Phytochemical and Biological Activity Studies on the Twigs of *Baikiaea Plurijuga* and the Tubers of *Ipomoea Bolusiana*

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Certification

The undersigned certify that they have read and hereby recommend, for the acceptance by the Department of Chemistry, University of Botswana, a dissertation entitled "Phytochemical Studies on the Twigs of *Baikiaea Plurijuga* and the Tubers of *Ipomoea Bolusiana*" as part of the work recommended in fulfilment of the requirements for a Master's of Science Degree in Chemistry of the University of Botswana.

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Copyright and Declaration

The work contained in this thesis was completed by the author at the University of Botswana between June, 2014 and August 2016. I hereby declare that the work is original except where due reference has been made. The work in this thesis has never been nor will ever be submitted for the award of any degree at any other University.

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Yaone Moabi Date

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List of Abbreviations

DEPT: Distortionless Enhancement by Polarization Transfer

HMQC: Heteronuclear Multiple Quantum Correlation

HMBC: Heteronuclear Multiple Bond Correlation

COSY: Correlation spectroscopy

NOESY: Nuclear Overhauser Enhancement Spectroscopy

HR: High Resolution

LR: Low Resolution

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

MIQ: Minimum Inhibitory Quantity

TPC: Total Phenolic Content

IC_{50}: 50 % Inhibitory Concentration

NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form)

$NADP^+$: Nicotinamide adenine dinucleotide phosphate (oxidized form)

ESI: Electrospray Ionization

TLC: Thin Layer Chromatography

MTT: Methylthiazolyldiphenyl-Tetrazoliumbromide

Abstract

Phytochemical studies on the twigs of *Baikiaea plurijuga* afforded four compounds, (+) – catechin, taxifolin, eriodictyol and sucrose. The dissertation describes the methods of their isolation and discusses their characterization. All the isolated compounds are being reported for the first time from this plant species. The isolated compounds and extracts from this plant were screened for their antioxidant activity using DPPH radical. Eriodictyol showed the highest radical scavenging activity among the tested flavonoid derivatives with an IC_{50} value of 0.047 ± 0.007 μM. The IC_{50} value was better than that of the standard ascorbic acid (0.090 ± 0.01 μM). The total phenolic content of the extracts was determined using the Folin-Ciocalteau method and the ethyl acetate extract exhibited the highest amongst the twigs of *Baikiaea plurijuga* extracts (350 mg GAE/g). Antimicrobial studies on both the extracts and pure isolates was done using the agar-overlay method and (+) – catechin exhibited the best inhibitory activity against gram-positive bacteria *S. aureus* at an MIQ of 5 μg. Eriodictyol on the other hand showed the best inhibition against gram-negative bacteria *E. coli* and *P. aeruginosa* at MIQs of 50 μg. Taxifolin exhibited the best activity against the test fungus *C. albicans* at an MIQ of 5 μg.

Phytochemical studies on the tubers of *Ipomoea bolusiana* afforded seven compounds, (*E*)-octadec-11-en-1-yl (*E*)-3-(4-hydroxyphenyl) acrylate, (*E*)-icos-13-en-1-yl (E)-3-(3,4-dihydroxyphenyl) acrylate, methyl (*E*)-octadec-11-enoate, sucrose, maltose, C4C and IBMF2, all of which are reported for the first time from this plant species. Trans esterification of C4C afforded caffeic acid methyl ester. The extracts and isolated compounds from these tubers were screened for antimicrobial as well as antioxidant activities. Amongst the cinnamic acid derivatives isolated, (*E*)-icos-13-en-1-yl (*E*)-3-(3,4-dihydroxyphenyl) acrylate showed better radical scavenging activity with an IC_{50} value of 0.023 ± 0.006 μM as compared to (*E*)-octadec-11-en-1-yl (*E*)-3-(4-hydroxyphenyl) acrylate which showed an IC_{50} value of 0.47 ± 0.06 μM. Antimicrobial studies of the isolated compounds as well as the extracts revealed that C4C exhibited the best activity against gram-negative bacteria *P. aeruginosa* with an MIQ of 10 μg, while (*E*)-icos-13-en-1-yl (*E*)-3-(3,4-dihydroxyphenyl) acrylate exhibited weak activity against gram-positive bacteria *S. aureus* with an MIQ of 50 μg. Extracts of the tubers of this plants exhibited moderate activity against all the test microorganism at an average MIQ of 50 μg. The total phenolic content of the extracts revealed that the aqueous methanol (80 %) extract showed the highest among the extracts at 150 mg GAE/g.

Table of Contents

List of Tables

List of Schemes

List of Figures

Chapter One

1.1 Introduction

Plants have been used in Africa and the world over for centuries to meet people's needs, be it as sources of building materials, transport, as source of food or as medicines. However, the use is dependent on the type of plants available in a particular area. For instance, Morama (*Tylosema esculentum*), a plant known for its high nutritional value and found in the Kalahari has been the main source of food for centuries (Bower *et al.,* 1988). Medicinally this plant is known to treat diarrhea and for general upkeep of human health (Chingwaru., *et al* 2011). It is worth mentioning that there is a large diversity of medicinal plants in the African continent and even here in Botswana. Plants such as *Cassia abbrivita, Hoodia gordonii, Harpagophytum procumbens, Ziziphus mucronata, Myrothamnus flabellifolius* and *Elephantorhriza goetzei* are some of the known medicinal plants used in Botswana (Hoareau and Da Silva, 1999). *Hoodia gordonii* products are even marketed in many western countries where obesity is a problem. This plant is known to suppress appetite. These few examples show that there is indeed quite a diversity of medicinal plants available in Botswana.

It is not surprising therefore that herbal or traditional medicines are commonly used in rural areas as compared to modern medicines. It is assumed that they are viewed to be affordable, easily accessible and with minimal side effects (Payyappallimana, 2010; George, 2011). Some sources even estimate that in South Africa for instance, more than 80 percent of households depend on traditional medicines (Mulholland, 2005). Official recognition of traditional medicines has been elusive despite the medicines being responsible for the treatment of illnesses throughout the developing world where modern day health care systems are not easily accessible. This recognition was done in the Beijing Declaration of 2008 and was endorsed by the World Health Organization (WHO) member states. The declaration encouraged amongst others, integration of traditional medicines into health care systems where appropriate and improve national policies on traditional medicines and also promote improved education, research and clinical inquiries into traditional medicines (Abbott, 2014).

Higher plants, especially medicinal plants are the main sources of medicines in addition to microbial sources like fungi (Katiyar *et al*., 2012). Plants are also the main providers of natural

products or secondary metabolites. The term natural products, is quite commonly understood to refer to products like herbs, concoctions from herbs, dietary supplements as extracts or powders from the stem wood, stem bark and roots (Holt and Chandra, 2002). Baker *et al*, 2007 however, defined natural products (secondary metabolites) as compounds with biological activities derived from natural sources like plants, animals and microorganisms. Examples include salicylic acid, a common natural product derived from the bark of the willow tree. This natural product is known to be effective against pains, aches and fevers.

Realizing that plants are the main sources of these important natural products as well as phytochemicals, careful review on the studies of these plants can help uncover new phytochemicals or shed some light on relationships between plant species and their phytochemicals. Studies have shown that plants from the same genus can have different secondary metabolites from different classes of compounds, although in many cases they share common secondary metabolites. For example, quercetin (**1**) and isoquercetin (**2**) have been isolated from the flowers of *Albizia julibrissin* whereas lupeol (**3**) and acacic acid (**4**) were isolated from the whole plant of *Albizia versicolor* (Kokila *et al*., 2013). Albizzine A (**5**) on the other hand has been isolated from the bark of *Albizia myriophylla* (Kokila *et al*., 2013). Traditional medicines however receive little attention in terms of modern research in Botswana as correlated by the few numbers of published articles (Setshogo, 2011). This is despite their contribution to rural societies especially. Moreover, less effort has been made to upgrade the practice of traditional medicinal use in our societies or even try and incorporate it into modern practices.

The observation opens an opportunity into the study of some species of plants that have not been explored extensively or have not been studied at all in an attempt to discover new and novel secondary metabolites. Studies like bio-guided assays on plant extracts are usually a good starting point as this can save time and money. The way we use medicines today should be such that we explore a combination of traditional and modern practices in an attempt to upgrade the health care system that will be beneficial to everyone.

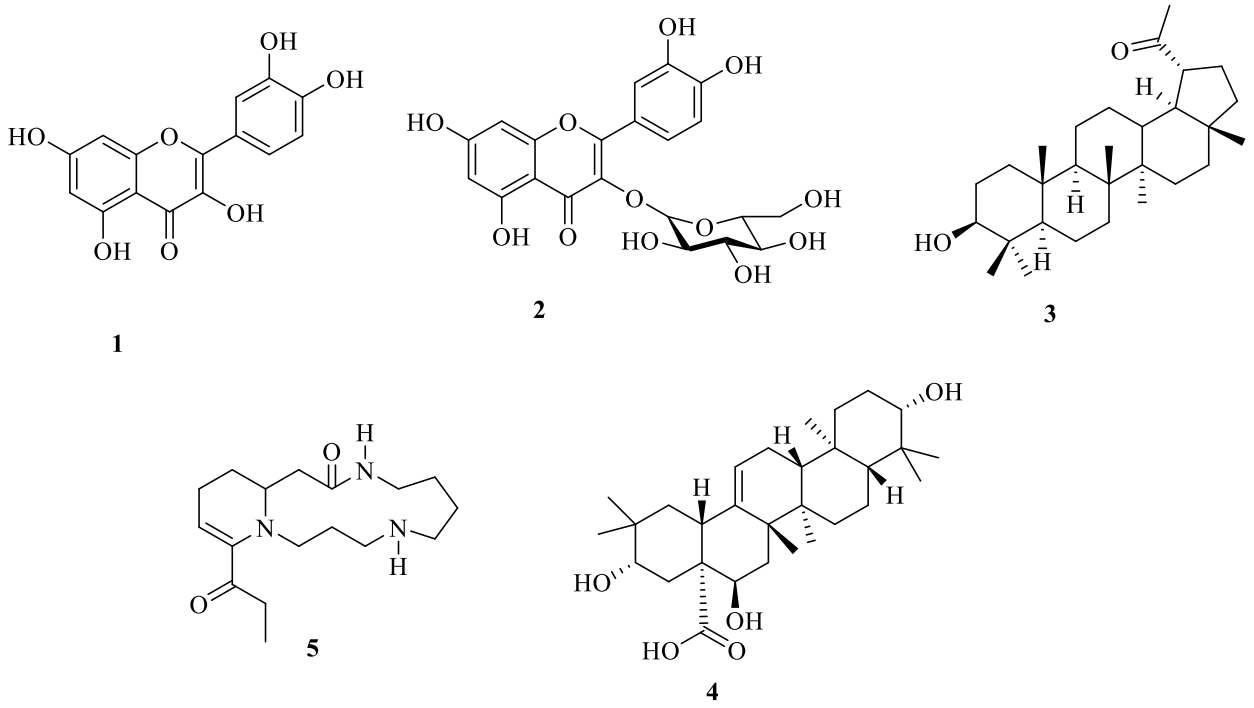


Figure 1.1: Examples of secondary metabolites isolated from different plant species but from the same genus *Albizia*.

As already indicated above, plants have been shown to possess medicinal properties which are helpful to the health and wellbeing of the human species especially the rural communities in most parts of the world. From literature survey, minimal research has been carried out on medicinal plants in Botswana (Setshogo, 2011) and also, much of what is known today about medicinal plants and their properties comes directly or indirectly from indigenous knowledge through prolonged usage and experience (Arzu and Thiagarajan, 2016). Moreover, not much is known about the secondary metabolites contained in these medicinal plants. These natural products and their derivatives are a major source of leads in the development of new drugs (Molinari, 2009). It is estimated that about 25 % of the drugs in the market are derived from natural sources (Rates, 2001). According to Baker however, pharmaceutical companies have decreased research in natural products (Baker *et al*., 2007). Reasons cited include difficulties working with natural products, complex nature of natural product science, limited sources of plant material and concerns about intellectual property rights (Harvey, 2008). As a result, a number of problems associated have emerged. These include amongst others failure to produce new lead drugs from some of the

available secondary metabolites in key therapeutic areas (Lahlou, 2007). Also, most of the available drugs are ineffective against most of the diseases as most of these disease causing microorganisms are becoming more resistant with prolonged use of the same drugs (Lahlou, 2013). This then limits the effectiveness of available drugs in those key therapeutic areas.

It is important therefore to engage in isolation, purification and characterization of compounds from plants in order to have new lead compounds to new drugs. Such a program of discovering lead compounds is cheaper when compared to others like combinatory chemistry (Molinari, 2009; http://www.who.int/intellectualproperty/submissions/Sauerbarriers.pdf). This report outlines the results of a research project that involved isolation, purification and characterization of natural products from the twigs of *Baikiaea plurijuga* and the tubers of *Ipomoea bolusiana*. The description and summary of previous phytochemical investigations of these plants will be discussed in the next sections of this chapter.

1.2 Plants Selected for The Study

Two plants, *B. plurijuga* and *I. bolusiana* were selected for this study. *B. plurijuga* belongs to the family Fabaceae and was selected for the study because it has never been phytochemically investigated and other plants in the family have proved to be a good source of interesting natural products with biological activity (Krishna and Banji, 2012; Hanganu *et al*., 2010). *I. bolusiana* on the other hand belongs to the family Convolvulaceae and was selected for the study because it has never been phytochemically investigated as well. Each of these plants will be discussed in the latter parts of this report. The two plants are closely related to medicinally important plants in their respective families, the Fabaceae and Convolvulaceae.

Baikiaea plurijuga belongs to the family Fabaceae subfamily Caesalpinioideae. Its complete scientific classification is as follows;

Kingdom: Plantae

Order: Fabales

Family: Fabaceae

Genus: *Baikiaea*

Species: *B. plurijuga*

The family Fabaceae or Leguminosae is the third largest in the plant kingdom consisting of 730 genera and over 19 000 species easily recognized by their fruit (legume) and their compound stipulated leaves (Stevens, 2001). There are three subfamilies of the Fabaceae namely the Mimosodeae, Caesalpinioideae and Papillinoideae. The Fabaceae is also the second largest family in terms of medicinal plants (Gao *et al*., 2010) after the Astragalus (Rahman and Parvin, 2014). The Leguminosae consists of trees, shrubs and herbaceous plants. These plants are distributed around the world and are important sources of food and medicines (Rahman and Parvin, 2014).

The genus *Baikiaea* consists of seven plant species namely *B. fragrantissima, B. ghesquiereana, B. plurijuga, B. robynsii, B. suzannae, B. zenkeri* and *B. insignis.* These are trees found in tropical lowland rain forests in countries like Equatorial Guinea and also in drier habitats in countries like Tanzania as well as on the Kalahari sands of Botswana.

B. plurijuga, also known as the Zambezi Teak or Mukusi in Shona, is a large tree with an upright stem. It is the dominant species in the Kalahari. It is also found in Zambia, southern Angola, northern Botswana, northern Namibia, and Zimbabwe and is regarded as one of the most valuable timber trees in these areas (Joker and Jepsen, 2003). The tree has attractive pink or purple flowers (Figure 1.2) found between December and March (Theilade *et al*., 2001). It has characteristic woody pods that have velvety hairs and are broadest near their hooked tips (Theilade *et al*., 2001). As with most trees, it is a source of food for wild animals as its young seedlings are fed on by wild animals. *B. plurijuga* belongs to the to the subfamily Caesalpinioideae. The subfamily is characterized by trees, shrubs and herbs (Childes and Walker, 1987).

Timber is the reason why *B. plurijuga* is exploited. The trunk and branches of the tree are widely used to make poles used in house building, doors, railway sleepers and furniture because of its high resistance to abrasion, low shrinkage and attractive appearance. The communities where this tree is found use it for making drums, dug-out canoes and for firewood. The bark and wood extracts of this tree have been used for leather tanning to produce reddish brown leather (Theilade *et al*., 2001; Joker and Jepsen, 2003).

1(a)

1(b)

Figure 1.2: 1(a) Leaves and dehiscent pods of *B. plurijuga* (Musgrave) and 1(b) Flowers of *B. plurijuga* (http://www.zimbabweflora.co.zw/speciesdata/image-display.php?species id=126740&image id=2).

Baikiaea plurijuga is a medicinally important plant as some communities use it even here in Botswana. Bark decoctions and infusions of this plant are used to treat eye diseases, syphilis and toothache (Theilade *et al*., 2001). The sap from this plant is used to treat stomach-ache and the gum is used to treat rabies. In some parts of northern Namibia, the tree is used to alleviate symptoms of malaria such as fever and head ache (Nafuka and Mumbengegwi, 2013). Here in Botswana, the Hambukushu use the boiled leaves of this plant to relieve stomach pains.

Ipomoea bolusiana belongs to the family Convolvulaceae subfamily *Ipomoea*. Its complete scientific classification is as follows;

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Solanales

Family: Convolvulaceae

Genus: *Ipomoea*

The genus *Ipomoea* comprises over 500 species and is the largest in the flowering plant family Convolvulaceae (Bhellum, 2012). Common names include morning glory, sweet potato,

bindweed, and moonflower. This is a large and diverse group made up of annual and perennial herbaceous plants, shrubs and small trees. Most of the species are twining climbing plants (Glimn-Lacy and Kaufman, 2006). The genus occurs throughout the tropical and subtropical regions of the world (Bhellum, 2012). The genus is not widely used medicinally in Botswana, however, it is commonly used in other parts of the world. This family of plants is distributed around the world in places like Asia, Africa and the Americas.

The genus *Ipomoea,* since time immemorial, has continuously been used for different purposes such as medicinal, ritual and in agriculture. An ethno medical survey of *Ipomoea* species revealed widespread and diverse folkloric uses throughout the world. Generally, these species are used in the treatment of infectious and parasitic diseases (Sahu and Gupta, 2014). For instance, *Ipomoea asarifolia* is used in Brazil to neutralize inflammation that is induced by the *Tityus serrulatus* scorpion venom (de Souza Lima *et al*., 2014). Some species of *Ipomoea* showed antimicrobial, analgestic hypotensive and anticancer activities (Meira *et al*., 2012). Other major applications include treatment of bacterial infections, respiratory diseases, diabetes and urinary tract infections (Meira *et al*., 2012). For instance, the ash of *Ipomoea carnea* is used to treat skin diseases and the milky juice of the same plant is used for treatment of leucoderma (Sharma and Bachheti, 2013). Despite extensive research on the other members of the genus *ipomoea*, little has been carried out on *Ipomoea bolusiana*, hence this research. The uses of plants of the genus *Ipomoea* in traditional medicine are summarised in Table 1.1

Table 1.1: Some species of *Ipomoea* used in traditional medicine.

Species	Common name(s)	Plant part used	Traditional uses	References
I. aquatic	Swamp morning-glory	Leaves Floral buds Whole plant	Diabetes, scorpion venom antidote, liver complaints, ringworm, leprosy and fever. Anthelmintic. Nosebleeds and high blood pressure.	Prasad *et al*., 2005
I. asarifolia	Ginger-leaf, morning glory	Leaves NS	Treatment of inflammation disorders such as scabies, symptoms of syphilis, skin ulcers and external wounds. Treatment of ophthalmias, neuralgias, headaches and stomach and arthritic pains.	Furtado *et al*., 2016 Jegede *et al*., 2009
I. cairica	Messina creeper, Cairo morning glory, coast morning glory and railroad creeper	NS Leaves	Lessens inflammation and as a carminative. Useful in fever, bronchitis and liver complaint Treatment of body rushes	Srivastava and Shukla, 2015 AhbiRami *et al*., 2014
I. carnea	Bush morning glory, pipe-cane and besharam behaya		Anti-inflammatory, Antidiabetic, wound healing, antifungal and cardiovascular activity	Sharma and Bachheti, 2013
I. digitata	Alligator yam and milky yam	Tubers Roots	Treat blood dysentery and used as astringent Promote weight gain, treatment of spermatorrhoea, fever, bronchitis and scorpion stings	Jain *et al*., 2011
I. muricata	Clove bean and purple moonflower	Seeds, stems and leaves	Treatment of various skin ailments such as chronic and dangerous wounds, cuts and blisters	Joseph and Markose, 2008.

I. murucoides	Foolish dove and pajaro bobo.	Tree Flowers, stem bark and leaves	Smoke from burnt tree used as mosquito repellent Skin disorders, antiseptic, anti-inflammation and muscle pain Itches and rashes	Rivera *et al*., 2013; Cherigo et al., 2009.
I. nil	Picotee morning glory, ivy morning glory and Japanese morning glory	NS NS	Treatment of liver disorders	Buga *et al*., 2013
I. oblongata	Bhogo, bhoqu, ubhogo and ubhoqo	NS	Cancer, stomach ailments and swollen feet.	Kose., *et al*., 2015
I. orizabensis	Mexican scammony root	NS Roots	Purgative, abdominal fever, dysentery, epilepsy, meningitis and tumors. Cathartic and hydragogue	Nataraya and Acharya, 2015
I. pes-caprae	Bayhops, beach morning glory or goat's foot	NS Leaves	Used in inhibition of vomiting and diarrhea. Used as stomachic and tonic and also as rheumatic. Juice from the leaves used as first aid to treat jelly fish stings.	Manigaunha, Ganesh and Kharya, 2010
I. purga	Jalap	Roots NS	Purgative and laxative Anti-cancer	Pereda-Miranda and Bah, 2003 Arnason *et al*., 2013
I. stans	Ornamental sweet potato vine	Roots	Treatment of anxiety Ophthalmic diseases and paralysis.	Guzman Gutierrez, 2014 Pereda-Miranda and Bah, 2003
I. stolonifera	Fiddle-leaf morning glory	NS	Treats rheumatism, arthritis, fish puncture wounds, sun stroke and escolitis	Cai *et al*., 2014

**NS-Not Stated*

Ipomoea bolusiana is admired for its beauty. It is a perennial dwarf shrub, growing from a thick tuberous rootstock, up to 20 cm in diameter. It is easily characterized by the hairless stems that erect from the base. Its leaves are narrow and about 7 cm long. Flowers are the most attractive part of the plant and are funnel-shaped with a pink or mauve color (Figure 1.3). This plant is native to Madagascar and South Africa but can also be found throughout Southern Africa such as in Botswana.

2(a) 2(b) 2(c)

Figure 1.3**:** Photographs of *I. bolusiana* plant (2(a)), flower and tuber (2(b)) and (2(c)) respectively (http://www.worldofsucculents.com/ipomoea-bolusiana/, http://www.cactus-art.biz/schede/IPOMOEA/Ipomoea bolusiana/Ipomoea bolusii/Ipomoea bolusiana.html).

