Methodology development of Quality control, Quality assurance and standards for *Moringa oleifera* seeds using Liquid chromatography

by

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DECLARATION

Student number: 36340227

I declare that "**Methodology development of Quality control and Quality assurance of *Moringa oleifera* seeds using Liquid chromatography"** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

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SIGNATURE DATE

DEDICATION

This dissertation is dedicated to my parents David and Rebecca Chokwe who are my pillars of strength and my number one supporters. Their love, prayers and belief in me kept me going when the work was just too much. I'm truly blessed to call you my parents.

To almighty God, without whom nothing is possible

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ABSTRACT

Natural products or traditional medicine has been used for centuries to treat various ailments by mankind. The World Health Organization estimates that in recent times 80% of people in emerging economies rely on traditional medicine as their primary health care [1]. However, unlike pharmaceutical products which have methods in place for quantification of the active compounds traditional medicine still requires a lot of focus in this area. *Moringa oleifera* is one of those species that have been used traditionally to cure various ailments for centuries. Even though extensive phytochemical and pharmacological studies have been conducted on the different parts of the plant, there is still no analytical method that enables the quantification of the compounds in the Moringa products in the market. The aim of this research was to develop an HPLC separation method that can be used to quantify the compounds found in the *Moringa oleifera* products.

Compounds were extracted from the seeds of *Moringa oleifera* using the maceration method with a mixture of water and ethanol (1:1 v/v). The compounds were isolated using preparative HPLC. The structures of the compounds were elucidated using FTIR, NMR and MS. An HPLC separation method for quantification of the isolated compounds was developed and validated. The method was applied to the crude extract to quantify the isolated compounds in the extract.

The following compounds 3-caffeoylquinic acid, 4-(α-L-Rhamnosyloxy) benzyl isothiocyanate, O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate and O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate were isolated at percentage purities ranging from 90 to 99%. The HPLC separation method for quantification showed a linear relationship between peak area and

concentration of the compounds with regression coefficients ranging from 0.9977 to 0.9994. The method is also precise with % RSD values between 0.01 and 1.16%. The method was shown to be specific to the compounds of interest. The percentage distribution of compounds in 50 mg of the seeds extract was between 0.25 and 1.10 % w/w.

This study successfully developed and validated an HPLC separation method for four compounds found in the seeds of *Moringa oleifera* and quantified them in the crude extract of the seeds found in Zambia. This method can be used for identification and quantification of these four compounds in any of the Moringa products. As far as it could be ascertained this is the first time that such a method has been developed for these compounds.

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ABBREVIATIONS

CC	**C**olumn **C**hromatography
DEPT	**D**istortionless **E**nhancement by **P**olarization **T**ransfer
DNA	**D**eoxyribo**n**ucleic **A**cid
DMSO	**D**i**m**ethyl **S**ulf**o**xide
DPPH	2,2 **D**i**p**henyl-1-**p**icryl**h**ydrazyl
EI	**E**lectron **I**onization
EDV	**E**pstein-**B**arr **V**irus-**E**arly **A**ntigen
FCC	**F**lash **C**olumn **C**hromatography
FTIR	**F**ourier **T**ransform **I**nfra**r**ed
HPLC	**H**igh **P**erformance **C**olumn **C**hromatography
IR	**I**nfra**r**ed
LLE	**L**iquid-**L**iquid **E**xtraction
LOD	**L**imit **o**f **D**etection
LOQ	**L**imit **o**f **Q**uantification
MAE	**M**icrowave **A**ssisted **E**xtraction
MO	***M**oringa **O**leifera*
MS	**M**ass **S**pectrometer
1D	One **D**imension
3D	Three **D**imensions
PVDF	**P**oly**v**inyl **D**i**f**luoride
PPM	**P**arts **P**er **M**illion
RSD	**R**elative **S**tandard **D**eviation
RF	**R**etardation **F**actor
SE	**S**oxhlet **E**xtraction
SFE	**S**upercritical **F**luid **E**xtraction
TLC	**T**hin **L**ayer **C**hromatography
UAE	**U**ltrasound **A**ssisted **E**xtraction
UV-VIS	**U**ltra**v**iolet **V**isible
WHO	**W**orld **H**ealth **O**rganization

CHAPTER ONE

1.1 Background

For centuries mankind has relied on the use of traditional medicine to cure various ailments. The most popular include African, Arabic, Chinese and Indian traditional medicine [1]. Natural products from plants have been and continue to be vital to mankind as a source of nutrition, for beauty treatment and medicine [2]. The World Health Organization estimates that 80% of people in emerging economies rely on traditional medicine as their primary health care [3]. This is mainly because of the lack of good health care facilities and the high cost of western medicine whereas traditional medicine is accessible and affordable. This is the case in South Africa where the Conserve Africa Foundation has reported that the ratio of traditional practitioners to patient is 1: 700-1200 and that of western doctors to patient is 1: 17 400 [4,5].

Natural products are described as pharmacologically and biologically active secondary metabolites found in nature or compounds produced by living organisms as a defence mechanism against microbial attack, animal predation and ultraviolet radiation [6,7]. These metabolites include groups of compounds such as alkaloids, terpenoids and phenolics. Table 1.1 shows some of the compounds derived from natural products and their uses.

Table 1.1 Chemicals derived from natural products and their uses [8, 9]

Chemical	**Scientific Source name**	**Clinical use**	**Traditional use**	**Traditional Source name**
Atropine	*Atropa belladonna*	Anticholinergic	headache, inflammation, menstrual symptoms	Deadly nightshade
Caffeine	*Camellia sinesis*	Central nervous system stimulant	Tonic, stomach disorder, diarrhoea	Tea plant
Cocaine	*Erythroxylum cola*	Local anaesthetic	Suppress pain, hunger, thirst	Cocoa
Danthron	*Cassia species*	Laxative	Used to treat various infections	Golden shower tree
L- Dopa	*Mucuna species*	Anti-parkinsonism	Snakebite, diarrhoea.	Velvet bean
Morphine	*Papaver somniferum*	Analgesic	To induce sleep, headache.	N/A
Penicillin	*Penicillium fungi*	Antibacterial	N/A	N/A
Quinine	*Cinchona ledgeriana*	Antimalarial/ antipyretic	Loss of appetite	N/A
Galantamine hydrobromide	*Galanthus nivalis*	Alzheimer's disease	Neurological conditions	Snowdrop
Artemotil	*Artemisia annua*	Antimalarial	To cure chills and fever	Sweet annie, Wormwood

1.2 Studies on traditional African medicinal plants

Traditional medicine was recognised as the source of primary health care in the Primary Health Care Declaration of Alma Ata 1978 [3]. That coupled with the developments in analytical techniques, development of drug-resistant micro-organisms, side effects caused by modern drugs and emerging diseases has led to renewed attention in traditional medicine so as to provide novel chemical entities [10]. There are ongoing phytochemical and pharmacological studies on African medicinal plants. These studies involve testing the crude extract for biological activities followed by isolating and characterizing the active compounds. Ongoing studies include those on plants such as the kola plant originally found in West African countries. The species *Cola acuminate* is used traditionally as an antidote for poisoning, to suppress appetite, increase alertness and to treat migraines as well as motion sickness [11].

The *Cajanue cajan,* also called pigeon pea, is found in Senegal, East Africa and South Africa. It is traditionally used as a painkiller, to treat skin irritation, measles and jaundice [12]. The following compounds found in the plant; hexadecanoic acid, α-amyrin, β-sitosterol, pinostrobin, lonistylin A and C have been found to possess anti-cancer properties. Another compound, cajanuslacton, isolated by Kong Y et al from the plant has anti-bacterial activity [13]. *Hoodia gordonii* is a leafless thorny plant that grows naturally in South Africa and Namibia. It has long been used to treat indigestion and mild infections. The compound P57, a glycoside with the molecular formula $C_{47}H_{74}O_{15}$ responsible for the appetite-suppressant effect, was isolated from the plant by the South African Council of Scientific and Industrial Research (CSIR) in 1977 [14].

Moringa is found in different communities across Africa, Asia and the Caribbean. The plant is used traditionally to cure ailments such as ulcer, digestive problems, fungal infections and for

wound healing [15]. Moringa contains compounds such as niazimicin that is reported to have anti-tumor activity [16]; benzyl isothiocyanate and benzyl glucosinolate were found to have anti-fungal and anti-bacterial activities [17]. The phytochemistry for most of these plants is complete; however there are no analytical methods in place to enable the quality control and quality assurance of the products made from them. This study focuses on *Moringa oleifera* which is one of the species of Moringa.

1.2 Motivation for study

There is continued interest in the Moringa plant because of its many uses especially the medicinal uses such as the treatment of urinary tract infections, diabetes and many more [18, 19]. Extensive phytochemical and pharmacological studies have been conducted on the different parts of the plant such as the leaves, seeds, bark and roots. However, there are no methods in place to determine the distribution of the compounds within the plant or the quantity of the compounds in the Moringa products in the market such as those shown in Figure 1.1. The challenge or limitation in developing quantification methods is the lack of commercial reference standards for the compounds found in the Moringa plant. Therefore, this study focuses on generating reference standards, developing and validating an HPLC separation method for quantification of the compounds found in the *Moringa oleifera* plant. The validated method will provide the platform for the quality control of the *Moringa oleifera* products in the market.

Figure 1.1 *Moringa oleifera* products in the South African market [20,21]

1.4 Aims and objectives

The aim of this study is to develop a quality control method for the *Moringa oleifera* plant. For this study, *Moringa oleifera* seeds were used.

1.4.1 Specific objectives

- To isolate and characterize the constituents of the seeds of Moringa oleifera;
- To develop and validate an HPLC separation method for the isolated compounds;
- To quantify the isolated compounds in the crude extract of the seeds of *M. Oleifera*.

CHAPTER TWO

2.1 Introduction to Moringa

Moringa is a medicinal plant that is the sole genus from the flowering plant family *Moringaceae*. The *Moringaceae* family is identified by its parietal placentation, 3-valved fruit, elongated, non-dehiscent berry and winged seeds [22]. Moringa is native to African and Asian countries and it is being cultivated in different parts of the world including South Africa because of its many uses [23]. It has thirteen species with *Moringa oleifera* being the most cultivated followed by *Moringa stenopetalla* [15]. The species are divided into three categories; bottle trees, slender trees and tuberous shrubs (see Table 2.1).

The species of interest in this study is *Moringa oleifera*, commonly called the drumstick tree, horseradish tree, Mupulanga in Zimbabwe, Haleko in Ethiopia [24]. The plant is a fast growing, small, hard tree producing tuberous taproot. It has a straight trunk, whitish bark, green leaves about 1 to 2 cm long and white coloured flowers that are 10 to 25 cm long. The pods are green or reddish in colour and the seeds are round, white with a brown cover see Figure 2.1. The plant grows in tropical and sub-tropical areas, thriving well in well-drained soil at a neutral pH. The *Moringa oleifera* plant can grow up to 12 m tall [25].

Table 2.1 The different species of Moringa [26, 15]

Species	**Country of origin**	**Morphology**
Moringa arborea	Kenya	Tuberous shrub
Moringa borziana	Kenya, Somalia	Tuberous shrub
Moringa concanensis	India, Pakistan	Slender tree
Moringa drouhardii	Madagascar	Bottle tree
Moringa hildebrandtii	Madagascar	Bottle tree
Moringa longituba	Ethiopia, Kenya, Somalia	Tuberous shrub
Moringa oleifera	India	Slender tree
Moringa ovalifolia	Angola, Namibia	Bottle tree
Moringa peregrina	Ethiopia, Eritrea, Djibouti, Israel, Jordan, Somalia, Sudan, Syria, Yemen, Iran, Pakistan	Slender tree
Moringa pygmaea	Somalia	Tuberous shrub
Moringa rivae	Ethiopia, Kenya	Tuberous shrub
Moringa ruspoliana	Ethiopia, Kenya, Somalia	Tuberous shrub
Moringa stenopetalla	Ethiopia, Kenya, Somalia	Bottle tree

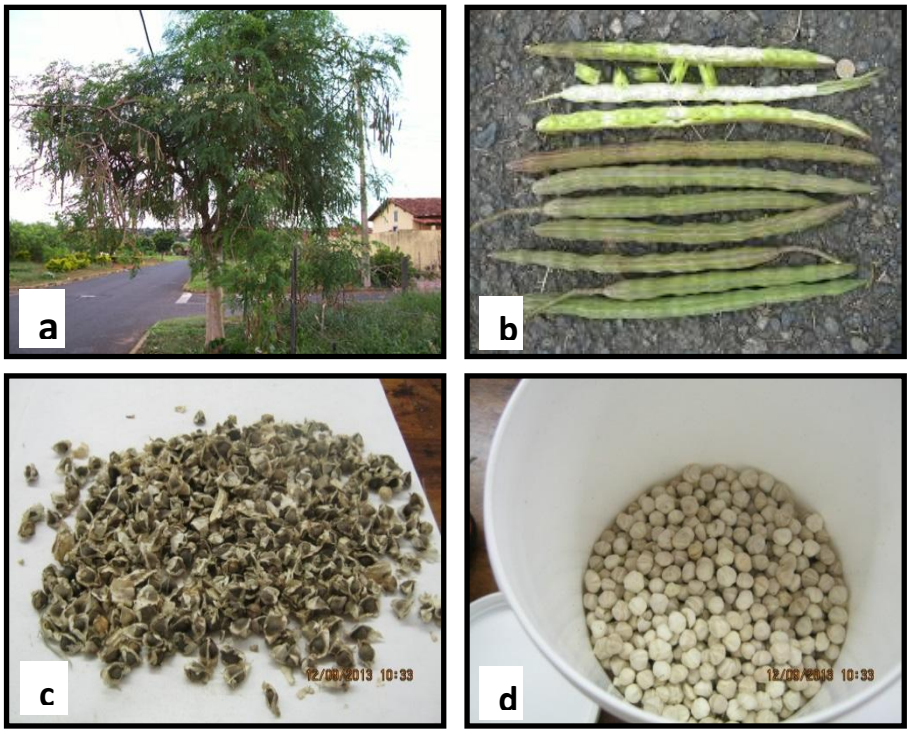


Figure 2.1 (a) *Moringa oleifera* tree [27] (b) *Moringa oleifera* pods [28] (c & d) Moringa *oleifera* seeds

2.2 Uses of Moringa

The use of Moringa as a medicinal plant dates back to ancient times. Evidence of the usage of the plant (by the Egyptians, Greeks, Indians and Romans) can be found from ancient writings dating back to 150 B.C. [29]. Moringa is often referred to as the 'magic bullet' or the 'miracle tree' because of its many uses, some of which have been proven scientifically. Every part of the plant is reported to have some use either nutritional, medicinal, environmental, and cosmetic use or all of them (Figure 2.2) **[**15]. Moringa is being used traditionally to treat various ailments such as anxiety, asthma, chest

congestion, cholera, cough, diabetes, diarrhoea, ear infections, fever, joint pains, scurvy, semen deficiency, skin infections, sore throat, tuberculosis, venous bites and more as summarized in Figure 2.3 [30, 15]. The knowledge on how to use Moringa to treat the mentioned ailments has been passed down from generation to generation.

Moringa oleifera has exhibited nutritional, therapeutic and prophylactic properties [16]. Studies of extracts from different parts of the plant have been found to have anti-inflammation, anticancer, antimalarial, antidiuretic, antispasmodic, antimicrobial properties etcetera [31, 32]. The leaves, flowers and green pods are eaten as vegetable and they are also used for the treatment of malnutrition in children and breast feeding mothers [30]. The seeds have proven effective for purification of water in areas where clean water is scarce [17]. The seed oil is used for cooking and for cosmetic purposes.

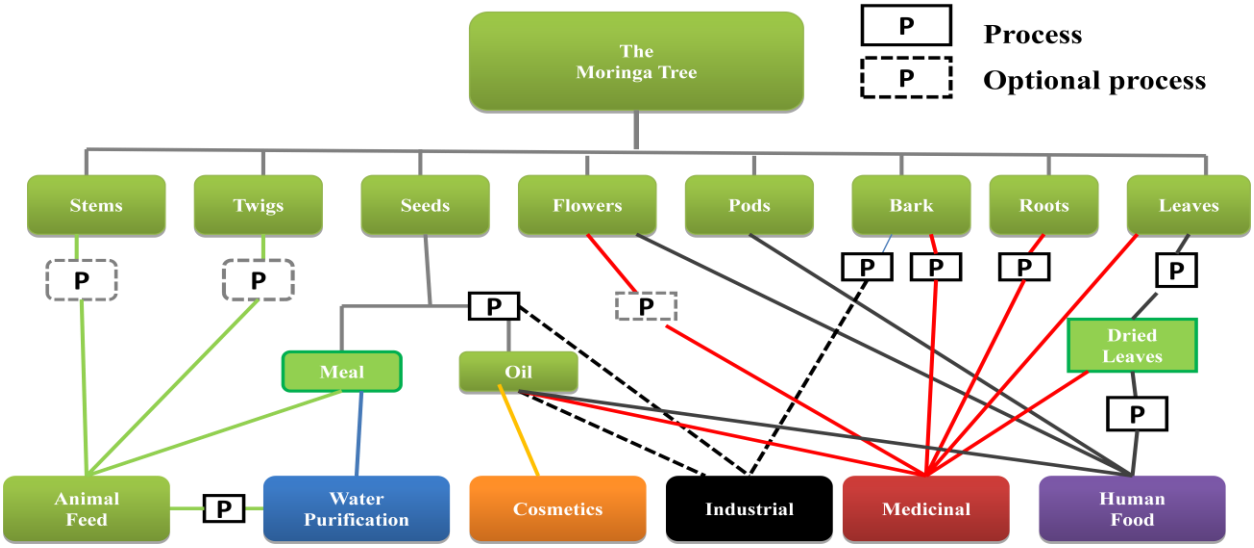


Figure 2.2 Uses of the different parts of Moringa tree **[**33]

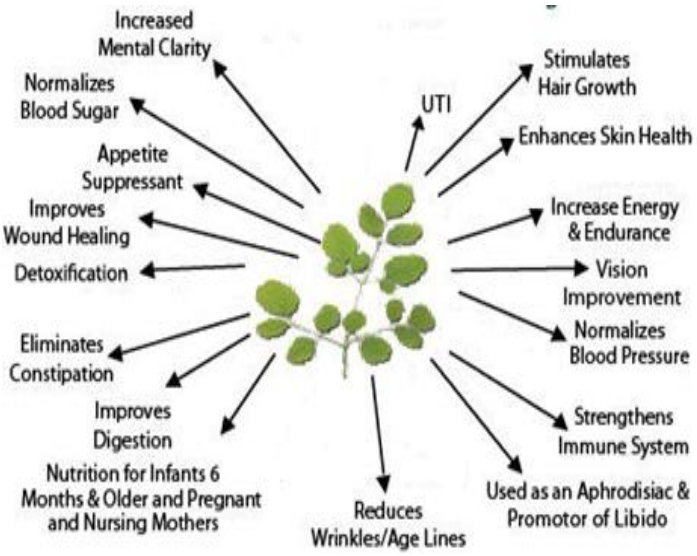


Figure 2.3 Traditional medicinal uses of Moringa [34]

2.2.1 *Moringa oleifera* in water treatment

The usefulness of *Moringa oleifera* seeds in water treatment has been shown in several studies. *Moringa oleifera* seeds yield a natural cationic polypeptide which is soluble in water. This positively charged protein acts as a coagulant by binding with the negatively charged particulate matter, reducing the water turbidity, the total water hardness, acidity and chloride content [24, 17]. A study using the two-stage clarifier tanks with the arrangement of natural filter media, coconut fiber, followed by sand media combined with powdered *Moringa oleifera* seeds as natural coagulant, resulted in significant improvement of the quality and appearance of tapioca starch waste water final effluent [35]. The water pH, biochemical oxygen demand, total suspended solids and total solids were also stabilized.

In another study *Moringa oleifera* seeds were found to remove up to 95% of the total bacteria in water. The bioactive steroidal glycoside-strophantidin isolated from the seeds is responsible for the activity. Steroidal glycoside-strophantidin is also responsible for the clarification and sedimentation of inorganic and organic matter in raw water [36]. Mangale S.M et al [17] identified the compounds benzyl isothiocyanate and benzyl glucosinolate to be responsible for the activity against bacteria and fungi in water. In their study, Ndabigengesere A. et al [37] recommended that *Moringa oleifera* seeds can be used to replace or as an alternative to alum for water treatment because they do not result in corrosion problems.

2.3 Scientific analysis of medicinal plants

Studies on medicinal plants follow the framework shown below in Figure 2.4. A crude extract is obtained from the plant and can be screened for biological activities. This is followed by separation and isolation of individual compounds from the extract. Finally the structures of the compounds are elucidated using spectroscopic techniques.

2.3.1 Extraction techniques for medicinal plants

Extraction of secondary metabolites from plant material is the most important sample preparation step in the analysis of medicinal plants [38, 39]. The extraction step involves the separation of secondary metabolites from the matrix. This is achieved by using solvents which the secondary metabolites are soluble in, whilst excluding most of the other components in the plant. There are several methods that have been developed and are used for the extraction of metabolites. First there are the traditional or conventional methods which include; Soxhlet extraction, maceration

and sonification. The modern techniques are supercritical fluid extraction, microwave assisted solvent extraction etcetera. Overall an ideal extraction method should be fast, simple and inexpensive. It should give high yields of the compounds of interest without loss or degradation and should have little or no solvent waste disposed into the environment [38,40].

The choice of solvent for extraction depends on the compounds of interest. The compound/s of interest should be highly soluble in the solvent. For example if the interest is in extracting non-polar compounds like oils, a non-polar solvent such as hexane is used as an extracting solvent. Whereas for polar compounds, a polar solvent (ethanol) is used for extraction [41]. The boiling point of the solvent is also a very important parameter to consider because the extract has to be concentrated by evaporating the solvent and hence the boiling point of the solvent should not be too high to avoid degradation of the extracted compounds. Additionally the extracting solvent should not react chemically with the extract resulting in different compounds being formed. The solvent should also be inexpensive, safe to use, environmentally friendly and suitable for reuse [42].

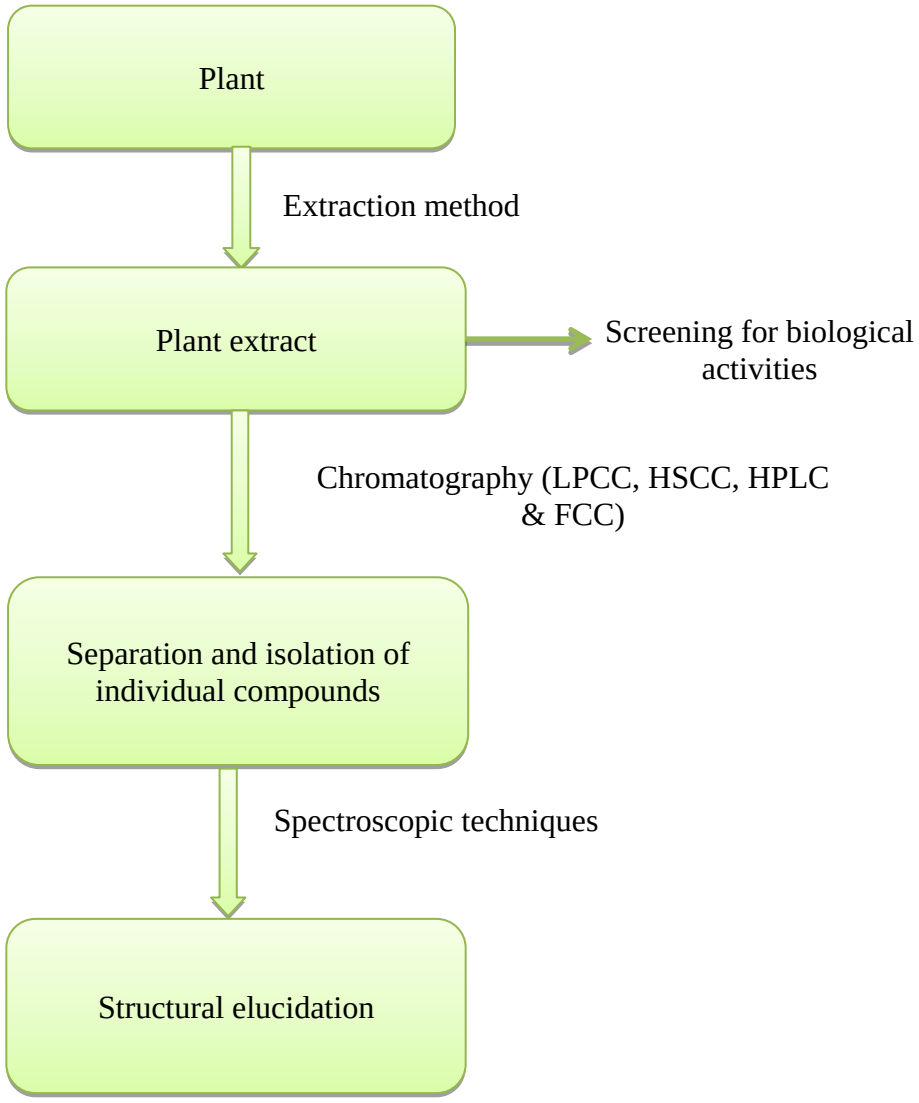


Figure 2.4 Framework followed for the analysis of medicinal plants, where LPCC= Low pressure liquid chromatography, HSCC= High speed counter current, HPLC= High pressure liquid chromatography and FCC= Flash column chromatography.

