent), added on the first day, with fresh media without the test agent.

The methanol extract did not display any cytotoxic effects on all cell lines over the 2 days, even at the highest concentration of 2mg/ml (stock concentration) (Figure 21 A/B). Similarly, the methanol/water (1:1) extract was also non-toxic at 2 mg/ml (Figure 22A/B). However, there was a marked decrease in the proliferation percentage of PNT2A cells, 48.1±2.9% at 2mg/ml (Day 1 reading), then the cells recovered without the test agent to 63.7±2.3% (Day 2 reading).

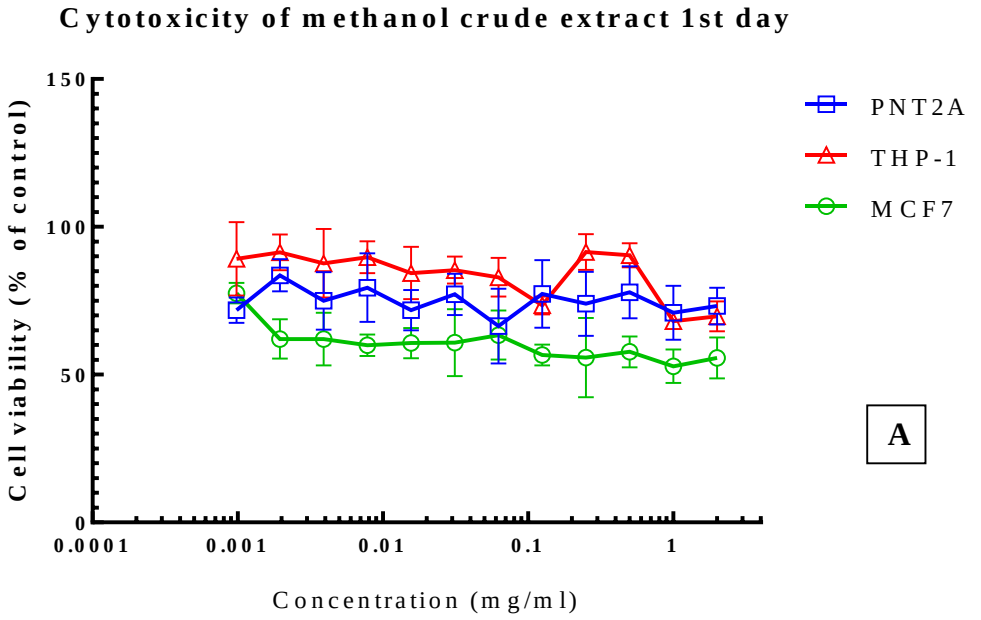
The crude ethyl acetate extract showed a marked decrease in proliferation percentage on the Day 1 measurements in MCF7 and PNT2A cell lines. The lowest concentration that showed a cytotoxic effect on the MCF7 cells was 0.25 mg/ml (45.2±3.2%), while cytotoxic effects was observed on PNT2A cells exposed to 2 mg/ml stock solution of ethyl acetate crude extract (Figure 23A). For the Day 2 readings the growth percentage was markedly decreased in all three cell lines at the higher concentrations (0.25-2 mg/ml) with proliferation percentages declining to approximately under 20% in all cell lines at concentrations 1 mg/ml and 2 mg/ml.

On the other hand, lower concentrations enhanced the proliferation of PNT2A and THP-1 cells to reach levels above 100% (Figure 23B).

Similarly, the lowest n-hexane crude extract concentration that showed cytotoxic effect at the Day 1 was at a concentration of 0.25 mg/ml with cell proliferations of 43.3±3.4%, 18.8±2.4% and 50.1±1.8% for PNT2A, THP-1 and MCF7 cells, respectively (Figure 24A). In the 2nd day PNT2A cells failed to show any proliferation at the highest four concentrations (0.25-2 mg/ml). Similar inhibition of proliferation was seen in THP-1 and MCF7 cells at concentrations between 0.5 mg/ml and 2 mg/ml, with a slight cell growth recovery seen at concentration 0.25 mg/ml for THP-1 cells. MCF7 cells showed above 55% cell proliferation percentage at a concentration of 0.25 mg/ml (Figure 24B). Table 8 presents a summary of the different crude extract IC_{50} on the three cell lines.

Table 8: The IC_{50} concentrations (mg/ml) of Lovage crude extracts on THP-1, MCF7 and PNT2A cells. Absence of toxicity at the highest concentration is indicated by (-).

	Crude Extract (mg/ml)			
Cell line	**Hexane**	**Ethyl acetate**	**Methanol**	**Methanol/water**
THP-1	0.081	-	-	-
MCF7	0.086	0.178	-	-
PNT2A	0.098	1.725	-	-



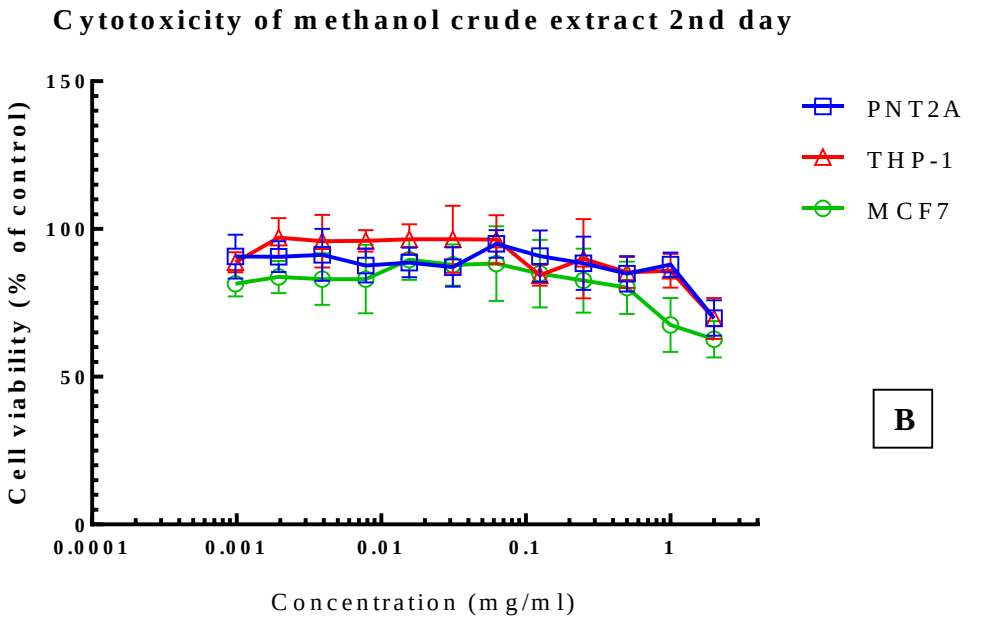


Figure 21: The 1st day (A) and 2nd day (B) percentage of cell proliferation of PNT2A, THP-1 and MCF7 cells exposed to crude Lovage methanol extract between (2-0.001 mg/ml) stock concentrations, each point represents the mean of triplicate readings ± SEM. The graph is representative of 3 separate experiments (n=3).