Ipomoea bolusiana is not commonly used medicinally in Botswana. However, where it is used, the plant is widely used in the treatment of sexually transmitted infections, toothache, sores and urinary tracts in some parts of South Africa (Mongalo *et al*., 2015). Extracts of the plant have shown both antioxidant as well as antimicrobial activities (Mongalo *et al*., 2015).

1.3 Previously Isolated Metabolites of *B. Plurijuga* and *I. bolusiana*

Previous research work on the heart wood of *B. plurijuga* was aimed at establishing the chemical constituents responsible for the reddening of the wood in the presence of light. The work resulted in the isolation of an amino L-1:2:3:6-tetrahydropyridine-2-carboxylic acid (**6**) also known as

baikiain and a tannin (**7**). Baikiain is also found in *Caesalpina tinctoria*, red algae and the seeds of *Ioquat* (*Eriobotrya japonica*) (Mazon and Nijera, 1997). It was concluded in the study that the presence of a leucofisetinidin polymer was responsible for the reddening of the wood when exposed to light. Tannins are important secondary metabolites with antibacterial, antifungal and antiviral activities (Sahayaraj and Ravi, 2008). Other secondary metabolites reported were D-pinitol (**8**) from the acetone extract, Hederagenin (**9**) a dihydrotriterpene acid from the ether extract, resorcinol (**10**), catechol (**11**) and *p*-hydroxybenzoic acid (**12**). D-pinitol (**8**) is known to prevent rat breast carcinogenesis through inhibition of cancer genes (Bcl-2 and p53) and also has free radical scavenging capabilities (Rajendran *et al*., 2013). Hederagenin (**9**) was also isolated from *Cephalaria aristata* by Sarikahya and many hederagenin containing saponins have been shown to possess haemolytic activity. These are natural triterpenoids widely distributed in higher plants (Chwalek *et al*., 2006). Resorcinol (**10**), also isolated from Awa-ban tea is known for its antioxidant capabilities (Hiasa *et al*., 2013). *p*-Hydroxybenzoic acid (**12**) is found in a wide variety of mushroom species (Heleno *et al*., 2013) and together with other phenolic acids are known to be powerful antioxidants with antibacterial, anti-inflammatory and antiviral activities (Meira *et al*., 2012).

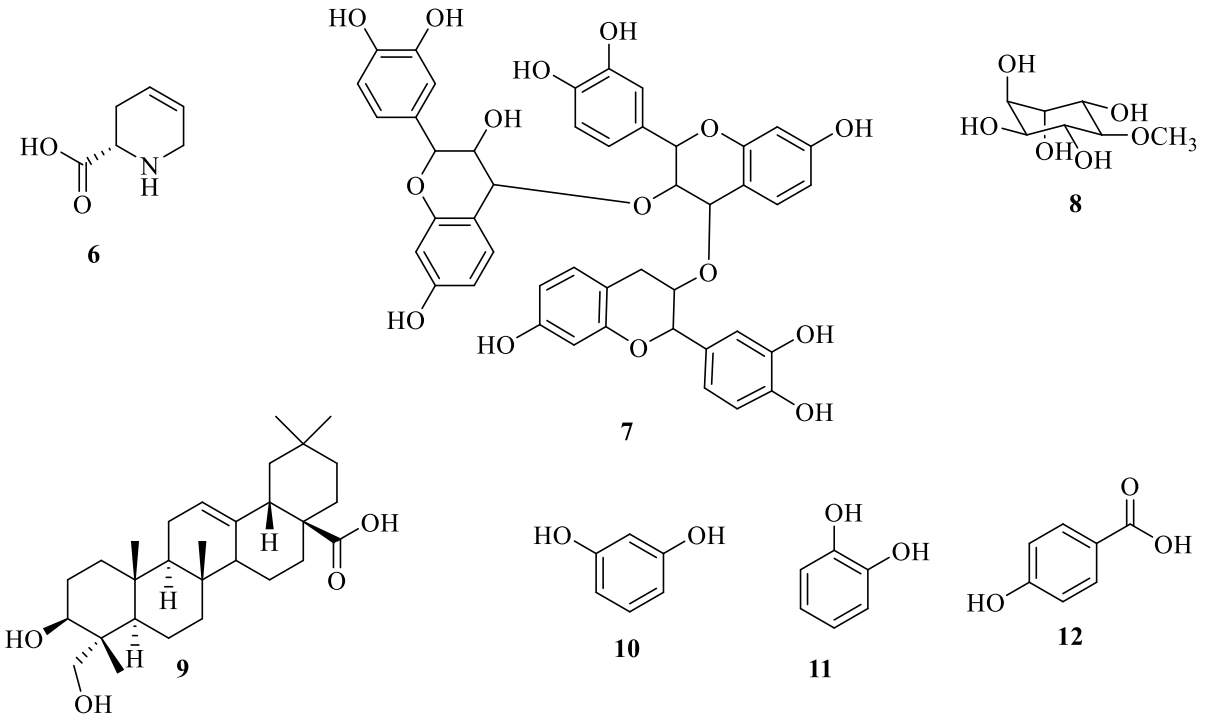


Figure 1.4: Some compounds isolated from the heartwood of *B. plurijuga*.

Phytochemical studies on some species of *ipomoea* resulted in the isolation and characterization of secondary metabolites belonging to different classes such as alkaloids, phenolic compounds, coumarins, norisoprenoids, diterpenes, flavonoids, lignans and triterpenes (Meira *et al*., 2012). Previous phytochemical work on some species of *ipomoea* lead to the isolation of some alkaloids. Alkaloids are heterocyclic compounds with at least one nitrogen atom in their ring system. They are defined by their basic properties, complex structures, physiological activities as well as biosynthetic pathways. Alkaloids exhibit diverse structural and biosynthetic pathway in terms of structural complexities and functionalities. Examples of alkaloids from some species of *ipomoea* include agroclavine (**13**), isolated from *I. fistulosa*, *I. mueller* and *I. tricolor*, chanoclavine (**14**) isolated from *I. asarifolia*, *I. hederacea* (Zia-Ul-Haq *et al*., 2012; Meira *et al*., 2012), *I. muelleri*, *I. corymbosa*, *I. tricolor* and *I. violacea* (Meira *et al*., 2012). Ergometrine or ergonovine (**15**) was isolated from *I. mulleri*, *I. corymbosa*, *I. tricolor* and *I. violacea* (Meira *et al*., 2012). Alkaloid (**15**) is known to exhibit psychotropic, psychotomimetic, oxytocic vasoconstrictor as well as haemostatic activities (Meira *et al*., 2012). Ergometrine (**15**) was found to have hypotensive effects (Joseph *et al*., 2011). One study even revealed that it can prevent pregnancy in female rats when

administered in a diet at nontoxic dosages (Finn and Mantle, 1969). Ergosinine (**16**) was isolated from *I. palmat* while penniclavine (**17**) was isolated from *I. hederacea, I. mulleri, I. corymbosa* and *I. violacea* (Meira *et al*., 2012). Alkaloid (**16**) has shown uterotonic and festuclavive activities (Fiserova and Pospisil, 1999) while alkaloid (**17**) has antimicrobial properties (Meira *et al*., 2012). Another alkaloid of interest, ipalbidine (**18**) was isolated from *I. alba, I. muricata* and *I. hardwickki* (Meira *et al*., 2012). This alkaloid exhibited analgesic and antioxidant activities (Honda *et al*., 2003). Both 2-epi-lentiginosine (**19**) and swainsonine (**20**) were isolated from *I. carnea*. 2-Epi-lentiginosine (**19**) is known for its α-glucosidase inhibitory activity (Kim and Jung, 2011) while swainsonine (**20**) is known for its immunomodulatory antimetastatic potent inhibitory activity towards rat α-mannosidase (Elbein *et al*., 1981; Hueza *et al*., 2005). Calystegine B1 (**21**) and calystegine B2 (**22**) were isolated from the herbal material and roots of *I. alba* (Schimming *et al*., 1998) while calystegine C1 (**23**) has been isolated from I. *alba, I. aquatic, I. batatas, I. carnea, I. hederifolia, I. eremnobrocha, I. pes-caprae, I. setifera* and *I.* violacea (Meira *et al*., 2012). Alkaloids (**21**), (**22**) and (**23**) have exhibited potent inhibitory activity towards rat lysosomal β-glucosidase (Asano *et al*., 1997). Calystegine B3 (**24**) isolated from *I. alba, I. aquatic, I. batatas, I. carnea, I. hederifolia, I. eremnobrocha, I. obscura, I. pes-caprae, I. seifera* and *I. violacea* (Meira *et al*., 2012) is known for its moderate inhibitory activity towards rat α-mannosidases and β-mannosidases (Asano *et al*., 1997). Agroclavine (**13**) has shown antimicrobial as well as cytostatic activities in some studies (Meira *et al*., 2012). Glycosylated agroclavine derivatives are known to enhance the cytotoxic activity of lymphocytes and are useful in the treatment of various tumours (Boichenko *et al*., 2003). Chanoclavine I (**14**) on the other hand is known for its cytostatic and antitumor properties (Eich and Pertz, 1999; Meira *et al*., 2012).

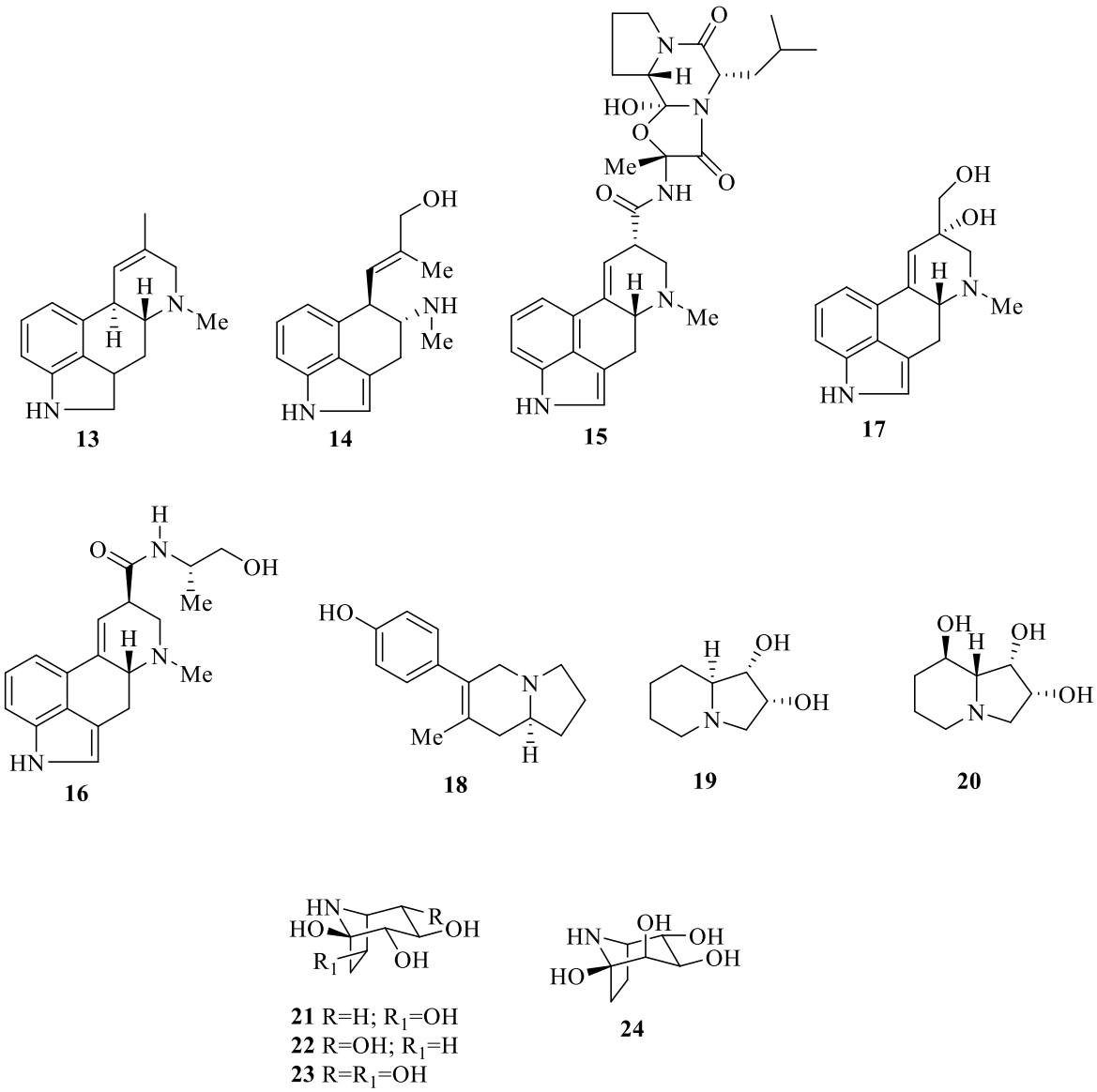


Figure 1.5: Examples of alkaloids isolated from other species of *Ipomoea* (Meira *et al*., 2012).

Further, some phenolic compounds have been isolated from some species of *ipomoea*. Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants with a common carbon skeleton: C6-C3 phenylpropanoid unit (Talhaoui *et al*., 2015). There is a wide variety of these compounds and they include cinnamic acids (C6-C3), benzoic acids (C6-C1), flavonoids (C6-C3-C6), proanthocyanidins [(C6-C3-C6) n], coumarins (C6-C3), stilbenes (C6-C2-C6), lignans (C6-C3-C3-C6) and lignins [(C6-C3) n] and

most of these compounds have antioxidant capabilities. Some phenolic compounds have been isolated from the genus *Ipomoea*.

N-cis-feruloyl tyramine (**25**) and N-trans-feruloyl tyramine (**26**) have been isolated from roots of *I. aquatic* (Meira *et al*., 2012). The compounds are known for their inhibition of prostaglandin synthesis and also show antitumor activities (Fang *et al*., 2007). Caffeic acid (**27**) has been isolated from *I. batatas* as well as *I. muricata* (Meira *et al*., 2012). It is known for its antioxidant and antimutagenic properties (Sud'ina *et al*., 1993). 3-O-caffeoyl-quinic acid (chlorogenic acid) (**28**) has been isolated from *I. batatas* as well as *I. fistulosa* (Meira *et al*., 2012). The compound is important as it exhibits a wide variety of activities such as antimutagenic, antioxidant (Wang *et al*., 2005) and inhibition of HIV replication (Islam *et al*., 2003).

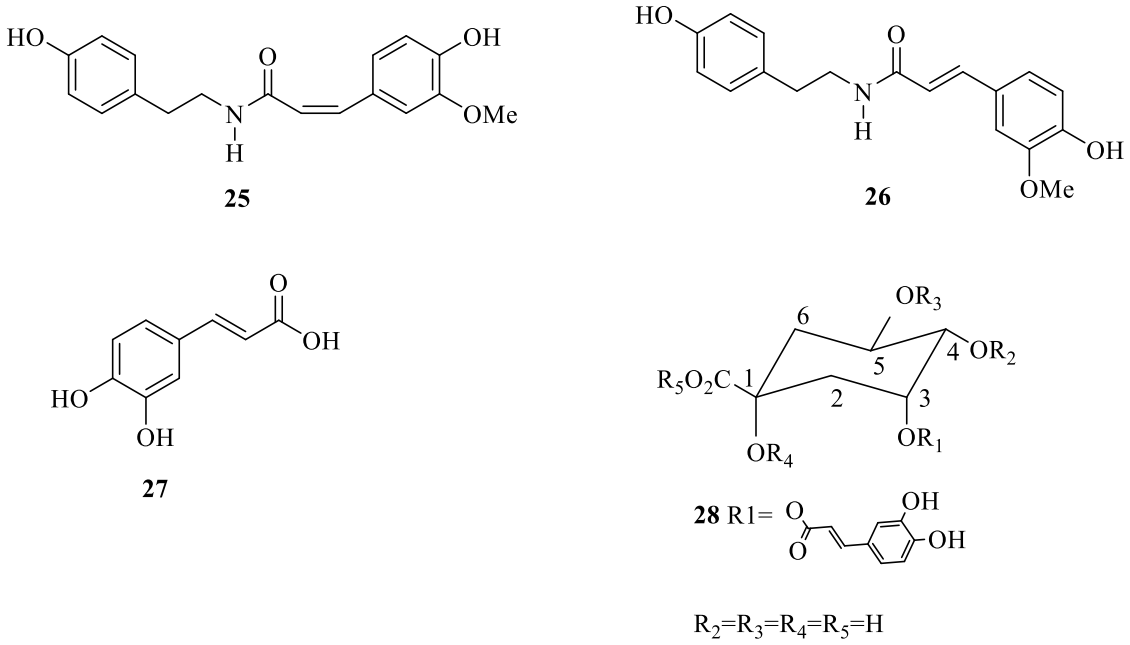
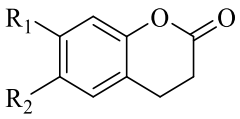


Figure 1.6: Examples of phenolics isolated from some species of *Ipomoea* (Meira *et al*., 2012).

Coumarins have also been isolated from some species of *ipomoea*. Coumarins are oxygen containing heterocyclic phytochemicals that occur naturally in several plant species. Some coumarins have been isolated from some species of *ipomoea*. Coumarin (**29**) has been isolated from *I. turpethum* (Meira *et al*., 2012). The compound has cytotoxic, antibacterial (Ojala *et al*., 2000) as well as antifungal properties (Souza *et al*., 2005). Coumarin (**30**), scopoletin has been

isolated from a number of species of *ipomoea* including *I. batatas, I. cairica, I. digitata, I. stans* and *I. turpethum* (Meira *et al*., 2012). The Coumarin exhibits a number of properties that include inhibition of prostate cancer proliferation, acetyl cholinesterase inhibition, antioxidant, anticoagulant and anti-HIV activities (Meira *et al*., 2012). Esculetin (**31**) has been isolated rom *I. batatas* (Meira *et al*., 2012). The compound has antioxidant, anticoagulant as well as anti-HIV properties (Lin *et al*., 2000; Meira *et al*., 2012). Umbelliferone (**32**) isolated from *I. batatas, I. cairica* and *I. digitata* is known for its anticoagulant as well as anti-HIV properties (Meira *et al*., 2012).



29 R_1=R_2=H
30 R_1=OH; R_2=OMe
31 R_1=R_2=OH
32 R_1=OH; R_2=H

Figure 1.7: Coumarins isolated from some species of *Ipomoea* (Meira *et al*., 2012).

Norisoprenoids, actinidol (**33**) was isolated from *I. pes-caprae* (Meira *et al*., 2012). The compound exhibits inhibition of ethyl phenylpropiolate induced rat ear oedema (Manigaunha *et al*., 2010). Another norisoprenoids trans-β-damascenone (**34**) and a diterpenes, E-phytol (**35**), have been isolated from *I. pes-caprae* (Manigaunha *et al*., 2010). The compounds are anti-microbial agents (Igwe and Abii, 2014). 3,4-dihydro-8-hydroxy-3-methylisocoumarin (**36**) and isocoumarin and benzenoids; eugenol (**37**), 4,4,7-trimethyl-1,4-dihydro-2-hydroxy-1-naftalenone (**38**) and 4-vinyl-guaiacol (**39**) have all been isolated from *I. pes-caprae* (Meira *et al*., 2012). All the compounds are known to exhibit inhibition of prostaglandin synthesis hence their importance (Meira *et al*., 2012).

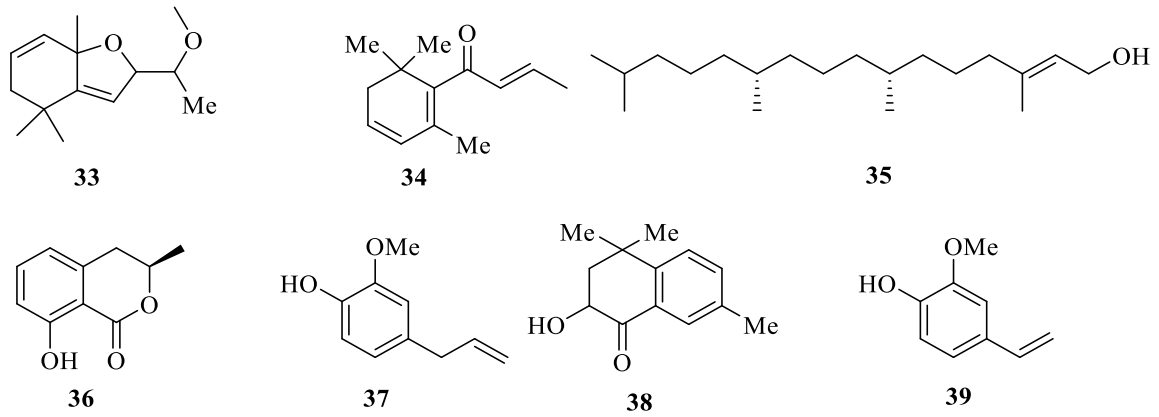
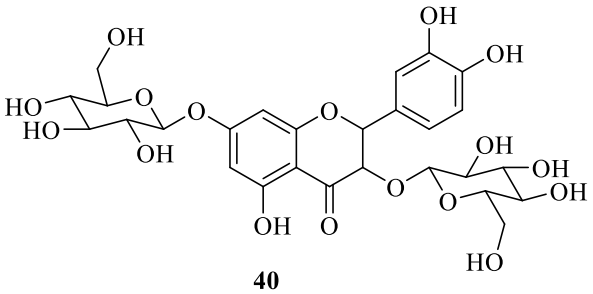


Figure 1.8: Examples of norisoprenoids, diterpenes, isocoumarin and benzenoids isolated from some species of *ipomoea* (Meira *et al*., 2012).

Phytochemical work on the same species *ipomoea* also lead to the isolation of some flavonoids and antocianosides. 3α,7β-O-Diglycopyranosyldihydroquercetin (**40**) has been isolated from *I. aquatic* (Meira *et al*., 2012). The compound has antioxidant and cytotoxic properties, in vitro (Meira *et al*., 2012). Peonidin (**41**), isolated from *I. batatas* and 3-O-(2-O-E-caffeoyl-β-D-glycopyranosyl)-(6-O-E-caffeoyl)-β-D-glycopyranosyl)-5-O-β-Dglycopyranoside-cianidin (**42**) isolated from *I. asarifolia*, *I. batatas* and *I. purpurea* are known for their antioxidant and anticancer properties (Chen *et al*., 2005; Meira *et al*., 2012). 3-O-Sophoroside-5-O-glycosil-cianidin (**43**) known for its antimutagenic properties together with flavonoids and antocianosides (**45**) to (**50**), isolated from *I. batatas* exhibit antioxidant properties (Meira *et al*., 2012). Compound (**50**) exhibits a number of properties such as antimutagenic and antihyperglycemic properties. Compound (**51**), isolated from *I. pes-caprae* has antinociceptive properties (Meira *et al*., 2012). Heavenly blue anthocyanin (**52**) isolated from *I. tricolor* and *I. nil* is known to protect against ultraviolet (medium wave) induced DNA damage (Meira *et al*., 2012; Mori *et al*., 2005).