2.3.1.1 Traditional methods of extraction

Maceration

This method uses an organic solvent such as methanol or a mixture of an organic solvent and water as an extracting solvent. Extraction is performed at room temperature with occasional stirring or shaking of the mixture. The amount of solvent that is used depends on the sample size and it is usually a ratio of 1:5 (sample: solvent). The time required for extraction is 3- 4 days. With this method repeated extraction yields better results [41].

Soxhlet extraction

Soxhlet extraction uses an organic solvent or a mixture of water and an organic solvent for extraction. The solvent is heated to its boiling point and extraction of the metabolites from the sample takes place after the solvent has condensed. The method uses 100-200 mL of the solvent. Extraction usually takes between 3-18 hours [38, 41].

Sonication

Sonification uses the same solvents as the two methods described above. Moderate heat is applied to the system and extraction requires about 50-100 mL of the extracting solvent. The extraction time can be as long as about an hour [41].

The advantage and disadvantages of these methods are given in Table 2.2. Even though these methods are simple and easy to use, they however, require large amounts of the solvent. The extraction time for maceration and soxhlet extractions is long.

Table 2.2 Advantages and disadvantages of traditional or conventional methods of extraction **[**43**,** 39**].**

Extraction method	**Advantages**	**Disadvantages**
Maceration	• Simple and easy to use • Economical in terms of glassware	• A large amount of the solvent is used • It is time consuming due to long extraction time • It is not selective to the analyte
Soxhlet extraction	• The sample is repeatedly brought into contact with fresh solvent • No filtration is required after extraction • Several extractions can be set up simultaneously because the glassware is not expensive	• High solvent consumption • Long extraction time • Increased temperature may lead to degradation of compounds
Sonification	• Less solvent consumption • Shorter extraction time	• May lead to degradation of the analytes

2.3.1.2 Modern methods of extraction

Supercritical fluid extraction

Compounds of interest are separated from the matrix using supercritical fluid as the extracting solvent. When the fluid is between the gaseous and liquid state (its critical point), temperature and pressure are manipulated so that it can solubilize the compounds of interest and extract them. Carbon dioxide is used as the extracting solvent because it has near ambient critical temperature of 31°C, which avoids degradation of the compounds. Carbon dioxide is sometimes modified by ethanol or methanol to increase its polarity as the extracting solvent [38].

Microwave assisted solvent extraction

The solvent is added to the sample and heated with microwave energy. The heating results in the rupture of the cell wall of the plant sample followed by the release of the compounds to the solvent. This technique is a combination of closed-vessel microwave acid digestion and solvent extraction of metabolites from the solid sample [^{44}].

There are advantages and disadvantages associated with these methods and they are given in Table 2.3. Unlike the traditional methods of extraction, these methods are quick and only use small amounts of the solvent.

Table 2. 3 Advantages and disadvantages of modern extraction methods **[**45–47**]**

Extraction method	**Advantages**	**Disadvantages**
Supercritical fluid extraction	• Environmentally friendly • Extraction is done at low temperatures which avoids degradation of the metabolite • Carbon dioxide (CO_2) is inert and nonflammable	• There is loss of the analyte during collection of the extracted analytes • Carbon dioxide used as the extracting solvent has limitations with regards to polarity
Microwave assisted solvent extraction	• Short extraction time • Reduced solvent consumption • The method is reproducibility	• Degradation of the compounds because of high temperature • A decrease in extraction yields because of overexposure to microwave radiation

The modern extraction techniques offer more and better advantages than the traditional methods with respect to green chemistry (Tables 2.2 and 2.3). However, most institutions and laboratories do not have the instruments required for these methods because of their high cost. Hence, most extractions are still carried out using traditional methods like maceration which is popular because it is convenient, simple and easy to use. Vongsak et al. [40] suggested that maceration is more applicable to small and medium enterprises and emerging economies because of the above-mentioned reasons. Several laboratory researchers have investigated the efficiency of different extraction methods by comparing their extraction yields. Bampouli et al. [48] determined the extraction efficiencies of ultrasound assisted extraction (UAE) (a modern extraction method), Soxhlet extraction (SE), microwave assisted extraction (MAE) and supercritical fluid extraction (SFE) on the leaves of *Pistacia lentiscus var. chia* by comparing their extraction yields and antioxidant activity of the extracts. MAE was found to have the highest extraction yield and antioxidant activity followed by UAE then SE. SFE had the least extraction yield and the extract had no antioxidant activity [48].

Maceration and Soxhlet extractions were compared for their extraction efficiency on *Bacopa monnieri* using different solvents. The highest extraction yield was found from maceration using methanol [49]. Extraction from the leaves of *Mentha spicata* using the Soxhlet technique resulted in higher crude extract yield as compared to supercritical fluid extraction. However, upon quantification using HPLC the SFE extract was found to have more flavonoid compounds than that of the Soxhlet extract [50]. This was attributed to SFE being selective to a group of compounds. Maceration gave maximum amounts of total phenolics and total flavonoids when it was compared to squeezing, percolation (both traditional methods) and Soxhlet extraction.

Hence, maceration was recommended as the method of choice because it is simple, convenient and economical [40]. Based on these various reports, it can be suggested that the choice of extraction method should be based on the objective of the study. That is, whether a high extraction yield is required or specific group of compounds are targeted.

2.3.2 Separation of compounds using chromatography

The crude extract contains a mixture of different types of secondary metabolites or phytochemicals. To be able to identify and characterize these phytochemicals, they first have to be separated and purified [41]. Chromatography is a powerful technique used for separation and purification of compounds. There are different chromatographic techniques that can be used for the isolation of phytochemicals from a crude extract (see Table 2.4). The chromatographic system consists of a stationary phase and a mobile phase. The analytes are transported through the stationary phase by the mobile phase and separation of the analytes within the sample is based on their different affinities for the mobile or stationary phase [2]. For separation and isolation of secondary metabolites from natural products, preparative chromatography is used which enables separation and collection of large quantities of the compounds ranging from milligrams to grams [42].

2.3.2.1 Thin layer Chromatography (TLC)

TLC is a quick, easy and cheap method for the separation of compounds in a mixture. It is mostly used to check the number of compounds in a mixture and to assess the purity of a compound. It can also be used to confirm the identity of a compound that is present in a mixture by comparing the retardation factor (R_f) of the compound in question with that of the reference standard. The separation is based on adsorption, partition or both depending on the adsorbent used. The adsorbent which is the stationary phase is a thin layer of silica applied to a support such as glass, aluminium or plastic plate. The mobile phase is the solvent used to transport the compounds through the stationary phase by capillary action. Separation occurs as the compounds travelling through the stationary phase are adsorbed and released at different rates, depending on their affinity for the stationary phase [[51], [52]].

Thin Layer Chromatography is one of the oldest separation techniques with several advantages that include:

- It is cheap and simple to use
- It requires less time for separation
- It is sensitive
- It is able to separate several samples simultaneously
- The method can be up-scaled to column separation

2.3.2.2 Column chromatography

Separation in column chromatography (CC) is also based on adsorption and partition similar to TLC. However in CC the stationary phase is packed in a column and the mobile phase moves down the column due to gravity or external pressure. Modifying the process of CC by applying external pressure results in a technique called Flash Column Chromatography (FCC). Flash column chromatography uses a pump to generate pressure higher than the gravitational pressure, but lower than the high pressure used in high pressure liquid chromatography (HPLC). FCC results in the reduction of time for separation of compounds from hours in normal column chromatography to minutes. This method allows separation, isolation and purification of large quantities of the compounds, if required for further studies such as identification and characterization of the compound as well as pharmacological studies [53].

2.3.2.3 High Performance Liquid Chromatography

This is a separation technique similar to CC and FCC, which uses very small particle size packing (3 µm, 5 µm and 10 µm) for the stationary phase. The smaller particle size packings lead to very high back pressure and hence the need for high pressure for the mobile phase. The separation is based on the adsorption, partition, ion exchange and size exclusion or gel permeation stationary phase. The separation of the components of a mixture is achieved due to difference of the component's affinity for the stationary phase. The advantages of this method are that it is quick, efficient, automated, highly accurate and reproducible [41]. Separation of most phytochemicals can be accomplished using isocratic mode where the mobile phase composition is kept constant throughout the separation. However, gradient elution is used to

separate compounds that do not separate well under isocratic conditions and have long separation time [41]. Several detectors that have been used with HPLC which include; diode array (DAD), fixed or variable Ultraviolet/visible (UV-VIS), fluorescence (FLD), mass spectrometer (MS), refractive index (RID), and electrochemical light scattering (ELSD) detectors. The most commonly used in this work is the diode array detector [[52]]. When performing quantitative and qualitative work on the HPLC, reference standards are required except when the aim is to separate and purify the compounds in a mixture.

2.4 Phytochemical studies done on *Moringa oleifera*

Phytochemical studies are conducted to discover bioactive secondary metabolites, which may be used to treat diseases [59, 60]. These studies have gained popularity in recent years to investigate natural compounds that can be used as an alternative to synthetic drugs which are associated with adverse side effects [[56]]. Extensive phytochemical studies have been conducted on the seeds of *Moringa oleifera*. Thirty compounds that have been identified from the seeds of the plant in different areas are shown in Table 2.5 and their structures in section 2.8.1. Guevara A.P et al [16] isolated eight compounds, seven of which were already known. The compounds were isolated using flash column chromatography (FCC), and preparative HPLC was used for purification of the isolated compounds. The purity of the compounds was assessed using TLC. The structures of the compounds were elucidated using MS, IR, ^{1}H and ^{13}C NMR.

A new compound in the Moringa species 4-(β-D-glucopyranosyl-1-4-α-L-rhamnopyranosyloxy)-benzylthiocarboxamide was isolated together with known compounds 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate and methyl-N-4(α-L-rhamnopyranosyloxy) benzyl carbamate using preparative HPLC [57]. Characterization of the compounds was carried out using ultraviolet/visible spectrometry, infrared spectrometry, mass spectrometry and NMR. Oleic, palmitic and behenic acids, β-sitosterol, stigmasterol, campesterol, α- and ϒ-, tocopherols were identified in *Moringa oleifera* seeds using gas chromatography-mass spectrometer [58]. Liquid chromatography-mass spectrometry was used for the detection and identification of 4-(α-L-rhamnopyranosylosy)-benzyl glucosinolate in the seeds [31]. 4(α-L-rhamnosyloxy) phenyl acetonitrile, stigmasterol, β-sitosterol, 4-(4'-O- acetyl-α-L-rhamnosyloxy) benzyl isothiocyanate, 4-(α-L-rhamnosyloxy) benzyl isothiocyanate, squalene and sitosterol were isolated using column chromatography and preparative HPLC. The compounds were characterized using FTIR, MS, NMR and UV-VIS. [64, 65].

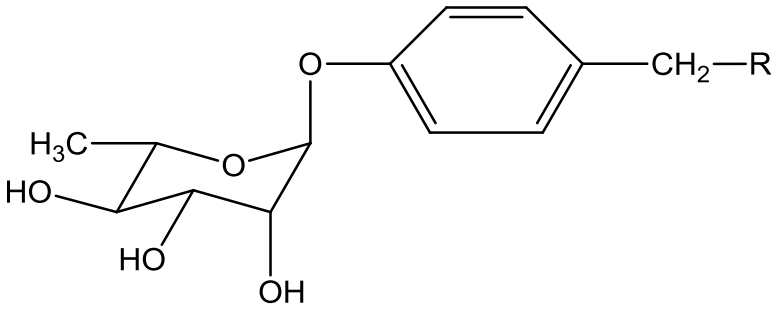
Table 2.4 Some of the compounds isolated from *Moringa oleifera* seeds

Phytochemicals	**Extracting solvent**	**Reference**
1. O-ethyl-4(α-L-rhamnosyloxy) benzyl carbamate 2. 4-(α-L-rhamnosyloxy) benzyl isothiocyanate 3. O-ethyl-4(α-L-rhamnosyloxy) benzyl carbamate 4. β- sitosterol 5. Glycerol-1-(9-octadecanoate) 6. 3-O-(6'-O-oleoyl-β-D-glucopyranosyl)-β-sitosterol 7. β-sitosterol-3-O-β-D-glucopyranoside	Ethanol	[16]
8. 4-(α-L-rhamnosyloxy) benzyl glucosinolate	Methanol	[31]
9. Moringyne 10. Mono-palmitoc	Ethanol	[61]
11. Di-oleic triglyceride		[62]

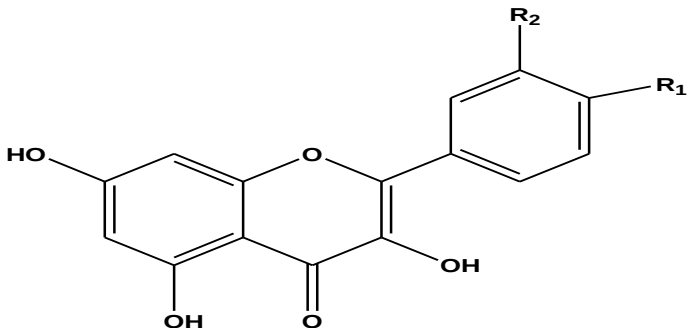
Table 2.4 …..continued

12. Oleic acid 13. Palmitic acid 14. Stearic acid 15. Behenic acid 16. Arachidic acid		[63]
17. 4(α-L-rhamnosylosy) phenylacetonitrile	Water or ethanol	[64, 16]
18. 4-(β-D-glucopyranosyl-1-4-α-L-rhamnopyranosyloxy)-benzylthiocarboxamide		[57]
19. Gallic acid 20. Quercetin	Methanol	[65]
21. Chlorogenic acid 22. Ferullic acid 23. Kaempferol 24. Vanillin	Water	66

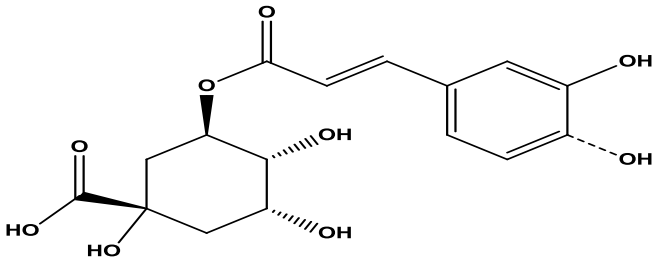
2.8.1 The structures for some of the isolated compounds are shown below



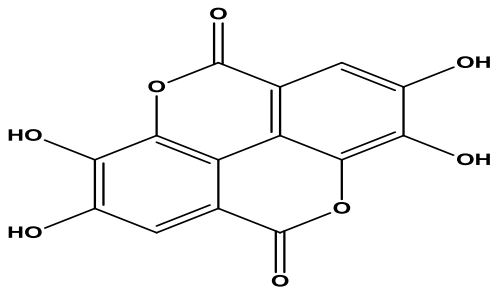
Compound name	**R**	**Reference**
4-(α-L-rhamnosyloxy) benzyl isothiocyanate	—N=C=S	[16]
O-ethyl-4(α-L-rhamnosyloxy) benzyl carbamate	—NH—C(=O)—O—CH2—CH3	[16]
O-ethyl-4(α-L-rhamnosyloxy) benzyl thiocarbamate	—NH—C(=S)—O—CH2—CH3	[16]
4-(α-L-rhamnosylosy) benzyl glucosinolate	—C(H)(=NOSO3K+)—S—Glc	[31]
4-(α-L-rhamnosylosy) phenylacetonitrile	—C≡N	[69]



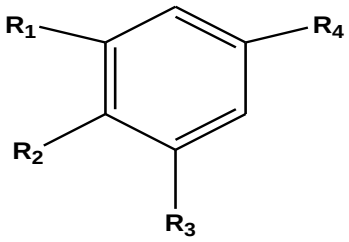
Compound name	R1	R2	Reference
Kampferol	—OCH3	—H	[71]
Quercetin	—OH	—OH	[70]



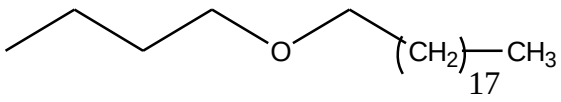
Chlorogenic acid [71]



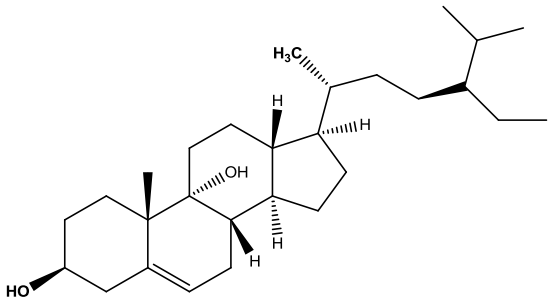
Ellagic acid [71]



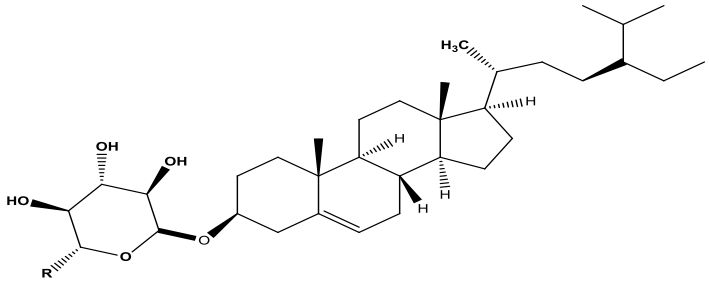
Compound name	**R1**	**R2**	**R3**	**R4**	**Reference**
Gallic acid	—OH	—OH	—OH	—C(=O)—OH	[70]
Ferullic acid	—OCH_3	—OH	—H	—CH=CH—C(=O)—OH	[71]
Vanillin	—OCH_3	—OH	—H	—C(=O)—H	[71]
Moringyne	—CH_3	—C(=O)—O—Glc	—CH_3	—H	[66]

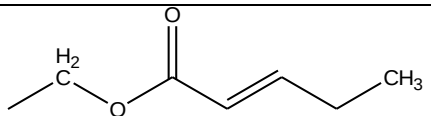
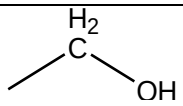


Glycerol-1-(9-octadecanoate) [16]



β- sitosterol [16]



Compound name	**R**	**Reference**
3-O-(6'-O-oleoyl-β-D-glucopyranosyl)-β-sitosterol		[16]
β-sitosterol-3-O-β-D-glucopyranoside		[16]

2.5 Biological activities of *Moringa oleifera* seeds

Several studies conducted on the seeds of *Moringa oleifera* plant have shown them to have many biological properties. Firstly *Moringa oleifera* seeds have exhibited antimicrobial activities against various species of microorganisms. They were found to have in vitro anti-fungal activities against *Trichophyton rubrum, Trichopyton mentagrophyte, Epidermophyton floccosum and Microsprorum canis* [67]. Jabeen et al. [68] reported strong activity of the crude extract from the seeds against *Fusariam solani, Bacillus subtilis* and *Staphlococcus aureus* with the following minimum inhibitory concentrations (MICs) 24.0 ± 1.50, 22.2 ± 1.15 and 24.0 ± 1.50 respectively.Moderate activity was observed against *Escherichia coli, Aspergillus niger* and *Metarhisium aniscoplae,* the MICs were 28.0 ± 1.50, 38.0 ± 0.02 and 32.0 ± 0.9 respectively. Similar work by Lar et al. [69] showed antibacterial activity of the seed extract against gram negative bacteria (*Stigella flexneri* and *Escherichia coli*). The group concluded that the *Moringa oleifera* seeds can be useful in the treatment of gastrointestinal and wound infections caused by gram negative bacteria. Oluduro et al. [70] also investigated the antimicrobial activity of the crude *Moringa oleifera* seeds extract against *E.coli, P. aeruginosa, S. aureus, Cladosporium cladosporioides* and *Penicillium sclerotigenum* using a thin layer chromatography bioassay. According to their results, 99.2 to 100% inbition against the bacteria was observed.

Secondly, *Moringa oleifera* seeds have also exhibited antioxidant property. This property was observed in a study which compared *Moringa oleifera* seeds oil with palm oil from south east Nigeria. The results showed that both oils have concentration dependent 2,2 diphenyl-1-picryhydrazy (DDPH) free radical scavenging (antioxidant) activity [71]. In their study Sighn et al. [72] demonstrated that the seeds extract is able to inhibit the OH^--dependent damage of polyvinyl chloride deoxyribonucleic acid (PVC) 18 plasmid DNA. Anticancer activity has also

been seen for *Moringa oleifera* seeds extract. Four compounds isolated from *Moringa oleifera* seeds namely:{4(α-L-rhamnosyl)-benzyl isothiocyanate; 3-O-(6'-O-oleoyl-β-D-glucopyranosyl)-β-sitosterol; β-sitosterol-3-O-β-D-glucopyranoside and niazimicin} exhibited *in vitro* inhibitory effects against Epstein-Barr Virus-early antigen (EDV-EA) activation. *In vivo* testing of niazimicin showed its antitumor activity in mouse skin [16]. *Moringa oleifera* seed oil was found to have cytotoxic activity against the following human carcinogenic cells: (breast carcinoma cells (MCF-7), colon carcinoma cells (HCT-116), hepatocellular carcinoma cells (HEG-2), larynx carcinoma cells (HEP-2) and cervical carcinoma cells (HELA) [73]. In addition, *in vitro* studies of the seeds extract, on lung cancer cells (A-549); liver (HEP-2), colon (502713HT-29) and neuroblastoma (IMR-32) cancer cells, resulted in percentage inhibition varying from 93% for neuriblastoma to 95% for liver cancer line[72]. Anti-inflammatory effects of the seeds extract were investigated by using experimentally induced acute and sub-acute inflammatory models such as (carrageenan induced paw edema method and cotton pellet granuloma method). Investigations, conducted by Fayazuddin et al. [74], Caeceres et al. [75] and Minaiyan et al. [76], found the seeds extract to have concentration dependent anti-inflammatory effects , the optimum activity was achieved at 1000 mg kg^{-1}.

Different ulcers were induced in rats to test the anti-ulcer activity of the *Moringa oleifera* seeds. Ethanol and pylorus ligation were administered in rats to induce gastric ulcers. The seeds extract was able to prevent the development of those ulcers [77]. In another study, rats were administered with 20% carbon tetrachloride to induce liver fibrosis. The rats were then treated with the seeds extract and the results showed that the extract was able to reduce liver damage as well as the symptoms of liver fibrosis[78]. The conclusion from the study was that the extract

acted against liver injury by a mechanism related to its antioxidant properties and anti-inflammatory effect [78]. Al-Malki and El Rabey [79] studied the antidiabetic effects of the seeds powder at low doses. Diabetic rats that were given a diet supplemented with the seed powder recovered to normal renal function, histology of the kidney and pancreas with no pathological changes. The activity was attributed to the antioxidant compounds found in the seeds. The authors suggested that the *Moringa oleifera* seed powder may have medical benefits if used as a food supplement for people with diabetes. Seed oil of *Moringa oleifera* has also proven to have anthelmintic properties. The anthelmintic activity is due to the presence of 72% oleic acid in the seed oil [85]. This activity was tested by administrating the Pheritima posthuma earthworm (which is similar to the worms in the intestines of humans) with the seed oil [80]. The death of the worms was observed after a short period. Rathi et al [81] reported the wound healing ability of *Moringa oleifera* seeds. The researchers suggested that the healing ability of the extract may be due to the high content of crude protein, zinc and antimicrobial compounds found in the seeds.

The ethanol extract from the seeds has exhibited analgesic effects in albino mice. This is supported by study conducted by Dipti S. [82]. In the study, he conducted the test using Eddy's Hot plate method. Mice were given the seeds extract and placed on a hot plate, an increase in pain threshold of the mice was observed. These studies show the importance of *Moringa oleifera* as a medicinal plant.

2.6 Separation methods developed for compounds in the other parts of the *Moringa oleifera* plant

Separation methods for quality control have been developed for some of the compounds in the different parts of the plant such as the leaves. Shanker et al. [83] developed and validated a separation method for niaziridin and niazirin found in the pods and leaves of *Moringa oleifera.* Reference standards used to develop this method were isolated from the plant because they cannot be obtained commercially. The method was validated for linearity, selectivity, precision and robustness. The percentage distributions were 0.015% for niaziridin in the leaves and 0.038% in the pods and 0.038% and 0.033% for niaziridin in the leaves and pods respectively[83]. A separation method for analgesics 1,3-dibenzyl urea and aurantiamide found in the roots of the plant was developed. The method was validated for accuracy, precision, limit of detection and limit of quantification [84]. Another separation method was developed for antioxidant compounds crypton-chlorogenic acid, isoquercetin and astragalin which are found in the leaves of *Moringa oleifera*. This method was validated for linearity, precision, accuracy, limit of detection and limit of quantification. Applying the method to the crude extract the percentage distribution of the compounds in the leaves was found to be 0.081, 0.120 and 0.153% (w/w) for crypto-chlorogenic acid, isoquercetin and astragalin respectively[85]. Reference standards in the development of these methods were isolated from the *Moringa oleifera* plant. The developed and validated methods can be used for quality control for pharmaceutical products of *Moringa oleifera* that contain those compounds [85,86,84]. The above-mentioned methods have been developed for quantification of those specific compounds and cannot be used for any other compound. However, the method that is being developed in this work will enable the simultaneous quantification of all the polar compounds in the extract of the *Moringa oleifera*

seeds. This is advantageous because it has been reported that compounds in an extract act in synergy to give better activity as compared to individual compounds at the same dose [87].

CHAPTER THREE

3. Experimental

This chapter outlines the materials used and the experimental procedures followed for this work. The seeds were extracted using the maceration method with a mixture of ethanol and water (1:1, v/v) followed by fractionation of the crude extract. Compounds were then isolated from the fractions and their structures were elucidated using spectrometric techniques and confirmed using published data. A separation method for the isolated compounds was developed and validated. The validated method was used to quantify the compounds in the crude extract. The experimental procedures followed in this work are shown in Figure 3.3.