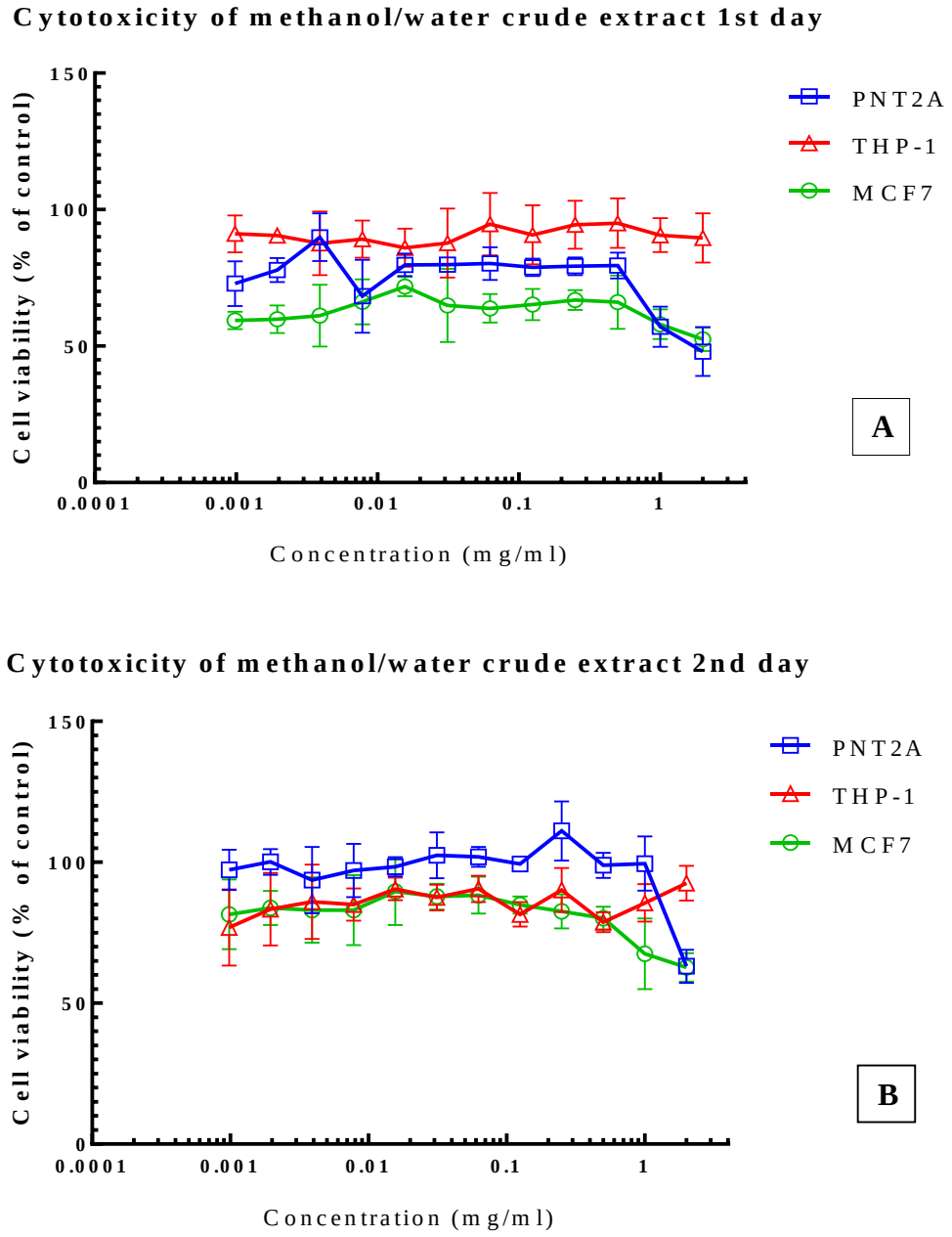


Figure 22: The 1st day (A) and 2nd day (B) percentage of cell proliferation of PNT2A, THP-1 and MCF7 cells exposed to crude Lovage methanol/water extract between (2-0.001 mg/ml) stock concentrations, each point represents the mean of triplicate readings ± SEM. The graph is representative of 3 separate experiments (n=3).