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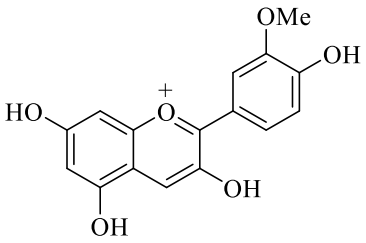

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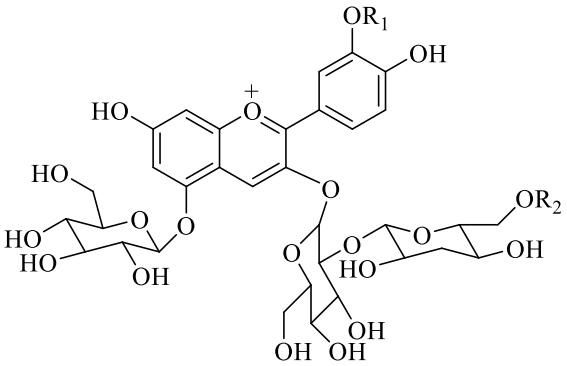
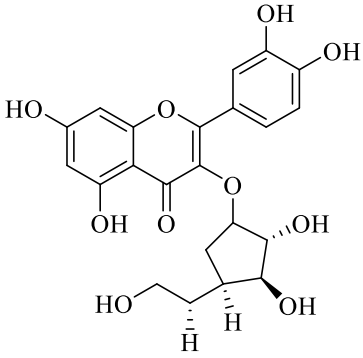
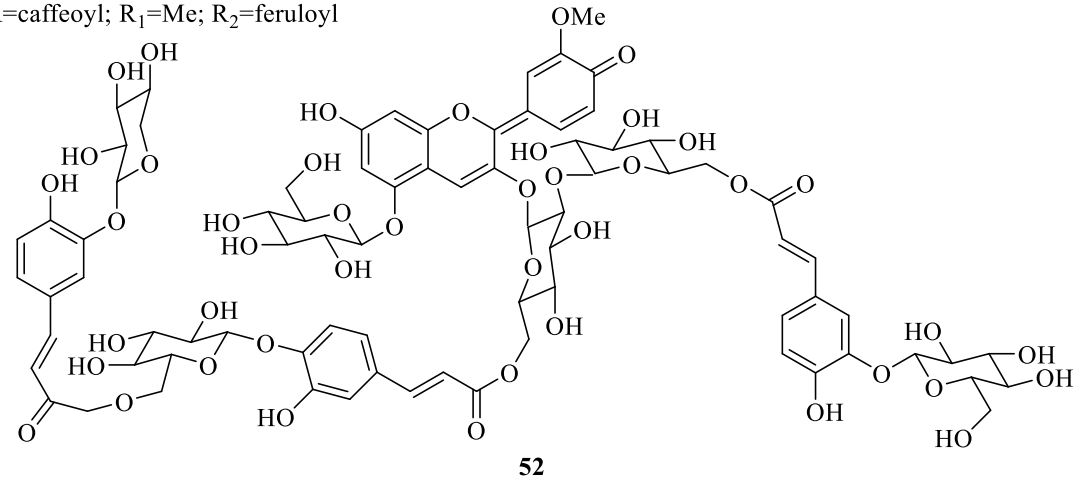




51

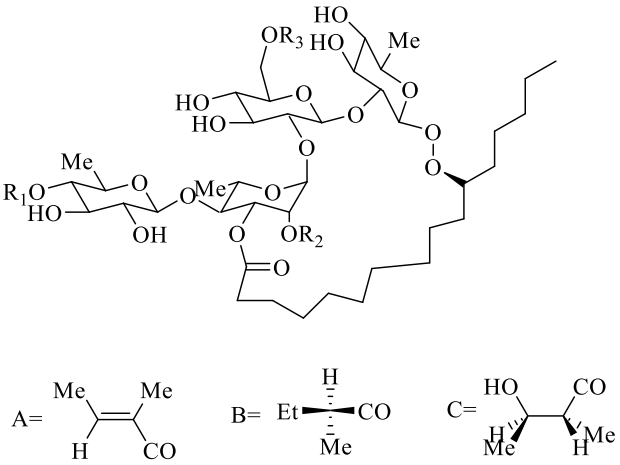
42 R=R_2=caffeoyl; R_1=H
43 R=R_1=R_2=H
44 R=caffeoyl; R_1=R_2=H
45 R=caffeoyl; R_1=Me; R_2=H
46 R=caffeoyl; R1=H; R_2=p-hydroxy-benzoyl
47 R=R_2=caffeoyl; R_1=Me
48 R=caffeoyl; R_1=H; R_2=p-hydroxy-benzoyl
49 R=caffeoyl; R_1=Me; R_2=p-hydroxy-benzoyl
50 R=caffeoyl; R_1=Me; R_2=feruloyl



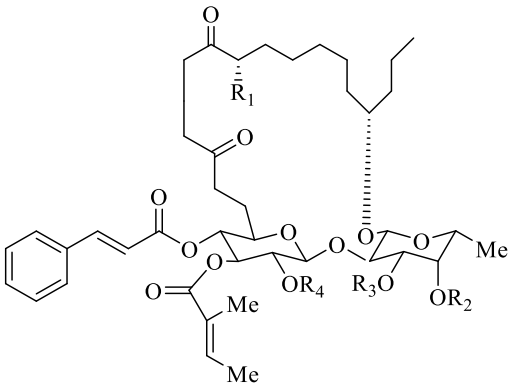
52

Figure 1.9: Examples of flavonoids and antocianosides isolated from some species of *Ipomoea* (Meira *et al*., 2012).

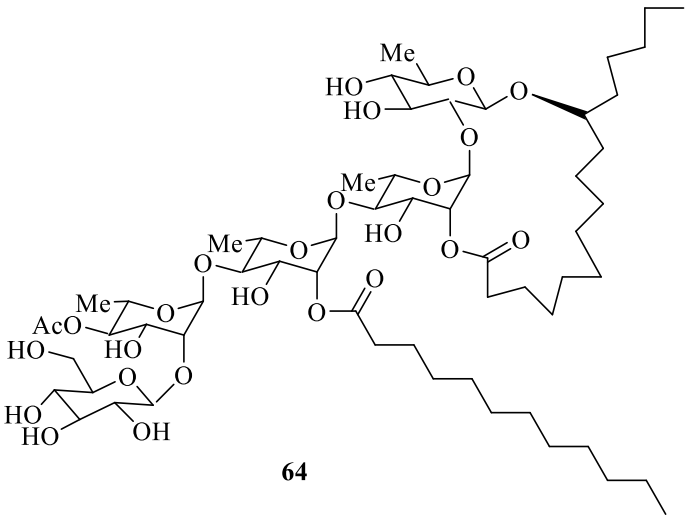
Glycolipids scammonine I (*53*) and and scammonine II (**54**) were isolated from *I. orizabensis* (Meira *et al*., 2012). Glycolipid (**53**) has shown weak cytotoxic activity against oral human epidermal carcinoma and also activity against methicillin resistant *Staphylococcal aureus* while compound (**54**) has shown weak cytotoxicity against oral human epidermal carcinoma (Meira *et al*., 2012). Glycolipids ipomoeassins A-E (**55**-**59**) were isolated from *I. squamosal* and all the compounds exhibit cytotoxic activity against ovarian carcinoma (Meira *et al*., 2012). Murucin 1 (**60**) isolated from *I. murucoides* also shares the cytotoxic activity against ovarian carcinoma (Meira *et al*., 2012).



53 R_1=A; R_2=B; R_3=H
54 R_1=H; R_2=B; R_3=H



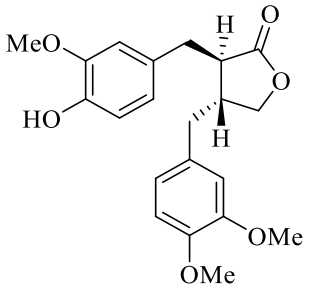
55 R_1=R_3=R_4=H; R_2=Ac
56 R_1=R_2=R_3=R_4=H
57 R_1=OH; R_2=Ac; R_3=R_4=H
58 R_1=OAc; R_2=Ac; R_3=R_4=H
59 R_1=OAc; R_2=R_3=R_4=H



64

Figure 1.10: Examples of glycolipids isolated from some species of *Ipomoea* (Meira *et al*., 2012).

A Lignan, arctigenin (**61**) was also isolated from *I. cairica* by previous phytochemical studies. It exhibits antioxidant, anti-inflammatory, anticancer (Awale *et al*., 2006; Meira *et al*., 2012) and it also inhibits HIV replication (Eich *et al*., 1996).



61

Figure 1.11: Lignan isolated from *I. cairica* (Meira *et al*., 2012).

Triterpene have also been previously isolated from this species. β-Amyrin acetate (**62**) was isolated from *I. batatas* and *I. pes-caprae* while α-amyrin acetate (**63**) was isolated from *I. pes-caprae* (Meira *et al*., 2012). Both compounds exhibit antinociceptive activities (Meira *et al*., 2012). Boehmeryl acetate (**64**), another triterpene, was isolated from *I. batatas* and is an insecticide used against the sweet potato weevil (*Cylas formicarius elegantulus)*. Betulinic acid (**65**) and glochidone (**66**) isolated from *I. pes-caprae* are known for their antinociceptive properties (Meira

et al., 2012). Friedelin (**67**) isolated from *I. batatas* is known to exhibit antibacterial activity against *Staphylococcus aureus* and it is also antifungal against *Psedallescheria boydii* (Meira *et al*., 2012). Taraxerol (**68**) isolated from *I. digitata* is known to exhibit anti-cancer activity as well as inhibit acetylcholinesterase, an enzyme that is responsible for the destruction of acetylcholine (Meira *et al*., 2012; Sharma and Zafar, 2015).

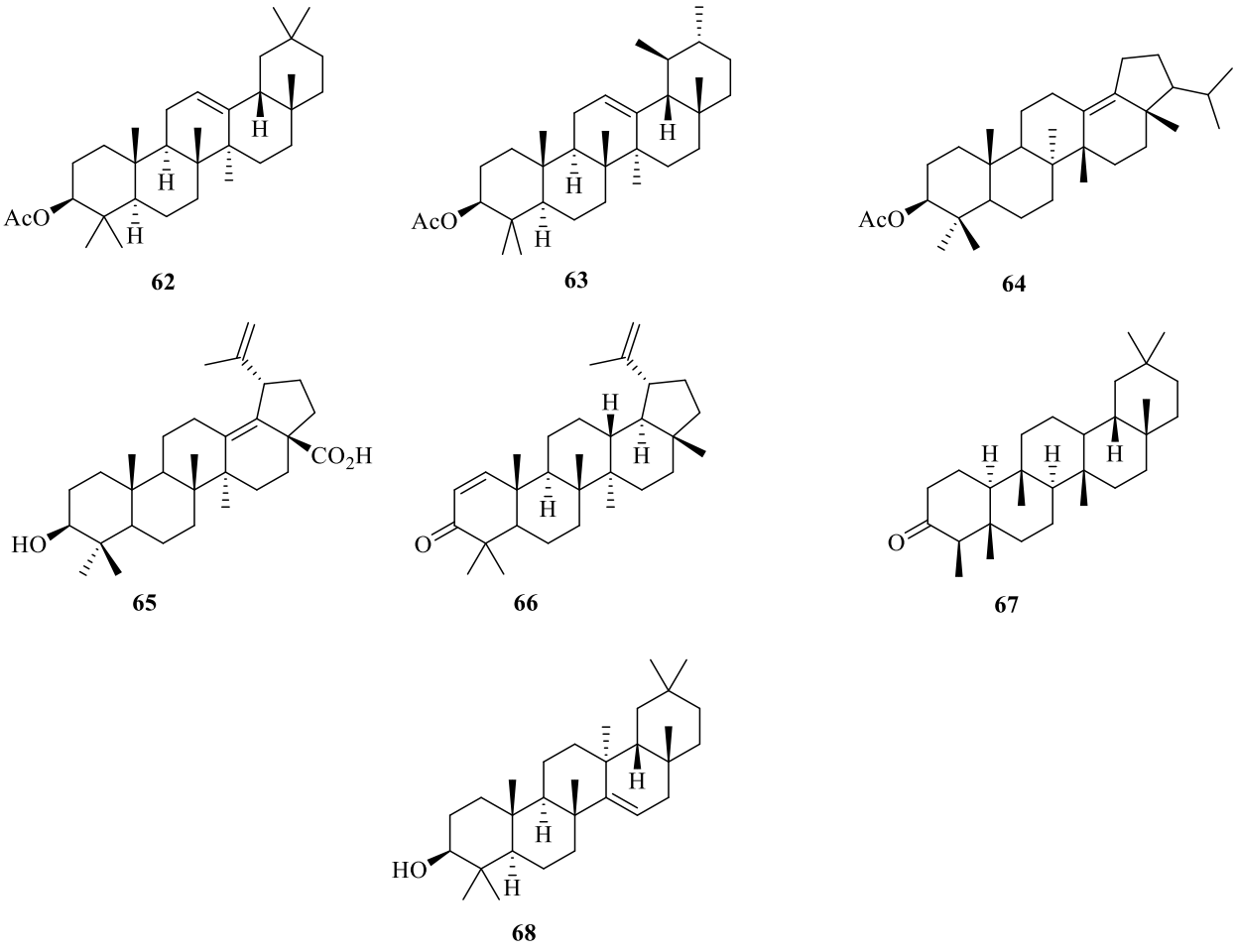


Figure 1.12: Triterpenes from some species of *Ipomoea* (Meira *et al*., 2012).

1.4 Aim of The Study

Despite the two plants being used as medicinal plants, there are no reports of any phytochemical work on *I. bolusiana* and *B. plurijuga*. Therefore, the aim of this study is to carry out phytochemical investigation on *I. bolusiana* and *B. plurijuga* with the hope of isolating novel secondary metabolites with interesting biological activities. The study also hopes to justify the folkloric uses of the two plants as medicinal plants.

1.5 Objectives

The research objectives are therefore;

1. To extract and isolate secondary metabolites from the tubers of *Ipomoea bolusiana* and from the twigs of *Baikiaea plurijuga*.
2. To identify and characterize the isolated compounds using spectroscopic techniques such as NMR, IR, UV-Vis and GC-MS/ GC-LC.
3. To determine the DPPH-free radical scavenging activities and antimicrobial activities of the plant extracts and pure isolates.

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Chapter Two

Materials and Methods

2 General Experimental Procedures

2.1 Physical and Spectroscopic Measurements

The 1D [^{1}H (300 and 600 MHz), ^{13}C (75.4 and 150.9 MHz), DEPT] and 2D [COSY, HMQC and HMBC] NMR spectra were recorded using a Bruker Advance 300 MHz spectrometer and a Bruker Advance 600 MHz spectrometer and referred to residual solvent signals. All the spectra were recorded at room temperature with all chemical shifts recorded against the internal standard, tetramethylsilane (TMS).

The IR spectra were recorded using a Perking Elmer Spectrum FT-IR Spectrometer Spectrum Two fitted with a universal attenuated total reflectance (UATR) sampling device.

Optical rotations were recorded using an Autopol IV automatic Polarimeter (Rudolph Analytical, NJ). All measurements were recorded at room temperature. Acetone, chloroform and methanol were used as solvents.

The HRMS experiments were carried out on an LTQ-Orbitrap spectrometer (Thermo Fisher, USA) equipped with an HESI. The spectrometer was equipped with an Agilent 1200 HPLC system including pump, PDA detector, column oven (30 ^{o}C) and auto-sampler (injection volume 5 µL). The spectrometer was operated in a positive and negative mode (with nominal mass resolving power of 60 000 at m/z 400 with a scan rate of 1 Hz) and LRESI-MS.

The UV spectra were recorded using a Shimadzu (UV 2101 PC) UV-Vis spectrometer. The UV spectra of the compounds dissolved in methanol were obtained first before obtaining those of the shift reagents and their UV spectra were recorded. Shift reagents were then used and they were prepared as follows;

- Sodium methoxide (NaOMe) was prepared by cutting 2.5g of sodium metal and dissolving it in 100 mL of dry methanol

- Aluminum chloride ($AlCl_3$) was prepared by dissolving 5g of anhydrous $AlCl_3$ in 100 mL of dry methanol.
- Hydrochloric acid (HCl) was prepared by diluting 50 mL of concentrated HCl in 100 mL of distilled water.
- Anhydrous powdered analytical reagent of sodium acetate (NaOAc) was used.
- Anhydrous powdered analytical reagent of boric acid (H_3BO_3) was used.
- Melting points were measured using a Stuart Scientific melting point apparatus.

2.2 Procedure for Acquisition of UV Spectra Using Shift Reagents

The procedure used was adapted from the one used by Mabry, 1970 and Markham, 1982. The flavonoid solutions were prepared by dissolving 1 mg of the flavonoid samples in 10 mL of dry methanol (analytical reagent). A 3 mL aliquot of each solution prepared was poured into a quarts cuvette and the UV spectra for each sample was acquired first for methanol and then the procedure repeated before adding shift reagents as follows;

- For sodium methoxide, three drops of the sodium methoxide solution were added to 3 mL of the original solution and the spectrum of the mixture was measured.
- For aluminum chloride, six drops of the aluminum chloride solution were added to 3 mL of the original solution and the spectrum of the mixture was measured.
- For hydrochloric acid, three drops of HCl were added to the aluminum chloride solution above and the $AlCl_3$/HCl spectrum was measured.
- For sodium acetate, about 1 mg of the anhydrous powdered analytical reagent of sodium acetate was added to 3mL of the original solution. This was allowed to settle at the bottom of the cuvette before acquisition of the spectrum.
- For the H_3BO_3, anhydrous powdered analytical reagent of boric acid (about half the amount of NaOAc) was added to the solution of NaOAc above and the spectrum for NaOAc/H_3BO_3 was measured.

2.3 Chromatographic and Separation Methods

Sephadex Columns

Using Sephadex LH-20, gel filtration was used to separate compounds with different molecular weights but with similar polarities. Elution was done using methanol and chloroform in the ratios (1:1) or (3:7) depending on the polarity of the compounds.

Silica Gel Columns

Normal liquid column chromatography was done on columns packed with Merck silica gel, 60-80 mesh with particle size 0.0400-0.0630 nm. Column elution was done using different solvent systems depending on the polarity of the different fractions.

Preparative Thin Layer Chromatography

Preparative thin layer chromatography was done on prep TLC plates coated on 20x20cm glass TLC plates with Merck silica gel $60HF_{254-366}$ with a coating thickness of 0.5mm. Different solvent systems were used to separate compounds with different polarities. Multiple developments were done in some cases.

Solvents

Analytical grade (AR) and general purpose reagents (GPR) were used for both column and prep-TLC elution. The solvents were sourced from different manufactures. GPR solvents were distilled before use by simple distillation process.

Monitoring, Detection and Visualization of Compounds

Column and prep-TLC eluents were monitored by observing under UV lamp (λ = 254 and 366) with marking of active compounds. The compounds were identified as being dark against a coloured background.

2.4 Extraction and Isolation Procedure for *I. Bolusiana*

Three medium sized tubers were obtained from Dr Tsopito of the Botswana University of Agriculture and Natural Resources. The wet tubers with a total mass of 1888.42g were cut into small cubes and totally extracted with a mixture of chloroform/ethyl acetate/ methanol (1:1:1, v/v/v) to afford an extract of 5.8815g. The tubers were then sequentially extracted with methanol and 80% aqueous methanol to afford extracts of 14.91g and 61.23g respectively. A total of 550 mL of water was recovered from extracting the wet tubers. The chloroform/ethyl acetate/ methanol extract was adsorbed on silica gel (6g) and subjected to normal column chromatography on a column packed with 150g of silica gel in n-hexane.

Column elution was carried out by gradient elution using n-hexane/ethyl acetate, ethyl acetate/ methanol and 100% methanol. A total of 11 fractions (1 to 11) were collected based on TLC analysis. Fraction 2 (45.5 mg) was subjected to Sephadex-LH20 column purification eluting with chloroform: methanol (7:3). A total of three fractions (A-C) were collected. Fraction B turned out to be compound **69** (3.9 mg). The compound could not be purified further because of its small quantities. Purification of fraction 4 (73.5 mg) on a silica gel column eluting with n-hexane/ethyl acetate (8:2) afforded compound **70** (73.5 mg). Fraction 6 (59.4 mg) was purified on a silica gel column eluting with n-hexane/ ethyl acetate (8:2) to afford compound **71** (52.2 mg). Crystals appeared upon concentration of fractions 8 to 11. The crystals were washed with methanol affording compound **72** (0.3202 g). A summary of the isolation procedure for the chloroform/ethyl acetate/methanol extract of the tubers of *I. bolusiana* is shown in figure 2.1 below.

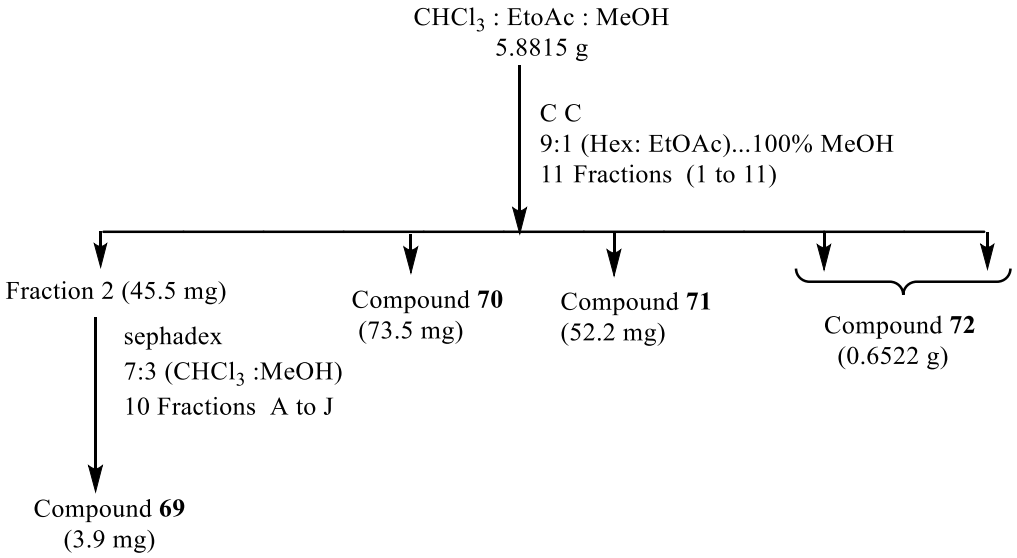


Figure 2.1: Summary of the isolation procedure for the chloroform/ethyl acetate/methanol extract of the tubers of *I. bolusiana*.