3.1 Reagents and chemicals

All chemical reagents used for this work were of HPLC grade with purity of at least 99%, the chemicals for HPLC separation were purchased from Sigma-Aldrich (St Louis, USA) and those for extraction were from Merck (Darmstadt, Germany). All aqueous solutions were prepared using ultra-pure water (18.2 mΩ) from a MilliQ water purification system (Molsheim, France). The standards for some of the compounds found in the seeds of *Moringa oleifera* cannot be obtained commercially hence they were isolated from the plant.

3.2 Sampling and sample treatment

Moringa oleifera seeds were bought from a farm in Chisamba, Zambia. They were de-coated and ground into a fine powder using traditional wood pestle and mortar, Figure 3.1. The powdered seeds were air dried, put in an air tight container and stored at room temperature until further use.

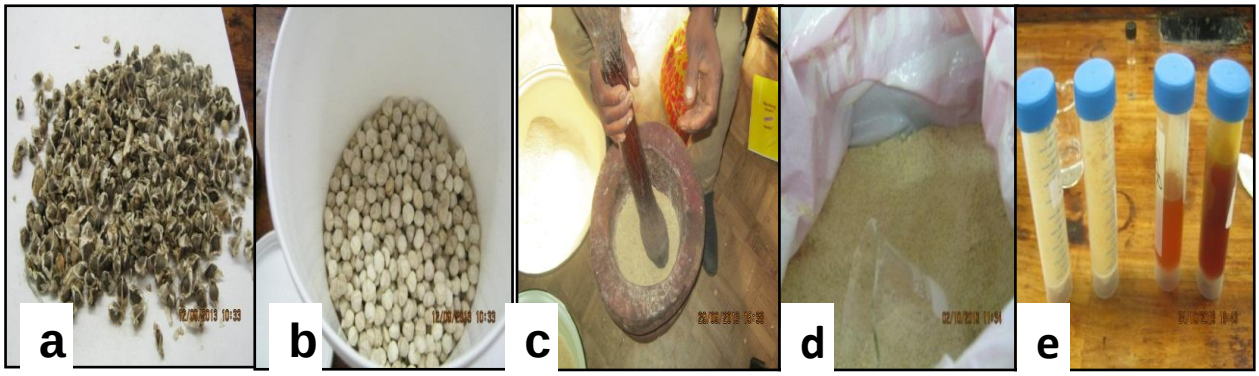


Figure 3.1 (a) *Moringa oleifera* seeds before de-coating (b) seeds after de-coating (c) Traditional wood pestle and mortar used for grinding (d) Grounded seeds (e) crude extract from the seeds

3.3 Preparation of the collected seeds for extraction

Two kilograms of dried powdered seeds were macerated in 1 L of ethanol: water (1:1, v/v) mixture for 72 hours with constant shaking. The extract was filtered using a Buchner funnel through a Whatman No. 1 filter paper under vacuum, and then concentrated under reduced pressure at 60 ºC using a rotary evaporator {Figure 3.1 (d)}. The same batch of seeds was extracted three times. The crude extract was dissolved in 100 mL of ultrapure water and

extracted using 100 mL of petroleum ether, dichloromethane, ethyl acetate or n-butanol successively. Extraction was repeated three times for each solvent. The resulting fractions were concentrated at 60 ºC using a rotary evaporator, weighed and put into labelled containers for storage at 4 °C.

3.4 Instrumentation

3.4.1 Instrumentation for preparative chromatography

An Ultimate 3000 Dionex preparative HPLC (Dionex softron GmbH, Germering, Germany) with an Ultimate 3000 binary pump, diode array detector and a fraction collector was used for chromatographic separation and isolation. The Chromeleon software (version 6.8) was used for instrument control and data processing. The isolated compounds were dried using a free Zone 2.5 freeze dryer (Labconco, Kansas City, USA). A photo of the Prep HPLC instrument is shown in Figure 3.2 (a).

3.4.2 Instrumentation for structural elucidation

A Vertex series FTIR spectrometer (Bruker optic GmbH, Hamburg, Germany) with a diamond ATR fitting was used for analysis. The compounds were analyzed in powder form and the Opus 7.3.139.1294 software was used to collect the data. The spectra were collected in transmittance mode with a resolution of 4 cm^{-1} and were averaged over 32 scans. An Agilent 500 MHz NMR spectrometer (Agilent Technologies, Waldbronn, Germany) using the Vnmrj 4.2 software was used for all the NMR spectra. The compounds were dissolved in dimethyl sulfoxide-d_6 to

acquire 1D (^{1}H and ^{13}C) data. The molecular masses of the compounds were acquired using a Bruker Impact II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The compounds were dissolved in a mixture of water and acetonitrile (1:1, v/v) prior to analysis.

3.4.3 Instrumentation for the HPLC separation and quantification method

An HPLC separation and quantification method of the isolated compounds was developed using an Agilent HPLC 1260 (Agilent Technologies, Waldbronn, Germany) which consisted of binary high pressure pump, autosampler, a thermostat column compartment, a diode array detector and a fluorescence detector. Instrument control, data collection and processing were achieved using the Chemstation (version 1.9.0) software. Chromatographic separation was achieved using XTerra C18 columns of different dimensions as specific in the following sections. A photo of this instrument is shown in Figure 3.2 (b).

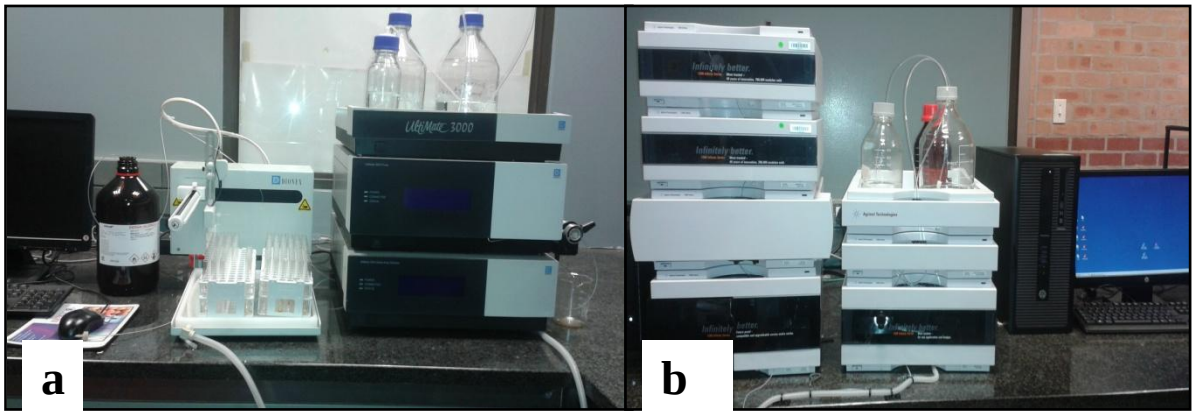


Figure 3.2 (a) The Ultimate 3000 preparative HPLC (b) The Agilent HPLC 1260

3.5 Experimental procedures

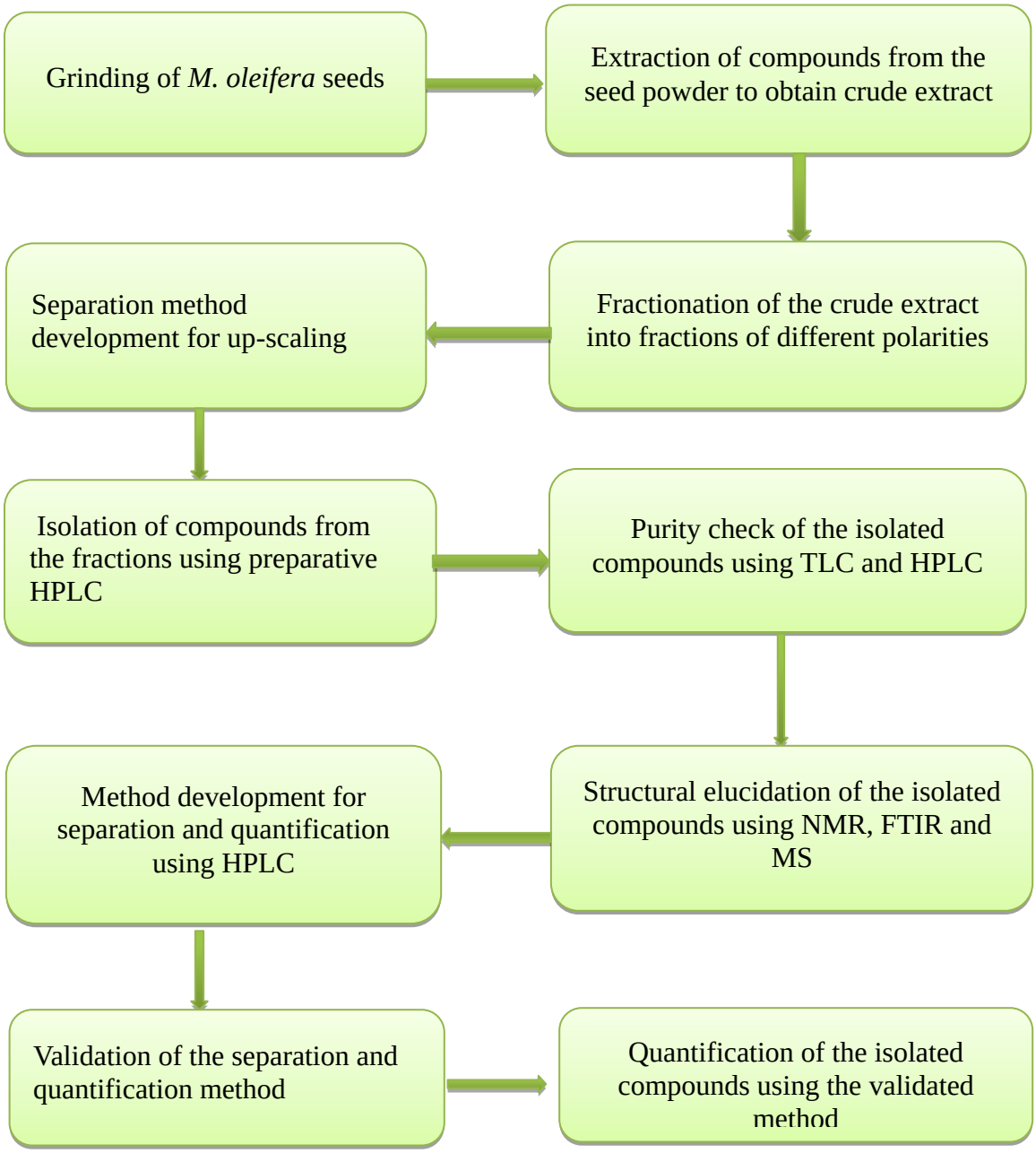


Figure 3.3 Schematic representation of the procedures

3.6 Isolation of the compounds from the different fractions

3.6.1 TLC method development

A TLC method was developed using plates with a silica gel 60 F_{254} (Merck, Darmstadt, Germany). Two milligrams of each fraction was dissolved in 5 mL of methanol and spotted on a marked TLC plate using a capillary tube. Hexane/chloroform, chloroform/methanol; chloroform/methanol/ethyl acetate mixtures at different ratios were tested as the mobile phase.

3.6.2 HPLC analytical method development for up-scaling

Compounds from different fractions obtained in Section 3.3 above were separated on a C18 XTerra column (50 mm x 4.6 mm, 5 µm) (Waters Corporation, Massachussets, USA) as shown in Table 3.1 using an Ultimate 3000 Dionex Preparative HPLC system described above. Five milligrams of each fraction were dissolved in 10 mL of acetonitrile, sonicated and filtered using a 0.22 µm PVDF (Sigma-Aldrich, St Louis, USA) syringe filter prior to injecting 10 µL on to the analytical column. Separation was achieved using chromatographic conditions as outlined in Table 3.1 and the gradient elution modes given in Table 3.2.

Table 3.1 Conditions of the HPLC analytical method for up-scaling

Column	XTerra C18 (50 mm x 4.6 mm; 5 µm)
Mobile phase	1% acetic acid ($C_2H_4O_2$) in ultrapure water (UHP H_2O) [**A**] and Acetonitrile (CH_3CN) [**B**]
Wavelength	254 nm
Injection volume	10 µL
Flow rate	1 mL/ min

Table 3.2 Gradient elution modes for the HPLC separation of the fractions for up-scaling. 1-3 are different fractions

1		**2**		**3**	
Dichloromethane fraction		**Ethyl acetate fraction**		**n-butanol fraction**	
Time (min)	**% mobile phase A**	**Time (min)**	**% mobile phase A**	**Time (min)**	**% mobile phase A**
0	90	0	95	0	95
3	82	5	88	4	88
17	68	7	73	6	80
20	65	9	36	9	65
24	50	28	28	19	15
27	36	30	14	24	10
37	9	35	14		
45	9				

3.6.3 Isolation of compounds from the fractions using preparative HPLC

The HPLC methods developed on the analytical column as described above were up-scaled to preparative HPLC methods for isolation of compounds from the fractions. An XTerra C18 (150 mm x 10 mm; 5 µm) preparative column was used for isolation. The equations below were used to calculate the different parameters for the preparative HPLC methods.

$$Fprep = Fanalytical \times \left(\frac{D^2 prep}{D^2 analytical} \right)$$

Where, **F prep** = Flow rate of the preparative column, **F analytical** = Flow rate of the analytical column, **D prep** = Inner diameter of the preparative column and **D analytical** = Inner diameter of the analytical column.

$$Preparative\ injection\ volume = Vanalytical \times \left(\frac{D^2 prep}{D^2 analytical} \right) \times \left(\frac{Lprep}{Lanalytical} \right)$$

Where **V analytical**, **D prep** and **L prep** are the analytical injection volume, inner diameter and the length of the preparative column respectively, **D analytical** and **L analytical** are the inner diameter and length of the analytical column respectively. The calculated parameters are shown in Table 3.3.

The gradient conditions from analytical to preparative methods were calculated in accordance to the Waters "Analytical to Prep Gradient Calculator" [88]. The computer gradient elution program is shown in Table 3.4; however this program was optimized to shorten the run time as shown in Table 3.5. To prepare the samples for isolation, 2 g of the dichloromethane and ethyl acetate fractions were dissolved in 10 mL of acetonitrile: water (1:1 v/v) individually and the n-butanol fraction was dissolved in 10 mL water.

Table 3.3 Conditions for isolation of compounds from the fractions

Column	XTerra C18 (150 mm x 10 mm; 5 µm)
Mobile phase	H_2O in 1% acetic acid (**A**) and CH_3CN (**B**)
Wavelength	254 nm
Injection volume	2 mL
Flow rate	4.7 mL/ min

Table 3.4 Up scaled gradient conditions for the fractions obtained using the Waters calculator

Dichloromethane		**Ethyl acetate**		**n-butanol**	
Time (min)	**% mobile phase A**	**Time (min)**	**% mobile phase A**	**Time (min)**	**% mobile phase A**
0	90	0	95	0	95
9	82	5	88	12	88
51	68	7	73	18	80
60	65	9	36	27	65
72	50	28	28	57	15
81	36	30	14	72	10
111	9	35	14		
135	9				

Table 3.5 Optimized gradient elution modes for preparative isolation of the fractions

n- butanol		Dichloromethane and Ethyl acetate	
Time (min)	**% mobile phase A**	**Time (min)**	**% mobile phase A**
0	95	0	90
15	85	6	80
21	65	30	60
30	47	33	55

The system was programmed to collect the fractions as they left the column after separation.

3.6.4 Purity of the isolated compounds

The collected compounds were tested for purity using TLC and the Agilent HPLC 1260 described in Section 3.4.3. The mobile phase for TLC was chloroform/methanol at a ratio of (60/40); the spots on the plate were visualized using iodine and a UV lamp (Camag, Muttenz, Switzerland) at a wavelength of 254 nm. An XTerra C18 column (150 mm X 4.6 mm, 3.5µm) was used for separation on the Agilent HPLC 1260 instrument with 1% acetic acid in water and acetonitrile at a ratio of (50:50) as the mobile phase under isocratic conditions. The separated compounds were monitored at a wavelength of 254 nm and were checked for percentage peak purity using Chemstation software (version 1.9.0).

3.7 HPLC method development for separation and quantification of the compounds

3.7.1 Separation conditions

Stock solutions were prepared at a concentration of 500 mg L^{-1} using standards obtained from the isolation process. An accurately weighed amount of 2.5 mg using a micro balance (Mettle Toledo,Im Langacher Greifensee, Switzerland) of each compound was dissolved in a 5 mL volumetric flask and diluted with a mixture of (1:1, v/v) acetonitrile and water. From the stock solutions, a 10 mg L^{-1} artificial mixture was prepared by taking 100 µL from each stock solution, diluting and making up to 5 mL with a mixture of (1:1, v/v) acetonitrile and water. An XTerra C18, 150 mm x 4.6 mm, 3.5µm analytical column was used to separate the compounds. Acetonitrile and 0.1% formic acid in water were the mobile phase under gradient elution mode. The injection volume of 10 µL was used while the separation was monitored at 254 nm. The separation method was optimized by varying the mobile phase composition, solvent flow rate from 0.8-1.6 mL min^{-1} and the column temperature (25- 40 ^{o}C). The optimum conditions were 1.4 mL min^{-1} and 40 ^{o}C for the flow rate and temperature respectively.

3.8. Method validation

The developed method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), precision and specificity. The calibration standard solutions were prepared using various isolated standards with purity greater than 90%. A nine point calibration curve was plotted with the standard solutions covering a concentration range of 0.3 to10 mg L^{-1} with seven replicates at each concentration level.

3.9 Quantification of the compounds in the crude extract

Fifty mg of the crude extract was dissolved in 50 mL of a mixture of acetonitrile and water (1:1, v/v), then filtered using a 0.22 µm PVDF syringe filter prior to injecting 10 µL onto a XTerra C18 (150 mm x 4.6 mm; 3.5 µm) analytical column. The prepared sample was analysed using the validated method to quantify the isolated compounds in the crude extract. The calibration data was used to calculate the percentage distribution of the isolated compounds in the seeds of *Moringa oleifera*.

CHAPTER FOUR

4. Introduction

This chapter presents the results of the work carried out on the extraction, isolation and structural elucidation of compounds from *Moringa oleifera* seeds. The use of the isolated compounds as reference standards in the development and validation of an HPLC separation and quantification method of these compounds in the seeds is also reported.

4.1 Extraction of compounds from Moringa seeds

Maceration method was used to extract compounds from the *Moringa oleifera* seed powder. A mixture of water and ethanol (1:1 v/v) was used as the extraction solvent. This combination was chosen because a mixture of an alcohol and water often results in optimum extraction of compounds from a complex matrix. These solvents are also environmentally friendly and cheap.

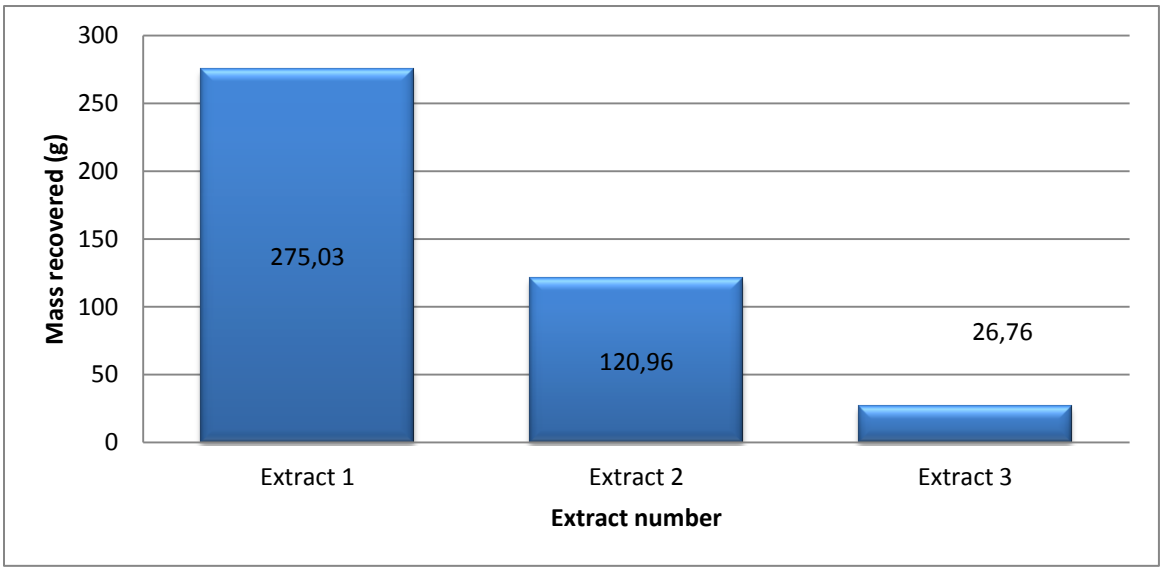


Figure 4.1 A histogram showing the different extraction yields from *Moringa oleifera* seeds.

The histogram (Figure 4.1) shows the yields of the crude extract for the three subsequent extractions. It shows that the extraction yield decreased with subsequent extractions. Based on the low extraction yield after the third extract, it was assumed quantitative extraction had been achieved. The percentage yield for the extraction was 21.15%. This is significantly higher than some extraction yields reported by other researchers. Chuang P-H et al only managed 6.4% extraction yield from Moringa seeds using 70% ethanol [67]. In another study, Paliwal R. et al achieved a 15.6% yield from the pods of *Moringa oleifera* using 80% ethanol as the extraction solvent [89].

4.2 Liquid-liquid extraction (LLE)

LLE was done in order to fractionate the extract into different polarities that are easier to work with when separating the compounds. Table 4.1 shows the percentage yields for each solvent from 400 g of the crude extract. Dichloromethane extracted most of the compounds followed by petroleum ether, ethyl acetate and finally n-butanol.

Table 4.1 Extraction yields from the crude extract using various solvents

Extraction solvent	Extraction yield (g)	% yield
Petroleum ether	48.9683	12.24
Dichloromethane	53.7426	13.44
Ethyl acetate	25.8916	6.47
n-butanol	23.8069	5.95

4.3 Isolation of pure compounds from the fractions

The process of isolating compound from the fractions usually starts with the development of a separation method using small quantities. For example to isolate compounds by preparative HPLC, a method is first developed using an analytical column which requires small volumes then up-scaled to preparative HPLC. A TLC method can be developed and up scaled to column chromatography or Flash Column Chromatography. Using these two approaches is advantageous because polar compounds are separated better on the polar stationary phase used in TLC and non-polar compounds are separated better on a non-polar stationary phase used in HPLC. In this work we report on TLC and HPLC separation methods that were developed, with the objective to upscale them to FCC and preparative HPLC respectively. However, only the up-scaling to preparative HPLC for isolation is reported.

4.3.1 TLC Method development

A TLC method was developed because the methods reported in literature are those for extraction carried out with different solvents as the ones used in this work. Different mobile phases were tested for the separation of the compounds in the fractions. The compounds had to have retardation factors (R_f) between 0.2 and 0.8 for them to be well separated when up scaled to flash column chromatography. Figure 4.2 shows some of the mobile phases that were tested. When a non-polar solvent such as 60/ 40, hexane/chloroform was used, the compounds remained at the bottom of the plate and did not move meaning that the compounds had a strong affinity for the polar stationary phase {Figure 4.2 (a)}. An attempt with ethyl acetate which is a medium polar solvent was then carried out. Different ratios of the three solvents were tested. The R_f values

achieved with 20/70/10 methanol/chloroform/ethyl acetate {Figure 4.2 (d)} ranged from 0.1 to 0.8 as indicated in Table 4.2. Compounds with smaller R_f values interact more with the polar stationary phase. This means that those compounds with smaller R_f values are more polar compared to those with higher R_f values.

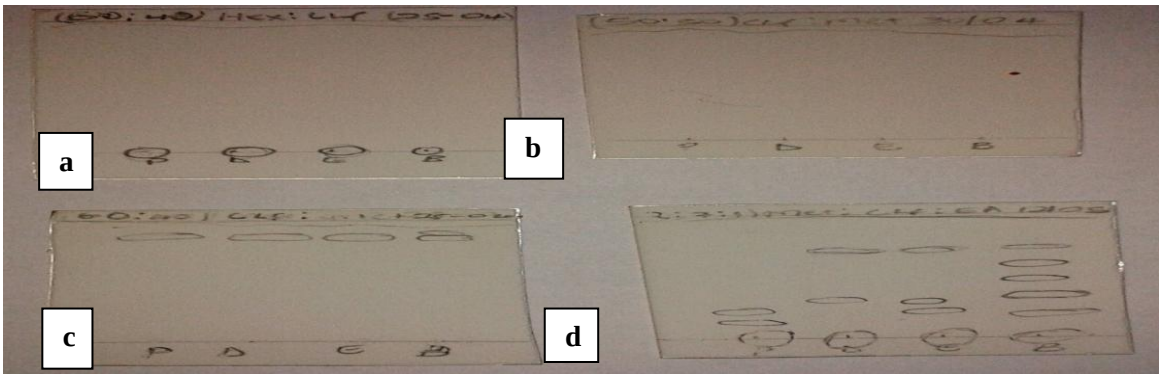


Figure 4.2 TLC separation of the fractions using different mobile phases (a) 60/40 hexane/chloroform (b) 50/50 chloroform/methanol (c) 70/30 chloroform/methanol (d) 20/70/10 methanol/chloroform/ethyl acetate

Table 4.2 R_f values using 20/70/10 methanol/chloroform/ethyl acetate

Petroleum ether fraction	Dichloromethane fraction	Ethyl acetate fraction	n-butanol fraction
0.1	0.3	0.3	0.2
0.2	0.7	0.4	0.3
		0.8	0.5
			0.6
			0.7

4.3.2 HPLC separation method development for up-scaling

The HPLC separation method was focused only on the individual fractions of the polar solvents, namely dichloromethane, ethyl acetate and n-butanol. The HPLC separation methods were developed to achieve conditions that could be up scaled for the isolation of the compounds using preparative HPLC. This approach in isolating compounds from *M. oleifera* has not been reported before. The usual approach starts by isolating the compounds using column chromatography and preparative HPLC is used to purify the compounds that could not be purified using column chromatography. Using acetonitrile and water, the compounds in the fractions (dichloromethane, ethyl acetate and n-butanol) were initially separated using a 50/50 aqueous/organic mobile phase. No separation was observed as the compounds were not retained by the non-polar stationary phase, meaning the compounds were very polar. The polarity of the mobile phase was then decreased by increasing the composition of the organic solvent. This resulted in some of the compounds being retained on the stationary phase but still without good separation. Increasing acetonitrile content to 70% resulted with two broad peaks for the n-butanol fraction which probably suggested co-elution of compounds. Gradient elution mode was then explored, starting with a higher composition of the aqueous solvent and decreasing the composition with an increase in time. The mobile phase and flow rate were optimized and their optimum values together with other conditions used are given under the experimental section in Tables 3.1 and 3.2.