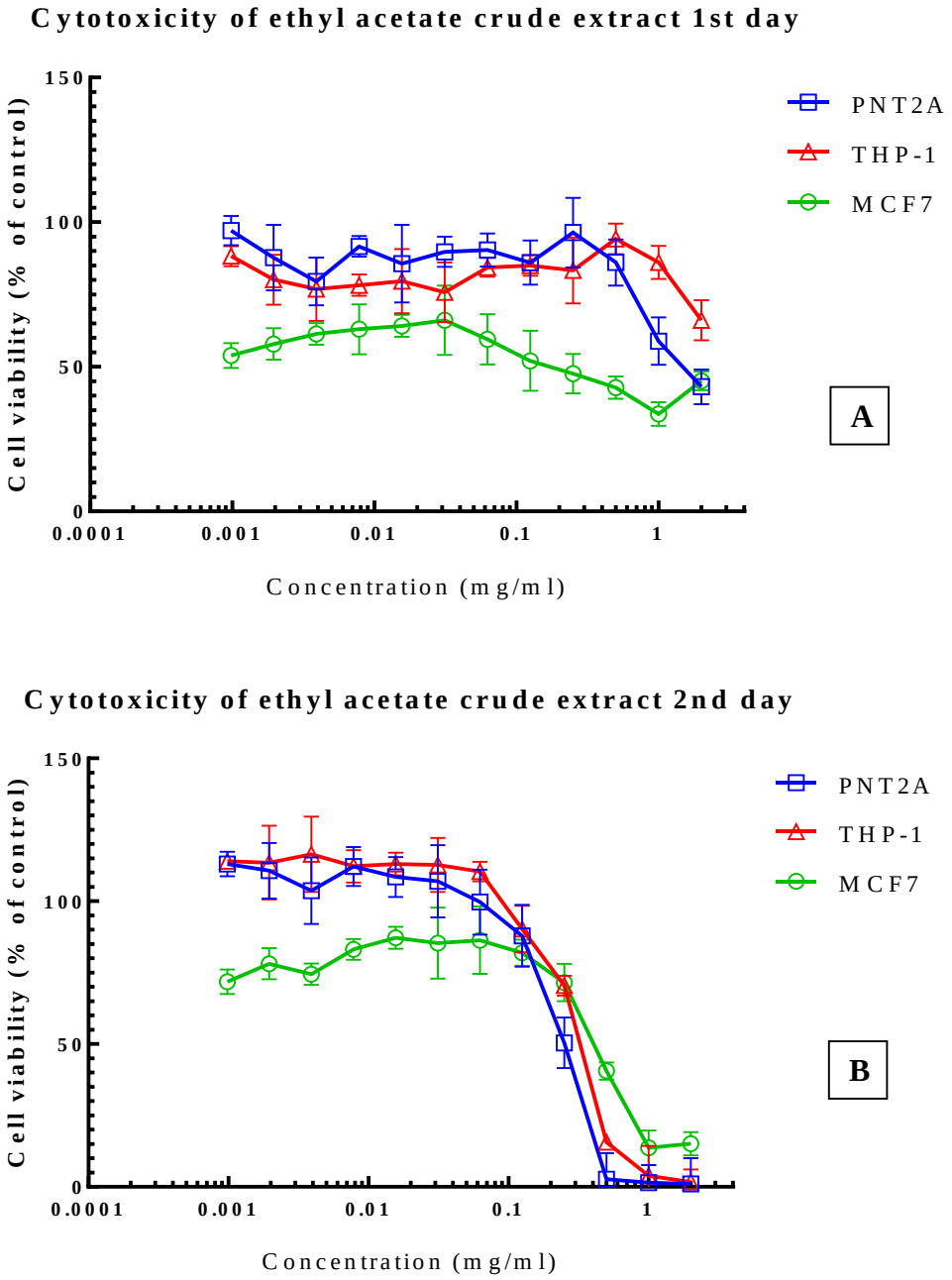


Figure 23: The 1st day (A) and 2nd day (B) percentage of cell proliferation of PNT2A, THP-1 and MCF7 cells exposed to crude Lovage ethyl acetate extract between (2-0.001 mg/ml) stock concentrations, each point represents the mean of triplicate readings ±SEM. The graph is representative of 3 separate experiments (n=3).

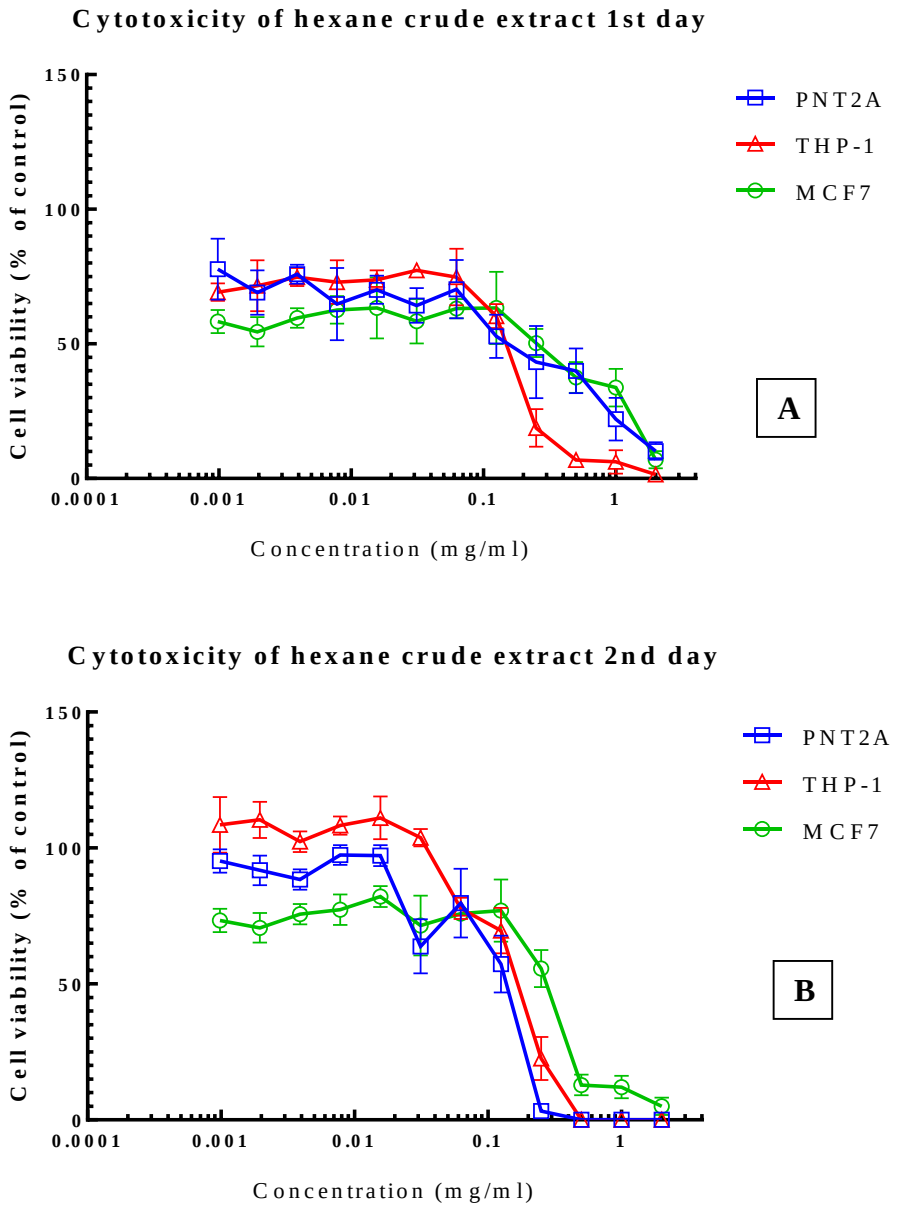


Figure 24: The 1st day (A) and 2nd day (B) percentage of cell proliferation of PNT2A, THP-1 and MCF7 cells exposed to crude Lovage n-hexane extract between (2-0.001 mg/ml) stock concentrations, each point represents the mean of triplicate readings ±SEM. The graph is representative of 3 separate experiments (n=3).

3.2.2 Cytotoxicity assessment of obtained fractions.

Fractions obtained from n-hexane and ethyl acetate crude extracts showed a dose-dependent cytotoxic effect on all cell lines. Therefore, attempts were made to further separate that crude extracts by CC and MPLC fractionation. Fractions were analysed by TLC and similar profiles were pooled. According to TLC and the observations under UV, compounds that may contain flavonoids were selected for further study. Only one compound from the ethyl acetate crude extract fractionation (LoE18-19) and two major compounds from the hexane crude extract fractionation (LoH26-36 and LoH49-60) were chosen for cytotoxicity assessments.

3.2.2.1 Cytotoxicity of ethyl acetate fractions (LoE18-19) on THP-1, MCF7 and PNT2A cell lines.

The ethyl acetate fractions (LoE18-19) showed slight cell growth inhibition of at the highest concentrations on day 1 (1 mg/ml and 2 mg/ml) on PNT2A cells. However, only 2 mg/ml was considered toxic at this time point (49.3±4.1%). THP-1 cells were more affected with 0.5 mg/ml as the lowest cytotoxic concentration (40.6±6.1%). On the other hand, MCF7 cells were inhibited by lower concentrations, which started at 0.004 mg/ml (42.8±4.5%) on the Day 1 measurements (Figure 25A).