The methanol extract (65.51g) was adsorbed onto 50.00 g of silica and subjected to normal column chromatography on a column packed with 200 g of column silica in n-hexane. Column elusion was carried out by gradient elusion using n-hexane/ethyl acetate, ethyl acetate/methanol and 100% methanol to afford 14 (1 to 14) fractions based on TLC analysis.

Fraction 1 (0.1045g), eluted using n-hexane/ethyl acetate (9:1) was further subjected to gel filtration chromatography (Sephadex LH-20) using chloroform/methanol (7:3) to afford a total of 4 fractions (A to D) based on TLC profiles. Fraction C (12.4 mg), a brown gum turned out to be compound **73** (8.3 mg).

Fraction 4 (0.4196g), was subjected to a Sephadex LH-20 column eluting with chloroform/methanol (1:1) to afford a total of 4 sub-fractions (A to D). Sub-fraction C (85.2 mg) was subjected to preparative TLC eluting with ethyl acetate/methanol/water (10:1:1) to afford 4 fractions (C1-C4). The most polar fraction, fraction C4 afforded compound **74** (11.2 mg) as a brown gum.

Fraction 7 (2.5363g) eluted using ethyl acetate/methanol (7:3) from the first column was adsorbed on to 3g of silica gel and subjected to normal column chromatography packed with 30g of column silica and eluted with ethyl acetate/methanol up to 100% methanol to afford 10 fractions (A-J).

Crystals appeared upon concentration of fractions F, G and H affording compound **75** (531 mg). Fraction 2 (65.3 mg) was subjected to a Sephadex column and eluted with chloroform/methanol (1:1) to afford 5 fractions (1-5) compound **75** was eluted as a yellow paste from fraction 2. A summary of the isolation procedure for the methanol extract of the tubers of *I. bolusiana* is shown in figure 2.2 below.

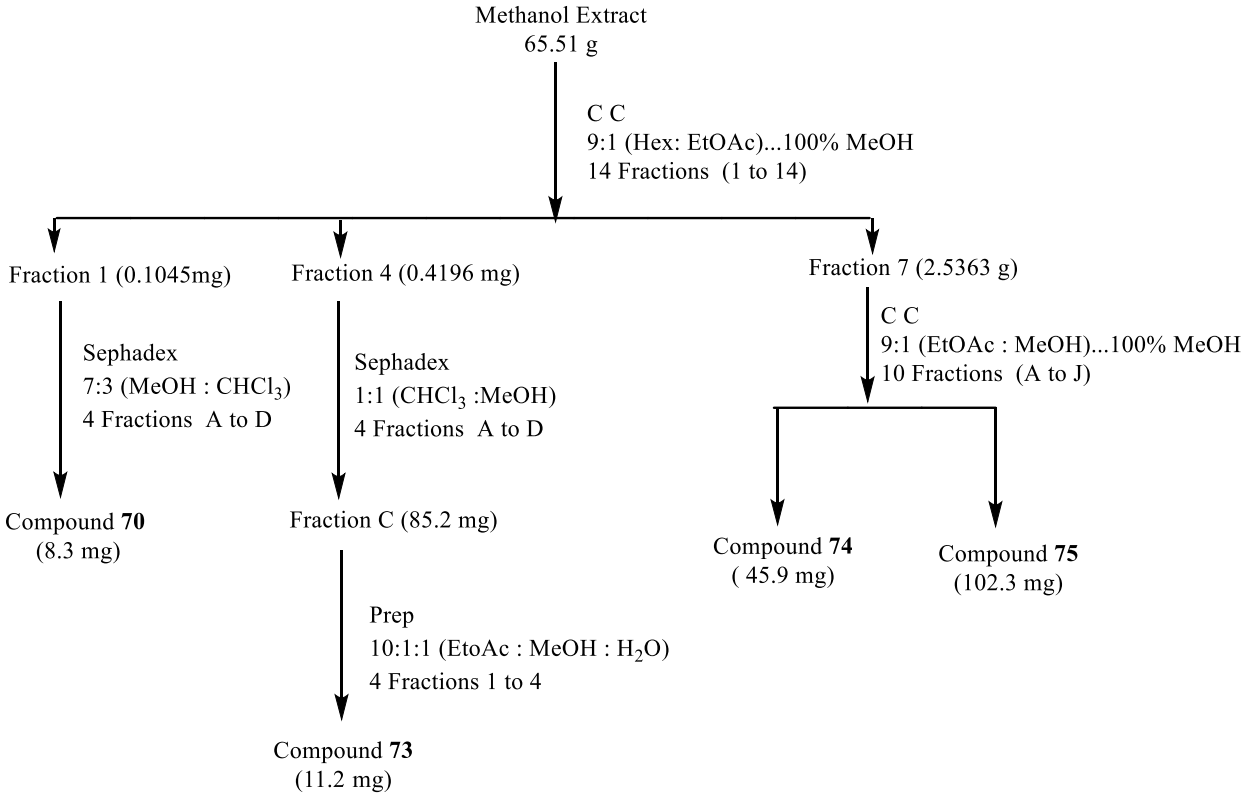


Figure 2.2: Summary of the isolation procedure for the methanol extract of the tubers of *I. bolusiana.*

Other fractions yielded residual sucrose in an effort to isolate more compounds and further analysis was stopped.

2.5 Trans esterification of Compound 73

Trans esterification of Compound **73** (6.1 mg) in acid afforded compound **76**. Esterification was carried out by first dissolving the compound in 10 mL of methanol in a 25 mL flask fitted with a condenser. 2 M hydrochloric acid (5 mL) was added drop wise and the resulting mixture was refluxed at 65 ^{o}C for 3 hours with constant stirring. The resulting reaction mixture was extracted

with ethyl acetate. The organic layer was then transferred into a 50 mL beaker for air drying. The resulting brown solid (4.2 mg) was subjected to preparative TLC eluting with ethyl acetate/methanol/water (10:1:1) to afford 2 sub-fractions (A and B), of which sub-fraction B turned out to be compound **76** (2.1 mg). The other sport was the starting material (compound **75**) indicating that the reaction was not complete.

2.6 Extraction and Isolation Procedure for *B. Plurijuga*

Different plant parts including twigs of *B. plurijuga* were collected from Kasane along the Zambezi river by Prof R T. Majinda in June 2012. Kasane is in the northern part of Botswana in the Chobe district. The plant was identified at the Botany unit, department of Biological Sciences at the University of Botswana. A specimen was deposited in the department of Biological Sciences Herbarium at the University of Botswana.

The dried and ground plant material of *B. plurijuga* (1057.47 g) was sequentially extracted with organic solvents in order of increasing polarity; n-hexane, chloroform, ethyl acetate, methanol and finally 80% aqueous methanol in 3 L conical flasks overnight in each case. The yields obtained for each solvent were, n-hexane (2.35 g), chloroform (2.43g), ethyl acetate (7.51 g), methanol (25.02 g) and 80% aqueous methanol (78.83 g).

The dried methanol extract (25.02 g) was adsorbed on silica gel 25.00 g) and subjected to a normal column chromatography on a column packed with silica gel (300 g) in n-Hexane. Column elusion was carried out by gradient elusion using n-hexane/ethyl acetate and ethyl acetate /methanol solvent with increasing polarity and finally 100% methanol. The resulting eluents were combined into 15 major fractions (A to P) based on TLC profiles. Crystals appeared during the concentration of fractions N to P using a rotary evaporator and these turned out to be compound **72** (551 mg).

Fraction E (153.7 mg) eluted using ethyl acetate/methanol (9:1) was subjected to Sephadex LH-20 and eluded with chloroform/methanol (7:3). A total of 10 fractions (A to J) were collected based on TLC profiles. Fraction I turned out to be compound **78** (7.3 mg).

Fraction F (118.2 mg) eluted using ethyl acetate/methanol (8:2) from the main column was subjected to Sephadex LH-20 and was eluted using chloroform/methanol (1:1). A total of 10

fractions (1 to 10) were collected based on TLC profiles. Fraction 10 turned out to be compound **77** (10.8 mg).

Fraction G (891 mg) was subjected to Sephadex LH-20 and eluted using chloroform/methanol (1:1). A total of seven fractions (1a to 7a) were collected based on TLC profiles. Sub-fraction 6a (0.0893 mg) was adsorbed onto silica gel (2 g) and further subjected to normal column chromatography on a column packed with 30 g of column silica in n-Hexane. Column elusion was carried out by gradient elusion using chloroform/methanol to afford 7 fractions (6A1 to 6A7) based on TLC profiles. Fraction 6A5 (30.2 mg) was subjected to preparative TLC eluting with chloroform/methanol (8.5:1.5) to afford 6 fractions (A to F) based on UV analysis of the preparative TLC plates. Fraction B turned out to be compound **79** (5.9 mg). A summary of the isolation procedure for the methanol extract of the twigs of *B. plurijuga* is shown in figure 2.3 below.

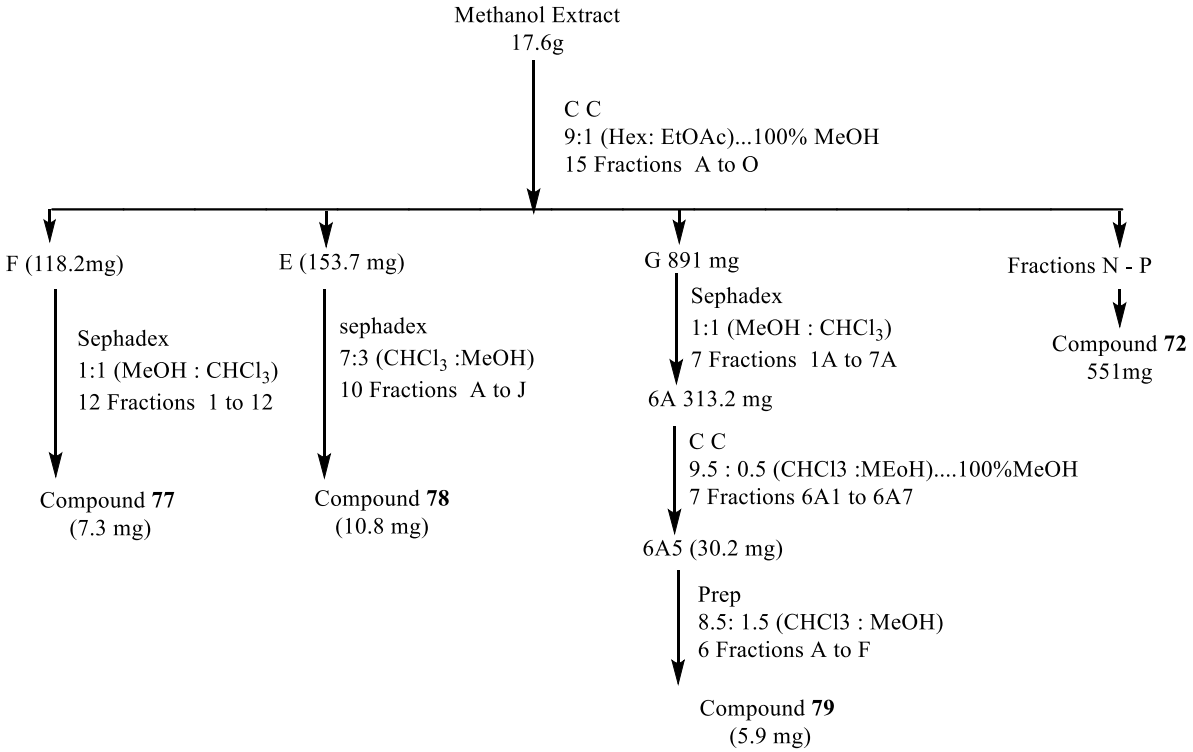


Figure 2.3: Summary of the isolation procedure for the methanol extract of the twigs of *B. plurijuga.*

Other extracts of the twigs of *B. plurijuga* were obtained in small quantities and TLC analysis showed many sports under UV lamp and also after spraying with vanillin spray suggesting that the crude extracts may have many compounds for the small amount of the crude extract, so they were not explored further.

2.6 Physical and Spectroscopic Data for The Isolated Compounds

(E)-octadec-11-en-1-yl (E)-3-(4-hydroxyphenyl) acrylate, 70

White crystals; Melting point 60-63 ºC; $[\alpha]_D$ = + 0.014 º (C = 0.0074, MeOH); UV λ_{max} (MeOH) nm: 227, 311; IR ν_{max} cm^{-1} 3355, 2922, 2854, 1682, 1633, 1604, 1585, 1515, 1451, 1438, 1338, 1327, 1304, 1276, 1201, 1167, 979, 721; HR-LTQ ESI-MS (m/z) calculated for $[M+H]^+$: 415.3212; found, 415.3210, (m/z) calculated for $[M+Na]^+$: 437.3032; found, 437.3027; ^1H and ^{13}C NMR (chloroform-d), refer to Table 3.1 and Appendices 1 and 2.

(E)-icos-13-en-1-yl (E)-3-(3,4-dihydroxyphenyl) acrylate, 71

White solid; Melting point 72-75 ºC; $[\alpha]_D$ = + 0.137 º (C = 0.0013, MeOH); UV λ_{max} (MeOH) nm: 207, 291, 327; IR ν_{max} cm^{-1} 3333, 2920, 2851, 1686, 1643, 1603, 1526, 1447, 1366, 1276, 1181, 1045, 972, 858, 816, 754; HR-LTQ ESI-MS (m/z) calculated for $[M+H]^+$: 459.3474; found, 459.3472, (m/z) calculated for $[M+Na]^+$: 481.3294; found, 481.3290; ^1H and ^{13}C NMR (chloroform-d), refer to Table 3.2 and Appendices 3 and 4.

C4C, 73

Brown solid; $[\alpha]_D$ = + 0.1 º (C = 3.53, MeOH); IR ν_{max} cm^{-1} 3339, 2926, 1688, 1601, 1520, 1445, 1276, 1183, 1164, 1115, 1031, 814; LR-ESI (m/z) 665.34 $[M - H]^-$, 503.29 (100) $[M- C_9H_7O_4]^-$, 341.18 $[C_{12}H_{21}O_{11}]^-$, 178.99 $[C_9H_7O_4]^+$; ^1H and ^{13}C NMR (methanol-d_4), refer to Table 3.3 and Appendices 5 and 6.

Caffeic acid methyl ester, 76

Brown solid; Melting point 156-161ºC; IR ν_{max} cm^{-1} 3349, 2962, 2924, 1700, 1602, 1518, 1443, 1284, 1197, 1113, 1047; LR-ESI (m/z) 193.29. $[M - H]^+$; ^1H and ^{13}C NMR (methanol-d_4), refer to Table 3.4 and Appendices 7 and 8.

Methyl (*E*)-octadec-11-enoate, 69

Yellow paste; $[\alpha]_D$ = + 6.45 º (C = 0.001, MeOH); UV λ_{max} (MeOH) nm: 208; IR ν_{max} cm^{-1} 3425, 2922, 2853, 1734, 1578, 1460, 1379, 1269, 1171, 1121, 1072, 962; LR-ESI (m/z) 284 $[M]^+$; ^1H and ^{13}C NMR (chloroform-d), refer to Table 3.3 and Appendices 9 and 10.

Sucrose, 72

Brown solid; Melting point 185-188 ºC; $[\alpha]_D$ = + 60.3 º (C = 0.003, H_2O); IR ν_{max} cm^{-1} 3326, 2929, 1643, 1416, 1103, 1049, 988, 906, 849; LR-ESI (m/z) 341.17 $[M - H]^-$; ^1H and ^{13}C NMR (D_2O), refer to Table 3.4 and Appendices 11 and 12.

Maltose, 75

Brown solid; Melting point 108-111 ºC; $[\alpha]_D$ = + 9.3 º (C = 10.62, MeOH); IR ν_{max} cm^{-1} 3306, 1639, 1016, 507; LR-ESI (m/z) 341.19 $[M - H]^-$; ^1H and ^{13}C NMR (D_2O), refer to Table 3.7 and Appendices 13 and 14.

IBMF2, 74

Brown gum; IR ν_{max} cm^{-1} 3339, 2498, 1647, 1451, 1014, 502; LR-ESI (m/z) 665.33 $[M - H]^-$; ^1H and ^{13}C NMR (methanol-d_4), refer to Table 3.8 and Appendices 15 and 16.

(+)-Catechin, 79

Brown solid; Melting point 174-176 ºC; $[\alpha]_D$ = + 12.3 º (C = 0.001, MeOH); UV λ_{max} (MeOH) nm: 282, + NaOMe (289), + NaOAc (281), + NaOAc/H_3BO_3 (, 285), + $AlCl_3$ (284), + AlCl3/HCl (279); IR ν_{max} cm^{-1} 3274, 2926, 2857, 1606, 1522, 1454, 1366, 1281, 1243, 1140, 1101, 1045, 1030, 816; HR-LTQ ESI-MS (m/z) calculated for $[M+H]^+$: 291.0869; found, 291.0864, (m/z) ($C_{15}H_{12}O_6Na$) calculated for $[M-2H+Na]^-$: 311.0543; found, 311.0539; ^1H and ^{13}C NMR (acetone d_6), refer to Table 3.5 and Appendices 17 and 18.

Eriodictyol, 78

Brown solid; Melting point 260-264 ^{o}C; $[\alpha]_D$ = - 0.007 ^{o} (C = 0.0027, MeOH); UV λ_{max} (MeOH) nm: 323, + NaOMe (323), + NaOAc (323), + NaOAc/H_3BO_3 (288), + $AlCl_3$ (290), + AlCl3/HCl (221, 290); IR ν_{max} cm^{-1} 3238, 2956, 1695, 1634, 1597, 1519, 1449, 1339, 1261, 1182, 1157, 1085, 1066, 1035, 1014, 962, 819; HR-LTQ ESI-MS (m/z) calculated for $[M+H]^+$: 289.0712; found, 289.0708, (m/z) ($C_{15}H_{10}O_6Na$) calculated for $[M-2H+Na]^-$: 309.0369; found, 309.0365; ^{1}H and ^{13}C NMR (acetone d_6), refer to Table 3.6 and Appendices 19 and 20.

Taxifolin, 77

Brown solid; Melting point 222-225 ^{o}C; $[\alpha]_D$ = + 39.5 ^{o} (C = 0.003, MeOH); UV λ_{max} (MeOH) nm: 325, + NaOMe (325), + NaOAc (326), + NaOAc/H_3BO_3 (290), + $AlCl_3$ (291), + AlCl3/HCl (228, 291); IR ν_{max} cm^{-1} 3303, 2922, 2847, 1634, 1519, 1467, 1361, 1279, 1254, 1182, 1161, 1118, 1085, 1027, 1001, 806; HR-LTQ ESI-MS (m/z) calculated for $[M+H]^+$: 305.0661; found, 305.0657, (m/z) calculated for $[M+Na]^+$: 327.0475; found, 327.0481; ^{1}H and ^{13}C NMR (acetone d_6), refer to Table 3.7 and Appendices 21 and 22.

References

Markham K. R. **1982**. Techniques of Flavonoid Identification. *Academic press INC*. London, Great Britain. Page 37 and 72.

Mabry T. J., Markham K. R. and Thomas B. M. **1970**. The systematic Identification of Flavonoids. *Springer-Verlag*, New York, Pages 165-166.

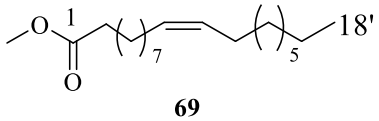
Chapter Three

Results and Discussion

3.1 Compounds from *Ipomoea Bolusiana*

Phytochemical investigation of the tubers of *I. bolusiana* afforded seven secondary metabolites comprising of a *p*-coumaric acid derivative (**70**), a caffeic acid derivative (**71**), a fatty acid (**69**) and a glycoside (**73**), sucrose (**72**) and two other oligosaccharides (**74**) and (**75**). Compounds **70**, **71** and **73** were isolated from the chloroform/ethyl acetate/methanol extract, whereas compounds **74,** and **75**, were isolated from the methanol extract. Hydrolysis of compound **73** afforded compound **76**. The structures of the compounds were elucidated using a combination of modern spectroscopic techniques such as UV, MS, NMR and IR.

Phytochemical work on the $CHCl_3$/EtOAc/MeOH extract of the wet tubers of *I. bolusiana* gave compound **69** as a yellow paste. This compound was possibly due to esterification of the natural product oleic acid during extraction with methanol. The UV absorption maxima at 209 nm indicated the presence of a double bond in the compound. Analysis of the IR spectrum of compound **69** showed the presence of an ester carbonyl functionality at 1734 cm^{-1}, a C-O stretch at 1269 cm^{-1} and an absorption band at 2922 cm^{-1} due to C-H aliphatic stretch. LR-MS gave an $[M]^+$ ion peak at m/z 296 consistent with a molecular formula $C_{19}H_{36}O_2$ implying two degrees of unsaturation.



69

Figure 3.1: Proposed structure of compound **69**.

The ^1H NMR spectrum of compound **69** (Appendix 1) exhibited a singlet at δ_H 3.78, integrating for three protons suggestive of a methoxy group in the compound. The presence of a long hydrocarbon chain in the compound was indicated by a singlet at δ_H 1.31 due to the 'methylene proton envelope' and the terminal methyl protons at δ_H 0.81 (3H, *t*, *J* = 6.9 Hz). Analysis of the ^{13}C NMR spectrum (Appendix 2) with the help of the MS spectrum showed the presence of twenty-

four carbon signals. Using the DEPT-135 and HMQC spectral data, these carbon signals were classified into one quaternary carbon, two methine, one methoxy (δ_C 51.4), a terminal sp^3 methyl carbon signal at δ_C 14.1 and fourteen methylene carbons embedded within the CH_2 envelope that resonated between δ_C 34.2 and 22.6 (Table 3.1). The carbonyl carbon at δ_C 174.4 and the IR absorption peak at 1734 cm^{-1} were suggestive of the presence of non-conjugated ester functionality in the compound. The two methine carbon signals δ_C 130.2 and 128.1 and two protons resonating at δ_H 5.40 together with the two degrees of unsaturation supported the presence of a double bond somewhere along the hydrocarbon chain. The HMBC spectrum was used to verify the above assignments where the methoxy protons at δ_H 3.71 showed correlation with the carbonyl carbon at δ_C 174.4 and the terminal methyl protons at δ_H 0.81 with the carbon at δ_C 22.7. HMBC correlations are shown in Figure 3.2 below. Table 3.5 below shows the ^1H and ^{13}C NMR data as well as HMBC correlations for compound **69**. Based on the spectral data presented above, compound **69** was identified as methyl (*E*)-octadec-11-enoate commonly known as oleic acid and the structure is presented in Figure 3.1 above. Oleic acid is a known anti-tumor agent (Oleico and De Accion., 2012).