Figures 4.3-4.5 show typical gradient separations of the compounds in the individual fractions. The peaks observed between 0.5 and 1 minute in Figures 4.3-4.5 represent those compounds in the fractions that are not retained by the non-polar stationary phase and hence cannot be

separated. In Figure 4.3 about ten peaks were observed probably meaning that there are ten compounds in the DCM fraction. In Figures 4.4 (ethyl acetate fraction) and 4.5 (n-butanol) eight and fourteen peaks were observed respectively. Some of the peaks in the chromatograms were showing effects of fronting, tailing and some were broad probably results from compounds being retained too long by the stationary phase and the sample being too concentrated. For this intended purpose this was not a problem because the aim at this point was to develop conditions for isolation and not for quantification of the compounds.

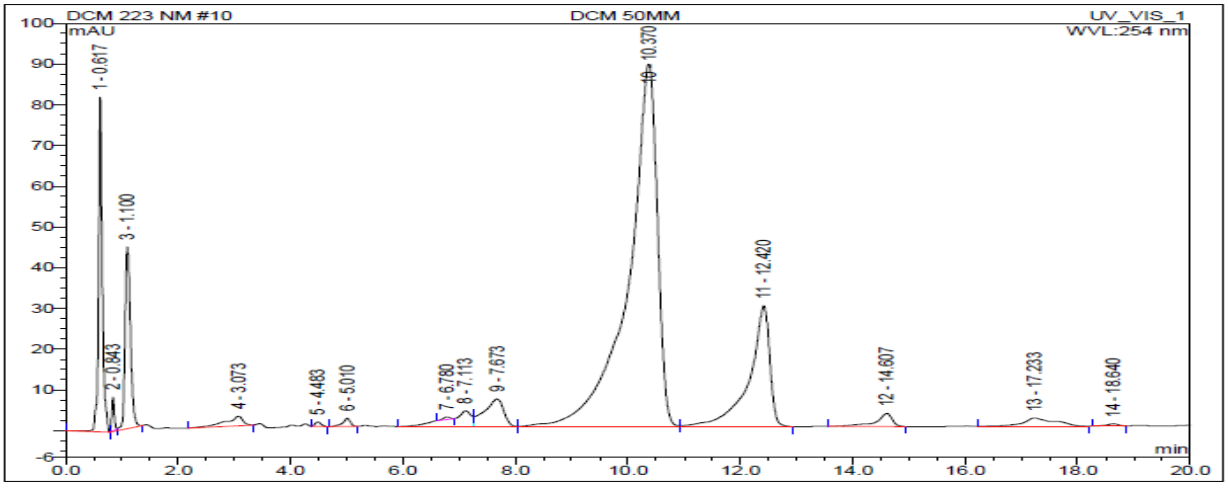


Figure 4.3 A chromatogram of a gradient separation of the dichloromethane fraction using XTerra C18 (50 mm x 4.6 mm; 5 µm) under the analytical condition given in Table 3.1 and the gradient elution mode in Table 3.2

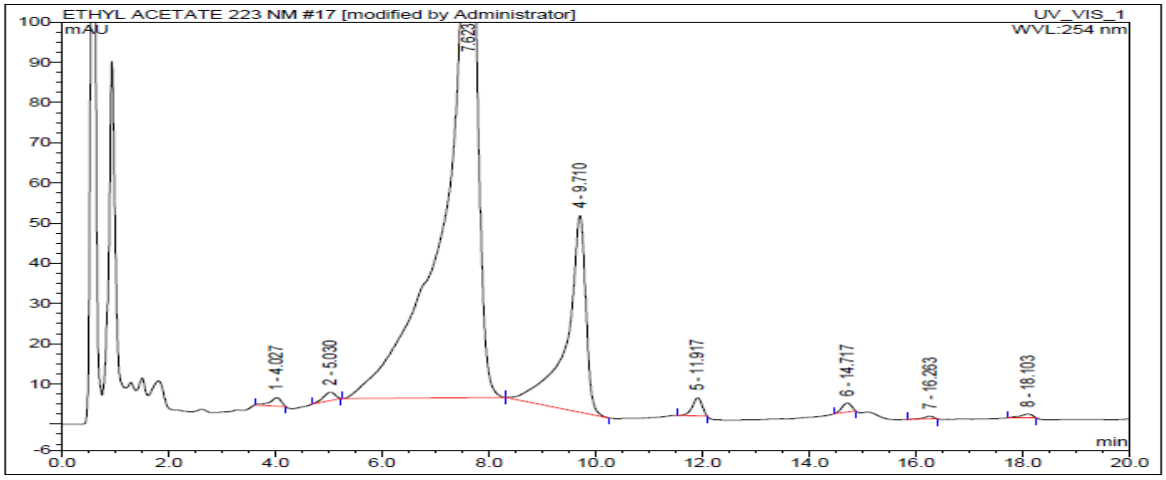


Figure 4.4 A chromatogram of a gradient separation of the ethyl acetate fraction using XTerra C18 (50 mm x 4.6 mm; 5 µm) under the analytical condition given in Table 3.1 and the gradient elution mode in Table 3.2

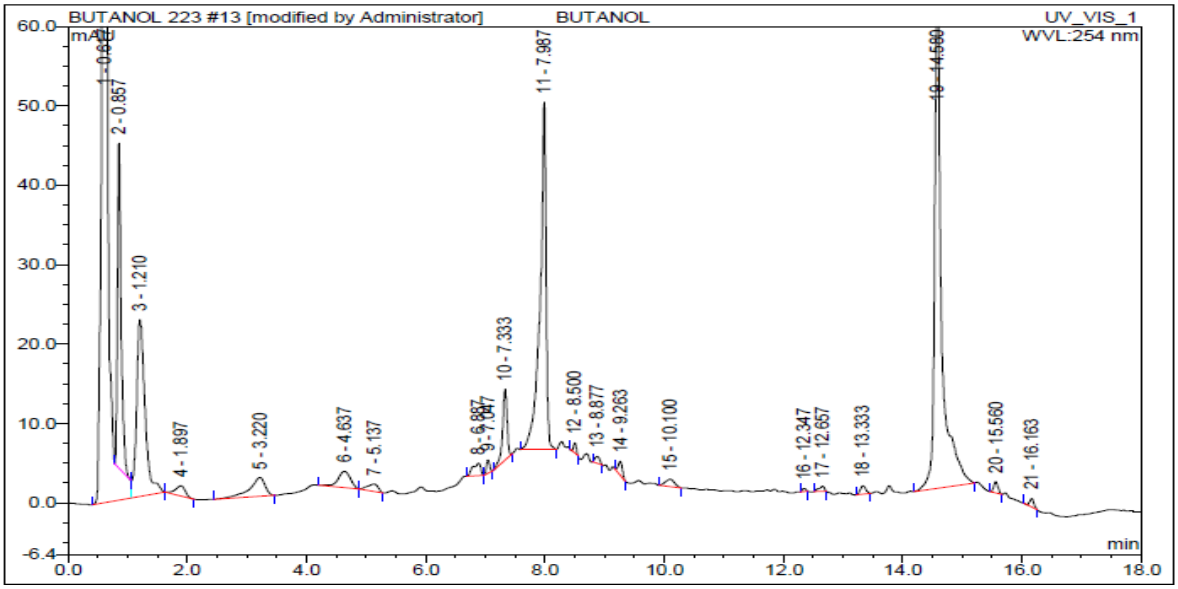


Figure 4.5 A chromatogram of a gradient separation of the butanol fraction using XTerra C18 (50 mm x 4.6 mm; 5 µm) under the analytical condition given in Table 3.1 and the gradient elution mode in Table 3.2

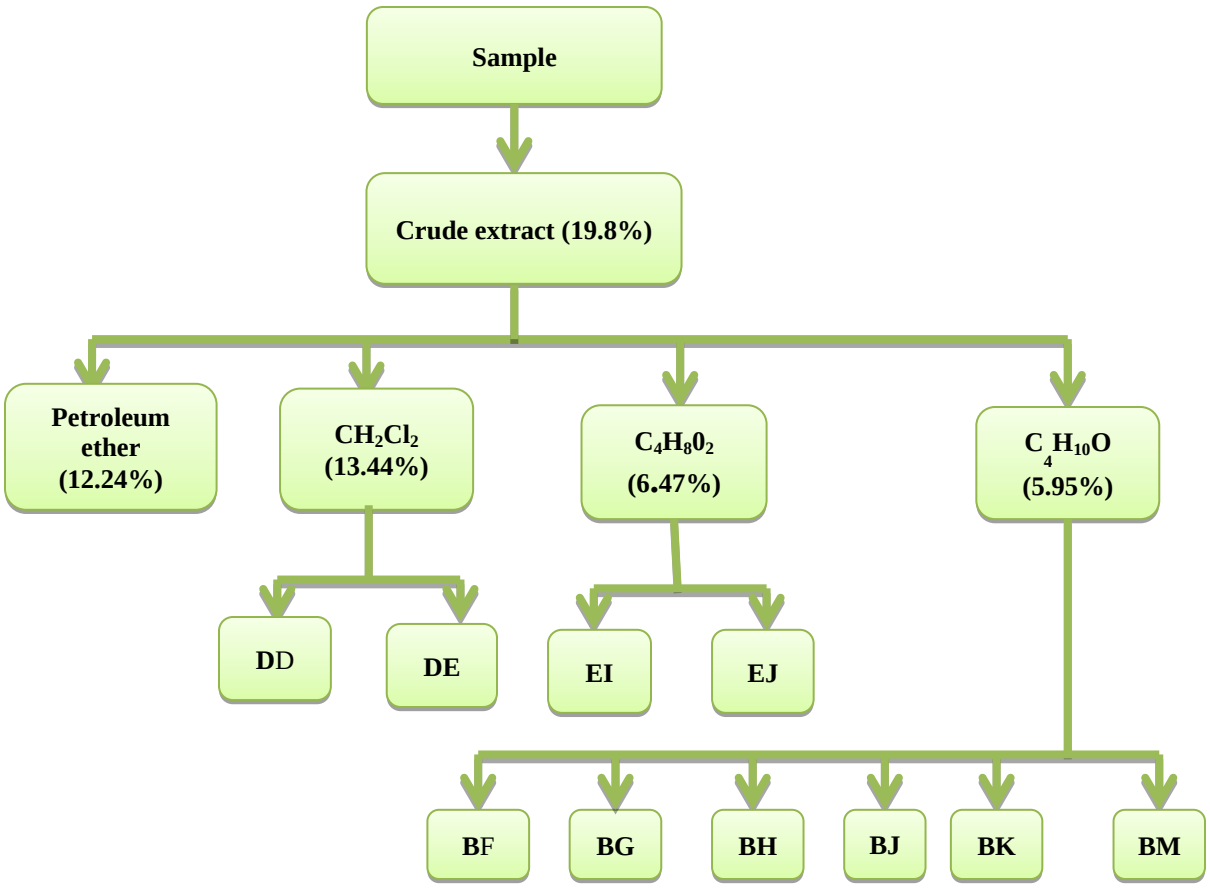


Figure 4.6 Schematic representation of the extraction and isolation results

4.3.3 Isolation of compounds using Preparative HPLC

The optimized parameters from the HPLC separation method were up scaled to preparative isolation as described in section 3.6.3. The scaled up methods were successful however the run times were too long. Therefore, the gradient conditions were optimized by adjusting the mobile phase composition to give shorter run times as shown in Table 3.5 under the experimental section. The method run times were reduced by more than 50% for all the fractions. Table 3.3 and Table 3.5 show the parameters and gradient elution modes used for preparative isolation of the compounds in the individual fractions.

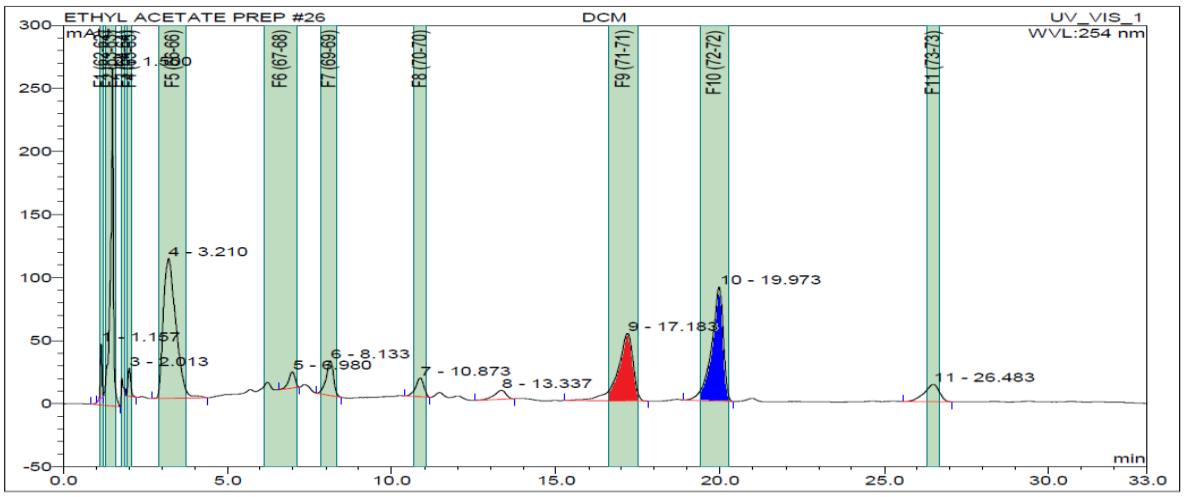


Figure 4.7 Chromatogram for the preparative isolation of compounds from the dichloromethane fraction. Using XTerra C18 (150 mm x 10 mm; 5 µm) with the conditions given in Table 3.3 and the gradient elution mode given in Table 3.5

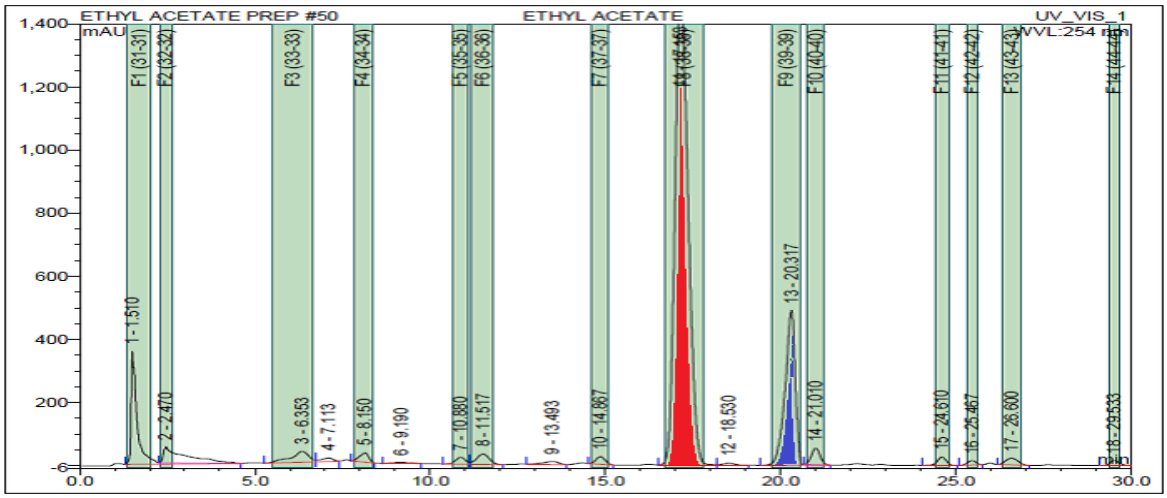


Figure 4.8 Chromatogram for the preparative isolation of compounds from the ethyl acetate fraction. Using XTerra C18 (150 mm x 10 mm; 5 µm) with the conditions given in Table 3.3 and the gradient elution mode given in Table 3.5

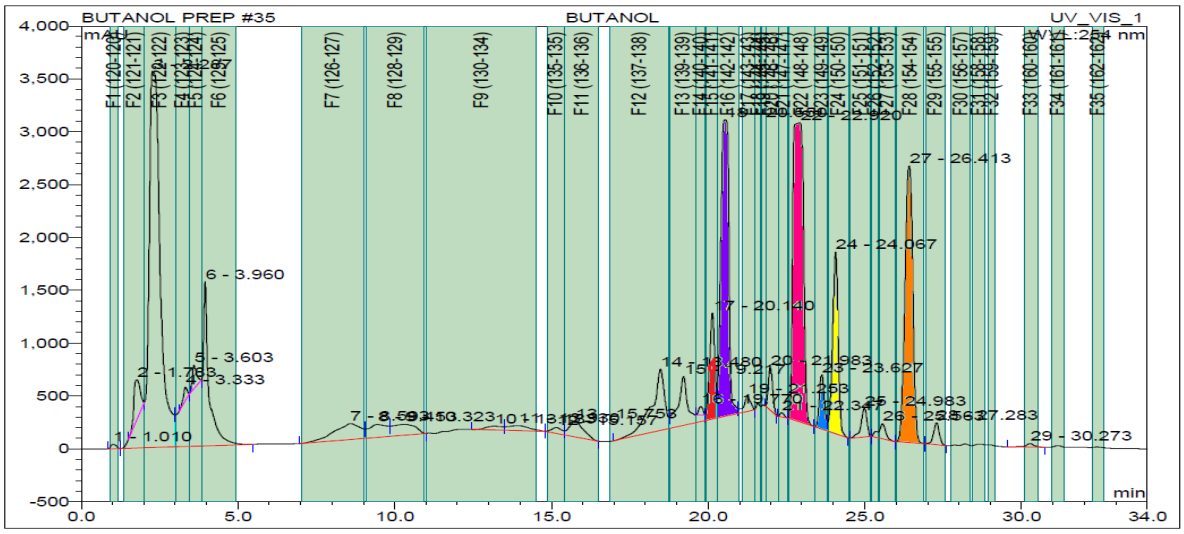


Figure 4.9 Chromatogram for the preparative isolation of compounds from the butanol fraction. Using XTerra C18 (150 mm x 10 mm; 5 µm) with the conditions given in Table 3.3 and the gradient elution mode given in Table 3.5

The same gradient elution mode (0 min 90% A (H_2O), 6 min 80% A, 30 min 60% A, 33 min 55% A) was used to isolate compounds from two fractions (dichloromethane and ethyl acetate fractions). This is because the two solvents have similar polarity and thus can extract compounds that have similar physicochemical properties. Peaks that were observed on the HPLC separation chromatograms could still be seen on the preparative HPLC separation chromatograms (Figures 4.3 and 4.4), (Figures 4.7 and 4.8). This was an indication that the HPLC methods were successfully scaled up. Some of the peaks that can be seen on the HPLC chromatogram for the n-butanol fraction (Figure 4.5) are not clearly resolved and were overlapping on the preparative HPLC chromatogram (Figure 4.9). This could be due to overloading of the column. The column was overloaded to increase the recovery of the isolated compounds.

4.3.4 Determining the purity of the isolated compounds

Once the compounds were isolated, thin layer chromatography (TLC) was used as a quick and easy method to assess the purity of the isolated compounds. Using this technique, a single spot on the TLC plate implies that the compound is pure unless there are co-eluting compounds that have the same retardation factor thus appearing as one spot. The compounds that appeared as one spot on the TLC plate Figure 4.10 (a) or those that appeared with minor impurities {Figure 4.10 (b) and (c)} were assessed further for their percentage purity using HPLC. The Chemstation software was used and had a tool to assess the purity of the peak; it also gives a 3D image of the peak to check for co-eluting peaks.

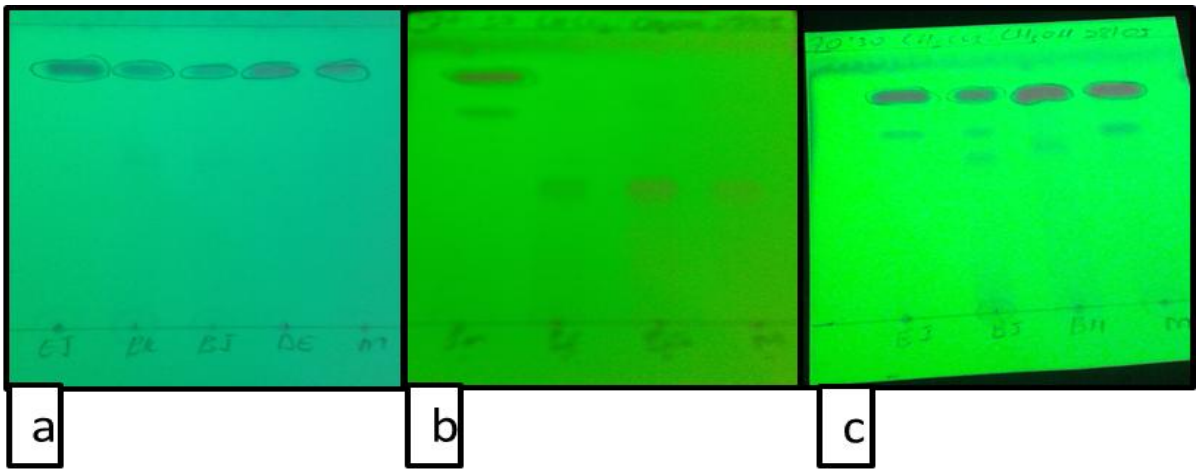


Figure 4.10 TLC plates showing the purity of the isolated compounds using 60/ 40 chloroform/ methanol viewed at a wavelength of 254 nm

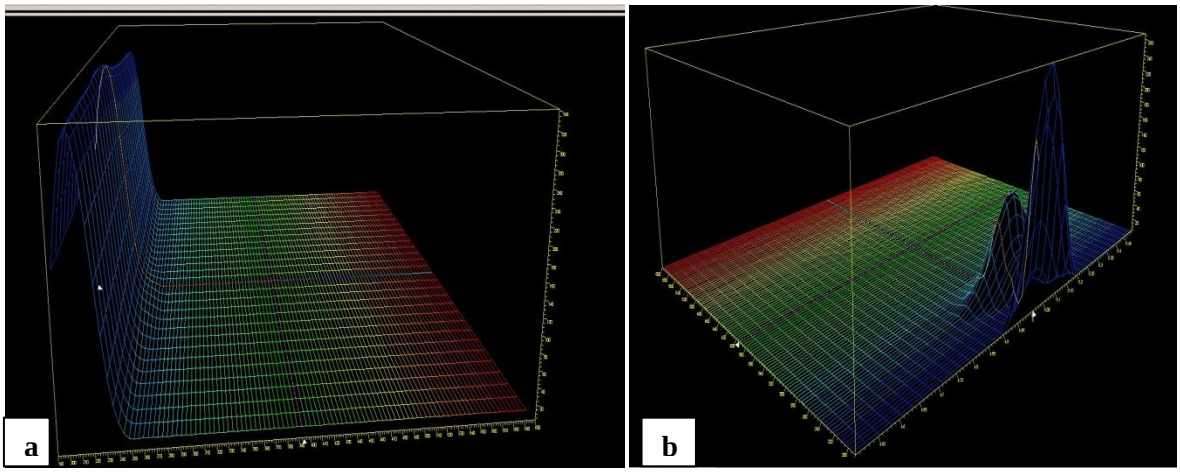


Figure 4.11 3D pictures of some peaks presenting the isolated compounds using the Chemstation software (a) Shows a peak that represents a pure compound (b) Shows a peak that represents two compounds that co-elute

The 3D image of the peak enables the viewing of the peak from all angles. As can be seen in Figure 4.11 (a) the peak is shown from the side and only one peak can be observed meaning that there is no co-elution and the compound is pure. However, in Figure 4.11 (b) shows the presence of two co-eluting peaks, where one peak is behind the other. On a one dimensional chromatogram these peaks are observed as one.

Only those compounds whose percentage purity met the set requirement of 90% or more for reference standards were characterized. The accepted compounds had percentage purities in the range 90-99% (see Table 4.3). Two compounds each were isolated from the ethyl acetate and dichloromethane fractions. The two compounds from the ethyl acetate fraction were labelled EI and EJ; from the dichloromethane fraction were labelled DD and DE. From the n-butanol

fraction, the six compounds isolated were labelled BF, BG, BH, BK, BJ and BM. These compounds are represented by the highlighted peaks in Figures 4.7, 4.8 and 4.9. Only these compounds were further characterized to elucidate their structures.