On the Day 2 measurements, both PNT2A and THP-1 cell lines showed marked reduction in cell proliferation at lower concentrations (0.25 mg/ml; 29.7±3.6% and 0.06 mg/ml; 30±4.6% for PNT2A and THP-1 cell lines, respectively). However, MCF7 cells recovered at lower concentrations, where the lowest cytotoxic concentration was 0.25 mg/ml with 40.5±3.7% proliferation (Figure 25B).

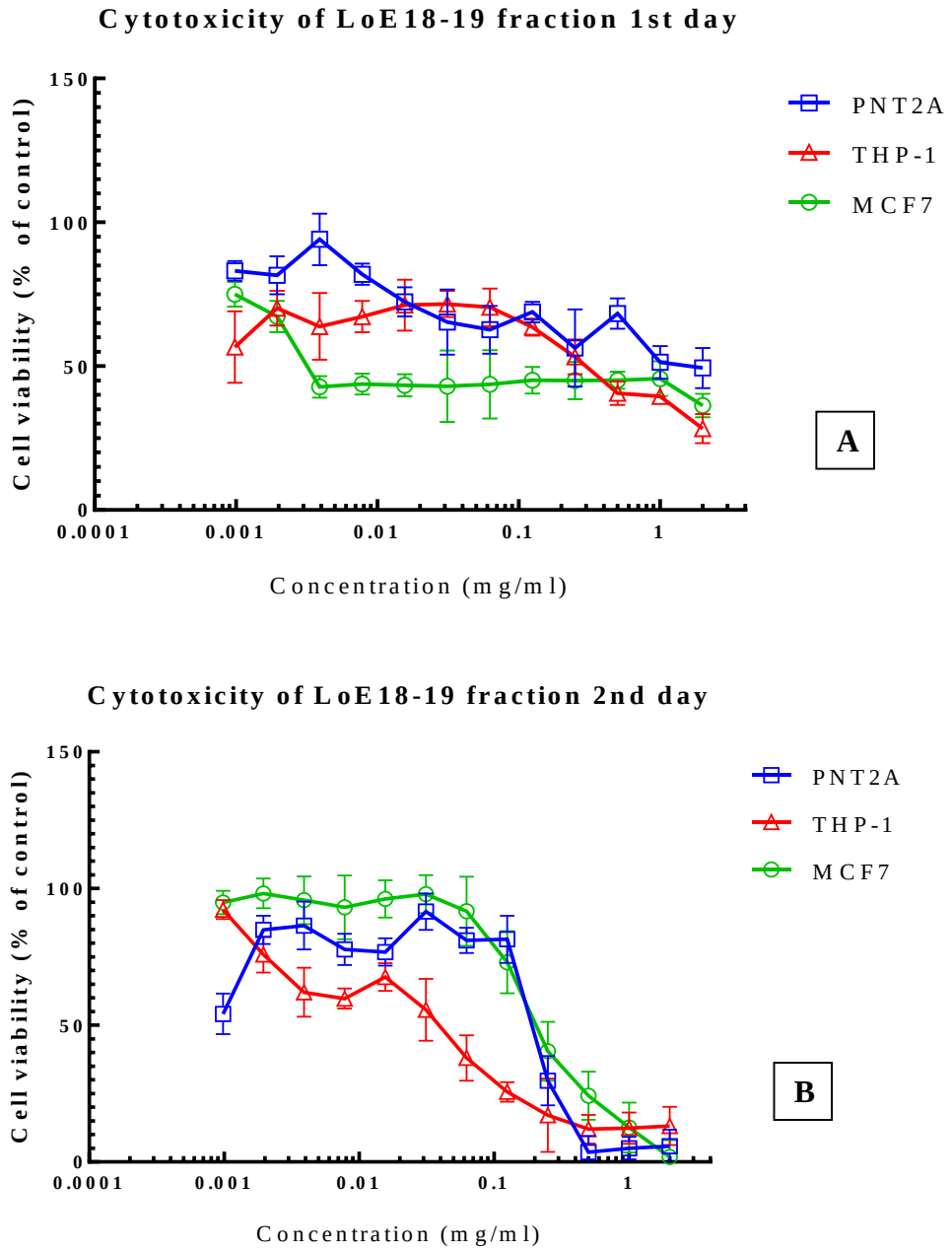


Figure 25: The 1st day (A) and 2nd day (B) percentage of cell proliferation of PNT2A, THP-1 and MCF7 cells exposed to ethyl acetate fraction (LoE18-19) between (2-0.001 mg/ml) stock concentrations, each point represents the mean of triplicate readings ±SEM. The graph is representative of 3 separate experiments (n=3).

3.2.2.2 Cytotoxicity of the n-hexane fractions (LoH26-36) on THP-1, MCF7 and PNT2A cell lines.

The n-hexane fractions (LoH26-36) caused PNT2A and MCF7 cells to reduce their proliferation with the lowest active concentration in measurements after 1 day (0.5 mg/ml; 33.2±3.7% and 1 mg/ml; 41.7±4% for PNT2A and MCF7 cell lines, respectively). While THP-1 cell lines were more affected when exposed to the fractions at all concentrations with only >50% growth proliferation (54.4±12.0%) seen at 0.03 mg/ml (Figure 26A).

In the Day 2 measurements, PNT2A cells showed reduction in cell proliferation at higher concentrations (0.5-2 mg/ml), but the growth percentage remained at the same level at 0.25 mg/ml. On the other hand, lower concentrations (0.001-0.06 mg/ml) appeared to enhance the growth of the PNT2A cells to reach ≥100% proliferation on the second day. Similarly, MCF7 cells recovered at lower concentrations, where the lowest active cytotoxic concentration was 0.5 mg/ml with 27.1±5.2% proliferation in 2^{nd} day measurements. A similar growth recovery was observed in THP-1 cells at the lower concentrations on the Day 2 with a minimal active cytotoxic concentration of 0.125 mg/ml with 40.7±1.2% proliferation observed in the 2nd day measurements (Figure 26B).