Table 3.1: ^1H (300 MHz) and ^{13}C (75.4) NMR data for Compound 69 in $CDCl_3$. (Refer to appendix 1 and 2).

Position	δ_H (ppm)	δ_C (ppm)	HMBC Correlations
1		178.7	
2	2.35 (2H, *t*, *J* = 7.5 Hz)	34.2	
3	1.19 (26H, *m*)*	25.7	
4	1.19 (26H, *m*)*	24.7-31.9	
5-15	1.19 (26H, *m*)*	31.9	
16	1.57 (2H, *m*)*	22.7	C17
17	0.81 (3H, *t*, *J* = 6.9 Hz)	14.1	C16
OCH_3	3.71 (3H, *s*)	51.4	C1
C=C	5.40 (2H, *d*, *J* = 5.7 Hz)	130.2	
C=C	5.40 (2H, *d*, *J* = 5.7 Hz)	128.1	

Assignments were confirmed by DEPT, COSY and HMQC experiments.
Overlapped peaks*

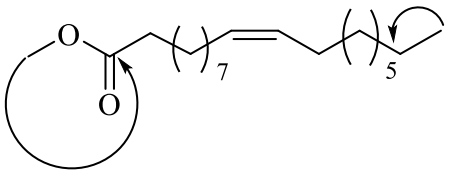
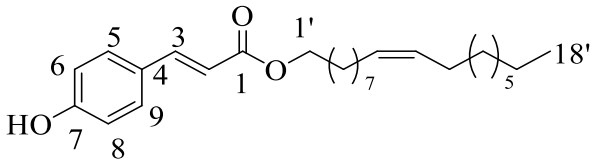


Figure 3.2: Some selected HMBC correlations of compound **69**.

Compound **70** was also isolated as white crystals with a melting point range of 60-63ºC from the $CHCl_3$/EtOAc/MeOH extract of the tubers of *I. bolusiana*. A broad band in the Infra-red spectrum at 3355 cm $^{-1}$ indicated the presence of phenolic -OH group in the compound. The other peaks which were apparent in the IR spectrum were those at 2922 and 2854 cm $^{-1}$ due to aliphatic C-H stretch; 1682 cm $^{-1}$ attributed to a conjugated carbonyl group; 1604 cm $^{-1}$ due to trans C = C stretch, 1585 -1515 cm $^{-1}$ due the aromatic C = C stretches and 1276 cm $^{-1}$ due to C-O stretch. The UV spectrum of the compound exhibited absorption maxima at 311 and 227 nm was indicative of an aromatic and α,β-unsaturated carbonyl functional groups, respectively and were in good agreement with the UV absorption maxima of a previously reported *p*-coumaric acid derivative 208 and 312 nm (Singh and Singh, 2014). The HR-MS spectrum gave an $[M+H]^+$ ion peak at *m/z* 415.3210 (Appendix 5) consistent with a molecular formula $C_{27}H_{43}O_3$, which implied seven degrees of unsaturation.



70

Figure 3.3: Proposed structure of compound **70**.

The ^{1}H NMR spectrum of compound **70** (Appendix 3) exhibited an AA'XX' aromatic proton spin system with resonances at δ_H 7.45 (2H, *d*, *J* = 8.4 Hz) and 6.94 (2H, *d*, *J* = 8.4 Hz) suggestive of a *para*-substituted benzene ring. Two *trans*-coupled protons observed at δ_H 7.69 (1H, *d*, *J* = 15.9 Hz) and 6.34 (1H, *d*, *J* = 15.9 Hz) were indicative of a conjugated α,β unsaturated system in this compound. The ^{1}H NMR spectrum also exhibited two (*cis*) olefinic protons resonating at δ_H 5.40 (*t*, *J* = 4.8 Hz); oxymethylene protons at δ_H 4.26 (2H, *t*, *J* = 6.9 Hz); allylic protons at δ_H 2.06 (2H,

m) and 1.75 (2H, m); a singlet at δ_H 1.31 integrating for twenty-six protons (13×CH_2) and the terminal methyl protons at δ_H 0.93 (3H, *t*, *J* = 7.2 Hz). The cluster of ethylene protons known as "the methylene envelope" at δ_H 1.31 and methyl protons at δ_H 0.93 were suggestive of the presence of a long hydrocarbon chain in this compound (Ngaski, 2015; May *et at*., 1986)

From the analysis of the ^{13}C NMR (Appendix 4, Table 3.2) and the MS spectral data it was deduced that there were twenty-seven carbons present in this compound. Using DEPT-135 and HMQC spectral data, these carbons were classified into one methyl, eight methines, fifteen methylenes and three quaternary carbons. The carbonyl carbon at δ_C 168.6 and an oxymethylene carbon at δ_C 65.1 together with the IR absorption peak at 1682 cm^{-1} were suggestive of a conjugated ester functional group in the compound. On the other hand, the presence of the long hydrocarbon chain in this compound was confirmed from the carbon signal at δ_C 14.2 due to a terminal methyl and a group of methylene carbon signals in the upper field between 20.0 and 30.0 ppm. In fact, from these carbon signals it was clear that the long hydrocarbon chain was attached via an ester linkage. Further examination of the carbon spectrum revealed an oxygenated aromatic carbon signal at δ_C 158.7; four aromatic methine carbon signals at δ_C 130.1×2; 116.1×2 and a quaternary aromatic carbon at δ_C 126.6. The carbon signals at δ_C 145.3 and 114.9 were assigned to the carbons of the conjugated α,β unsaturated system. The two methine carbon signals at δ_C 129.9 and 130.0 and the two proton triplet at δ_H 5.40 suggested that there was a double bond somewhere along the hydrocarbon chain. From this spectral data it was apparent that compound **72** was a derivative of *p*-coumaric acid. These assignments were further confirmed by the HMBC spectrum where the oxymethylene protons signal at δ_H 4.26 showed a correlation with the carbonyl carbon at δ_C 168.6 and a methylene carbon at δ_C 32.0. Furthermore, the proton δ_H 7.69 showed cross peaks with carbons at δ_C 168.6 and 130.1, whereas the proton at δ_H 6.34 showed correlations with carbons at δ_C 168.6 and 126. Other pertinent HMBC correlations are shown in Figure 3.4 below.

The position of the double bond situated along the hydrocarbon chain could not be ascertained due to clustering of the methylene proton and carbon signals in the ^1H and ^{13}C NMR spectra (Refer to Appendices 3 and 5). However, from the chemical shifts of the two carbons it was apparent that the double bond was far from the ester functionality as well as from the methyl end of the

hydrocarbon chain. It has been reported in the literature that if the double bond is sufficiently far from the acyl function as well as from the end methyl group, the two olefinic carbon atoms have almost identical chemical shift at approximately 129.9 and 130.4 ppm for both *trans* and *cis* configurations (Bus ,1976, 1977; Gunstone ,1993; Lie Ken Jie, 1995). Thus, the presence of this double bond was further confirmed by the seven degrees of unsaturation, where four degrees of unsaturation were due to the benzene ring, one due to carbonyl double bond, one due to the α,β trans double bond, leaving one for this double bond along the hydrocarbon chain. The long hydrocarbon chain which consists of eighteen carbons was most likely derived from oleic acid, that is, the reduction of oleic acid to give a fatty alcohol (refer to section 3.2). In fact, oleic acid is one of the isolated compounds from this plant (refer to section 3.2).

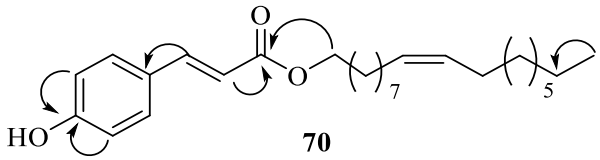
From the spectral data presented above, compound **70** was identified as an ester resulting from the condensation of *p*-coumaric acid and the fatty alcohol derived from the reduction of oleic acid. The proposed structure of compound **70** is presented in Figure 3.3 above. This is the first report on the occurrence of this *p*-coumaric acid ester in the tubers of *I. bolusiana* or any other plant species. However, other *p*-Coumarates have been reported in high concentrations from a variety of plants and have been shown to exhibit antioxidant properties (Sharma, 2011; Singh and Singh, 2014;).

Table 3.2: ^{1}H (300 MHz) and ^{13}C (75.4) NMR data for Compound 70 in $CDCl_3$. (Refer to appendix 1 and 2).

Position	δ_H (ppm)	δ_C (ppm)	HMBC Correlations
1		168.6	
2	7.69 (1H, d, J = 15.9 Hz)	114.9	C1, C4
3	6.34 (1H, d, J = 15.9 Hz)	145.3	C1, C4
4		126.6	
5	7.45 (2H, *d*, *J* = 8.4 Hz)	130.1	C7, C9
6	6.94 (2H, *d*, *J* = 8.4 Hz)	116.1	C4, C5, C7
7		158.7	
8	6.94 (2H, *d*, *J* = 8.4 Hz)	116.1	C4, C9, C7
9	7.45 (2H, *d*, *J* = 8.4 Hz)	130.1	C7, C5
1′	4.26 (2H, *t*, *J* = 6.9 Hz)	65.1	C16′
2′	1.75 (2H, *m*)	32.0	
3′	1.31 (26H, *br s*)*	29.8	
4′	1.31 (26H, *br s*)*	29.7	
5′-15′	1.31 (26H, *br s*)*	29.6	
16′	1.31 (26H, *br s*)*	27.3	
17′	2.06 (2H, *m*)	26.0	C18′
18′	0.93 (3H, *t*, *J* = 7.2 Hz)	14.2	C17′
C=C	5.40 (2H, *t*, *J* = 6.9)	129.9	
C=C	5.40 (2H, *t*, *J* = 6.9)	130.0	

Assignments were confirmed by HMQC, HMBC and DEPT experiments.

*Overlapped peaks.

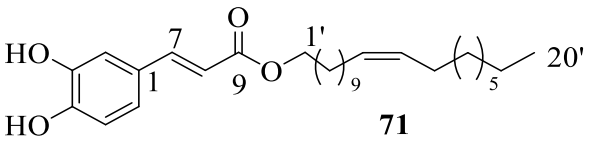


70

Figure 3.4: Key HMBC correlations of compound **70**.

Continued work on the $CHCl_3$/EtOAc/MeOH extract of the wet tubers of *I. bolusiana* lead to the isolation of compound **71** as a white solid with a melting point range of 72-75^{o}C. The UV spectrum of **71** exhibited maxima at 327, 291 and 207 nm due to the presence of an aromatic (C =C), α,β-unsaturated carbonyl and C=C functional groups respectively. The spectrum is characteristic of caffeic acid derivatives (Sarker *et al*., 2005). The IR spectrum displayed absorption bands at 3333 and 1526 cm-^{1} due to phenolic hydroxyl groups and aromatic C = C functionalities. A broad band

at 1643 cm-^{1} was attributed to the trans C = C functionality in the compound. A conjugated carbonyl functionality appeared as a broad band at 1686 cm^{-1}. The HR-MS spectrum gave an $[M+H]^{+}$ ion peak at m/z 459.3472 (Appendix 8) consistent with a molecular formula $C_{29}H_{46}O_{4}$, which implied seven degrees of unsaturation.



71

Figure 3.5: Proposed structure of compound **71**.

Analysis of the ^{1}H NMR spectrum of compound **71** (Appendix 6) revealed an aromatic ABM proton spin system at δ_H 6.92 (1H, *d*, *J* = 8.1 Hz); 7.05 (1H, *dd*, *J* = 8.1, 2.1 Hz) and 7.15 (1H, *d*, *J* = 2.1 Hz), suggestive of a 1,3,4-trisubstituted benzene ring. Apart from the substitution pattern of the benzene ring and two extra methylenes the ^{1}H and ^{13}C NMR spectral data of compound **71** (Appendix 7, Table 3.3) resembled that of compound **70**. Thus, it was apparent from the spectral data that compound **71** is an ester of caffeic acid and a C20 fatty alcohol (eicos-13-en-1-ol). The unsaturated fatty alcohol eicos-13-en-1-ol or 20:1 fatty alcohol is commonly found in nature (Mudge, 2005). Key HMBC correlations for compound **71** are shown in Figure 3.6 below. The carbonyl carbon resonating further upfield at δ_C 168.2 and an oxymethylene carbon at δ_C 65.0 together with the IR absorption peak at 1686 cm^{-1} were suggestive of a conjugated ester functionality in the compound.

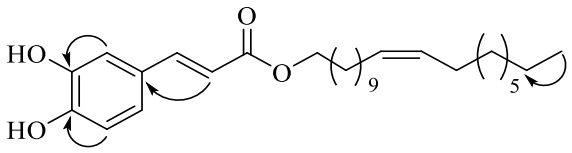
Thus, the structure of compound **71** was identified as caffeic acid eicocyl ester, which is a new natural product. The proposed structure of compound **71** is presented in Figure 3.5 above. This is the first report on the occurrence of this caffeic acid ester in the tubers of *I. bolusiana* or any other plant species.

Table 3.3: ^{1}H (300 MHz) and ^{13}C (75.4) NMR data for Compound 71 in $CDCl_3$. (Refer to appendix 3 and 4).

Position	δ_H (ppm)	δ_C (ppm)	HMBC Correlations
1		127.5	
2	7.15 (1H, *d*, *J* = 2.1 Hz)	114.4	C3, C4
3		144.0	
4		146.5	
5	6.92 (1H, *d*, *J* = 8.1 Hz)	115.5	C3, C4
6	7.05 (1H, *d*, *J* = 8.1, 2.1 Hz)	122.4	C3, C4
7	7.63 (1H, *d*, *J* = 15.9)	145.1	
8	6.31 (1H, *d*, *J* = 15.9)	115.6	C1
9		168.2	
1′	4.28 (1H, *t*, *J* = 6.9 Hz)	65.0	
2′	1.75 (2H, *q*, J = 6.9 Hz)	32.0	
3′	1.31 (24 H, *br s*)*	29.8	
4′	1.31 (24 H, *br s*)*	29.7	
5′-17′	1.31 (24 H, *br s*)*	29.6-28.7	
18′	1.31 (24 H, *br s*)*	27.2	
19′	2.05 (2H, q, J = 6.0Hz)	26.0	C20′
20′	0.93 (3H, t, *J* = 6.9 Hz)	14.8	C19′
C=C	5.40 (1H, t, J = 4.8 Hz)	130.0	
C=C	5.40 (1H, t, J = 4.8 Hz)	129.9	

Assignments were confirmed by HMQC, HMBC and DEPT experiments

Overlapped peaks*

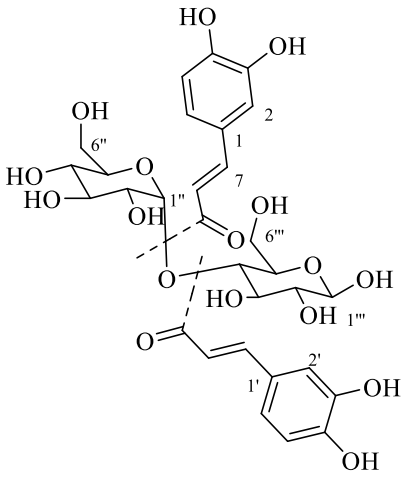


71

Figure 3.6: The key HMBC correlations of compound **71**.

Subjection of the methanol extract of the tubers of *I. bolusiana* to column chromatography gave compound **73** (Figure 3.7) as a brown gum. The UV spectrum of the compound exhibited absorption maxima at 325, 290 and 211 nm attributed to the presence of aromatic (C =C), α,β-unsaturated carbonyl and C=C functionalities. The IR spectrum exhibited absorption bands at 3339 and 1688 cm^{-1} attributed to hydroxyl and ester carbonyl functionalities respectively. The C-O

acyclic stretch appeared at 1276 cm^{-1} while the conjugated alkene absorption band was observed at 1601 cm^{-1}. The LR-ESI spectrum in negative ionization mode gave an $[M-H]^-$ ion peak at 665.34 (Appendix 11) suggesting the molecular formula $C_{30}H_{34}O_{17}$. Cleavage of one the caffeic acid units (162 amu) from molecular ion a) (Scheme 3.1) led to the fragment $[M-C_9H_6O_3]^-$ ion at m/z 503.29 (100) which turned out to be the base peak. Cleavage of the second caffeic unit from the resultant caffeate diglycoside gave the fragment ion at m/z 341.18 due to two sugar moieties.



73

Figure 3.7: Proposed structure of compound **73**.

The ^{13}C NMR spectrum (Appendix 10) exhibited thirty carbon signals and using DEPT-135 and HMQC. The signals were classified as eight quaternary, two oxymethylene and twenty methine carbon signals (Table 3.4). From the analysis of the ^1H and ^{13}C NMR and HMQC spectra it was evident that there were two sugar units in this compound where the anomeric carbons observed at 92.6 and 96.9 ppm were correlating with the anomeric protons at δ_H 5.14 and 4.50 ppm due to the β-D- and α-D-glucose moieties, respectively (Bubb, 2003). Furthermore, there were eight methine carbons resonating at 76.6, 74.9×2, 73.4, 72.4, 70.7, 70.4 and 69.4; two oxymethylenes at 63.4 and 63 5, characteristic of sugar moieties. The remaining eighteen carbon signals resembled those of the caffeiate moiety (refer to discussion on compound **71** above) and it was apparent from the doubling of the carbon signals that there were two caffeic acid derivatives in this compound. Thus, the sp^2 carbon signals observed at δ_C 145.4 and 113.5 were assigned to the α,β unsaturated system. The carbon signals at δ_C 113.7/113.6, (115.2 x 2) and 121.7/121.6 showed J^1 coupling with the

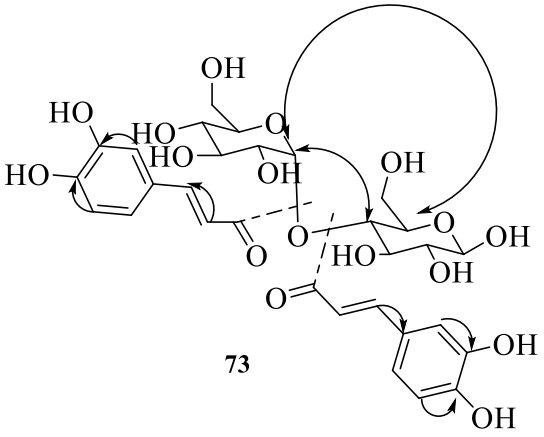
protons at δ_H 6.80 (2H, *d*, *J* = 8.1 Hz); 6.96 (2H, *dd*, *J* = 8.1, 1.8 Hz) and 7.07 (2H, *d*, *J* = 1.8 Hz) suggesting a 1, 3, 4 tri-substituted benzene ring in the compound and these were assigned positions C-2, C-8 and C-6, respectively. The quaternary carbons at δ_C 126.3 were assigned as the points of attachment of the α,β unsaturated system to the benzene ring.

The ^{1}H NMR spectrum of compound **73** (Appendix 9) showed a set of trans coupled protons at δ_H 7.59 (2H, *d*, *J* = 15.9 Hz) and 6.30 (2H, *d*, *J* = 15.9 Hz) indicative of a trans ethylenic system in the compound and from integration, it was apparent that there are two caffeate moiety units in the molecule. The presence of the two caffeate moiety units was also evident in the aromatic region of the spectrum where proton signals at δ_H 7.13 (*d*, *J* = 1.8 Hz); 6.96 (*dd*, *J* = 8.1, 1.8 Hz and 6.8 (*d*, *J* = 8.1 Hz) were also integrating for two protons each. The spectral data discussed shows that there are two caffeate units and two glucose units. The attachment of all four units could not be determined beyond reasonable doubt as the HMBC spectrum was inadequate due to the malfunctioning of the instrument as well as minute quantities of the compound. The HMBC spectrum however showed correlations between the protons at δ_H 7.07 and 6.30/6.29 with the quaternary carbons at δ_C 126.3 and the carbonyl carbons at δ_C 167.9/167.8, whereas the protons at δ_H 6.80 were correlating with the quaternary carbon at δ_C 126.3. HMBC spectrum also shows long range correlation between an anomeric proton at 5.14 ppm with a carbon signal at C-4′′(69.4 ppm). There is also correlation between a proton resonating at 3.20 ppm with a carbon signal at 96.9 ppm (C-1′) suggesting the point of attachment between the two sugar units at that position Other key HMBC correlations are shown in Figure 3.8 below. Taking into consideration steric effects as well as the changes chemical shifts of the carbon signal in the carbon spectrum, the point of attachment for the caffeic acid units on the sugar unit could not be ascertained due to poor HMBC correlations between the sugar unit and the caffeic acid units. The tentative structure for compound **73** was assigned as shown in Figure 3.7 above.

Table 3.4: ^{1}H (300 MHz) and ^{13}C (75.4) NMR data for Compound 73 in methanol-d_4. (Refer to appendix 5 and 6).