The remaining compounds (represented by peaks that are not highlighted on the chromatograms Figure 4.7, 4.8, and 4.9) had purity less than 90% and were not purified further because of the small quantities that were recovered. This will be focused on in future.

Table 4.3 Percentage purity of the isolated compounds

Compound designation	**Retention time on the preparative chromatogram**	**Mass isolated from 6 g of the fraction**	**% Purity**
DD	17.18 (Fig. 4.7)	20 mg	95.4
DE	19.97 (Fig. 4.7)	18 mg	94.9
EI	17.14 (Fig. 4.8)	12 mg	97.9
EJ	20.32 (Fig. 4.8)	30 mg	95.9
BF	19.25 (Fig. 4.9)	100 mg	91.8
BG	20.14 (Fig. 4.9)	182 mg	94.3
BH	22.92 (Fig. 4.9)	136 mg	90.3
BJ	23.63 (Fig. 4.9)	24 mg	90.5
BK	24.07 (Fig 4.9)	66 mg	89.7
BM	26.41 (Fig 4.9)	38 mg	98.9

4.4 Characterization of the isolated compounds

4.4.1 Structural elucidation using NMR, FTIR and MS.

The NMR spectra showed that compounds DD, EI, BH, BJ and BK were the same. Compounds DD and EJ were also the same. The occurrence of the same compound/s in different fractions could be due to incomplete extraction of the compound/s by the first solvent and hence they were also extracted by subsequent solvents.

Compound BG was isolated as a white powder {see Figure 4.13 (a)}. The ^{13}C NMR spectrum of this compound indicated the presence of sixteen carbon atoms (Table 4.4). The ^{1}H spectrum showed the presence of a tri-substituted aromatic ring. This is indicated by three different peaks in the aromatic region δ 6.15, 6.74 and 6.97 which were assigned to C_2, C_5 and C_6. A trans-substituted ethylene moiety was indicated by peaks at δ5.51 and 7.39. This was supported by peaks at δc 115.50 and 146.80 on the ^{13}C spectrum representing olefinic carbons bonded to an aromatic ring. The presence of two carbonyl carbons was observed at δc 166.95 and 176.17. This is supported by two strong peaks on the FTIR spectrum at 1638.47 and 1685.27 cm^{-1}, this similar stretch for carbonyl carbon in chlorogenic acid was reported by Prabhu et al.[90]. One of the carbons was assigned to an ester group observed at δc 71.52 and is bonded to $C_{5'}$. The other carbonyl carbon was assigned to the carboxylic acid group $C_{7'}$, the presence of the carboxylic acid group is supported by a peak at δ 12.34 on the ^{1}H spectrum. The presence of three carbons bonded to hydroxyl groups was observed at δc 69.23, 72.12 and 74.65, this were assigned to $C_{1'}$, $C_{3'}$and $C_{4'}$. The methylene groups assigned $C_{2'}$and $C_{6'}$ were indicated by peaks at δc 37.40 and

38.44.. This data corresponds with literature published by Suarez-Quiroz M.L et al [91] and Durust N et al [92] for the compound 3-caffeoylquinic acid. Using the mass spectrometer Q-ToF, the mass of the compound was found to be 355.1029 *m/z* $[M + H]^+$ (Appendix), thus confirming that the molecular formula of the compound to be $C_{16}H_{18}O_9$. The structure of the compound is shown in Figure 4.12.

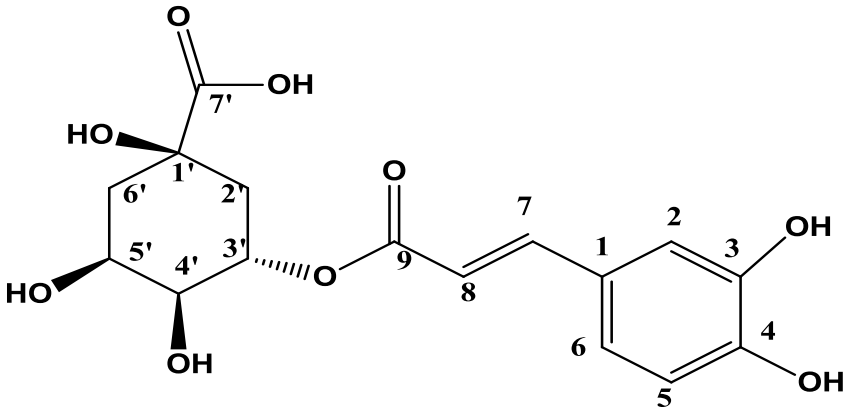
DD was isolated as a light yellow solid {see Figure 4.13 (c)}. Its ^{13}C NMR spectrum showed the presence of fourteen carbon atoms in the molecule (Table 4.4). The ^1H NMR spectrum showed the presence of an aromatic ring showing a AA'XX'spin system at δ_H 7.15 and 6.95. The double peaks were symmetrical meaning that the aromatic ring is para-substituted. The ^1H NMR spectrum also showed the presence of a sugar moiety indicated by peaks at δ 5.31, 3.78, 3.60, 3.26 and 3.42 (see Table 4.5). The presence of three hydroxyl groups on the sugar ring were indicated by peaks at δ 4.85, 4.66 and 5.02 on the ^1H NMR spectrum; and was also shown by a broad peak on the FTIR spectrum at 3297.37 cm^{-1} (Appendix). The peak on the sugar moiety (1'-H Table 4.5) is shifted downfield and appears at δ 5.31 meaning it is affected by the presence of an electronegative atom that is present in its environment. This is supported by the downfield shift of the peak representing one of the carbons on the sugar moiety at δc 98.81. These results corresponds with those of the sugar bonded to the aromatic ring via an α- glycosidic bond (α-L-rhamnosyloxy benzyl) [93].The aromatic ring is bonded at the anomeric carbon in a trans position hence the it is named α rather than β.

A peak on ^{1}H NMR spectrum at δ 9.54 indicates the presence of an NH group; this is supported by a strong peak at 1509 cm^{-1} on the FTIR spectrum, representing an NH stretch. The presence of a thiocarbonyl carbon was shown by a peak at δc 190. The double signals at δc 66.61 and 65.84; 14.74 and 14.53 indicate the presence of an ethoxy group The molecular mass of the compound using a Q-ToF mass spectrometer showed a peak at *m/z* 358.1406 $[M + H]^{+}$ (Appendix). The NMR and MS data for this compound is in agreement with the data published by Faizi et al. for the compound O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate, with the molecular formula $C_{16}H_{23}NO_6S$ [93]. This compound was reported to exist as a hybrid at a ratio of 2:1, that is indicated by the presence of double signals representing the benzyl ring and the ethoxy group; where the NH group can be either cis/trans (DD_1 and DD_2) to the thiocarbonyl group see Figure 4.12 with DD_1 being the major form.

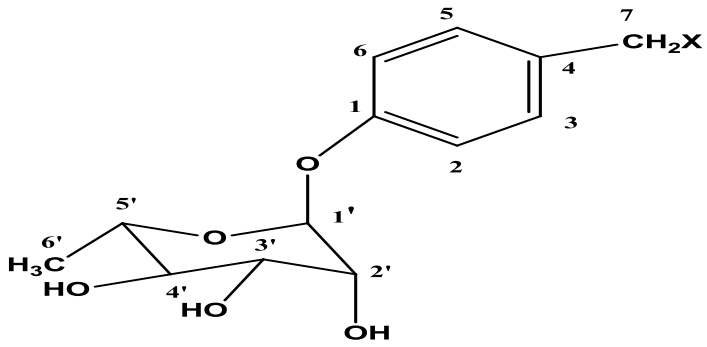
Compound BM was isolated as a yellow solid {see Figure 4.13 (d)}. The NMR data for the compound showed that it has the same parent structure as DD (α-L- rhamnosyloxy benzyl), however the ^{13}C spectrum for BM showed the presence of sixteen carbon atoms which is two more than those of O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate. The molecular mass of BM given by mass spectrometry was 386.1720 *m/z* $[M + H]^{+}$ (Appendix). The data corresponds with that of O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate with the molecular formula $C_{18}H_{27}NO_6S$ [94, 95]. This compound, like DD also exists as a hybrid (BM_1 and BM_2). O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate has never been reported in *Moringa oleifera* species but Ayyari M. et al [94] isolated it from *Moringa peregrina* species the structure is shown in Figure 4.12. O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate and O-

butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate are reported to have moderate antitrypanosomal activity [94].

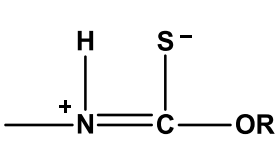
Compound DE was isolated as a colourless solid {Figure 4.13 (b)}. This compound also has the same parent structure as DD which is (α-L-Rhamnosyloxy) benzyl). However the peak representing the NH group observed in the other two spectra is missing here. Instead a peak at δc 129.25 representing the isothiocyanate group is observed. The same chemical shift for an isothiocyanate group is reported by Eilert et al. [96]. This is supported by a medium peak at 2169 cm^{-1} in the FTIR spectrum which is characteristic of the stretching frequency of an isothiocyanate group.The peak at δc 129.25 ppm represents an isothiocyanate group. The presence of the isothiocyanate group is also shown by the strong peak at 2081 cm^{-1} in the FTIR spectrum. The molecular mass of the compound was found to be 334.0816 *m/z* $[M + Na]^+$ (Appendix) corresponding to the molecular formula $C_{14}H_{17}SO_5N$. The NMR data together with the mass spectrometry data corresponds to that published by Ragasa et al[60] and Eilert et al [96] for the compound 4(α-L-rhamnosyloxy)-benzyl isothiocyanate with the structure shown in Figure 4.12. 4(α-L-rhamnosyloxy)-benzyl isothiocyanate, O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate and 3-caffeoylquinic acid are reported to possess antitumor activity [60, 97].

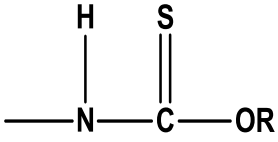


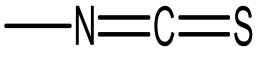
BG



X







DD_1: R= $-CH_2CH_3$ DD2: R= $-CH_2CH_3$ DE

BM_1: R = $-CH_2CH_2CH_2CH_3$ BM2: R= $-CH_2CH_2CH_2CH_3$

Figure 4.12 Structures of the isolated compounds from different fractions of *Moringa oleifera* seeds

Table 4.4 ^{13}C NMR data for the compounds BG in DMSO- d_6 at the operational frequency of 125 MHz

Carbon	**BG (δ ppm)**
1	126.81
2	116.00
3	146.20
4	149.58
5	116.96
6	122.61
7	146.80
8	115.50
9	166.95
1’	74.65
2’	38.44
3’	69.23
4’	72.12
5’	71.52
6’	37.40
7’	176.17

Table 4.5 ^{13}C NMR data for the compounds BG in DMSO- d_6 at the operational frequency of 125 MHz

Carbon	DD (δ ppm)	DE (δ ppm)	BM (δ ppm)	Carbon	DD (δ ppm)	DE (δ ppm)	BM (δ ppm)
1	155.61	156.36	155.56	5’	69.89	69.99	69.86
	155.58		155.01				69.69
2,6	116.71	117.13	116.71	6’	18.35	18.36	18.29
	116.78				18.36		18.32
3,5	129.17	128.40	129.18	$-OCH_2CH_3$	65.84		
	129.26		129.02		66.61		
4	131.84	129.27	132.11	$-OCH_2CH_3$	14.53		
	132.01		131.86		14.74		
7	45.54	47.964	47.56	$-OCH_2CH_2CH_2CH_3$			70.44
	47.60		45.54	$-OCH_2CH_2CH_2CH_3$			30.76
8	188.39		190.60				30.56
	190.45		188.53	$-OCH_2CH_2CH_2CH_3$			19.00
1’	98.81	98.72	98.77				18.95
			98.74	$-OCH_2CH_2CH_2CH_3$			14.06
2’	70.65	70.81	70.60				13.96
3’	70.85	70.58	70.79				
4’	72.22	72.16	72.19				

Table 4.6 ^{1}H chemical shifts (ppm) and the coupling constant (Hz) given in brackets for the compounds BG in DMSO- d_6 at the operational frequency of 500 MHz

Proton	**BG**
2-H	6.97 dd (1.76, 1.76 ,1.76)
5-H	6.75 s
6-H	6.73 s
7-H	7.42 s
8-H	5.51 s
2'-H	1.96 m
3'-H	6.54 t (7.03)
4'-H	3.89 s
5'-H	4.91 d (5.27)
6'-H	1.75 dd (3.81, 3.81)
3-OH	9.57 s
4-OH	9.13 s
1'-OH	5.04 dd (7.03, 6.44)
3'-OH	4.09 s
4'-OH	4.75 d (5.85)
COOH	12.34 s

Table 4.7 ^{1}H chemical shifts (ppm) and the coupling constant (Hz) given in brackets for the compounds DD, DE and BM in DMSO-d_6 at the operational frequency of 500 MHz

Proton	**DD**	**DE**	**BM**
2,6-H	6.95 t (1.47)	7.04 tt (2.69, 2.46)	6.95 m
	6.97 d (3.91)		6.97 dd (3.92, 3.91)
3,5-H	7.15 dt (3.92, 1.96)	7.29 dd (2.45, 2.45)	7.13 d (8.31)
	7.20 tt (1.71, 2.20)		7.19 d (8.31)
7-H	4.21 m	4.82 s	4.20 d (6.36)
	4.54 m		4.53 d (5.87)
1'-H	5.31 s	5.36 d (1.47)	5.31 s
2'-H	3.78 s	3.80 s	3.78 s
3'-H	3.60 m	3.60 dd (2.93, 2.93)	3.60 t (4.65)
4'-H	3.26 dd (3.42, 3.42)	3.42 m	3.25 ddd (5.38, 5.38, 5.38)
5'-H	3.42 dd (3.42, 4.18)	3.26 t (6.20)	3.42 ddt (2.40, 2.03, 4.90)
6'-H	1.07 m	1.07 d (6.36)	1.07 d (6.36)
			1.06 d (6.36)
2'-OH	5.02 s	5.05 s	5.02 d (4.30)
3'-OH	4.66 m	4.87 s	4.72 d (5.38)
4'-OH	4.85 d (7.33)		4.85 d (5.38)
$-OCH_2CH_3$	4.37 m		
$-OCH_2CH_3$	1.24 ddd (4.40, 4.40, 4.40)		
	1.19 dt (2.54, 3.42)		
$-OCH_2CH_2CH_2CH_3$			4.30 d (6.36)
			4.33 d(6.36)
$-OCH_2CH_2CH_2CH_3$			1.56 m
$-OCH_2CH_2CH_2CH_3$			1.33 tt (7.58. 7.34)
			1.20 tt (7.34, 7.34)
$-OCH_2CH_2CH_2CH_3$			0.88 t (7.34)
$-OCH_2CH_2CH_2CH_3$			0.81 t (7.34)
-NH	9.54 d (4.89)		9.56 tt (6.11, 6.12)

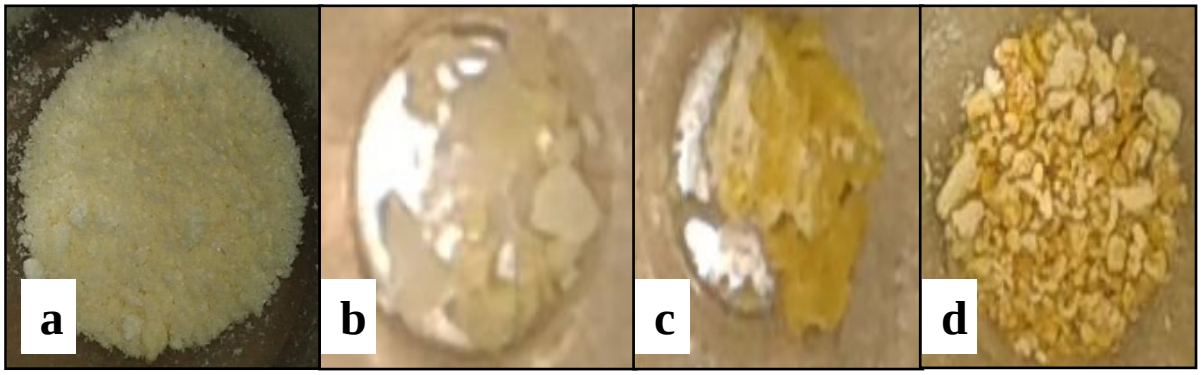


Figure 4.13 (a) BG (b) DE (c) DD (d) BM

4.5 HPLC separation method development for quantification of the isolated compounds

The pure isolated compounds were used as reference standards to develop an HPLC separation method for their quantification in the *Moringa oleifera* seeds. When developing an HPLC separation method, certain parameters have to be optimized. The parameters optimized in this study are the mobile phase composition, flow rate and the column temperature. The starting point for method development was with isocratic conditions using 50/50 (0.1% formic acid in water and acetonitrile) and a flow rate of 1 mL min^{-1} under ambient temperature (25ºC). The compounds were injected individually to check their retention times under these conditions. The retention times of the compounds were as shown in Table 4.6.

Table 4.8 Retention times of the compounds under isocratic condition (50/50), (water/acetonitrile)

Compound name	Retention time (min)
3-caffeoylquinic acid	3.57
4(α-L-rhamnosyloxy)-benzyl isothiocyanate	7.36
O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate	11.46
O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate	23.85

The observed retention times show that 3-caffeoylquinic acid was more polar than all the compounds and hence had less interaction with the non-polar stationary phase as compared to the other compounds. O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate was the least polar because it was retained for a longer period by the non-polar stationary phase than the other compounds. The compounds had different retention times meaning it was possible for them to be separated under isocratic conditions. The next objective was to reduce the analysis run time. This was achieved by optimizing each of the following parameters individually: mobile phase composition, mobile phase flow rate and the column temperature.

4.5.1 Optimization of the mobile phase composition

Mobile phase composition was the first parameter to be optimized and it was observed that increasing the composition of the organic solvent decreased the analysis run time. A mobile phase composition of 60/ 40 (water/ acetonitrile) had a run time of 18 min which was still long (chromatogram not shown). Gradient elution mode was then investigated in order to reduce the elution time. Figures 4.14- 4.18 are chromatograms obtained when using different mobile phase {0.1 % formic acid in water (A) and acetonitrile (B)} compositions that were explored. In all the

chromatograms: BG= 3-caffeoylquinic acid, DE= 4-(α-L-Rhamnosyloxy) benzyl isothiocyanate, DD= O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate and BM= O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate.

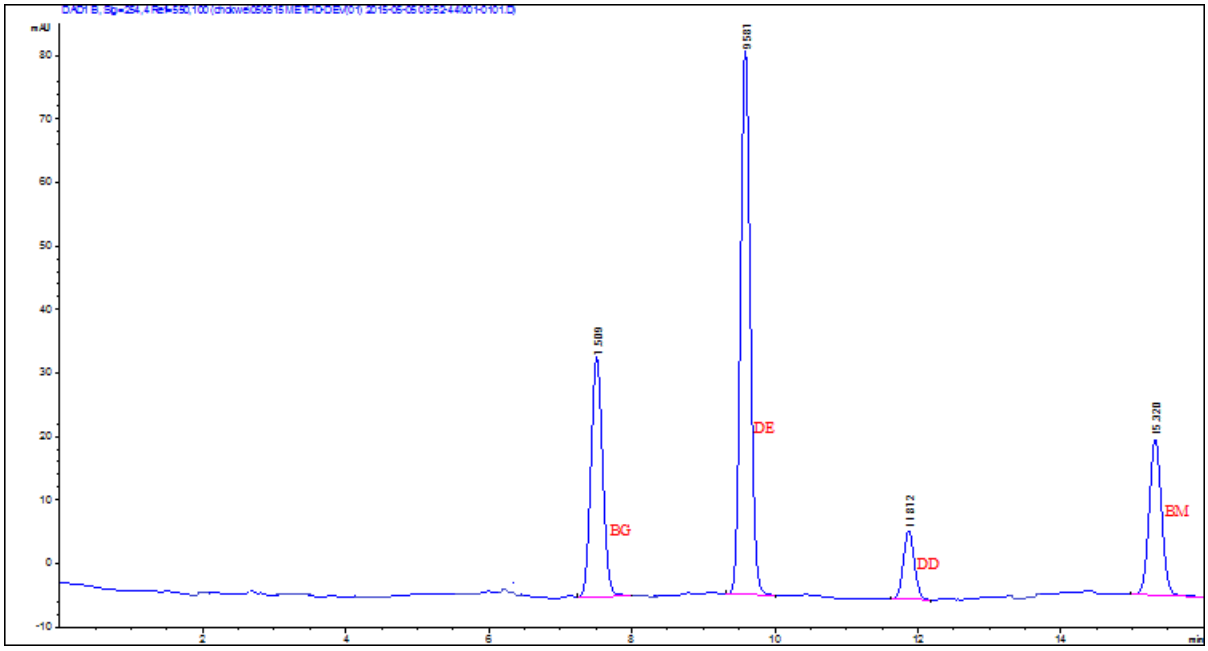


Figure 4.14 Chromatogram for the standard compounds under the following conditions. Gradient elution mode commenced at 0 min 90% (A), 6 min 60% (A), 12 min 50% (A), 14 min 40% (A), 20 min 10% (A). Flow rate: 1 mL min^{-1}, temperature: 25 ºC.

In Figure 4.14 the run time was still significantly long with the first peak eluted at 9.94 minutes and the most retained compound at 15.96 minutes. Increasing the organic solvent content in the mobile phase, the gradient elution mode shortened the separation time with the first compound eluting earlier, just after 5 minutes. The separation time was reduced from 15 min to about 13 min (Figure 4.15).

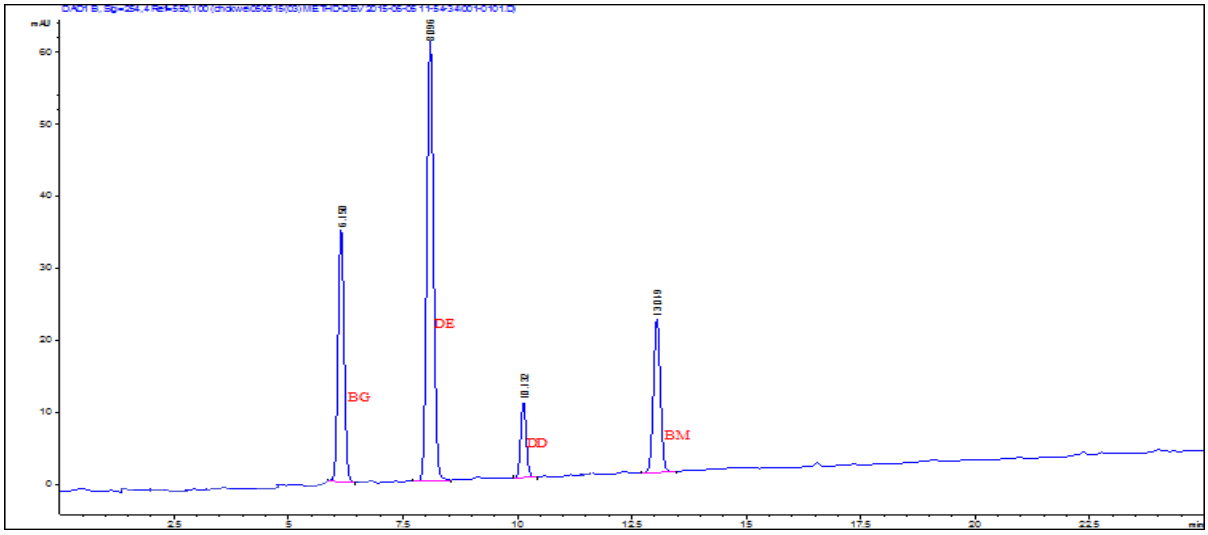


Figure 4.15 Chromatogram for the standard compounds under the following conditions. Gradient elution mode commencing at 0min 75% A, 3 min 70% (A), 8 min 55% (A), 12 min 50% (A), 20 min 35% (A). Flow rate: 1 mL min^{-1}, temperature: 25 ºC.

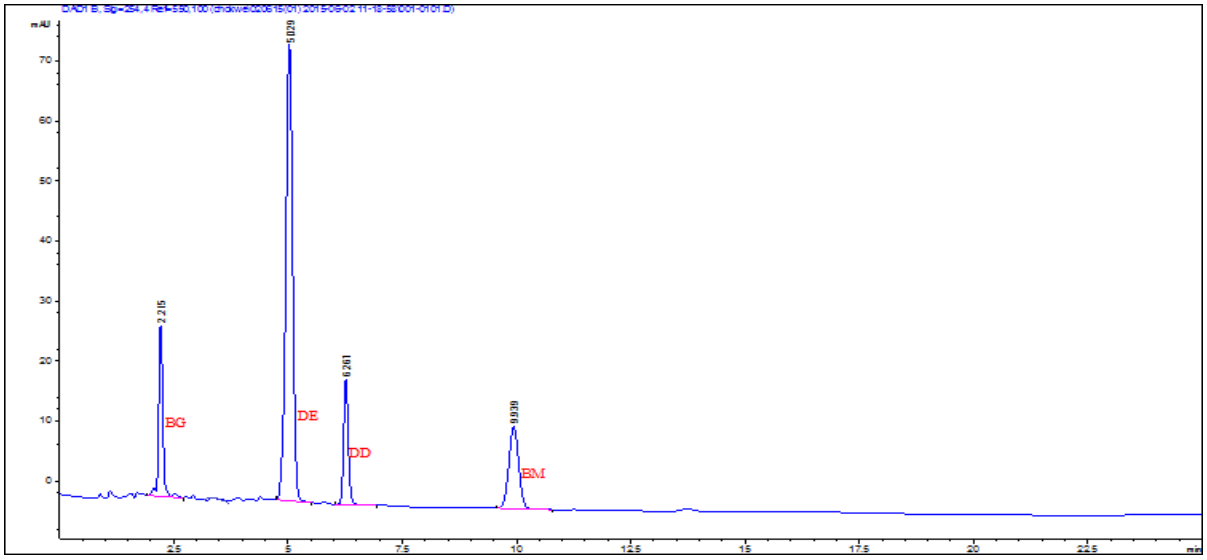


Figure 4.16 Chromatogram for the standard compounds under the following conditions. Gradient elution mode commencing at 0 min 70% (A), 2 min 65% (A), 5 min 55% (A), 8 min 45% (A), 10 min 40% (A). Flow rate 1 mL min^{-1}, temperature 25 ºC.