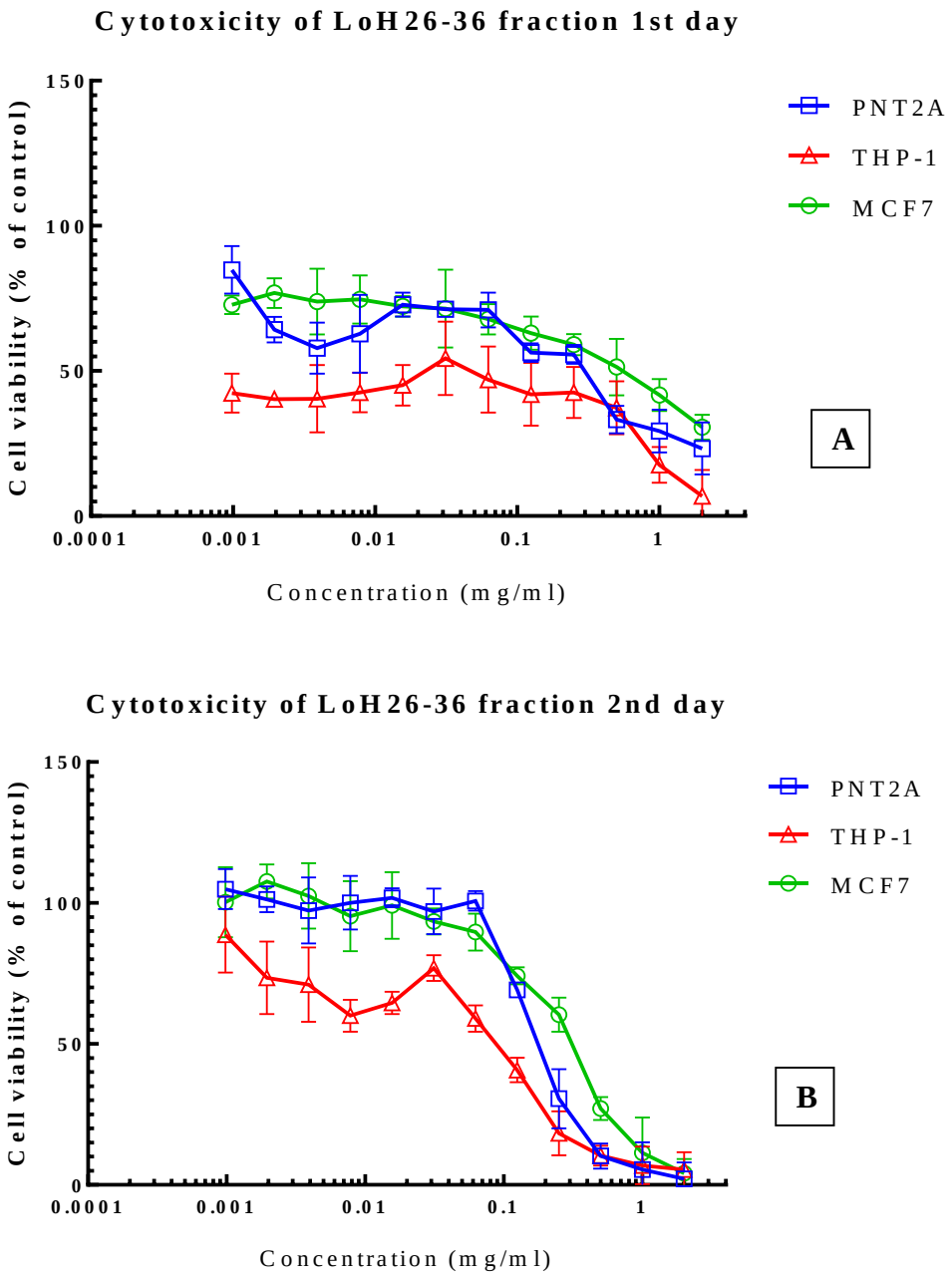


Figure 26: The 1st day (A) and 2nd day (B) percentage of cell proliferation of PNT2A, THP-1 and MCF7 cells exposed to n-hexane fractions (LoH26-36) between (2-0.001 mg/ml) stock concentrations, each point represents the mean of triplicate readings ±SEM. The graph is representative of 3 separate experiments (n=3).

3.2.2.3 Cytotoxicity of hexane fractions (LoH49-60) on THP-1, MCF7 and PNT2A cell lines.

The hexane fractions LoH26-36 and LoH49-60 on PNT2A and MCF7 cells showed reduction of proliferation in measurements after 1 day with the lowest active concentration of 1mg/ml at growth proliferations of 48.7±6.1% and 48.0±4.2% for PNT2A and MCF7 cell lines, respectively. THP-1 cells were more affected when exposed to the fractions at all concentrations (0.001-2 mg/ml) with growth proliferation <50% (Figure 27A).

In the Day 2 measurements, PNT2A cells showed a reduction in cell proliferation at higher concentrations (0.5-2 mg/ml). However, a similar growth proliferation enhancing effect was observed with lower concentrations between 0.001-0.25 mg/ml to reach 80-100% proliferation on the second day. Similarly, MCF7 cells recovered at most concentrations, where the lowest active cytotoxic concentration was 1mg/ml with 28.4±3.2% proliferation in Day 2 measurements. A lower level of growth recovery was observed in THP-1 cells at the lower concentrations on the 2nd day with a minimal active cytotoxic concentration of 0.03mg/ml with 37.5±5.5% proliferation (Figure 27B).