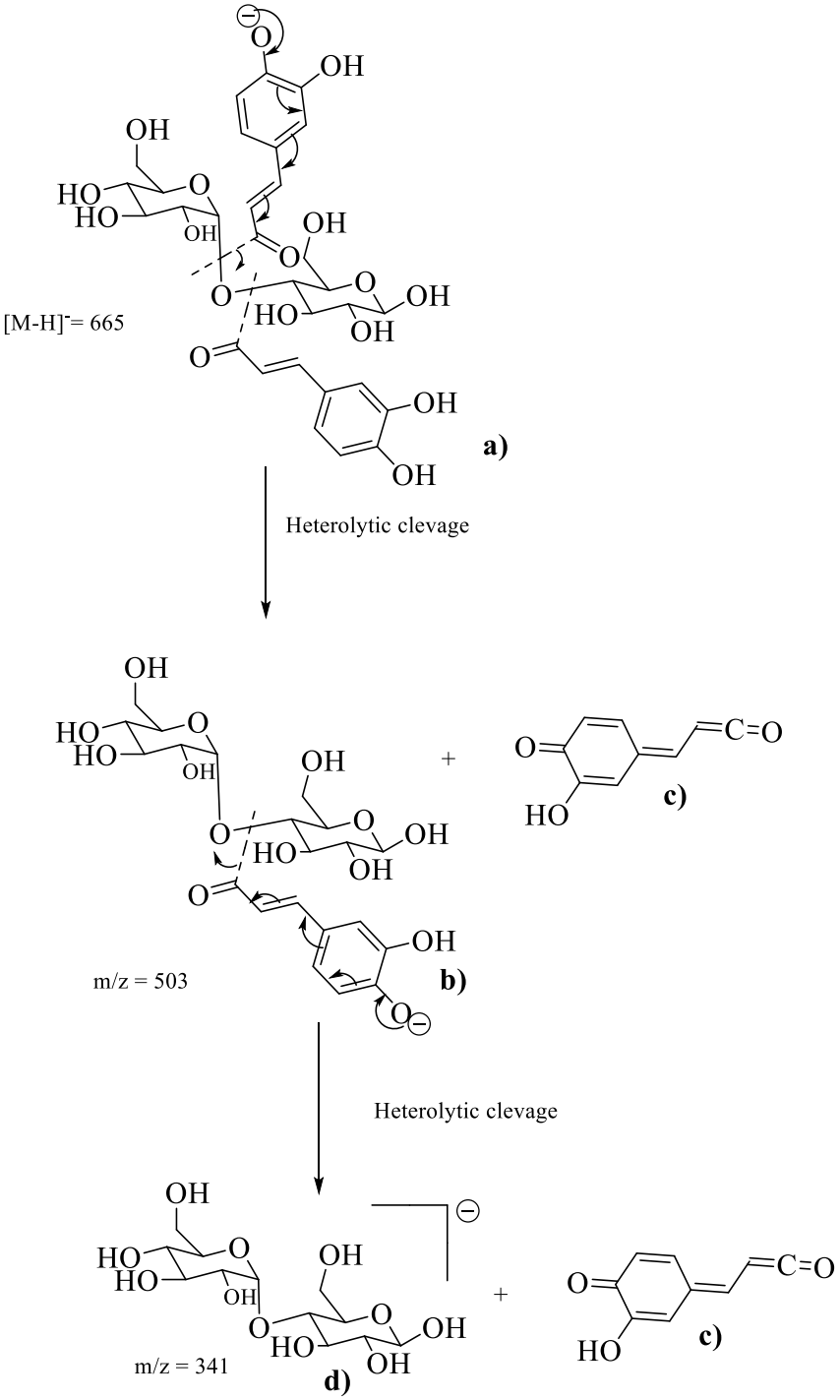
Position	δ_H (ppm)	δ_C (ppm)	HMBC Correlations
1		126.3	
2	7.07 (1H, *d*, *J* = 1.8 Hz)	113.7	C1, C3
3		145.7	
4		148.3	
5	6.80 (1H, *d*, *J* = 8.1 Hz)	115.2	C4
6	6.96 (1H, *dd*, *J* = 8.1, 1.8 Hz)	121.6	C1
7	7.59 (1H, *d*, *J* = 15.9)	145.4	
8	6.30 (1H, *d*, *J* = 15.9)	115.2	C1
9		167.9	
1′		126.3	
2′	7.07 (1H, *d*, *J* = 1.8 Hz)	113.7	C1′, C3′
3′		145.8	
4′		148.3	
5′	6.80 (1H, *d*, *J* = 8.1 Hz)	115.2	C4′
6′	6.96 (1H, *dd*, *J* = 8.1, 1.8 Hz)	121.7	C1′
7′	7.59 (1H, *d*, *J* = 15.9)	145.4	
8′	6.30 (1H, *d*, *J* = 15.9)	115.2	C1′
9′		167.8	
Sugar unit			
1″	5.14 (1H, *d*, *J* = 3.6 Hz)	96.9	C-4‴
2″	3.40 (4H, *m*)*	72.4	
3″	3.71 (3H, *m*)*	74.1	
4″	3.40 (4H, *m*)*	70.4	
5″	3.40 (4H, *m*)*	76.6	
6″	4.48 (2H, *m*)*	63.5	
1‴	4.49 (1H, *d*, *J* = 7.8 Hz)	92.6	
2‴	3.40 (4H, *m*)*	70.7	
3‴	3.71 (3H, *m*)*	73.4	
4‴	4.06 (1H, *m*)*	69.4	
5‴	3.20 (1H, *t*, *J* = 9.0 Hz)	74.9	C-1″
6‴	4.35 (2H, *m*)*	63.5	

Assignments were confirmed by HMQC, HMBC and DEPT experiments.
Overlapped peaks*



73

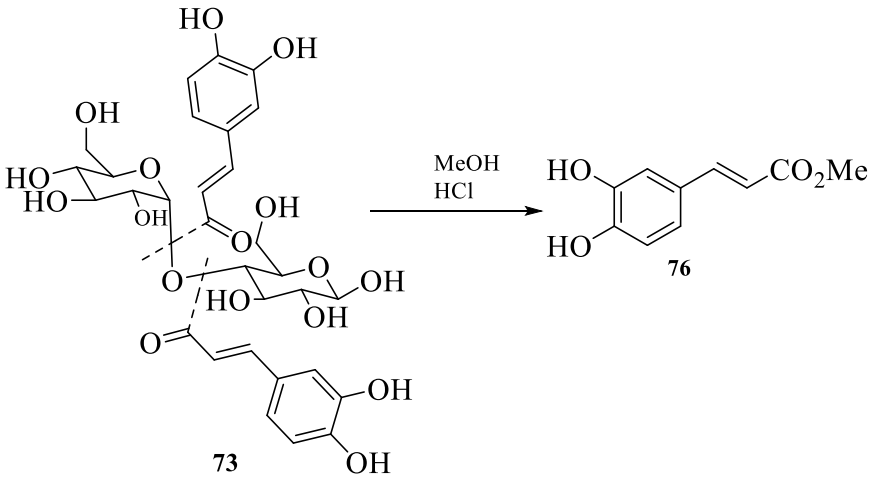
Figure 3.8: Key HMBC correlations of compound **78**.



Scheme 3.1: Proposed mass fragmentation of compound **73**.

In an effort to elucidate the structure of compound **73**, it was reacted with MeOH in the presence of concentrated HCl under reflux conditions (Scheme 3.2). TLC analysis of the reaction mixture

showed the presence of three products. Two sports were visible under UV radiation while the other was identified upon spraying with vanillin reagent appearing as a brown sport. This sport was probably the sugar unit. Of the two UV active sports, one was the starting material (compound **73**) while the other was the desired product (compound **76**). Compound **76** was afforded as a brown solid with a melting point of 156-161^{o}C. The UV spectrum exhibited bands at 326, 290 and 210 nm due the presence of aromaticity, α,β-unsaturated and C = C in the compound. The IR spectrum of the compound displayed absorption bands at 3349 and 1700 cm-^{1} which were attributed to hydroxyl and α,β-unsaturated carbonyl stretches. The acyclic C-O stretch was observed at 1284 cm-^{1}. The LR-ESI spectrum gave an [M-H]$^{-}$ ion peak at m/z 193.29 (Appendix 14).



Scheme 3.2: Trans-esterification of compound **73**.

The ^{1}H NMR spectrum of compound **76** (Appendix 12) showed an ABM aromatic spin system similar to that of the caffeate moiety of compound **71** resonating at δ_H 6.80 (1H, *d*, *J* = 8.1 Hz); 6.97 (1H, *dd*, *J* = 8.1, 1.8 Hz) and 7.06 (1H, *d*, *J* = 1.8 Hz). A set of trans coupled protons were observed at δ_H 7.57 (1H, *d*, *J* = 15.9 Hz) and 6.29 (1H, *d*, *J* = 15.9 Hz) indicative of an AX spin system in the compound. The above spectral data (Table 3.5) suggest that there are similarities between compounds **71** and **76**. A singlet at δ_H 3.78 integrating for three protons suggested the presence of a methoxy group in the compound. From the COSY spectrum, protons at δ_H 6.97 correlated with protons at δ_H 6.80 while protons at δ_H 7.57 were showing long range correlation with protons at δ_H 6.29.

The ^{13}C NMR spectrum of compound **76** (Appendix 13) displayed nine carbon signals (Table 3.5) and using the DEPT-135 spectral data, these were sorted into one methoxy, three quaternary and five methine carbons. The quaternary carbon signal around 168.0 ppm was not visible in the spectrum due to minute quantities of the compound. However, the IR spectrum exhibited a band at 1700 cm-^{1} indicative of the presence of an ester carbonyl functional group. The carbon signal at δ_C 50.6 as well as a band at 1284 cm^{-1} in the IR spectrum for a C-O acyclic stretch supported the presence of an ester functional group in the compound. The sp^2 carbon signals observed at δ_C 145.4 and 113.4 were attributed to the trans ethylenic system in the compound matching those of the α,β unsaturated system of caffeic acid isolated by Chang et al (δ_C 145.7 and 113.7). The quaternary carbon at δ_C 126.3 was assigned as the point of attachment of the α,β unsaturated system to the benzene ring as it showed close resemblance to that of compound **71** which was resonating at δ_C 127.5. Based on the available spectral data as well as literature data, the tentative structure for compound **76** was assigned as caffeic acid methyl ester. HMBC correlations are shown in Figure 3.9 below.

Caffeic acid methyl ester is a well-known antioxidant. It has been reported from the methanol extract of the aerial parts of *Bistorta manshuiensis* by Chang *et al*, 2009.

Table 3.5: ^{1}H (300 MHz) and ^{13}C (75.4) NMR data for Compound 76 in methanol- d_4. (Refer to appendix 7 and 8).

Position	Compound **76**		Literature (Chang *et al*, 2009)	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1		126.3		126.6
2	7.06 (1H, *d*, *J* = 1.8 Hz)	113.7	7.03 (1H, *d*, *J* = 2.0 Hz)	114.0
3		145.5		145.8
4		148.2		148.4
5	6.80 (1H, *d*, *J* = 8.1 Hz)	115.1	6.78 (1H, *d*, *J* = 8.0 Hz)	115.3
6	6.97 (1H, *dd*, *J* = 8.1, 1.8 Hz)	121.5	6.94 (1H, *dd*, *J* = 8.0, 2.0 Hz)	121.8
7	7.57 (1H, *d*, *J* = 15.9 Hz)	145.4	7.53 (1H, *d*, *J* = 15.5 Hz)	145.7
8	6.29 (1H, *d*, *J* = 15.9 Hz)	113.4	6.25 (1H, *d*, *J* = 15.5 Hz)	113.7
9				168.6
OCH_3	3.75 (3H, *s*)	50.8	3.75 (3H, *s*)	50.8

Assignments were confirmed by COSY and DEPT experiments.

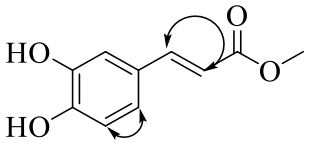
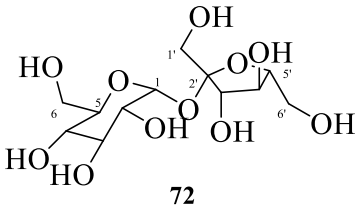


Figure 3.9: Key COSY correlations of compound **76**.

Further work on the methanol extract of the tubers of *I. bolusiana* lead to the isolation of compound **72** (Figure 3.10) as brown crystals with melting a point range of 185-188^{o}C (literature value; 185-190^{o}C, Hurta *et al*, 2004). The IR spectrum of the compound showed a broad band at 3326 cm $^{-1}$ (-OH) and 1103 cm^{-1} (C-O acyclic stretch). LR-ESI gave an [M-H] $^{-}$ ion peak at m/z 341.17 consistent with a molecular formula $C_{12}H_{22}O_{11}$ implying two degrees of unsaturation.



72

Figure 3.10: Proposed structure of compound **72**.

The ^{13}C NMR spectrum (Appendix 16) showed twelve carbon signals resonating between δ_C 103.8 and 60.3 ppm with the anomeric carbon signals resonating at δ_C 92.3 and 103.8 (Table 3.6). From DEPT-135 spectrum the signals were resolved to comprise of seven oxymethine carbon signals resonating at δ_C 92.3, 81.5, 76.6, 74.2, 72.7 72.5, 71.2, three oxymethylene carbons signals resonating at δ_C 62.5, 61.6, 60.3 suggesting the presence of pyranose and furanose conformers. A quaternary carbon was observed at δ_C 103.8 typical of a C-O-C functionality in sucrose (Mushibe, 2007).

The ^{1}HNMR spectrum (Appendix 15) showed a doublet signal resonating at δ_H 5.40 (1H, *d*, *J* = 3.6) characteristic of an anomeric proton of β-D-glucose in sucrose. Methylene proton signals were observed at δ_H 3.86 (2H, *m*) for the glucose unit and at δ_H (3.64 (2H, *br s*) and 3.39 (2H, *m*) for the fructose methylenes (Table 3.6). The HMBC spectrum confirmed the above assignments. Correlations between the anomeric carbon at δ_C 103.8 on the glucose unit with the protons at δ_H 5.40 (1H, *d*, *J* = 3.6 Hz) and 3.64 (2H, *br s*) were observed in the spectrum. Key HMBC

correlations are shown in Figure 3.11 below. The above data was found to correlate with data reported from literature (Mushibe, 2007), therefore compound **72** was thus identified as sucrose.

Table 3.6: ^{1}H (300 MHz) and ^{13}C (75.4) NMR data for Compound 72 in D_2O. (Refer to appendix 11 and 12).

Position	Compound **72**		Literature (Mushibe, 2007)	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1	5.40 (1H, *d*, *J* = 3.6 Hz)	92.3	5.19 (1H, *d*, *J* = 5.1 Hz)	92.2
2	3.17 (1H, *m*)	71.2	3.17 (1H, *m*)	72.1
3	4.04 (1H, *t*, *J* = 8.4 Hz)	74.2	3.78 (1H, *m*)	74.7
4	3.46 (1H, *t*, *J* = 9.3 Hz)	72.5	3.49 (1H, *m*)	73.3
5	4.21 (1H, *d*, *J* = 9.0 Hz)	76.6	3.88 (1H, *m*)	77.5
6	3.86 (2H, *m*)	62.5	3.56 (1H, *m*)	62.6
1′	3.64 (2H, *br s*)	61.6	3.41 (1H, *m*)	62.5
2′		103.8		104.5
3′	3.74 (1H, *d*, *J* = 9.6 Hz)	72.7	3.64 (1H, *m*)	73.4
4′	3.55 (1H, *dd*, *J* = 9.9, 3.9 Hz)	69.4	3.16 (1H, *m*)	70.3
5′	3.86 (1H, *m*)	81.5	3.56 (1H, *m*)	83.0
6′	3.39 (2H, *m*)	60.3	3.39 (1H, *m*)	60.9

Assignments were confirmed by DEPT, COSY and HMQC experiments.

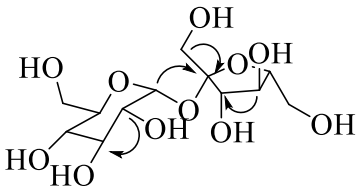
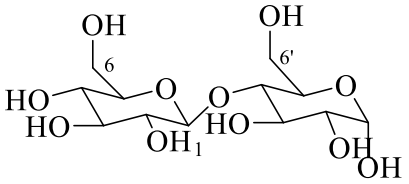


Figure 3.11: Key HMBC correlations of compound **72**.

Further phytochemical work on the methanol extract lead to the isolation of compound **75** as a brown solid with a melting point of 108-111^{o}C. The melting point differed from that obtained from literature (102-103 ^{o}C, pubchem.ncbi.nlm.nih.gov/compound/439186#section=Melting-Point) perhaps due to unforeseen contaminants. The Infra-red spectrum of the compound exhibited absorption bands at 3306 and 1016 cm^{-1} due to the presence of hydroxyl and C-O functionalities respectively. The LR-MS spectrum of compound **75** showed an [M-H]$^{-}$ ion peak at m/z 341.19 consistent with the molecular formula $C_{12}H_{22}O_{11}$ implying two degrees of unsaturation.



75

Figure 3.12: Proposed structure of compound **75**.

The ^{13}C NMR spectrum (Appendix 19) of the compound showed twelve signals and using DEPT-135 and HMQC, the signals were classified as two oxymethylene (δ_C 61.5 and 61.4), ten oxymethine carbons resonating at δ_C 76.7, 76.6, 74.9, 73.5, 72.5, 71.6, 70.5 and 70.4 with the anomeric carbon signals resonated at δ_C 96.8 and 92.3 (Table 3.7). Usually, the non- reducing anomeric carbon signal of the disaccharide with an α-glycosidic linkage resonates at a higher field than the β-linked disaccharide. In the case of compound 75, the non-reducing anomeric carbon resonates at a lower field indicating a β -linked disaccharide.

The ^{1}H NMR spectrum (Appendix 18) showed well resolved proton signals at δ_H 5.14 (1H, *d*, *J* = 3.6 Hz) and 4.50 (1H, *d*, *J* = 7.8 Hz) characteristic of anomeric protons (Rosland *et al*., 2008). For a given disaccharide, the *alpha* anomeric proton usually resonates further downfield from the *beta* proton making these two distinguishable by H-1 spectral data, thus configurations around the anomeric centres can be assigned from the magnitude of $J_{1,2}$, with values around 7.8 Hz associated with β-configuration and 3.6 Hz indicative of couplings of α-anomers (Bubb, 2003). From the ^{1}H-NMR spectral data (Table 3.7), it was possible to determine the linkage present by looking at the resonance in the H-1 spectrum. The absence of resonance around 5.00 ppm suggested that the two monomer units were not (1→6) α linked but were linked through the (1→4) α linkage. This linkage is observed by the presence of resonance around 5.20 ppm as observed in the ^{1}H-NMR spectrum of compound **75** (Appendix 18). Furthermore, the two anomeric protons δ_H 5.14 and 4.50 showed J^1 coupling with the anomeric carbons at δ_C 92.6 and 96.8 respectively. From HMBC spectral data, two oxymethylene protons at δ_H 3.71 and 3.86 were coupling with carbons at δ_C 61.5 and 61.4 while oxymethine protons further upfield at δ_H 3.15 (1H, *J* = 9.0 Hz) appeared as a triplet and correlated with the carbon at δ_C 74.9. From NOESY, the anomeric proton at δ_H 4.50 showed

correlation with protons at δ_H 3.34 (H-3′) and a triplet at δ_H 3.15 (H-5′) while the anomeric proton at δ_H 5.14 showed correlation with the protons at δ_H 3.34 (H-5′). HMBC showed weak correlations between a cluster of protons at δ_H 3.34 and carbons at δ_C 70.5, 71.6 and 72.5 and are shown in Figure 3.13 below. Based on the available spectral data, the tentative structure for compound **75** (Figure 3.12) was proposed as maltose which is a known disaccharide.

Table 3.7: ^{1}H (300 MHz) and ^{13}C (75.4) NMR data for Compound 75 in methanol- d_4. (Refer to appendix 13 and 14).

Position	Compound **75**		NOESY	HMBC
	δ_H (ppm)	δ_C (ppm)		
1	5.14 (1H, *d*, *J* = 3.6 Hz)	92.3	H-5, H-2, H-4′	
2	3.34 (5H, *m*)*	76.7	H-1	
3	3.69 (1H, *br s*)	73.5		
4	3.34 (5H, *m*)*	70.5		C-2
5	3.34 (5H, *m*)*	76.6	H-1	
6	3.86 (2H, *m*)	61.4		
1′	4.50 (1H, *d*, *J* = 7.8 Hz)	96.8	H-5′, H-3′	
2′	3.79 (1H, *m*)*	71.6		C-1′
3′	3.34 (5H, *m*)*	72.5		C-1′
4′	3.34 (5H, *m*)*	70.4	H-1	
5′	3.15 (1H, *t*, *J* = 9.0 Hz)	74.9	H-1′	
6′	3.71 (2H, *m*)	61.5		

Assignments were confirmed by DEPT, COSY and HMQC experiments.

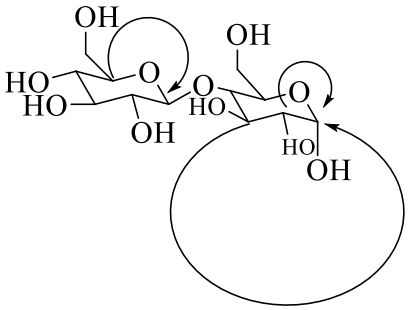
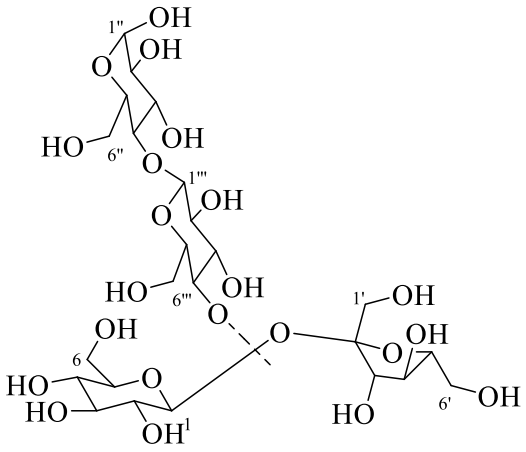


Figure 3.13: Key HMBC correlations of compound **75**.

In addition to compounds **73**, **72** and **75**, an oligosaccharide, compound **74** was isolated as a brown gum from the methanol extract of the tubers of *I. bolusiana*. The IR spectrum showed a broad band at 3339 cm^{-1} attributed to the presence of hydroxyl groups, a band at 2498 cm^{-1} due to alkane CH stretches. An acyclic C-O stretch appeared at 1014 cm^{-1}. The LR-MS spectrum of compound **74**

showed an $[M-H]^-$ ion peak at m/z 665.33 (Appendix 23), consistent with the molecular formula $C_{24}H_{42}O_{22}$, suggesting four degrees of unsaturation.



74

Figure 3.14: Proposed structure of compound **74**.

The ^{13}C NMR spectrum (Appendix 22) of compound **74** showed 24 carbon signals (Table 3.8). From DEPT 135 and HMQC, the signals were sorted out to consist of five sp^3 oxymethylene resonating at δ_C 62.4, 61.2, 60.8, 60.6 and 60.1, eighteen oxymethine carbons inclusive of anomeric carbons resonating at δ_C 95.9, 92.9 92.1 and one quaternary carbon signal at δ_C 103.7. From this spectral data it was apparent that the compound consisted of four hexose units. The presence of five oxymethylenes suggested that one of the four hexoses was fructose where the anomeric carbon becomes quaternary resonating at δ_C 103.7, thus characteristic of C-O-C glycosidic linkage in sucrose (Mushibe, 2007).

Analysis of the ^1H NMR (Appendix 21) and HMQC spectrum revealed a cluster of methylene protons at δ_H 3.83 (12H, *m*)* correlating with the methylene carbons at δ_C 62.4, 61.4, 60.8, 60.6 and 60.2. The anomeric protons at δ_H 5.42, 5.14 and 4.51 showed J^1 correlation with the carbons at δ_C 92.1, 92.2 and 96.0 respectively (Table 3.8). From NOESY, the anomeric proton at δ_H 4.51 showed correlation with the proton at δ_H 3.12(H-5′′), while the anomeric protons at δ_H 5.42 and 5.14 showed correlation with the protons at δ_H 3.32 (H-6) of the sucrose unit. Some of the NOESY correlations are shown in Figure 3.15 below. From the available spectral data, the linkage of the

sucrose unit to the other two sugar units could not be determined due to insufficient 2D NMR data as the instrument was malfunctioning at the time.