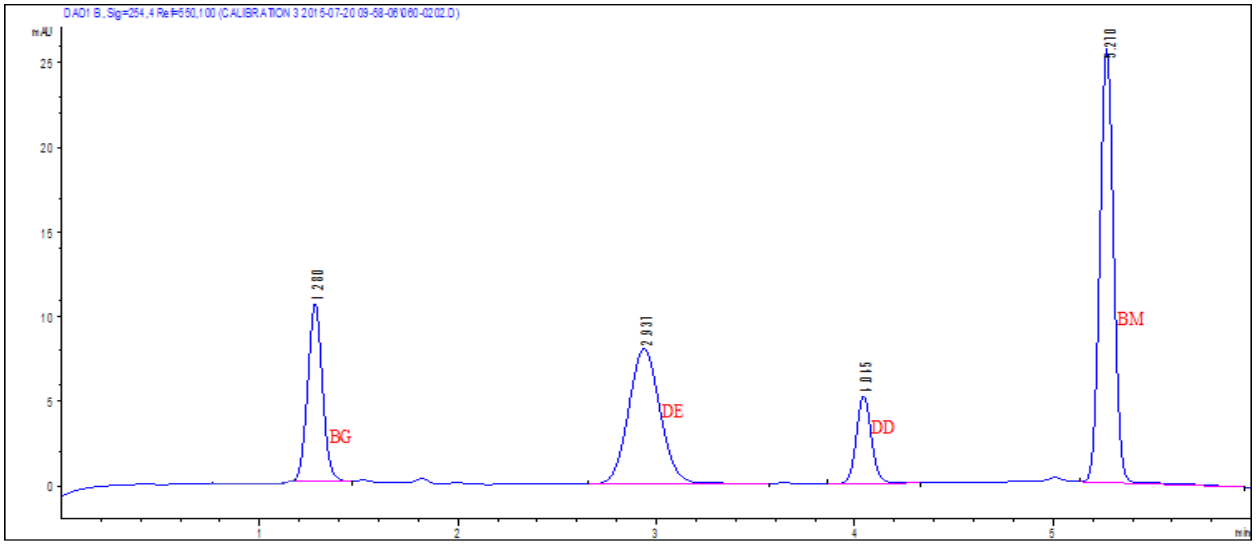


Figure 4.17 Chromatogram the standard compounds under the following conditions. Gradient elution mode commencing at 0 min 65% (A), 1 min 65% (A), 2 min 55% (A), 3 min 55% (A), 6 min 45% (A). Flow rate 1 mL min^{-1}, temperature 25 ºC.

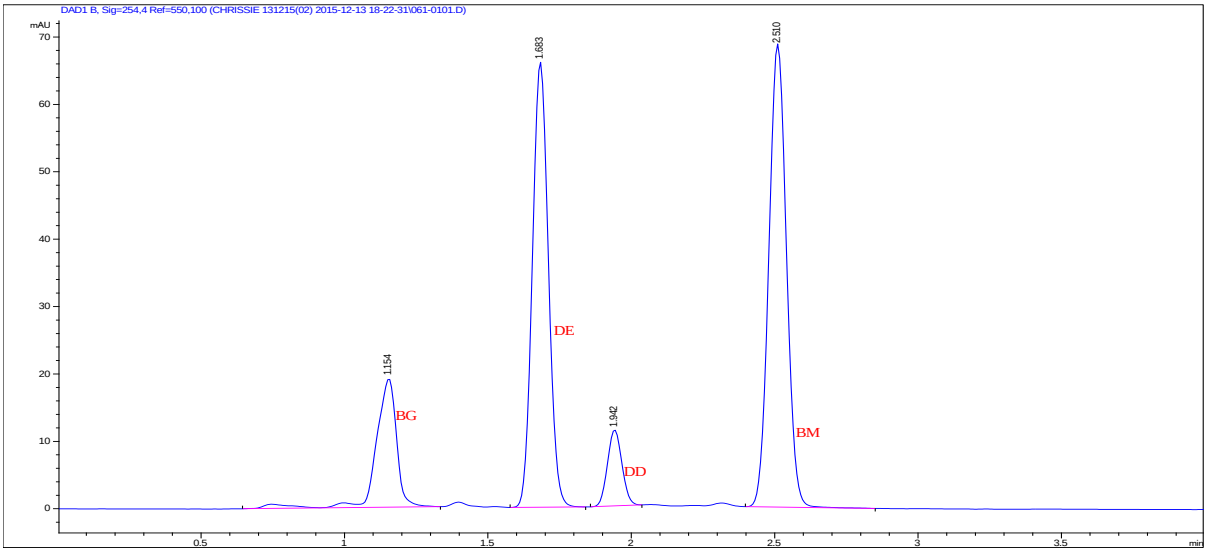


Figure 4.18 Chromatogram representing the separation of the compounds under the following conditions. Gradient elution mode commencing at 0 min 65% (A), 1 min 55% (A), 3 min 55% (A). Flow rate 1.4 mL min^{-1}, temperature 40 ºC.

The mobile phase composition was optimized further by initially starting with a higher percentage of the organic solvent and increasing it over a short period to give the separation within 10 minutes (Figure 4.16). However, the second compound was eluting 4 minutes after the first compound indicating that there was still a reasonable window for the later eluting compounds to be eluted faster without compromising the resolution. Further manipulation of the mobile phase by increasing the organic content in the mobile phase resulted with shorter separation times of less than 6 minutes (Figure 4.17) and 3 minutes (Figure 4.18). Despite the fact that the shortest separation time of 3 minutes was favourable and most economical, it was noted that when applied to the crude extract, it had limitations since the first eluting compound (3-caffeoylquinic acid) was overlapping with interferences thus making it impossible to quantify. Therefore the gradient elution mode in Figure 4.18 was used.

4.5.2 Optimization of mobile phase flow rate and column temperature

|Flow rates between 0.7 and 1.6 mL min^{-1} were examined in optimizing the mobile phase flow rate. Generally, an increase in the flow rate resulted in a decrease in the elution time of the compounds. The optimum mobile phase flow rate was taken as 1.4 mL min^{-1}. Temperature as a parameter was also investigated at 25, 30, 35 and 40 ºC. It was observed that an increase in temperature resulted in a decrease in the elution time of the compounds. A temperature of 40 ºC was selected as the optimum temperature for the separation of these compounds. Temperatures higher than 40 ºC were avoided in fear of the possible degradation of the compounds. A chromatogram for the separation of the compounds under the optimized conditions is shown in Figure 4.19. The peaks representing the compounds are well separated and sharp meaning the

method is highly selective to separate and quantify the compounds. The method was then applied to the crude extract and it was observed that the compounds were well resolved.

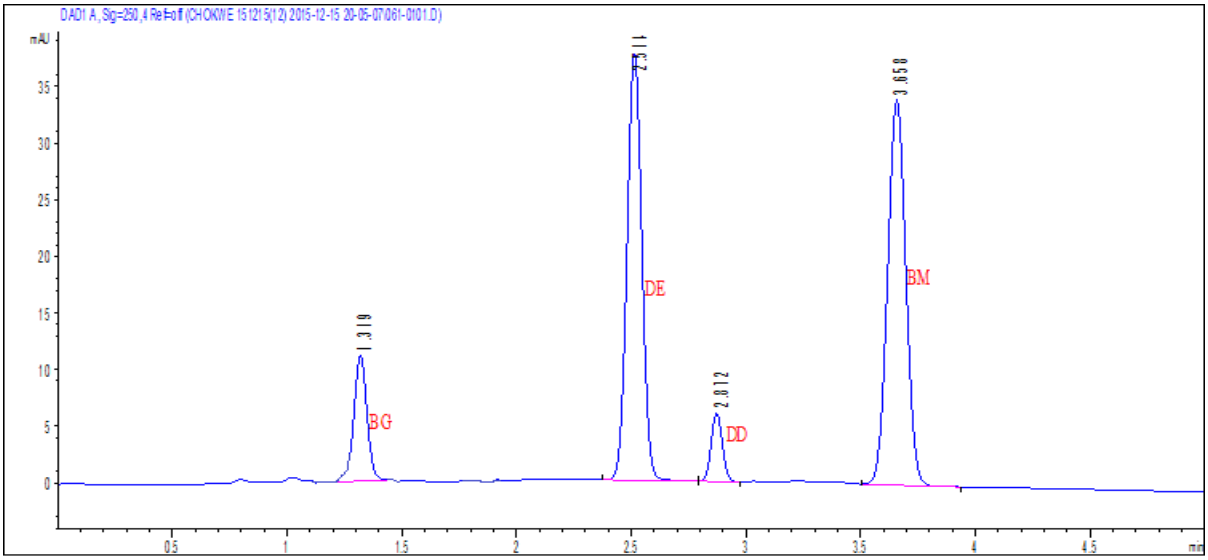


Figure 4.19 Chromatogram representing the separation of the compounds under the optimized conditions. Gradient elution mode commencing at 0 min 65% (A), 1 min 55% (A), 5 min 45% (A), flow rate 1.4 mL min^{-1}, temperature 40 ^{o}C

4.6 HPLC separation method validation

The method is validated to make sure that it is reproducible so that it can be used by any laboratory to quantify the isolated compounds.

4.6.1 Linearity

An external standard calibration method was used to determine the linearity of the method. Linearity is the ability of a method to provide a response that is directly proportional to the concentration of the analyte in the sample within a given concentration range [98]. Nine concentration levels (n = 9) with seven replicates (m = 7) were used. The number of injections per vial (x) was 3 for all the compounds. The calibration curves were obtained by plotting the average peak area versus the concentration of the compounds. The calibration curves showed good linear relationship between the peak area and the concentration in the concentration range 0.3-10 mg/ L for all the compounds. The equations of the curves were obtained using the least-squares method as shown in Table 4.7. The Table also shows the regression coefficients of the compounds were in the range 0.998-0.999, which indicates good linearity. Therefore, the calibration curves were used for quantification of the compounds in the crude extract.

Table 4.9 Linearity data for the compounds

Compound name	Equation	Regression coefficient (r^2)	Standard deviation (SD)	Percentage relative standard deviation (% RSD)
3-caffeoylquinic acid	y= 3.2065x - 0.2879	0.9990	0.55×10^3	0.056
4(α-L-rhamnosyloxy)-benzyl isothiocyanate	y = 11.324x + 1.551	0.9990	0.87×10^3	0.087
O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate	y = 1.915x + 0.5136	0.9977	0.73×10^3	0.074
O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate	y = 13.49x - 2.373	0.9994	1.7×10^3	0.17

4.6.2 Limit of detection (LOD) and Limit of Quantification (LOQ)

LOD is the lowest concentration of the analyte in the sample that can be detected by the instrument. LOQ is the lowest concentration of the analyte in the sample that can be quantified with accuracy and precision [99]. Both LOD and LOQ were determined using the linear regression equation according to ISO11843 method, where LOD is based on signal-to-noise ratio (S/N) of 3, and LOQ is based on signal-noise-ratio of 10.

The following equations were used:

$$LOD = \frac{3S_{Y/X}}{b} \text{ and } LOQ = \frac{10S_{Y/X}}{b}$$

$S_{y/x}$, is the standard error of regression and b is the slope of the calibration curve. LODs and LOQs for the compounds were ranging from 0.27 to 0.54 mg L^{-1} and from 0.91 to 1.80 mg L^{-1} respectively (Table 4.8). These values mean that the compounds can be detected and quantified respectively at these levels using this method.

Table 4.10 LOD and LOQ values for all the compounds using the developed method where n= 9 concentration levels

Compound	LOD (mg/L)	LOQ (mg/L)
3-caffeoylquinic acid	0.35	1.17
4(α-L-rhamnosyloxy)-benzyl isothiocyanate	0.36	1.18
O-ethyl-[4-(α-L-Rhamnosyloxy)benzyl] thiocarbamate	0.54	1.80
O-butyl-[4-(α-L-Rhamnosyloxy)benzyl] thiocarbamate	0.27	0.91

4.6.3 Precision

Precision measures the ability of a method to give reproducible and repeatable results. Precision is concentration dependent and was therefore measured at low, medium and high concentrations.

Intra-day variability was assessed using 0.3 mg L^{-1}, 3 mg L^{-1} and 10 mg L^{-1} concentrations. Each concentration was injected 10 times from a single vials (three vials for every concentration were used). The results were expressed as percentage relative standard deviation (% RSD). The % RSD (Table 4.9) values for all the compounds were below 2%, which is considered acceptable in analytical methodology as it shows that the method is repeatable. Inter-day variability was assessed for 6 consecutive days. Similar to the intra-day tests, three vials for each concentration were injected 10 times on each day. The % RSD values (Table 4.10) for inter-day variability were below 5%, which is considered acceptable and shows that the method is precise.

4.6.4 Specificity

This is the ability of the method to accurately quantify the compound of interest in the presence of other compounds [[99]]. Specificity of this method was assessed by comparing the retention times of the standards over six days. Their % RSD values were below 5% (Table 4.11). This was also assessed by calculating the percentage difference between the retention times of the standards with those of the compounds in the crude extract (see Table 4.12).

The % difference was calculated as follows:

$$\% \text{ difference} = \frac{R_T std - R_T sample}{\frac{R_T std + R_T sample}{2}} \times 100$$

Table 4.11 Repeatability (Intra-day precision) data, where m= 3 and x= 10 injections for each concentration level

	3-caffeoylquinic acid	4(α-L-rhamnosyloxy)-benzyl isothiocyanate	O-ethyl-[4-(α-L-rhamnosyloxy)benzyl] thiocarbamate	O-Butyl-[4-(α-L-rhamnosyloxy) benzyl] thiocarbamate
0.3 mg/ L (m=3)				
Mean peak area	2.48	6.98	1.18	8.37
SD	0.03	0.04	0.009	0.45
% RSD	1.16	0.57	0.77	0.53
3 mg/L (m= 3)				
Mean peak area	24.61	69.45	11.82	84.44
SD	0.22	0.33	0.099	0.65
% RSD	0.90	0.48	0.84	0.76
10 mg/L (m= 3)				
Mean peak area	82.45	232.51	39.18	280.56
SD	0.22	0.28	0.09	0.23
% RSD	0.27	0.12	0.24	0.08

Table 4.12 Intermediate precision data by comparing the peak areas of the standards over six days, where m= 3 and x= 10 injections

0.3 mg/L	BG	DE	DD	BM	3 mg/L	BG	DE	DD	BM	10 mg/L	BG	DE	DD	BM
Day 1	2.46	6.95	1.18	8.45	**Day 1**	24.61	69.45	11.82	84.44	**Day 1**	82.45	232.51	39.18	280.56
Day 2	2.47	6.96	1.17	8.52	**Day 2**	24.46	69.90	11.77	84.63	**Day 2**	82.83	232.87	39.46	280.58
Day 3	2.47	6.92	1.18	8.46	**Day 3**	24.22	69.79	11.75	84.63	**Day 3**	82.69	232.84	39.74	280.38
Day 4	2.45	6.94	1.17	8.51	**Day 4**	24.78	69.76	11.56	84.71	**Day 4**	82.41	232.76	39.56	280.38
Day 5	2.44	6.94	1.18	8.48	**Day 5**	24.71	69.59	11.78	84.69	**Day 5**	82.42	232.79	39.09	280.71
Day 6	2.46	6.97	1.17	8.45	**Day 6**	24.64	69.65	11.79	84.64	**Day 6**	82.43	232.24	39.56	280.38
Mean (peak area)	2.46	6.95	1.18	8.48	**Mean (peak area)**	24.57	69.69	11.74	84.62	**Mean (peak area)**	82.54	232.67	39.43	280.50
SD	0.01	0.01	0.002	0.03	**SD**	0.18	0.14	0.89	0.08	**SD**	0.16	0.22	0.23	0.13
%RSD	0.40	0.21	0.19	0.37	**%RSD**	0.74	0.21	0.75	0.10	**%RSD**	0.20	0.10	0.58	0.04

BG: 3-caffeoylquinic acid

DE: 4(α-L-rhamnosyloxy)-benzyl isothiocyanate

DD: O-ethyl-4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate

BM: O-butyl-4-[(α-L-rhamnosyloxy) benzyl]thiocarbamate

Table 4.13 Specificity assessment by comparing retention times over six days. n= 1, m=1 and x=10 injection

Compound name	Day 1 RT	Day 2 RT	Day 3 RT	Day 4 RT	Day 5 RT	Day 6 RT	Mean	SD	%RSD
BG	1.33	1.33	1.33	1.33	1.33	1.33	1.33	0.0008	0.5699
DE	3.08	3.07	3.06	3.08	3.07	3.06	3.07	0.0058	0.1886
DD	4.15	4.15	4.14	4.15	4.14	4.14	4.15	0.0038	0.0910
BM	5.32	5.32	5.32	5.32	5.32	5.32	5.32	0.0025	0.0478

BG: 3-caffeoylquinic acid

DE: 4(α-L-rhamnosyloxy)-benzyl isothiocyanate

DD: O-ethyl-4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate

BM: O-butyl-4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate

Table 4.14 Specificity assessment by comparing percentage difference of the retention times of the standard compounds

Compound name	Mean standard	Mean sample	% difference
BG	1.33	1.28	3.83
DE	3.07	3.04	0.98
DD	4.15	4.13	0.24
BM	5.32	5.28	0.76

BG: 3-caffeoylquinic acid

DE: 4(α-L-rhamnosyloxy)-benzyl isothiocyanate

DD: O-ethyl-4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate

BM: O-butyl-4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate

4.7 Quantification of the compounds in the crude extract

The validated method was applied to the crude extract for quantification of the compounds in the extract (Figure 4.20). The peaks of interest in the sample were identified by comparing their retention times with those of the standards. The unidentified peaks on the chromatogram represent the other compounds that are found in the seeds. Identification of these compounds required for them to be pure. They were not purified at the current stage because additional steps were required for their purification that will be done at a later stage. Quantification results are shown in Table 4.13. It was found that the percentage distribution of the compounds in 50 mg of the seeds extract range from 0.25- 1.10%. This means that the remaining unidentified peaks represent ± 98% of the compounds in the seeds.

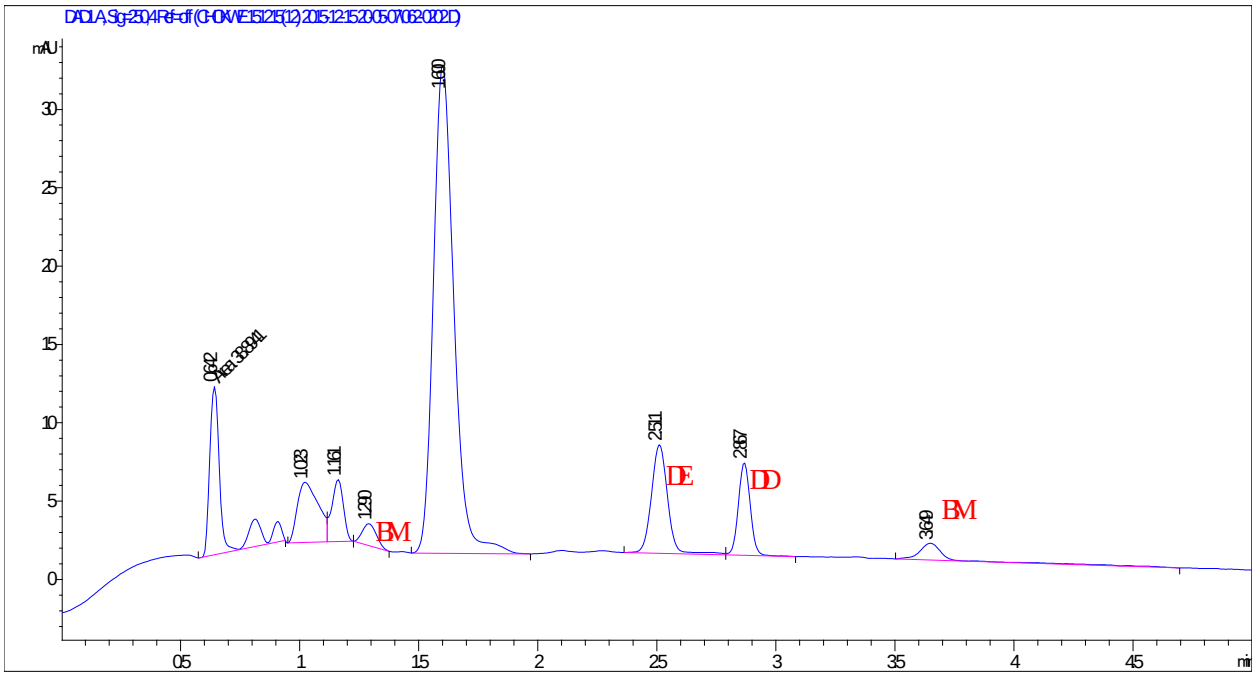


Figure 4.20 HPLC separation of the compounds in the crude extract using the validated method

Table 4.15 Percentage distribution of the isolated compounds in 50 mg of the crude extract

Compound name	% ± SD	% RSD
BG	0.190 ± 0.064	1.10
DE	0.353 ± 0.114	0.27
DD	1.605 ± 0.800	0.25
BM	0.027 ± 0.048	3.62

BG: 3-caffeoylquinic acid

DE: 4(α-L-rhamnosyloxy)-benzyl isothiocyanate

DD: O-ethyl-4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate

BM: O-butyl-4-[(α-L-rhamnosyloxy) benzyl]thiocarbamate

4.7.1 Spike and identification

To confirm the peaks that were identified in the crude extract represent the compounds of interest, the sample was spiked with the 10 mg L^{-1} of the reference standards for the individual compounds. An increase in peak area of the identified peaks was observed {Figures 4.21(a) to (d)} which meant that the identified peaks were indeed the peaks of interest. This was also confirmed by comparing the UV spectra of the identified peaks to those of the reference standards. The chemstation software was used to check for co-elution with interferences and there was none for all the peaks of interest. The changes in peak areas are shown in Table 4.14.