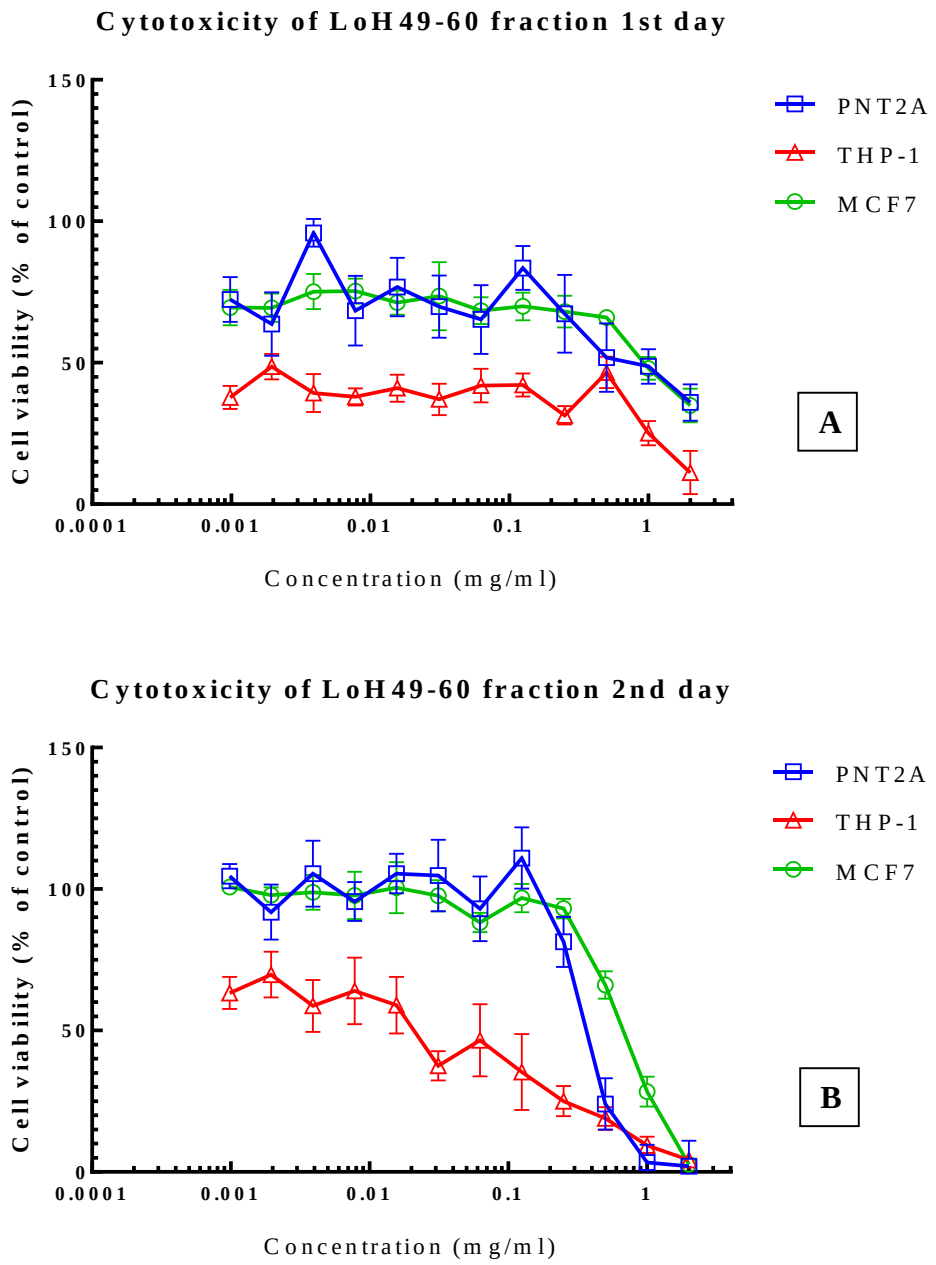


Figure 27: The 1st day (A) and 2nd day (B) percentage of cell proliferation of PNT2A, THP-1 and MCF7 cells exposed to n-hexane fractions (LoH49-60) between (2-0.001 mg/ml) stock concentrations, each point represents the mean of triplicate readings ±SEM. The graph is representative of 3 separate experiments (n=3).

The cytotoxicity of THP-1 cell line observed upon exposure to the hexane fractions were significant ($p < 0.001$) when compared to the other cell lines' cytotoxicity. Therefore, to investigate the cell death mechanism, the THP-1 cell line was used in the assay of detection of apoptosis. A summary of half maximal inhibitory concentration (IC_{50}) of different fractions on proliferation of the three cell lines is presented in Table 9.

Table 9: The IC_{50} (mg/ml) of fractions on THP-1, MCF7 and PNT2A cells.

	Fraction (mg/ml)		
Cell line	**LoH26-36**	**LoH49-60**	**LoE18-19**
THP-1	0.001	0.001	0.287
MCF7	0.466	0.635	0.022
PNT2A	0.145	0.572	1.833

3.2.3 Detection of apoptosis (DNA ladder assay)

DNA was recovered from THP-1 cells exposed to different concentrations (0.25, 0.5 and 1 mg/ml) of Lovage crude solvent extracts (ethyl acetate, methanol, methanol/water and hexane crude extracts) for 18h. Apoptotic DNA fragments were prominently detected in cells exposed to 1 mg/ml ethyl acetate crude extract and cells exposed to 1mg/ml of the hexane extract (Figure 28). While there was no DNA fragmentation observed from cells exposed to methanol and methanol/water crude extracts. Similarly, apoptotic DNA fragments were prominently detected in samples obtained from THP-1 cells incubated with different concentrations of fractions (LoE19-18, LoH26-36 and LoH49-60) for 18h (Figure 29).

M 1 2 3 4 5 6 7 8 9 10 11 12 C

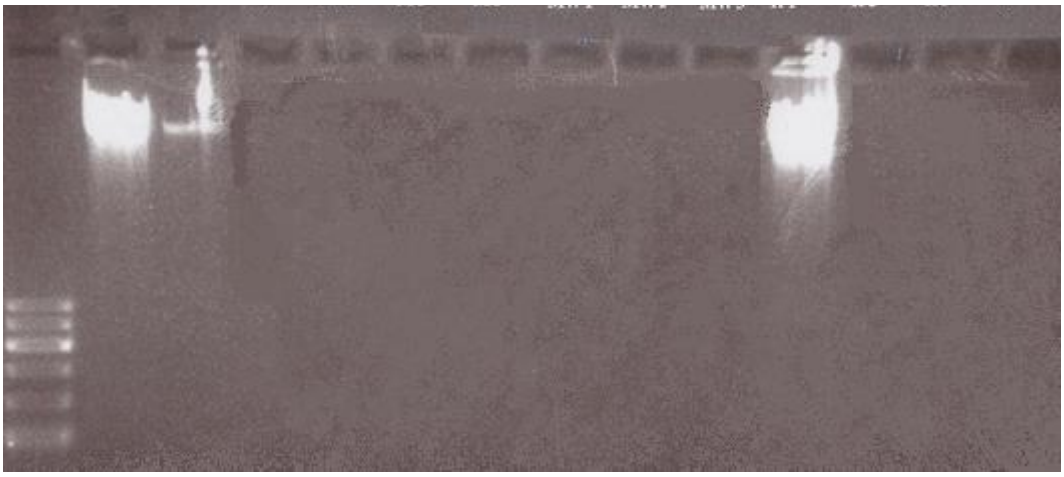


Figure 28: Gel electrophoresis image obtained after DNA fragmentation assay for apoptosis detection (cells exposed to crude extract). THP-1 cells were incubated with 0.25, 0.5 and 1 mg/ml of Lovage crude solvent extracts (ethyl acetate, methanol, methanol/water and n-hexane) for 18h.

M= Marker 100bp,

Lanes 1, 2 and 3= 1, 0.5 and 0.25 mg/ml of the ethyl acetate crude extract, respectively.

Lanes 4, 5 and 6= 1, 0.5 and 0.25 mg/ml of the methanol crude extract, respectively.

Lanes 7, 8 and 9= 1, 0.5 and 0.25 mg/ml of the methanol/water crude extract, respectively.

Lanes 10, 11 and 12= 1, 0.5 and 0.25 mg/ml of the hexane crude extract, respectively.

C = DNA obtained from negative control cells (no treatment).