The mass spectrum and the NMR data of compound **74** suggested the possible joining together of sucrose **72** and compound **75** units. Taking into consideration steric effects as well as the changes chemical shifts of the carbon signal in the carbon spectrum, the point of attachment between sucrose and maltose was at positions 4 on the sucrose unit while at position 1' on the sucrose unit. From the available spectral data, the proposed tentative structure for compound **73** was thus identified as shown in Figure 3.14 above.

Table 3.8: ^{1}H (300 MHz) and ^{13}C (75.4) NMR data for Compound 74 in methanol- d_4. (Refer to appendix 15 and 16).

Position	Compound **74**		NOESY
	δ_H (ppm)	δ_C (ppm)	
Sucrose unit			
1	5.42 (1H, *d*, *J* = 3.9 Hz)	92.2	
2	3.83 (12H, *m*)*	71.4	
3	4.05 (1H, *t*, *J* = 7.5 Hz)	74.2	H-5
4	3.83 (12H, *m*)*	72.6	
5	4.13 (1H, *d*, *J* = 8.4 Hz)	76.7	H-3
6	3.83 (12H, *m*)*	62.4	
1′	3.83 (12H, *m*)*	61.4	
2′		103.7	
3′	3.83 (12H, *m*)*	72.8	
4′	3.32 (10H, *m*)*)	69.6	
5′	3.83 (12H, *m*)*	81.4	
6′	3.83 (12H, *m*)*	60.2	
Compound **75** unit			
1″	5.14 (1H, *d*, *J* = 3.9 Hz)	92.1	H-5″
2″	3.32 (10H, *m*)*	76.0	
3″	3.83 (12H, *m*)*	72.8	
4″	3.83 (12H, *m*)*	71.1	
5″	3.32 (10H, *m*)*	75.8	H-1″
6″	3.83 (10H, *m*)*	60.6	
1‴	4.51 (1H, *d*, *J* = 7.8 Hz)	96.0	H-5‴
2‴	3.83 (12H, *m*)*	71.5	
3‴	3.83 (12H, *m*)*	72.4	
4‴	3.32 (10H, *m*)*	69.7	
5‴	3.12 (1H, *t*, *J* = 9.0 Hz)	74.1	H-1‴
6‴	3.83 (12H, *m*)*	60.8	

Assignments were confirmed by DEPT, COSY and HMQC experiments.

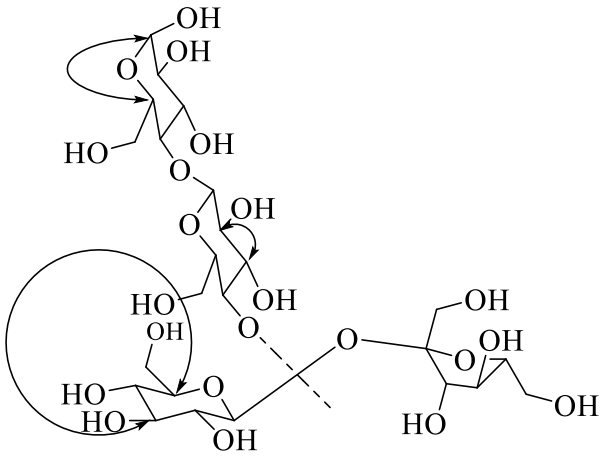


Figure 3.15: Key NOESY correlations of compound **74**.

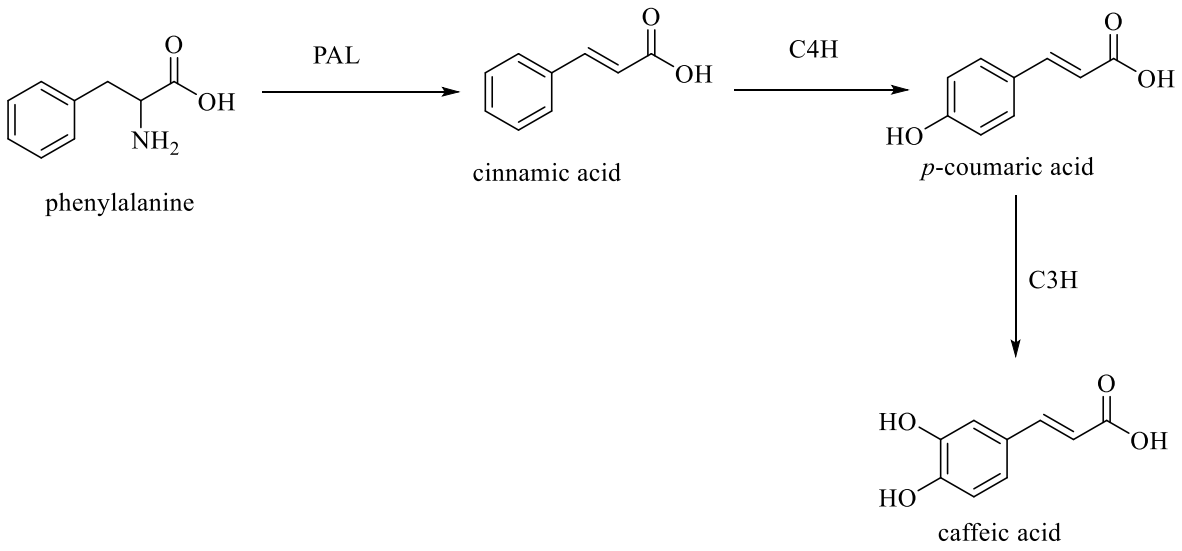
3.2 Proposed Biosynthesis of Compounds 70 And 71

Compound **70** and **71** are esters of cinnamic acid derivatives and fatty alcohols fused together via the ester linkage. The presence of the cinnamic acid unit was characterized by a set of *trans* coupled protons on the ^{1}H NMR together with the presence of a conjugated carbonyl carbon resonating around 168 ppm in the carbon spectrum of the two compounds (**70** and **71**). The presence of the fatty alcohol unit on the other hand was characterized by a broad singlet centered around 1.30 ppm indicative of a cluster of methylene protons for a long hydrocarbon chain. Isolation of compound

`69, a fatty acid suggests the biosynthesis of the two units (cinnamic acid unit as well as the fatty alcohol unit) separately and then fused together at a later stage. However, a cinnamic acid unit was not isolated during this study. Thus the biosynthesis of the cinnamic acid unit and later the fatty alcohol unit is discussed below.

Cinnamic acid and its derivatives can be biosynthesized by either the shikimate or the mevalonate pathway in higher plants. Through the shikimate pathway, aromatic amino acids including the amino acid phenylalanine are biosynthesized. Cinnamic acid and its derivatives including caffeic acid and *p*-coumaric acid are biosynthesized in plants through the intermediate phenylalanine as the precursor unit. The first step involves deamination of the precursor unit to afford cinnamic acid through the action of the enzyme phenylalanine ammonia lyase (PAL). The hydroxylation of cinnamic acid at position 4 of the benzyl ring through the action of the enzyme cinnamate 4-hydroxylase (C4H) affords the *p*-coumaric acid intermediate. The *p*-coumaric acid is then

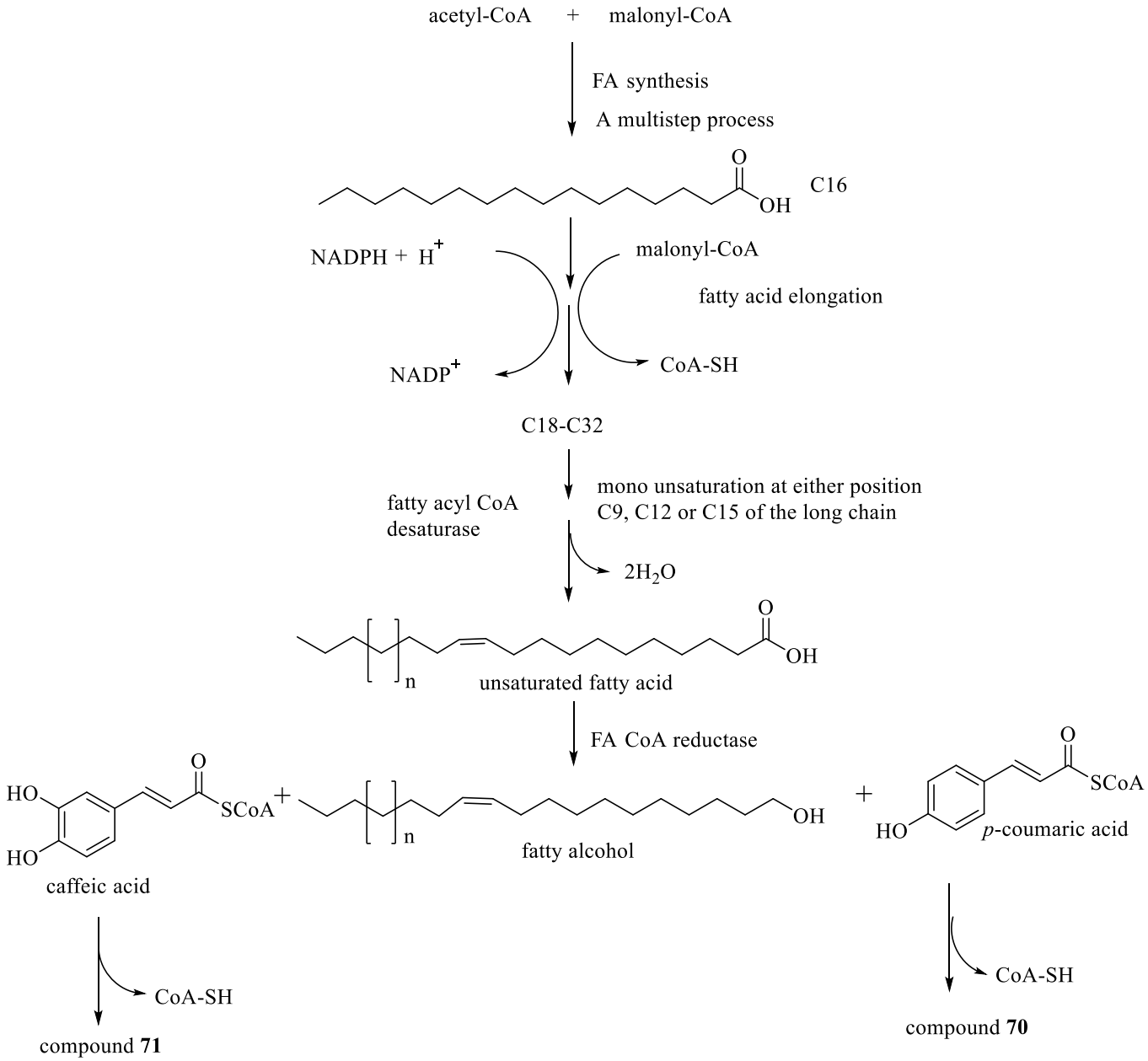
hydroxylated at position 3 to afford caffeic acid through the action of the enzyme *p*-coumarate 3-hydroxylase (C3H).



Scheme 3.3: Proposed biosynthesis of *p*-coumaric acid and caffeic acid (Kang *et al*., 2012).

Fatty alcohols are obtained from the reduction of fatty acids. The building blocks of fatty acid biosynthesis are the acetyl-CoA (the starter unit) and the malonyl-CoA which provides the 2 carbon subunits. A common example is phytol, derived from the side chain of chlorophyll. Briefly, the process proceeds via a four step process involving condensation, reduction, dehydration and reduction and adding two carbon units each time. A condensation reaction occurs between malonyl-CoA and Acetyl-CoA to give a four carbon chain β-ketide. Stereospecific reduction of the keto group by β-ketoacyl synthase using NADPH affords an alcohol with an *R* configuration. Dehydration of the alcohol by the enzyme 3-hydroxyacyl dehydratase gives a double bond with an (*E*) configuration which undergoes further reduction with NADPH to afford a saturated alkyl chain. The enzyme responsible for catalysing this reduction is enoyl reductase. The process is repeated by addition of 2 carbon units until a 16 carbon chain is formed. Chain elongation is achieved through modifications of the C16 carbon unit. Elongation is catalyzed by the elongases that use malonyl-CoA to add 2 carbon subunits until the appropriate chain length is reached. The synthesized fatty acid then undergoes mono-unsaturation at either position 9, 12 or 15 via the

enzyme fatty acid-CoA desaturase to afford the unsaturated fatty acid. A straight chain C20 alcohol with a double bond at the ω9 position is a common example. The Reduction of the fatty acid directly or via an aldehyde intermediate affords the appropriate fatty alcohol by the enzyme fatty acid-CoA reductase. The appropriate fatty alcohols then react with the *p*-coumaric acid and caffeic acid biosynthesized earlier to afford compounds **70** and **71**.

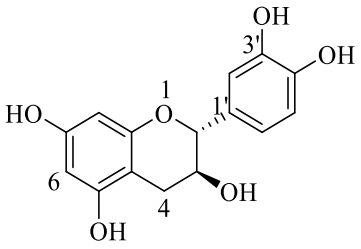


Scheme 3.4: Proposed biosynthesis of fatty alcohols and compounds **70** and **71** (Mudge, 2005).

3.3 Compounds Isolated from The Twigs of *Baikiaea Plurijuga*

Phytochemical investigation of the twigs of *B. plurijuga* afforded four known flavonoid derivatives namely; (+)-catechin **79**, eriodictyol **78**, taxifolin **77** and sucrose **72** all isolated from the methanol extract of the twigs. The spectral data of these known compounds were compared with the literature to confirm the identity of the isolated compounds.

Compound **79** was isolated as a brown solid with a melting point range of 174-176 ^{o}C (lit 174-175^{o}C, (Hyt *et al*, 2009). The IR spectrum of the compound showed a broad band at 3274 cm^{-1} indicative of a phenolic hydroxyl groups, 2926 cm^{-1} due to -CH stretch and 1606 cm^{-1} due to C = C aromatic stretch. The HR-MS spectrum gave an $[M+H]^{+}$ ion peak at m/z 291.0864 (Appendix 26) consistent with a molecular formula $C_{15}H_{15}O_{6}$.



79

Figure 3.16: Proposed structure of compound **79**.

The ^{13}C NMR spectral data of compound **79** (Appendix 25, Table 3.9) showed the presence of fifteen carbon signals. From the DEPT-135 and HMQC spectra, it was established that there were seven quaternary carbon signals resonating at δ_C 156.5, 156.3, 156.0, 144.8, 144.7, 131.3, and 99.8; seven methine carbon signals resonating at δ_C 119.2, 114.8, 114.4, 95.2, 94.5, 81.8, 67.4 and a methylene carbon resonating at δ_C 31.8. The absence of a carbonyl carbon together with the methine carbon at δ_C 67.4 and the methylene carbon at δ_C 31.8 were suggestive of a flavan-3-ol skeleton. This assignment was supported by the ^{1}H NMR and HMQC spectral data. Thus, analysis of the ^{1}HNMR spectrum (Appendix 24) revealed a set of diastereosopic methylene protons at δ_H 2.55 (1H, *dd*, *J* = 15.9, 8.1 Hz) and 2.93 (1H, *dd*, *J* = 15.9, 5.4 Hz) which were correlating with the methylene carbon at δ_C 31.8 and were thus assigned to position 4 of the flavan-3-ol skeleton.

Whereas the protons at δ_H 4.01 (1H, *ddd*, *J* = 15.9, 8.1, 5.4 Hz) and 4.54 (1H, *d*, *J* = 8.1 Hz) were correlating with carbons at δ_C 67.4 and 81.8 respectively and were assigned to positions 3 and 2 respectively, of the flavan-3-ol moiety. Further analysis of the ^{1}H NMR spectral data showed a set of two meta coupled protons resonating at δ_H 5.88 (1H, *d*, *J* = 2.1 Hz) and 6.03 (1H, *d*, *J* = 2.1 Hz) assignable to positions 6 and 8 of the flavanol skeleton. The ABX proton spin system at 6.91 (1H, *d*, *J* = 1.8 Hz); 6.82 (1H, *d*, *J* = 8.1 Hz) and 6.79 (1H, *dd*, *J* = 8.1, 1.8 Hz) were suggestive of 1,3,4-substituted benzene ring. These protons were assigned to positions 2′, 5′ and 6′ of the flavanol skeleton.

The above spectral assignments were confirmed by the HMBC spectral data, where protons at δ_H 2.93 (1H, *dd*, *J* = 15.9, 5.4 Hz) and 2.55 (1H, *dd*, *J* = 15.9, 8.1 Hz) were correlating with carbons at δ_C 67.4 and 99.8, whereas the proton at δ_H 4.54 (1H, *d*, *J* = 8.1 Hz) was correlating with carbons at δ_C 131.3, 114.4 and 67.4. Other key HMBC correlations are shown on figure 3.17 below. The optical rotation of compound **79** was found to be + 12.3 ^{o} (c = 0.001, MeOH). Based on the spectral data discussed above compound **79** was found to be (+)-catechin (Figure 3.16).

The above spectral data correlated with the data reported in literature (Kim *et al*., 2012). There are some differences in chemical shifts with those in literature probably due the use of different solvents when running the NMR spectra. This compound is reported for the first time from *Baikiaea plurijuga* as well as from other *Baikiaea* species. (+)-Catechin, is found in high concentrations in a variety of plant-based foods especially the colored ones such as apples, blackberries, cherries, black grapes and pears. Catechin and its derivatives have been associated with a variety of beneficial health effects including increased plasma antioxidant activity which involves the plasma's ability to scavenge free radicals aa well as thermogenic properties (Dulloo *et al*., 2000).

Table 3.9: ^{1}H (300 MHz) and ^{13}C NMR (75.4 Hz) data for Compound 79 in acetone d_6. (Refer to appendix 17 and 18).

Position	Compound **79**		Literature (Kim *et al*., 2012)	
	δ_H (ppm)	δ_C	δ_H (ppm)	δ_C
2	4.54 (1H, *d*, *J* = 8.1 Hz)	81.8	4.56 (1H, *d*, *J* = 7.6 Hz)	83.0
3	4.01 (1H, *ddd*, *J* = 15.9, 8.1, 5.4 Hz)	67.4	3.97 (1H, m)	69.0
4a	2.93 (1H, *dd*, *J* = 15.9, 5.4 Hz)	31.8	2.85 (1H, *dd*, *J* = 16.0, 5.3 Hz)	28.7
4b	2.55 (1H, *dd*, *J* = 15.9, 8.1 Hz)	31.8	2.50 (1H, *dd*, *J* = 16.0, 5.3 Hz)	28.7
5		156.3		157.8
6	5.88 (1H, *d*, *J* = 2.1 Hz)	95.2	5.92 (1H, *d*, *J* = 2.3 Hz)	96.4
7		156.5		157.1
8	6.03 (1H, *d*, *J* = 2.1 Hz)	94.5	5.85 (1H, *d*, *J* = 2.3 Hz)	95.6
9		156.0		158.0
10		99.8		100.9
1′		131.3		132.3
2′	6.91 (1H, *d*, *J* = 1.8 Hz)	114.4	6.83 (1H, *d*, *J* = 1.8 Hz)	115.4
3′		144.8		146.4
4′		144.7		146.5
5′	6.82 (1H, *d*, *J* = 8.1 Hz)	114.8	6.76 (1H, *d*, *J* = 8.2 Hz)	116.2
6′	6.79 (1H, *dd*, *J* = 8.1, 1.8 Hz)	119.2	6.79 (1H, *dd*, *J* = 8.2, 1.8 Hz)	120.2

Assignments were confirmed by DEPT, COSY and HMQC experiments.

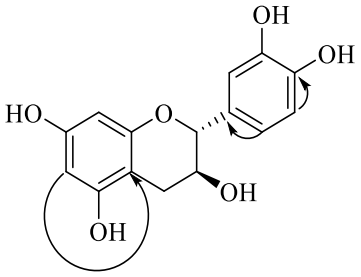
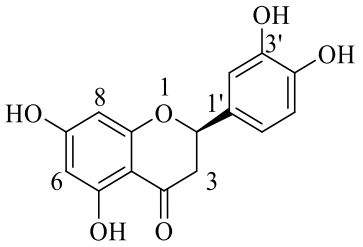


Figure 3.17: Some selected HMBC correlations of compound **79**.

Compound **78** (Figure 3.18) was also isolated from the methanol extract of the twigs of *B. plurijuga as* a brown powder with a melting point of 260-264ºC (literature value; 256-258 ºC, Vijay *et al*, 2014). The UV spectrum showed absorption maxima at 323 and 211 nm for bands II and I of the cinnamoyl and benzoyl moieties of the flavanone skeleton respectively. The IR spectrum of the compound showed a broad band at 3238 cm^{-1} indicating the presence of phenolic hydroxyl groups in the compound; a sharp strong peak at 1695 cm^{-1} indicative of a conjugated carbonyl group and C = C aromatic stretches were observed at 1596 and 1519 cm^{-1}. The molecular formula C_{15} H_{12}

O_6, of compound **78** was deduced from the HR-MS spectrum where $[M+H]^+$ ion was observed at m/z 289.0708 (Appendix 29).



78

Figure 3.18: Proposed structure of compound **78**.

The ^{13}C NMR spectral data of compound **78** (Appendix 28, Table 3.10) showed the presence of fifteen carbon signals. From the DEPT-135 spectral data the signals were resolved to comprise of eight quaternary carbons resonating at δ_C 196.3(C=O), 166.4, 164.4, 163.5, 145.5, 145.1 and 102.4; six methine carbon signals resonating at δ_C 118.4, 115.1, 113.8, 95.9, 95.0 and 79.1 and one sp^3 methylene carbon signal at δ_C 42.7. The carbon signals resonating at δ_C 145.5 and 145.1 are indicative of a diOH substitution in ring B of a flavanone skeleton, because they are resonating below 150 ppm due to mutual shielding (Geiger *et al*., 1993).

Analysis of the ^1H NMR spectral data of compound **78** (Appendix 27, Table 3.10) revealed a set of aliphatic protons at 2.72 (1H, *dd*, *J* = 17.1, 3.0 Hz); 3.09 (1H. *dd*, *J* = 17.1, 12.6 Hz) and 5.30 (1H, *dd*, *J* = 12.6, 3.0 Hz). The proton signal at δ_H 5.30 ppm appeared as a doublet of doublets suggesting that the neighboring protons were diasteriotopic in nature and these protons resonating at δ_H 2.72 and 3.09 ppm were assigned positions H-3a and H-3b respectively, of a flavanone skeleton. Further examination of the proton spectral data showed a chelated proton at 12.19 suggestive of the presence of a 5-OH in the flavanone skeleton. Other protons were observed at δ_H 6.90 (2H, *s*), 5.97 (2H, *s*) and 7.06 (1H, *s*). The peaks were not resolved and so they appeared as singlets. The optical rotation of compound **78** was obtained as - 0.007 ^{o} (C = 0.0027, MeOH). Key HMBC correlations for compound **78** are shown on Figure 3.19 below.