Table 4.16 Spike and identification data

Compound name	Mean peak area of sample	Mean peak area of spiked sample	Difference (peak area)
BG	5.86	20.06	14.20
DE	41.64	126.48	84.84
DD	31.38	41.48	10.10
BM	1.25	107.775	106.52

BG: 3-caffeoylquinic acid

DE: 4(α-L-rhamnosyloxy)-benzyl isothiocyanate

DD: O-ethyl-4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate

BM: O-butyl-4-[(α-L-rhamnosyloxy) benzyl]thiocarbamate

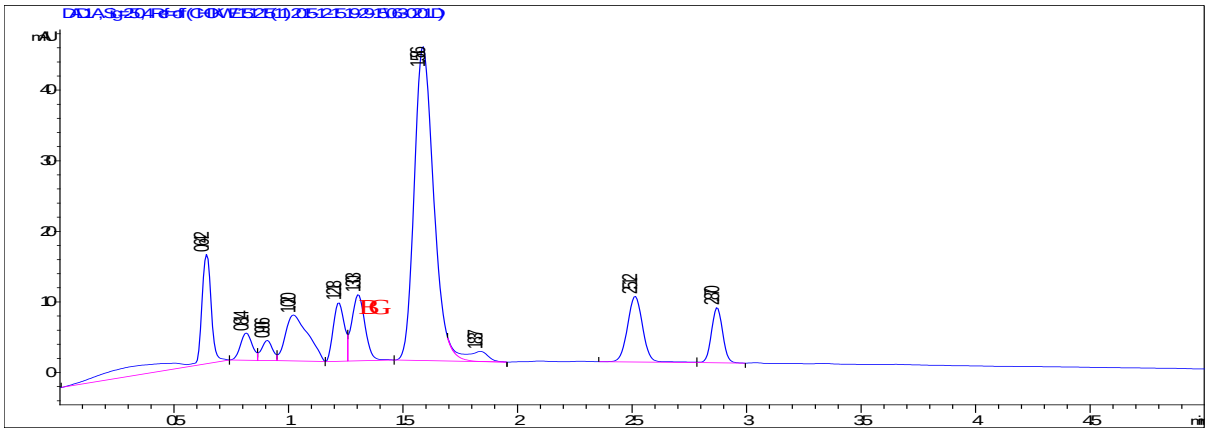


Figure 4.21 **(a)** Sample spiked with 10 mg/L of 3-caffeoylquinic acid (BG)

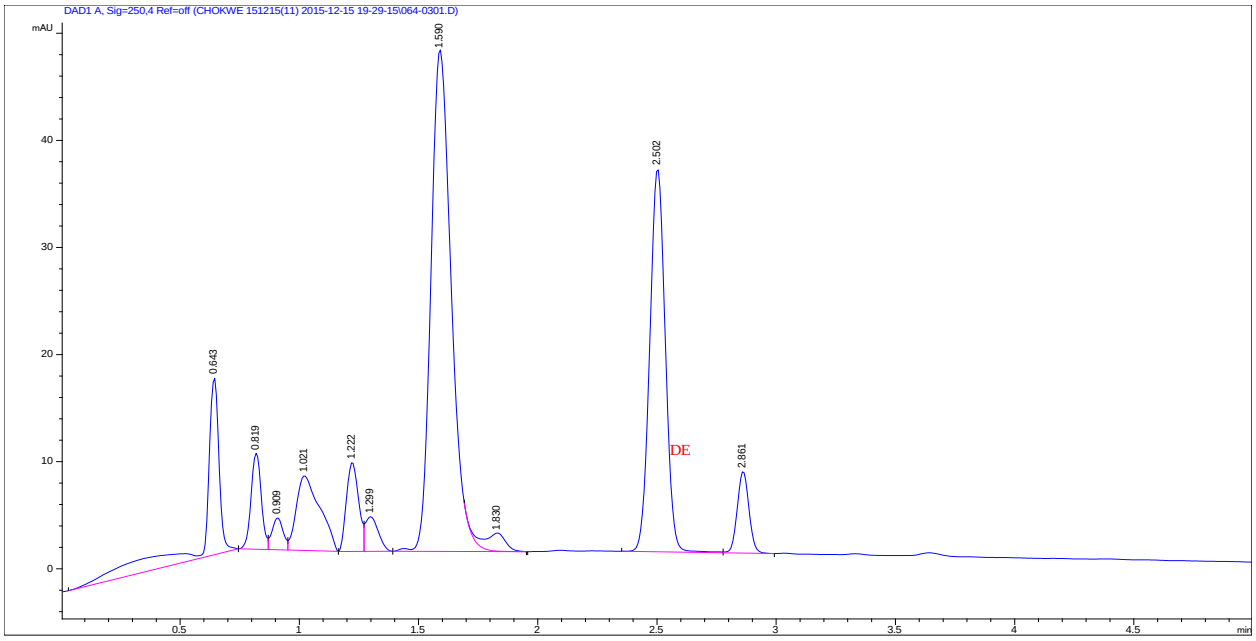


Figure 4.21 **(b)** Sample spiked with 10 mg/L of 4(α-L-rhamnosyloxy)-benzyl isothiocyanate (DE)

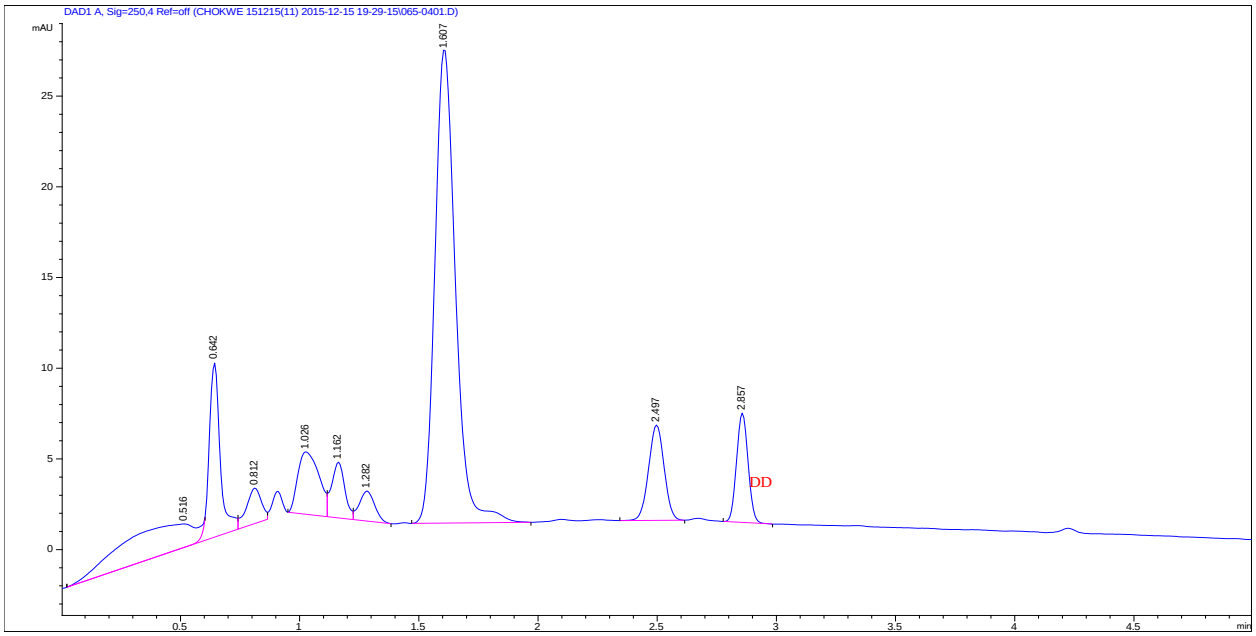


Figure 4.21 **(c)** Sample spiked with 10 mg/L of O-ethyl-4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (DD)

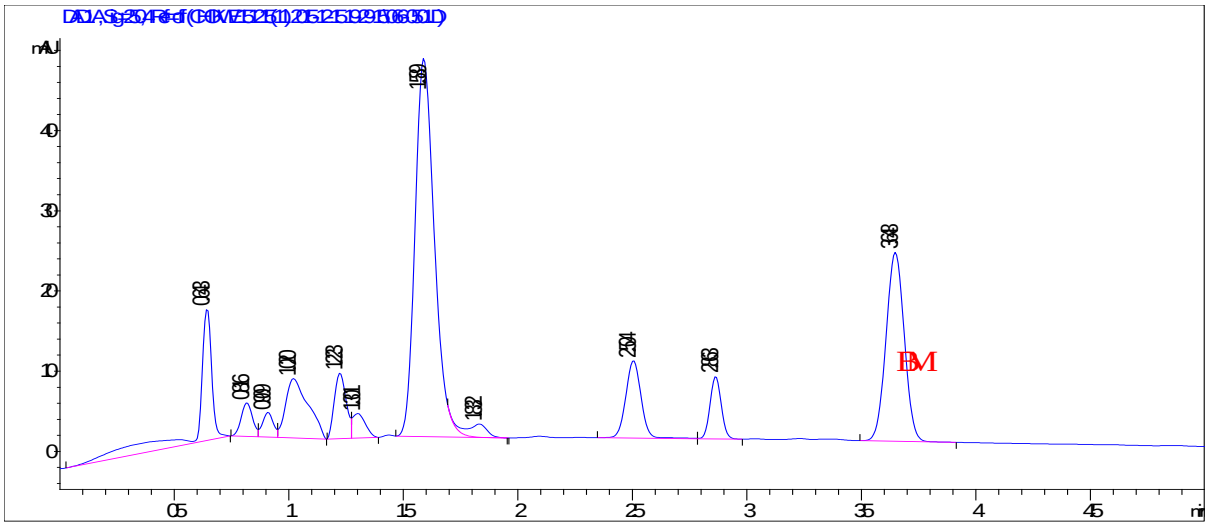


Figure 4.21 **(d)** Sample spiked with 10 mg/L of O-butyl-4-[(α-L-rhamnosyloxy) benzyl] thiocarbamate (BM)

CHAPTER FIVE

Summary, conclusions and future work

5 Introduction

The need for a quality control method for the Moringa products in the market led to this work. In this chapter, a summary of the results and conclusions based on the objectives that were set at the beginning are given. This work has been able to address all the set objectives, however further work still needs to be done. Recommendations are thus made with regards to the work that needs to be done.

5.1 Summary and conclusions

- Extraction from the seeds of *Moringa oleifera* was carried out successfully using the maceration method with a mixture of water and ethanol as the extracting solvent. The crude extract was recovered at 21.15%. The crude extract was further separated into fractions of different polarities using petroleum ether, dichloromethane, ethyl acetate and n-butanol. The polar fractions were recovered at a higher percentage yield as compared to the non- polar fraction.

- HPLC methods for the separation of the compounds within the fractions were developed. These methods were successfully scaled up to preparative HPLC methods which enabled quantitative isolation of the compounds from the fractions. Four compounds of

percentage purity between 90 and 99% were isolated. Preparative HPLC offered the advantage of being quick as compared to the traditional column chromatography.

- The compounds were characterized using NMR, FTIR and MS. The isolated compounds were identified as phenolic glucosides; O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate, O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate, 4-(α-L-Rhamnosyloxy) benzyl isothiocyanate and a polyphenol, 3-caffeoylquinic. O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate has not been reported in the *Moringa oleifera* species before but has been reported from *Moringa peregrina*.

- The isolated compounds were used successfully as reference standards to develop an HPLC separation method for their quantification. A fast separation method of less than 4 minutes was achieved using an optimum gradient elution programme, flow rate and column temperature. The developed method was validated and showed a linear relationship between peak area and concentration of the compounds with regression coefficients ranging from 0.998 to 0.999. The limit of detection and limit of quantification for the method were between 0.27 and 0.54 mg/L and 0.91 and 1.80 mg/L respectively. The method was found to be precise with %RSD values ranging from 0.53 to 1.16 at the low concentration, 0.48 to 0.90 at medium concentration and 0.08 to 0.27 at high concentration for intraday variability. For inter-day precision the %RSD values were ranging from 0.19 to 0.40 at low concentration, 0.10 to 0.73 at medium concentration and 0.014 to 0.58 at high concentration. Therefore it can be concluded that the developed method is linear, precise and specific to the compounds of interest.

- The method was applied to the crude extract to quantify the four identified compounds. The percentage compositions of the compounds in 50 mg of the seeds crude extract range between 0.25- 1.10% w/w. Therefore this method can be used for the quality control and quality assurance of these compounds in *Moringa oleifera* extracts and products.

5.2 Further work

It has been reported that compounds or a group of compounds in an extract act in synergy to give better activity as compared to an individual compound. It is therefore necessary to develop a method that is able to quantify all the compounds in the extract simultaneously. For further work it is suggested to develop a separation method that is capable of isolating the compounds that were not isolated with enough quantities and acceptable percentage purity for their further identification. Upscale the TLC method that was developed to FCC to isolate the compounds that can be separated better on a polar stationary phase. Modify the developed HPLC separation method for quantification to include all the compounds found in the *Moringa oleifera* seeds. This will enable the quantification and determination of the percentage distribution of all the compounds within the Moringa extracts and products on the market.

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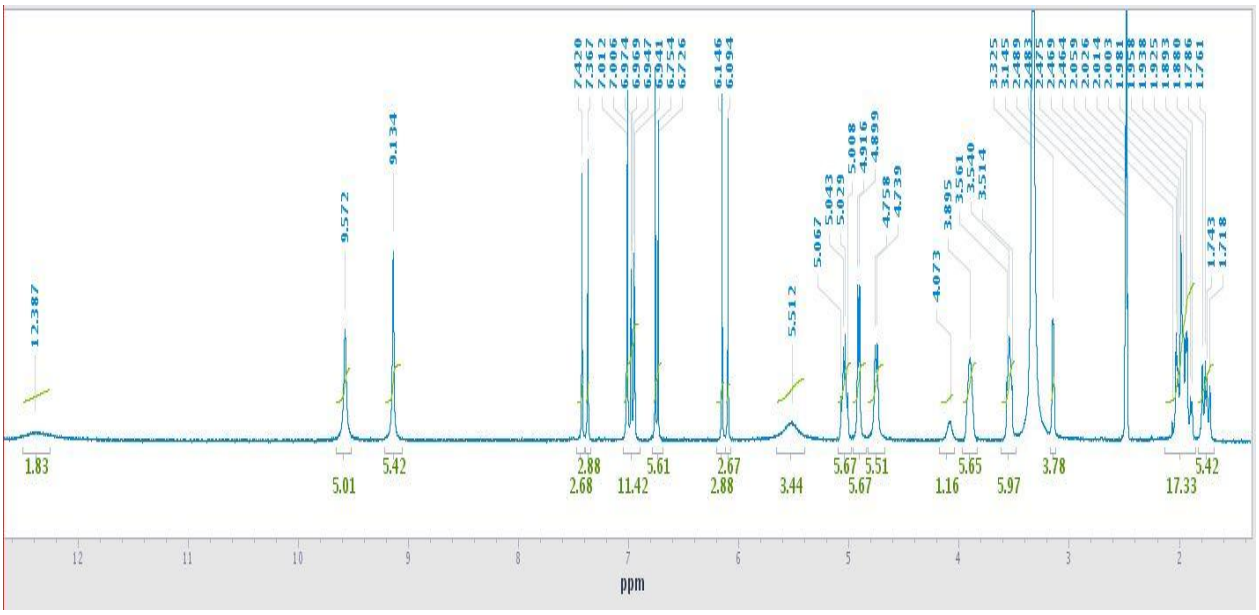
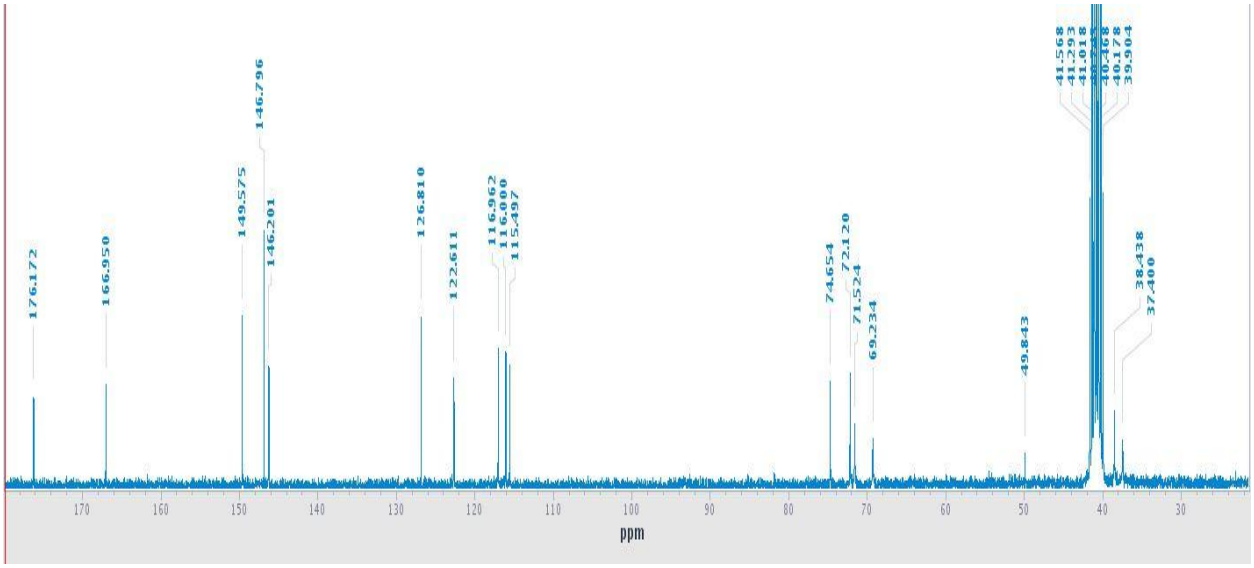
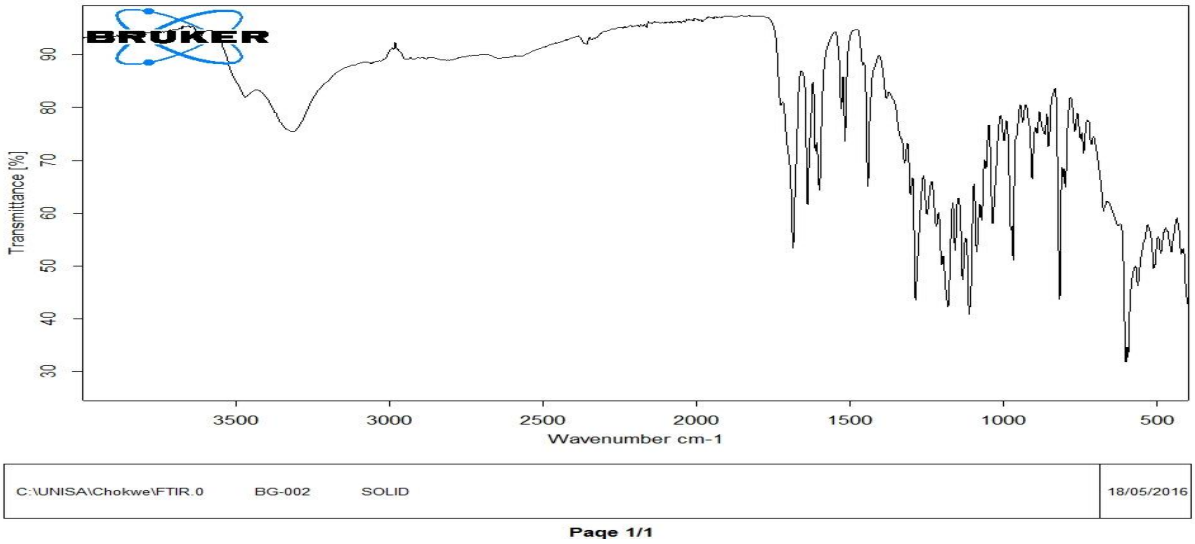
APPENDIX



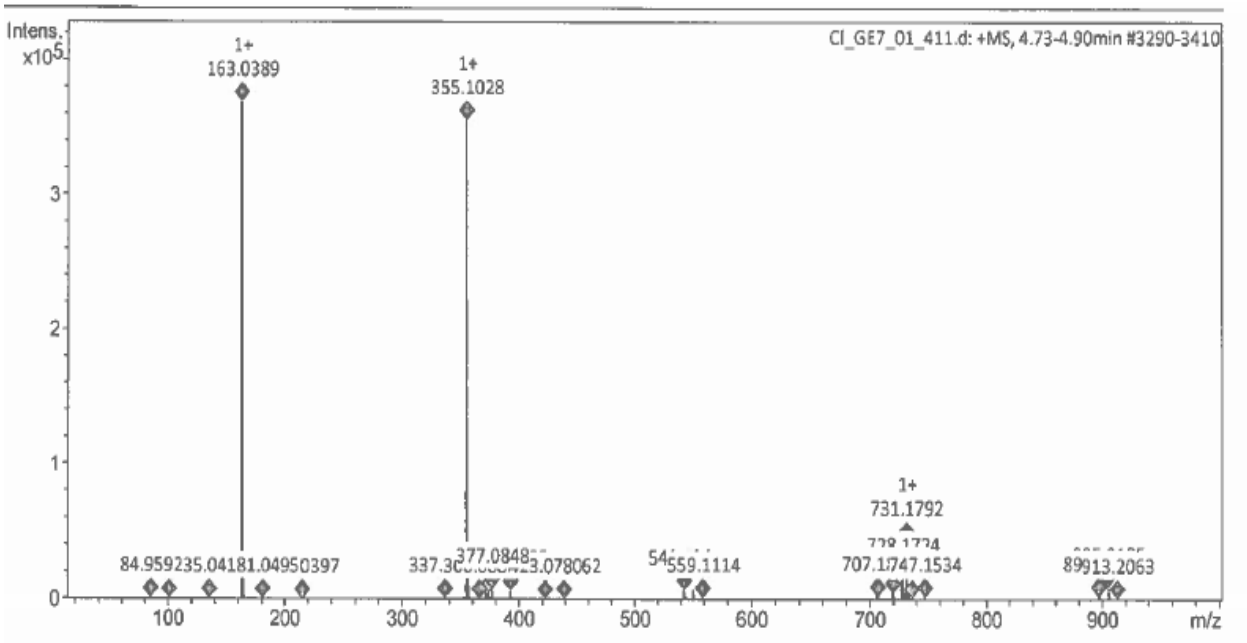
[1]H NMR spectrum of 3-caffeoylquinic acid



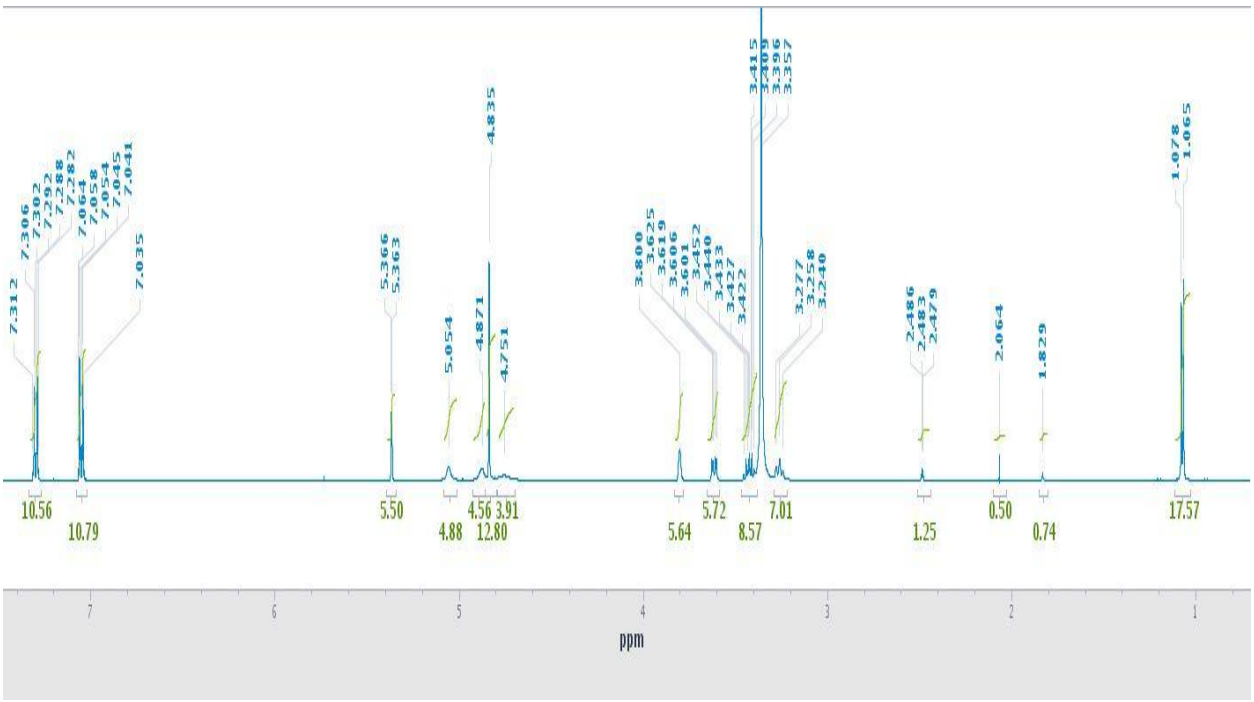
[13]C NMR spectrum of 3-caffeoylquinic acid