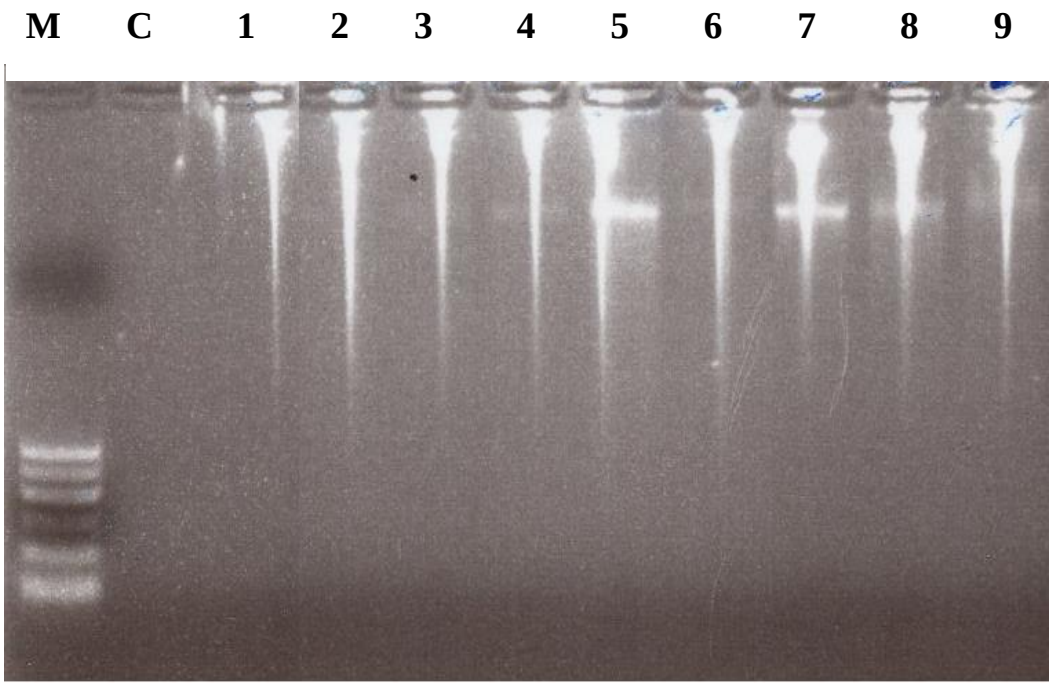


Figure 29: Gel electrophoresis image obtained after DNA fragmentation assay for apoptosis detection (cells exposed to fractions). THP-1 cells were incubated with 0.25, 0.5 and 1 mg/ml of fractions LoE19-18, LoH26-36 and LoH49-60 for 18h.

M= Marker 100bp.

C = DNA obtained from negative control cells (no treatment).

Lanes 1, 2 and 3= 1, 0.5 and 0.25 mg/ml of LoE18-19, respectively.

Lanes 4, 5 and 6= 1, 0.5 and 0.25 mg/ml of LoH26-36, respectively.

Lanes 7, 8 and 9= 1, 0.5 and 0.25 mg/ml of LoH49-60, respectively.

Chapter Four: Discussion and conclusions

The roots of *L. officinale* (Apiaceae) are traditionally used in the treatment of many disorders, in food as a spice and in perfume manufacturing. Alongside with known medicinal properties of plant extracts, the antioxidant properties might indicate the presence of compounds that can be of use for development of new anticancer drugs. However, there is a lack of studies on compounds isolated from Lovage root as possible natural drugs to treat cancer.

4.1 Extraction and yield

Previous studies used the seeds, stems, flowers, leaves and roots of *L. officinale* for isolation of bioactive compounds [92]. In the current research study, the dried roots were milled and solvent extracted. The yields obtained from extraction showed the highest to be with methanol (11.6%). Methanol is a solvent that is able to extract both polar and nonpolar compounds. On the other hand, the yield with n-hexane was mostly of nonpolar compounds such as waxes, fats and fixed oil with a yield of 5.5%. While the ethyl acetate extract yielded only 2.7%. These yields were greater than that reported by Guzman *et al*. (2013) using Soxhlet apparatus and n-hexane, chloroform and methanol, where, 1.5% of an oily the n-hexane extract, 0.22% of the chloroform extract and 2.6% of the methanol extract were obtained from 490 g of Lovage roots [93]. The greater yields obtained in this research could probably have been due to the growing conditions of the plant which may affect the quantity and quality of particular compounds [94]. The extraction method used in this research was successful in obtaining Lovage root compounds according to their polarity. However, to recover

pure nonpolar compounds it would have been better to apply further partitioning of the crude methanol extract with hexane and ethyl acetate [95].

Column chromatography was applied on the hexane extract for isolation of compounds, while MPLC was used for the ethyl acetate extract; the use of MPLC is due to the complexity on TLC. A smaller column was then used for some of collected fractions from the extracts and further purification was carried out. However, further purification of compounds were not obtained using this method, possibly because the amounts of compounds in these fractions were too small or they may have degraded and/or become stuck on the column. Using this technique, trilinolein was obtained according to NMR prediction. Therefore, better alternative isolation methods are recommended for future use which separates compounds based on size exclusion for example gel filtration or sephadex and HLPC technique [96].

4.2 Structure elucidation

NMR spectroscopic analyses of the fractions was carried out to try identifying the compounds present. LoE19-18 was examined by ^{1}H-NMR, from which it was possible to elucidate it as mainly ferulic acid as compared to previously reported by Sajjadi *et al*., [89]. LoH26-36 was examined by ^{1}H-NMR, from which it was possible to elucidate it as mainly trilinolein associated with a steroid ester. ^{1}H NMR spectrum described by Nieva-Echevarría *et al*., supported the presence of trilinolein [97]. The spectral data of ^{13}C of LoH26-36 also suggested trilinolein as described by Ragasa *et al*., [98]. The COSY spectrum showed the non-equivalent protons of CH_2-OAcyl groups and the deshielded protons of the other two CH_2 with the same *J* values. Therefore, trilinolein is a compound isolated from this fraction. LoH49-60 was

examined by ^{1}H-NMR, COSY, DEPT, HMBC and HMQC, from which it was possible to elucidate it as mainly falcarindiol as described by Fujioka *et al*. [99]. In a recent study, 3(R)-falcarinol and 3(R)-8(S)-falcarindiol were identified, from a dichloromethane extract of lovage [100, 101].

Liquid Chromatography Mass Spectrometry (LC-MS) was carried out to tentatively identify the compounds in the purified fractions. For future work, Temperature Programming Gas Spectrometry could be justified for identification which might help in reducing fragmentation and help to elucidate the structures of the compounds [102].

4.3 Cytotoxicity of crude extracts.

The crude methanol and methanol/water extracts showed no cytotoxic activity against all cell lines used in this study. The only exception was the PNT2A cell line, which at the highest concentration of 2 mg/ml showed a reduction in cell proliferation. This was at the marginal end of cytotoxicity (48.1±2.9%). However, the cells recovered on the 2nd day, (in the absence of test agent), which indicates that the methanol/water crude extract may have a cytostatic and not a cytotoxic effect on this type of cell line. These results are similar to those obtained by MacAskill (2009), Elbadawy (2008) and Gustafsson (2006), who used the same assays to determine cytotoxicity of Lovage leaf extracts [59, 103, 104]. In contrast, higher concentrations of the ethyl acetate and the hexane crude extracts showed a cytotoxic effect on all cell lines indicated by inhibition and reduction of cell growth on the first day that continued on the second day of assessment in the absence of test agent. Furthermore, DNA fragmentation was detected in cells treated with high concentrations of the ethyl acetate and the hexane crude extracts. The DNA fragmentation appeared as a smear with 2 groups of bands and a separated band. In another study using the same apoptotic DNA ladder detection kit a

similar DNA fragmentation profile was observed in a human teratocarcinoma cell line treated with retinoic acid [105]. In this research study, combined results of cytotoxicity and apoptotic DNA fragmentation indicate that the ethyl acetate and the hexane crude extracts of Lovage root have anti-proliferation activity against treated cell lines. The mechanisms of this activity could be by arresting the cells in the G1/M phase at low doses and promoting apoptosis at high doses. Similar results were reported by [106] where bioactive compounds isolated from plants belonging to the Lovage family (Apiaceae) had an inhibitory effect on cancer cells at the higher concentrations.

4.4 Cytotoxicity of isolated fractions.

Cell treatment with individual isolated fractions after the 1st day showed that THP-1 cells were significantly inhibited by the n-hexane isolated fractions (LoH26-36, p = 0.0001 and LoH49-60, $p < 0.0001$) at very low concentrations. In contrast, cell proliferation of MCF7 cells was significantly inhibited by ethyl acetate fraction (LoE18-19, p = 0.0001). While PNT2A cells showed growth inhibition only significant inhibition by the n-hexane isolated fraction (LoH26-36, $p < 0.001$) when compared to the inhibition observed by the other two fractions. On the 2nd day assessment, in the absence of test agents, recovery of cell proliferation at low concentrations was observed compared with a decrease in cell viability at higher concentrations. This indicated that cells exposed to the higher concentrations had already gone through apoptosis on the 1st day and died on the 2nd day. This was confirmed by the detection of DNA fragmentation in cells incubated for 18h with high concentrations of isolated fractions. Interestingly, MCF7 cells treated with low concentrations showed enhanced proliferation on the 2nd day which is normal in this

cell type due to its requirement to adhere to the plate surface, allowing it to recover and grow [59]. The results obtained from this research are comparable to those obtained in other studies that studied the anticancer activity of proposed compounds. For instance, the concentration-dependent activity of the polyacetylene falcarinol ((9Z)-heptadeca-1,9-dien-4,6-diyn-3-ol), isolated from carrots, was investigated in a bioassay with primary mammary epithelial cells in collagen gels and compared with that of β-carotene, the orange pigment in carrots. Falcarinol showed biphasic activity, having stimulatory effects between 0.01 and 0.05 µg/ml and inhibitory effects between 1 and 10 µg/ml, whereas β-carotene showed no effect in the concentration range 0.001-100 µg/ml [107]. Falcarinol and falcarindiol exhibited medium level cytotoxicity against leukaemia, lymphoma and myeloma tested cell lines in the range of IC_{50} approximately 30 µM [108]. Young *et al.* (2007) showed that falcarinol at low concentrations (0.5-10 µM) considerably increased the proliferation of the Caco-2 cell line and reduced expression of caspase-3 with decreased basal DNA strand breakage. On the other hand, higher concentrations of falcarinol prompted an increase of caspase activity and decreased proliferation of the Caco-2 cell line [106]. This compound may have an essential role to play in future cancer-drug discovery, and efforts in this direction are being made [109].

Previous studies reported that ferulic acid (major compound in LoE18-19) can prevent the apoptosis process by pathways [110-114]. However, in the current research study, cell growth inhibition with detection of DNA fragmentation at high doses was observed. These findings were also reported in a study carried out on the cytotoxicity of synthesised lipophilic caffeic and ferulic acid derivatives on cultured breast cancer

cells [115]. Six compounds were initially evaluated: caffeic acid, hexyl caffeate, caffeoylhexylamide, ferulic acid, hexyl ferulate, and feruloylhexylamide. Cell proliferation, cell cycle progression, and apoptotic signalling were investigated in three human breast cancer cell lines, including oestrogen-sensitive MCF-7 and insensitive MDA-MB-231 and HS578T cells. The results showed that although the parent compounds presented no cytotoxicity, the new compounds inhibited cell proliferation and induced cell cycle alterations and cell death, with a predominant effect on MCF-7 cells. Interestingly, in that study, cell cycle data indicates that effects on non-tumor fibroblasts were predominantly cytostatic and not cytotoxic. From this point, it could be anticipated that LoE18-19 fraction in this study has similar cytostatic properties, as it is not totally a pure compound. Therefore, more investigation is required to identify the impurities in this fraction and determine their possible effect in the "ferulic acid" anticancer activity.

Conclusions and future work

Solvent extraction of Lovage roots using heat-based extraction was used in this study. The amount of the crude extracts obtained after the extraction varied with the solvent polarity. However, it would be of interest to apply cold extraction methods using ethanol as the solvent in order to limit the effect of exposure to heat on the chemical compounds properties. The crude extracts obtained in this study were screened for cytotoxic properties using two cancer cell lines. Crude extracts that showed anticancer properties were further fractionated using different techniques in order to isolate the active compounds. Although some of the crude extracts showed the existence of several compounds, they were mostly sugars and fats hence they were excluded from

any biological assays. The isolated fractions in this study that showed anticancer activities were tested against 2 cancer cell lines. While different cells were affected by different degree by the isolated fractions, the results obtained suggest that THP-1 cells are more sensitive to the fractions isolated in n-hexane while MCF-7 cells are more sensitive to the fraction isolated in ethyl acetate solvent. However, PNT2A cells showed less variation in effect of different fractions. It would be worth investigating whether these variations in response affect other types of cancer cells and normal cell lines. It would also be worth studying those fractions or the identified active compounds in *in vivo* experiments. The cell death pathway was expected to be through activation of apoptosis pathways. The DNA ladder assay used was generally specific for detection of DNA fragmentation which is the hallmark of apoptotic cell death. However, the kit used did not provide a positive apoptotic DNA to be used in comparison with the DNA extracted from the treated cells. In future studies, the use of other apoptosis detection assays such as TUNNEL or Annexin V assays could give more details about the cell death pathways [116, 117]. In this research study, different methods and techniques have been applied for the prediction of compounds in the isolated fractions. However, the identified compounds in this research thesis were previously reported with the exception of impurities associated with ferulic acid in the LoH49-60 fraction. This fraction may contain a new isomer or form of ferulic acid that resulted in the existence of anticancer properties in this fraction. The results from this study suggest that future work could focus on isolation of the pure compounds from the n-hexane extract which may have anticancer properties and could be used for production of safer, natural and active anticancer candidates.

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