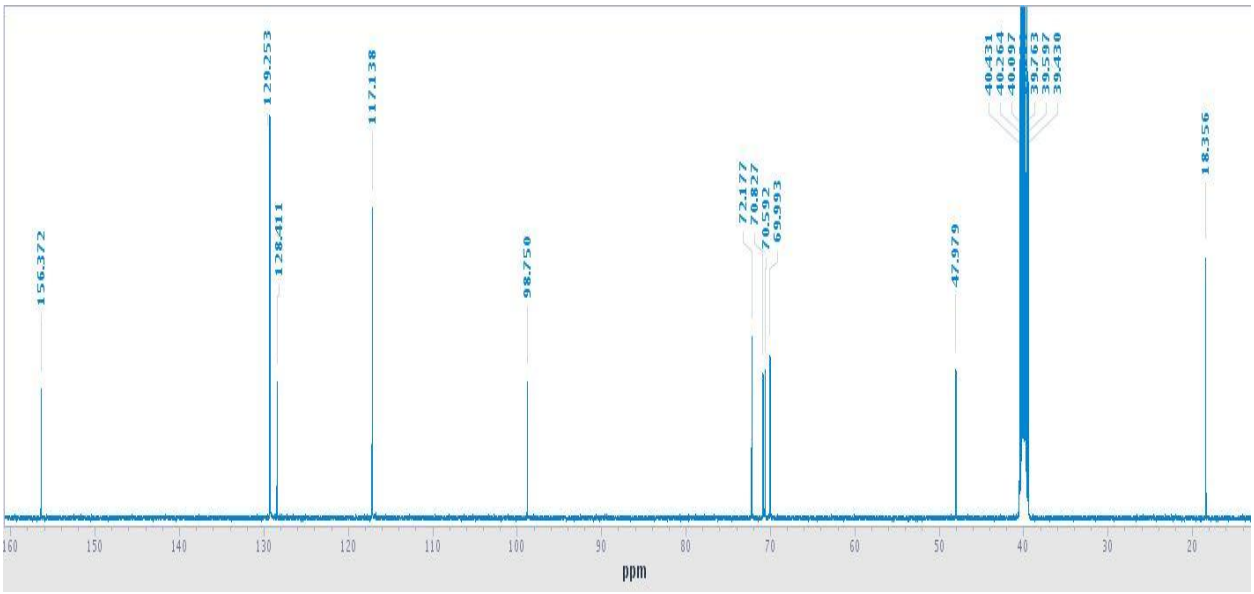
FTIR spectrum of 3-caffeoylquinic acid



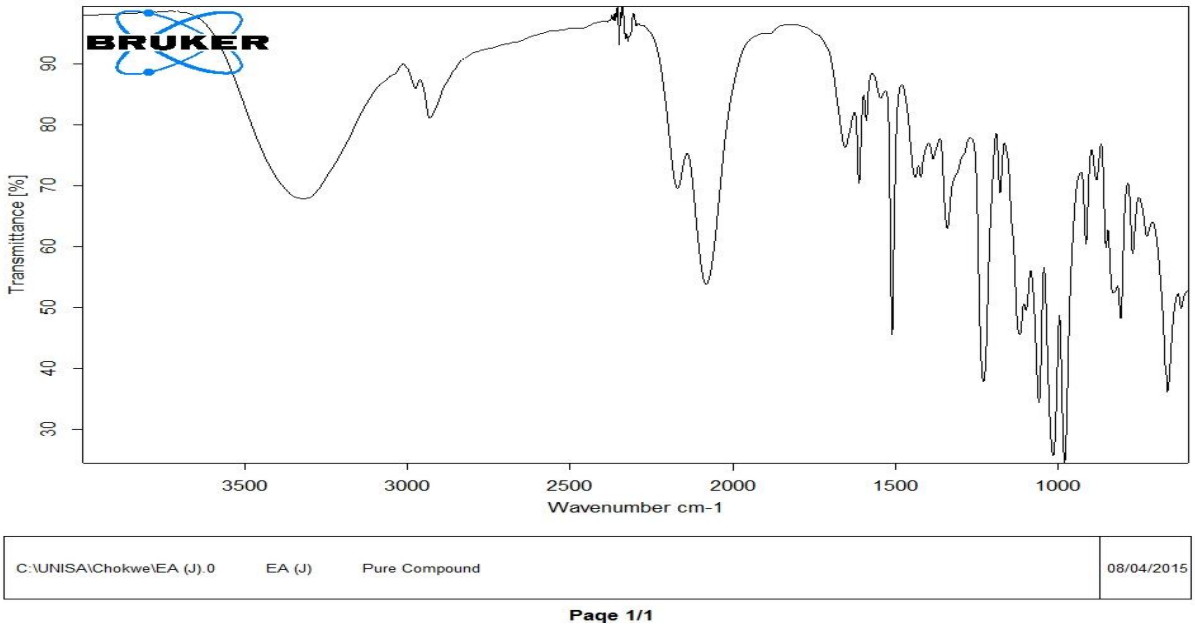
Mass spectrum of 3-caffeoylquinic acid



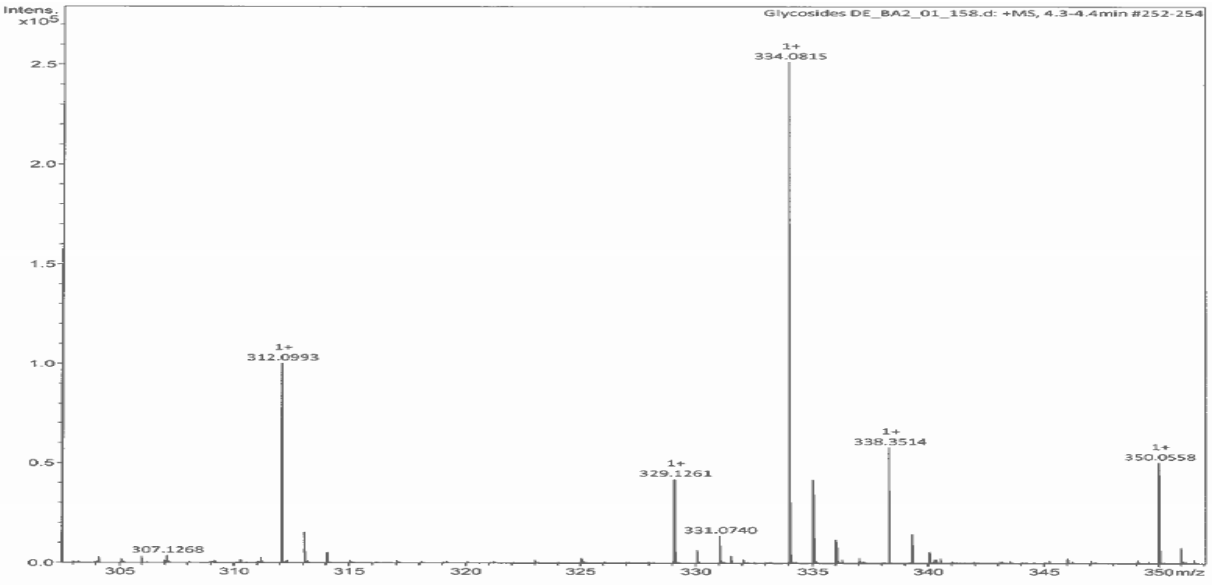
[1]H NMR spectrum of 4-(α-L-Rhamnosyloxy) benzyl isothiocyanate



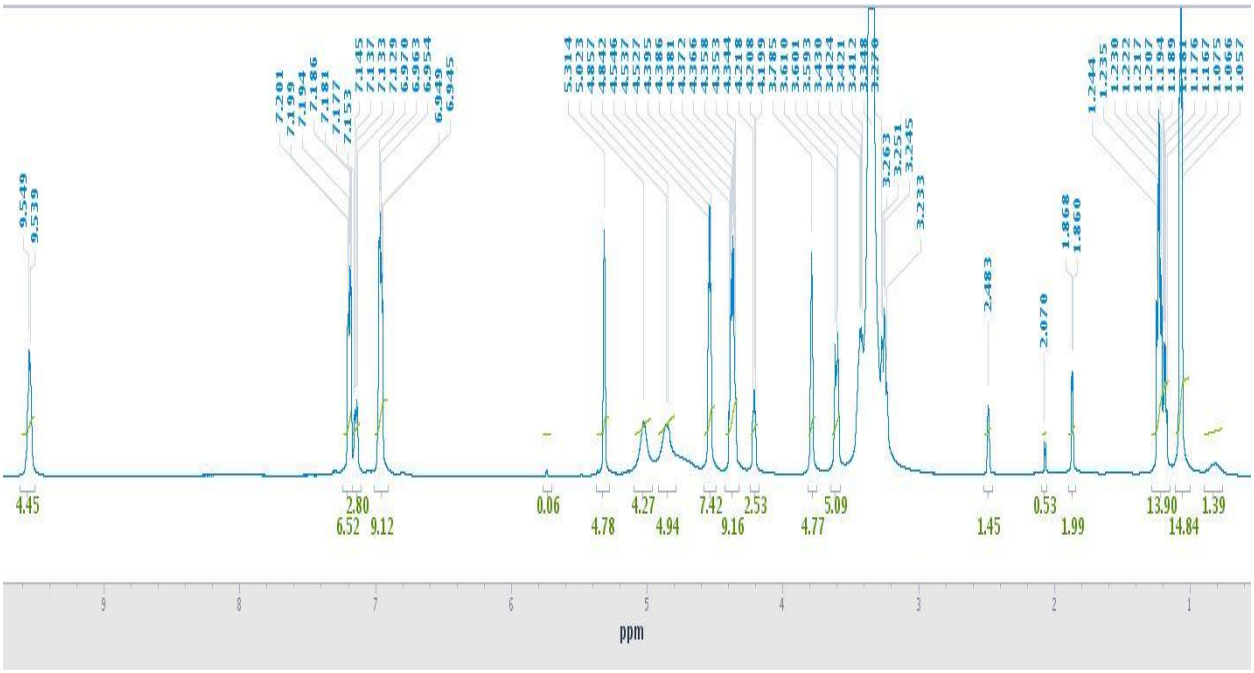
[13]C NMR spectrum of 4-(α-L-Rhamnosyloxy) benzyl isothiocyanate



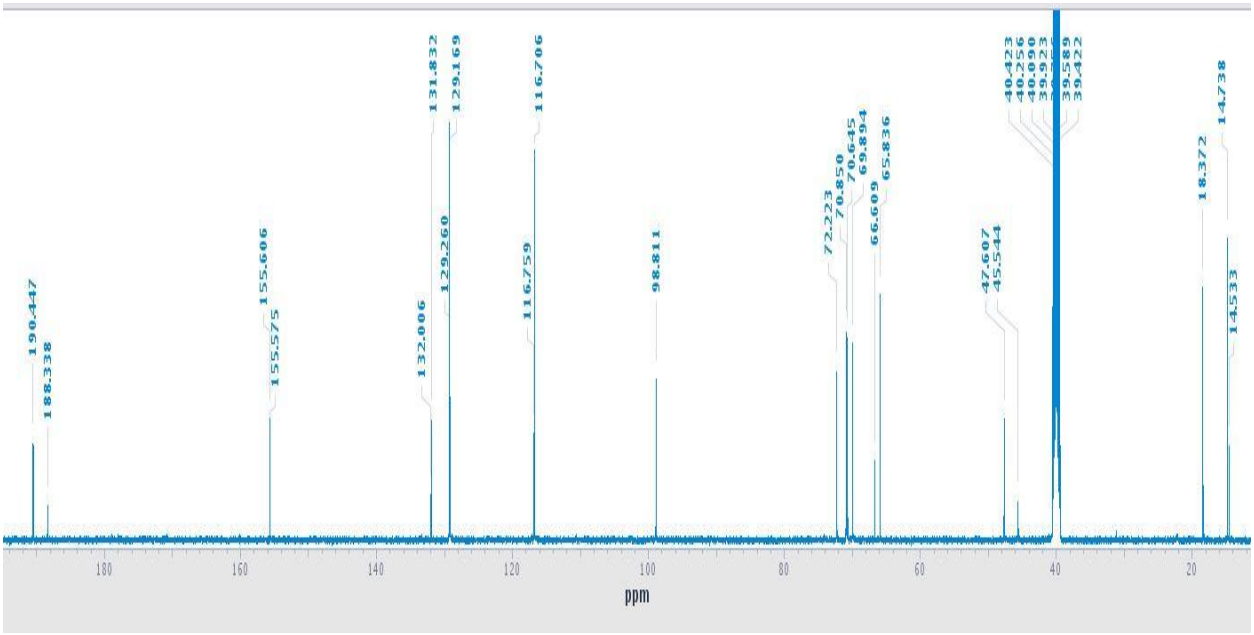
FTIR spectrum of 4-(α-L-Rhamnosyloxy) benzyl isothiocyanate



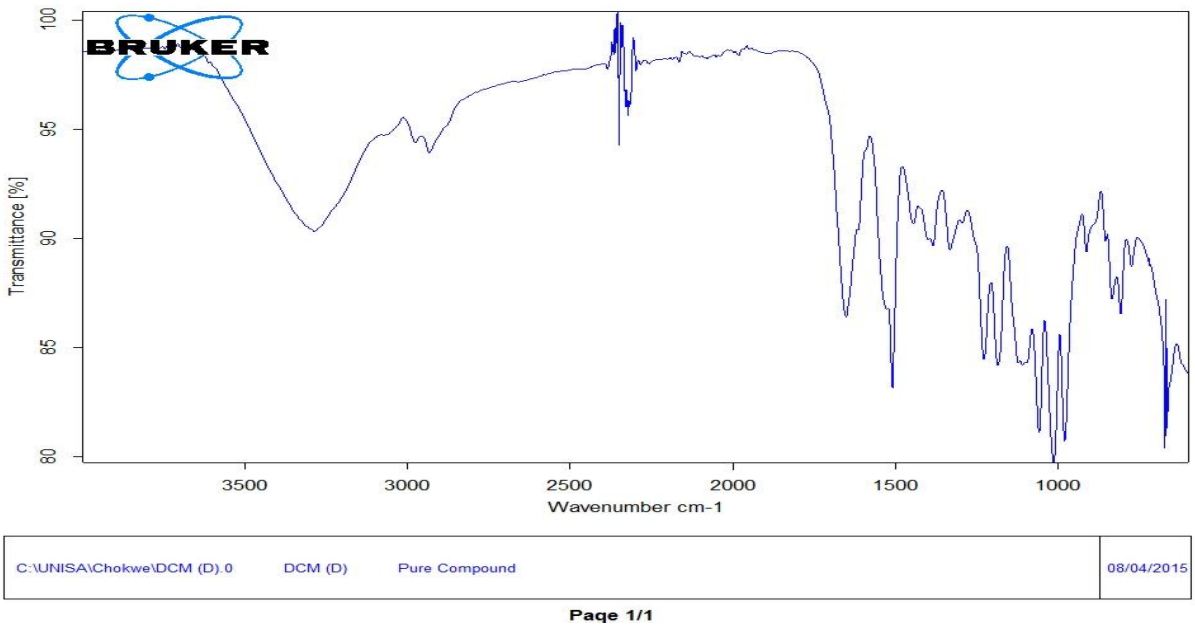
Mass spectrum of 4-(α-L-Rhamnosyloxy) benzyl isothiocyanate



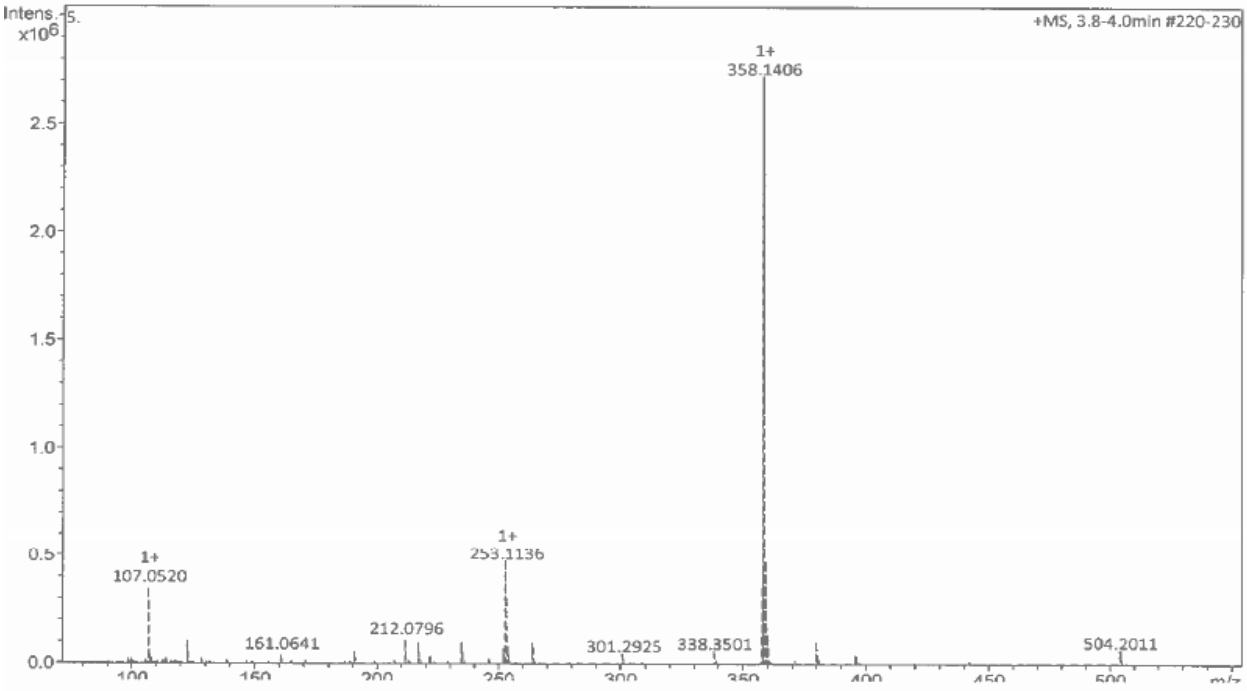
[1]H NMR spectrum O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate



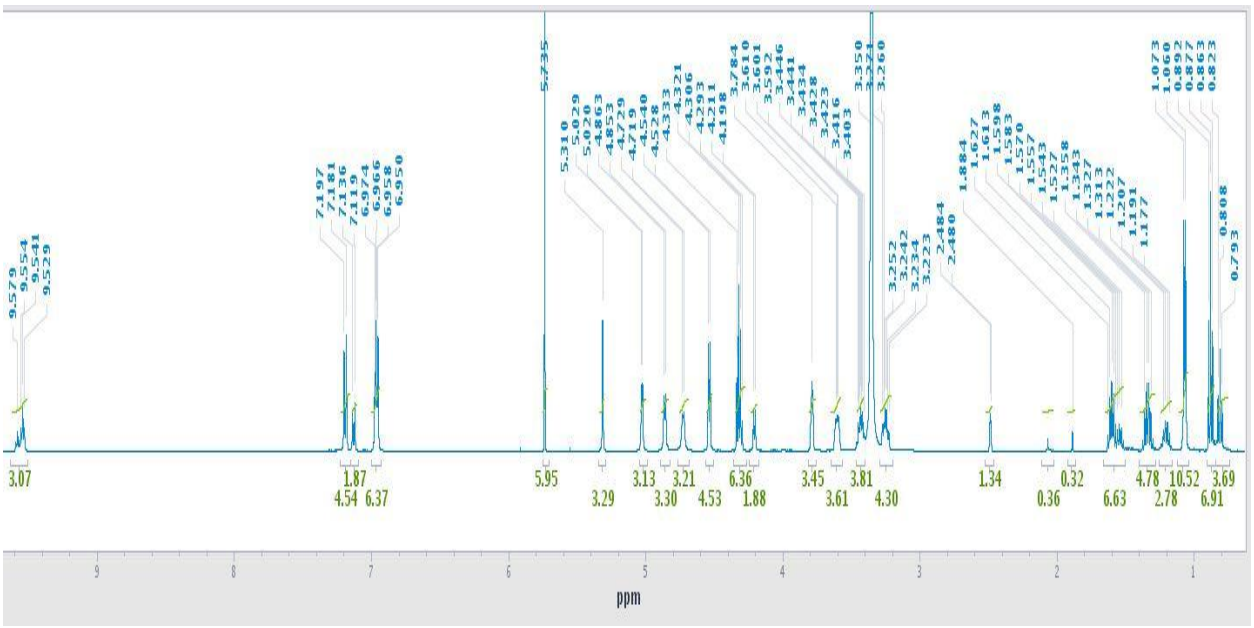
[13]C NMR spectrum of O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate



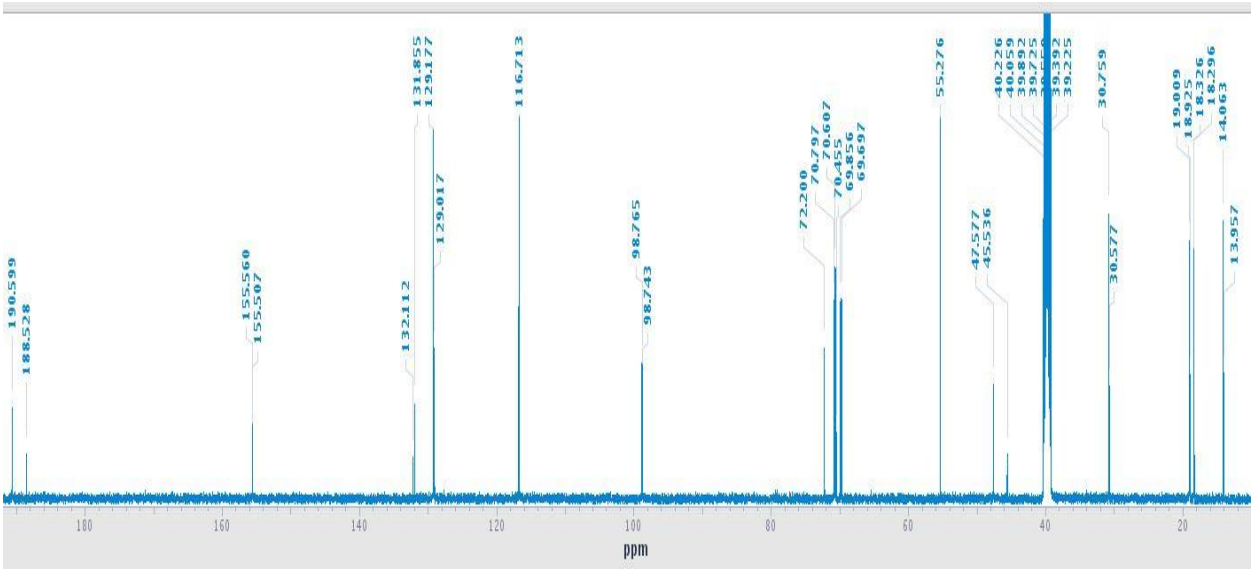
FTIR spectrum of O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate



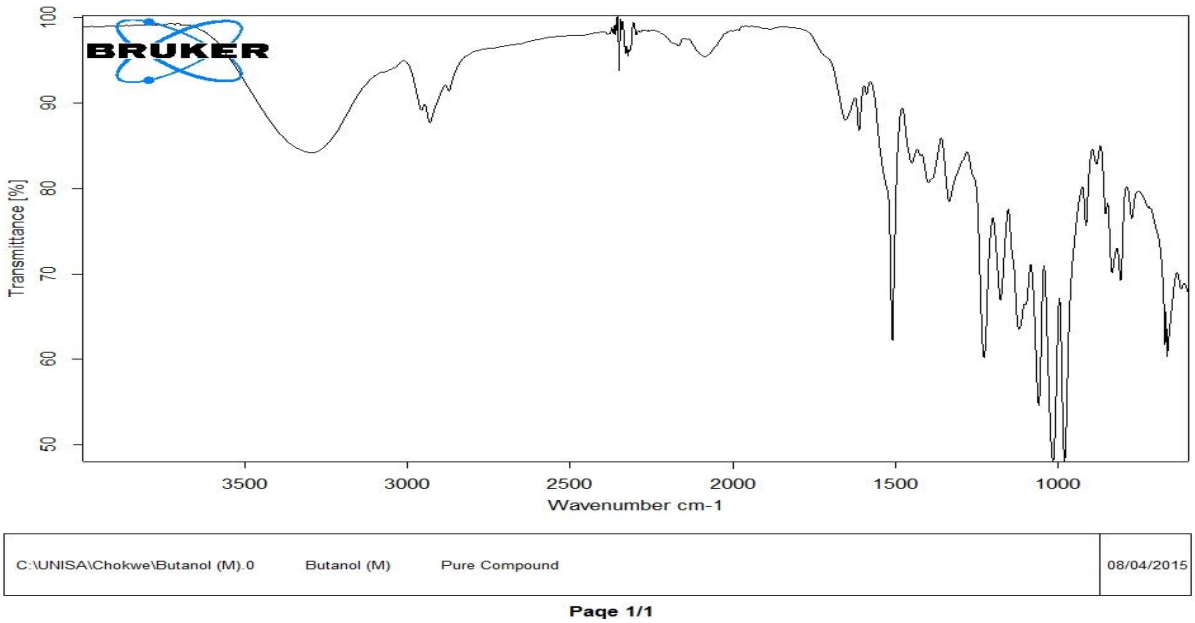
Mass spectrum of O-ethyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate



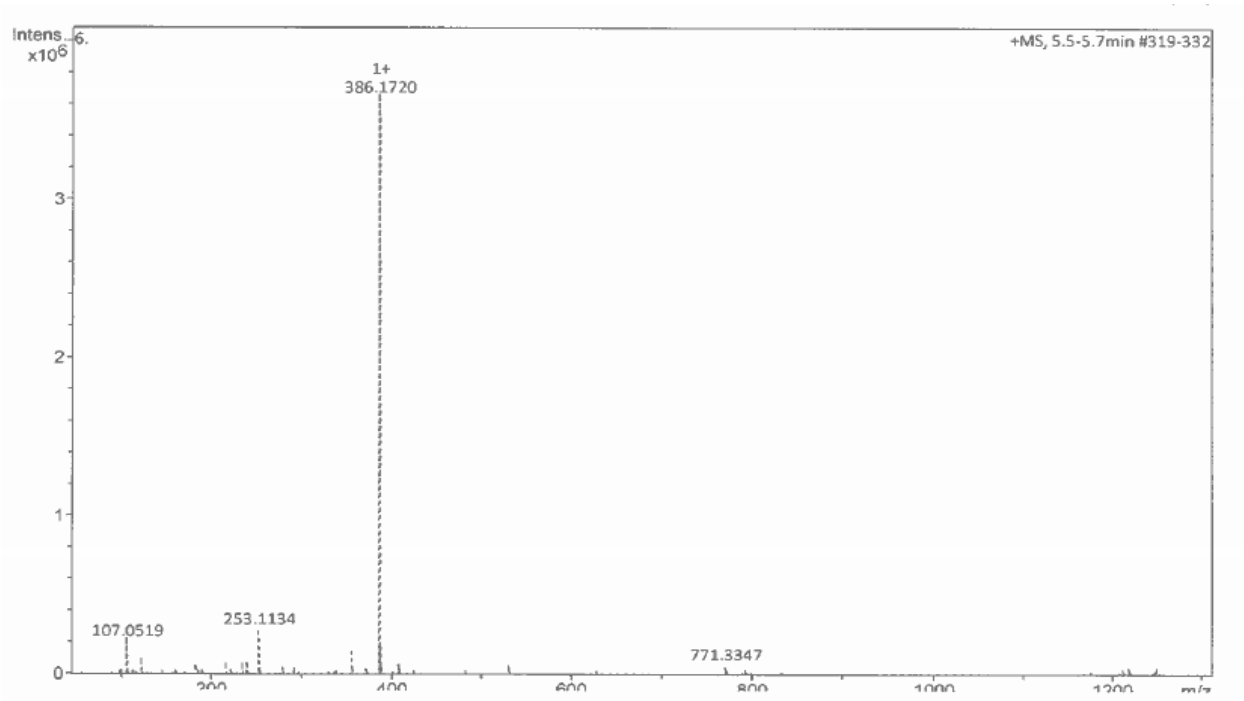
[1]H NMR spectrum of O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate



[13]C NMR spectrum of O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate



FTIR spectrum of O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate



Mass spectrum of O-butyl-[4-(α-L-Rhamnosyloxy) benzyl] thiocarbamate