Careful review of the literature confirmed that the spectral data of compound **78** were in full agreement with those reported for a known compound, eriodictyol (Encarnacion *et al*., 1999). This compound is reported for the first time from *Baikiaea plurijuga*. In-vivo studies have revealed that

eriodictyol could prevents early retinal and plasma abnormalities in streptozotocininduced diabetic rats (Bucolo *et al*., 2012).

Table 3.10: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR data for Compound 78 in acetone-d$_6$. (Refer to appendix 19 and 20).

Position	Compound **78**		Literature (Encarnacion *et al*., 1999)	
	δ_H (ppm)	δ_C	δ_H (ppm)	δ_C
2	5.30 (1H, *dd*, *J* = 12.6, 3.0 Hz)	79.1	5.40 (1H, *dd*, *J* = 12.6, 3.1 Hz)	79.3
3a	2.72 (1H, *dd*, *J* = 17.1, 3.00 Hz)	42.7	3.14 (1H, *dd*, *J* = 17.1, 12.6 Hz)	42.9
3b	3.09 (1H, *dd*, *J* = 17.1, 12.6 Hz)	42.7	2.73 (1H, *dd*, *J* = 17.1, 3.1 Hz)	42.9
4		196.3		196.5
5		164.4		164.6
6	5.97 (2H, *s*)	95.9	5.94 (1H, *d*, *J* = 2.2 Hz)	95.2
7		166.4		166.6
8	5.97 (2H, *s*)	95.0	5.95 (1H, *d*, *J* = 2.2 Hz)	96.1
9		163.5		163.7
10		102.4		102.0
1′		130.6		129.7
2′	7.06 (1H, *s*)	113.8	7.04 (1H, *d*, *J* = 1.7 Hz)	115.4
3′		145.5		145.7
4′		145.1		145.4
5′	6.90 (2H, *s*)	115.1	6.87 (1H, *d*, *J* = 8.0 Hz)	114.1
6′	6.90 (2H, *s*)	118.4	6.88 (1H, *dd*, *J* = 8.0, 1.7 Hz)	118.6
5-OH	12.19 (1H, *s*)		12.17	
7,3′,4′-OH			8.5 and 2.98, *br s*	

Assignments were confirmed by DEPT, COSY and HMQC experiments.

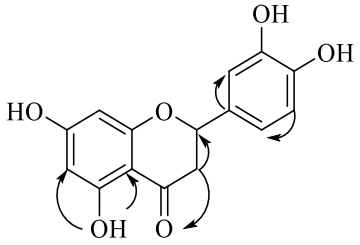
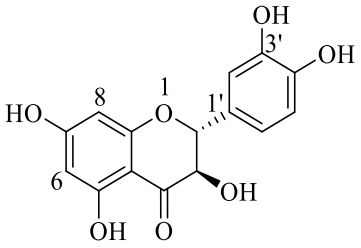


Figure 3.19: Some selected HMBC correlations of compound **78**.

Further work on the methanol extract lead to the isolation of compound **77** as a brown solid with a melting point range of 222-225^{o}C (literature value; 231 ^{o}C, Joo *et al*, 2014). The UV-Vis

spectrum showed absorption maxima in methanol at 325 and 213 nm. The optical rotation of compound **79** was found to be + 39.5 ^{o} (c = 0.003, MeOH). The IR spectrum of the compound showed a broad band at 3303 cm^{-1} and a sharp strong band at 1519 cm^{-1} indicating the presence of phenolic hydroxyl and C = C aromatic stretches in the compound. The absence of a conjugated carbonyl around 1700 cm^{-1} could be attributed to instrumental error. The HR-MS spectrum gave an $[M+H]^{+}$ ion peak at m/z 305.0657 (Appendix 32) consistent with a molecular formula $C_{15}H_{12}O_{7}$.



77

Figure 3.20: Proposed structure of compound **77**.

Examination of the ^{1}H NMR spectrum of compound **77** (Appendix 30) revealed a chelated proton at δ_H 11.73 (1H, *s*). In addition, an ABX spin system involving aromatic protons was observed at δ_H 7.09 (1H, *d*, *J* = 1.8 Hz); 6.94 (1H, *dd*, *J* = 8.1, 1.8 Hz) and 6.89 (1H, *d*, *J* =8.1 Hz) assignable to positions 6′, 2′ and 3′ of a dihydroflavonol moiety. A set of *meta*-coupled protons appeared at δ_H 6.02 (1H, *J* = 2.1 Hz) and 5.97 (1H, *J* = 2.1 Hz) and these were assigned to positions 6 and 8 respectively (Table 3.11), of a dihydroflavonol moiety. Further analysis of the proton spectral data revealed a set of one proton doublets in a *trans*-orientation at δ_H 5.05 (1H, *d*, *J* = 11.4Hz) and 4.64 (1H, *d*, *J* = 11.4 Hz) assignable to H-2 and H-3 respectively, of a dihydroflavonol skeleton.

The ^{13}C NMR spectrum revealed fifteen carbon signals (Appendix 31) and using the DEPT and HMQC spectral data these carbon signals were classified into eight quaternary and seven methine carbons. The methine carbon signal at δ_C 72.3 and a strong IR absorption band at 1161 cm^{-1} due to a C-O stretch for a secondary alcohol were supportive of a dihydroflavonol skeleton. The assignments above were confirmed by the HMBC spectral data where the proton at δ_H 5.05 (1H, *d*, *J* = 11.4 Hz) was correlating with the carbons at δ_C 128.9 and 197.3, whereas the proton at δ_H

6.89 (1H, *d*, *J* = 8.1 Hz) showed correlations with carbon atoms at δ_C 120.0 and 144.9. Key HMBC correlations are shown in Figure 3.21 below.

It was concluded from the spectral data discussed above that compound **77** was a known dihydroflavonol, dihydroquercetin or taxifolin as shown in Figure 3.20 above. Taxifolin is a known potent antibacterial agent (Khatik and Sharma, 2015).

Table 3.11: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR data for Compound 78 in acetone-d_6. (Refer to appendix 21 and 22).

Position	Compound **77**		Literature (Kim *et al*., 2012)	
	δ_H (ppm)	δ_C	δ_H (ppm)	δ_C
2	5.05 (1H, *d*, *J* = 11.4 Hz)	83.6	4.91 (1H, *d*, *J* = 11.5 Hz)	85.3
3	4.64 (1H, *d*, *J* = 11.4 Hz)	72.3	4.50 (1H, *d*, *J* = 11.4 Hz)	73.8
4		197.3		198.6
5		164.1		168.9
6	6.02 (1H, *d*, *J* = 2.1 Hz)	96.2	5.92 (1H, *d*, *J* = 2.1 Hz)	97.4
7		167.0		165.5
8	5.97 (1H, *d*, *J* = 2.1 Hz)	95.2	5.88 (1H, *d*, *J* = 2.1 Hz)	96.4
9		163.3		164.7
10		100.7		102.0
1′		128.9		130.0
2′	6.94 (1H, *dd*, *J* = 8.1, 1.8 Hz)	114.9	6.96 (1H, *d*, *J* = 1.8 Hz)	116.0
3′		145.7		147.3
4′		144.9		146.5
5′	6.89 (1H, *d*, *J* = 8.1 Hz)	115.0	6.80 (1H, *d*, *J* = 8.0 Hz)	116.2
6′	7.09 (1H, *d*, *J* = 1.8 Hz)	120.0	6.85 (1H, *dd*, *J* = 8.0, 1.8 Hz)	121.0
5-OH	11.73 (1H, *s*)			

Assignments were confirmed by DEPT, COSY and HMQC experiments.

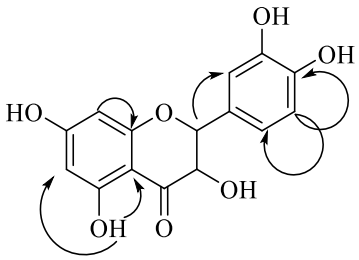


Figure 3.21: The key HMBC correlations of compound **77**.

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Chapter Four

Biological Activity Studies

4.1 Antioxidant Studies

Chemical species with unpaired electrons in their outer most shell are called free radicals and the species are highly reactive. The chemical species can be found in the environment we live in. They occur due to a number of factors like processes taking place in our bodies, which are internal thereby leading to reactive oxygen species (ROS) (Droge, 2002) such as superoxide anions ($O_{2}^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) as examples. Free radicals can also be caused by exogenous sources like cigarette smoking, ultra violet radiation especially from the sun, environmental pollution and the use of chemicals in food production. Most of the common ailments such as malaria, acquired deficiency syndrome, heart diseases, stroke diabetes, cancer, lung damage liver toxicity cardiovascular disorder are directly or indirectly linked to the harmful effects of these reactive oxygen species (Liao and Yin, 2000; Oke and Hamburger, 2002; Droge, 2002). Because of their reactivity, free radicals attack cell membranes and other important constituents of the cell leading to most of the ailments mentioned above (Wu and Cederbaum, 2003). The human body, however, naturally possesses antioxidants that counteract the destructive nature of these free radicals.

Antioxidants are chemical substances that are radical scavengers and by so doing protect the body against the harmful effects of these free radicals. They are found in many food stuffs and supplements that form a large part of our diet. Some of the commonly known antioxidants include vitamin A **81**, E **82**, C **83**, β-carotene **84** (Figure 4.1), selenium and polyphenols from plants such as stilbenes, lignans, flavonoids and phenolic acids. Plants are the main source~~s~~ of antioxidants (Sies and Stahl, 1995; Fang *et al*., 2002). Polyphenols are aromatic hydroxylated compounds and are common among secondary metabolites derived from plants although there are synthetic ones like butylated hydroxytoluene and butylated hydroxyanisol (Haslam, 1996; Marmesat *et al*., 2010).

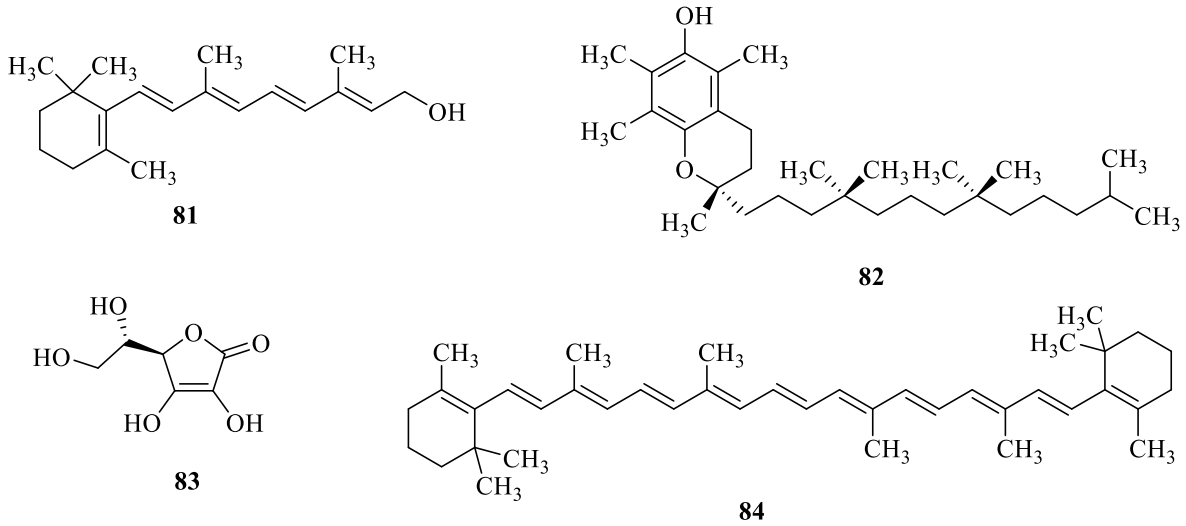


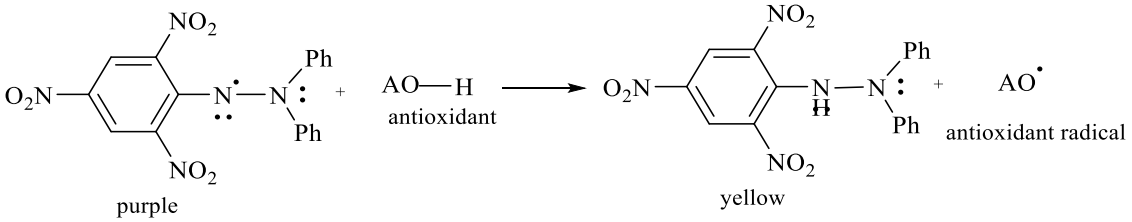
Figure 4.1: Some common antioxidants from medicinal plants.

As mentioned earlier, plants especially medicinal ones are the main sources of natural antioxidants and because of this, keen interest has shifted towards acquisition of plant derived antioxidants as opposed to synthetic sources (Rice Evans *et al*., 1996; Molyneux, 2004). Studies on some of these plants have revealed that the antioxidants contribute significantly towards the healing properties of these plants (Biapa *et al*., 2007). The use of parts of some of these plants such as intake of decoctions or extracts like oils can relieve or manage some of the deleterious effects associated with free radicals. As an example, the essential oils extracted from the pot marigold (*C. officinalis*) are used as medicines for soothing central nervous system and exhibits other useful healing properties (Miliauskas *et al*., 2004). The oils are also rich in carotenoids which are potential antioxidants with healing properties (Miliauskas et al., 2004; Edge *et al*., 1997).

Many methods such as ferric reducing-antioxidant power (FRAT) assay, hydrogen peroxide scavenging (H_2O_2) assay, nitric oxide scavenging activity (Badu *et al*., 2013; Sanchez-Moreno, 2002) and DPPH have been developed to evaluate the efficacy of extracts and pure isolates to scavenge free radicals. Among these methods, the DPPH (1, 1-diphenyl-1-picrylhydrazyl) method has been the most preferred method used to evaluate the antioxidant properties of both natural and synthetic compounds as it is considered easy and economical with minimal sources of errors when

performed. The method is however limited to compounds or extracts soluble in organic solvents, especially alcohols (Kedare and Singh, 2011).

A molecule of 1, 1-diphenyl-2-picrylhydrazyl; (DPPH) contains a stable free radical by virtue of the delocalization of the spare electron over the whole molecule resulting in the molecules not dimerizing. In its radical form, DPPH is easily recognizable by its purple colour giving a strong absorption band in methanol solution centred at 517 nm. In the presence of an antioxidant, the purple colour decays to yellow (Scheme 4.1) because the radical odd electron pairs with an unpaired electron from the antioxidant to form a reduced DPP-H (Pietta, 2000). The DPPH radical also acts as an indicator for the above reaction. The changes in absorbance from 517 nm as the radical DPPH is reduced can be followed spectrophotometrically.



Scheme 4.1: The reaction of DPPH radical with an antioxidant.

The reaction aims to provide the link with the reactions taking place in an oxidizing system, such as the auto-oxidation of a lipid in the body. The DPPH molecule is thus intended to represent the free radicals formed in the bodily system whose activity is to be suppressed by the antioxidants (Arti *et al*., 2014). The lowest minimum quantity applied is taken as the measure of the extent of the radical scavenging potency (Yeboah and Majinda, 2009).

The antioxidant activity of *I. bolusiana* and *B. plurijuga* extracts and pure isolates were evaluated using both the semi-quantitative as well as the spectrophotometric methods using gallic acid and ascorbic acid used as standards.

4.2 DPPH Radical Scavenging Abilities of crude extracts and isolated compounds from *B. Plurijuga* and *I. bolusiana*.

The ability of the isolated compounds and crude extracts to scavenge free radicals was tested. The semi quantitative TLC DPPH-RSA assay revealed that the crude extracts exhibited free radical scavenging activity. All the isolated compounds were tested except for sucrose, **72**. The compound has been isolated before and biological studies on the compound reveal that it is a possesses antioxidant capabilities (Reiser, 1994; Van den Ende and Valluru, 2009).

Flavonoids **79, 78** and **77** isolated from the methanol extract of the twigs of *B. plurijuga* were subjected to the DPPH radical scavenging test and the results are summarized in Table 4.1. Flavonoid **79** was found to possess radical scavenging activity against DPPH radicals with minimum inhibition quantity (MIQ) of 10 µg instantly after spraying with DPPH solution. The activity of the compound did not change after thirty minutes of reaction and after 2 hours the activity improved to an MIQ of 5 µg. Compared to the standard gallic acid, less quantities of compound **79** are needed to scavenge DPPH radicals with time after 24 hours (Table. 1). Flavonoids **78** and **77**, however showed better activity at an MIQ of 1 µg instantly after spraying. The activity did not improve after thirty minutes of spraying. Compared to **79**, the two compounds have a carbonyl functionality at position 4. Hydroxyl groups in the presence of this functionality (4 oxo) in A and C rings are known to enhance free radical scavenging ability of a flavonoid (Kurek-Gorecka *et al*., 2013). Moreover, the presence of hydroxyl groups in a compound are known to enhance the radical scavenging ability of a particular compound (Kurek-Gorecka *et al*., 2013). The number and positioning of hydroxyl groups on the isolated flavonoids is however similar which might explain comparable activities between the three compounds. The free radical scavenging ability of the isolated flavonoids might to some extent explain and justify the use of this plant in folk medicine.

Table 4.1: MIQ values for the flavonoids over a 24-hour period.

Name of compound	Minimum inhibitory quantities (MIQ) µg					
	Instantly	30 min	1-Hr	2-Hrs	3-Hrs	24-Hrs
Gallic acid	0.5	0.5	0.5	0.5	0.5	0.5
Ascorbic acid	1	0.1	0.1	0.1	0.2	0.2
Compound **79**	10	10	5	5	5	5
Compound **78**	1	1	1	0.1	0.1	0.1
Compound **77**	1	1	1	0.1	0.1	0.1

The crude extract of both plants showed free radical scavenging activity especially the polar ones as compared to the least polar extracts (Table 4.2). The ethyl acetate, methanol and methanol/water extracts from the twigs of *B. plurijuga* showed similar free radical scavenging activity with a minimum inhibitory quantity of 10 µg after 30 minutes. The activity of these extracts did not improve even after 24 hours. As for the least polar extracts, the n-hexane extract showed the least activity at 100 µg instantly after spraying and the result was consistent throughout the 24-hour period. The chloroform extract was however more active than the latter with an MIQ of 50 µg after 30 minutes of spraying with DPPH solution. The result improved to an MIQ of 10 µg after one hour and remained unchanged even after 24 hours. The extracts were not as active as the standard gallic acid which was active up to 0.5 µg loading. The observed trend of activity for the extracts was not surprising as phenolic compounds, which are generally polar, are concentrated in polar extracts like the methanol extract.

Table 4.2: MIQ values for the crude extracts of *B. plurijuga* over a 24-hour period.

Name of extract	Minimum inhibitory quantities (MIQ) µg					
	Instantly	30 min	1-Hr	2-Hrs	3-Hrs	24-Hrs
Gallic acid	0.5	0.5	0.5	0.5	0.5	0.5
Ascorbic acid	1	1	1	1	0.1	0.1
B. plurijuga						
B.P n-Hexane	100	100	100	50	50	50
B.P $CHCl_3$	50	50	10	10	10	10
B.P EtOAc	10	10	10	10	10	10
B.P MeOH	10	10	10	10	10	10
B.P Aqueous MeOH (80%)	10	10	10	10	10	10

Cinnamic acid and its derivatives, isolated from various plant species have been found to have antioxidant properties (Sharma, 2011) as shown in Table 4.3. *P*-Coumaric acid derivative **70**

isolated from the tubers of *I. bolusiana* exhibited an MIQ of 50 µg after 1 hour. The activity improved to 10 µg after two hours of spraying with the DPPH solution. *p*-Coumaric acid derivatives have one hydroxyl group on the aromatic ring. The presence of this group could be attributed to the scavenging activity of this compound. Caffeic acid derivative **71**, showed activity up to 1 µg loading instantly after spraying with DPPH spray. The activity did not improve after thirty minutes but improved to 0.1µg after three hours and remained unchanged. The caffeic acid derivative **71** has an extra hydroxyl group as compared to coumaric acid derivative **70** and more hydroxyl groups in a compound are known to enhance activity of the compound (Topal *et al*., 2016). This might explain why compound **71** showed better activity than compound **70**.

Compound **73** exhibited an MIQ of 5 µg immediately after spraying with the DPPH solution**.** The activity of the compound did not change after thirty minutes of reaction time. This compound is a glycoside derivative isolated from the tubers of *I. bolusiana*. The presence of a sugar moiety could somehow explain the lower potency of the glucoside as compared to the caffeic acid derivative **71**. Since cinnamic acid and its derivatives have been shown to have antiviral, anti-diabetic and cytotoxic abilities (Sharma 2011), free radical scavenging abilities of compounds **71** and **70** may, to some extent explain their use in as medicines in some communities.

Table 4.3: MIQ values for compounds isolated from *I. Bolusiana* over a 24-hour period.

Name	Minimum inhibitory quantities (MIQ) µg					
	Instantly	30 min	1-Hr	2-Hrs	3-Hrs	24-Hrs
Gallic acid	0.5	0.5	0.5	0.5	0.5	0.5
Ascorbic acid	1	1	1	1	0.1	0.1
Compound **70**	10	10	5	5	5	5
Compound **71**	1	1	1	1	0.1	0.1
Compound **73**	5	5	5	1	1	1
Compound **74**	50	10	10	10	10	10
Compound **75**	100	100	50	50	50	50
Compound **76**	10	5	5	5	5	1

As for other compounds, compound **74** exhibited an MIQ of 10 µg observed after thirty minutes of spraying. This is a relatively moderate activity compared to the already discussed compounds.

Compound **75,** which is a disaccharide, however showed an MIQ of 100 µg instantly after spraying and 50 µg after 1 hour. This is a low MIQ compared to the other compounds. Even though the activity of this oligomer is low, certain carbohydrate polymers have been shown to possess free radical scavenging activity (Tsiapali *et al*., 2001)

The crude extracts from the tubers of *I. bolusiana* portrayed a similar trend as observed for the twigs of *B. plurijuga*. The chloroform /ethyl acetate/methanol extract showed the least activity amongst the extracts of this plant with an MIQ of 50 µg after thirty minutes and remained unchanged. The methanol and methanol water extracts however showed similar activity with an MIQ of 10 µg after thirty minutes and remained unchanged (Table 4.4). The reason for the observed activity of the extracts might be due to the same reasons explained for the latter plant.

Table 4.4: MIQ values for the crude extracts of *I. bolusiana* over a 24-hour period.

Name of extract	Minimum inhibitory quantities (MIQ) µg					
	Instantly	30 min	1-Hr	2-Hrs	3-Hrs	24-Hrs
Gallic acid	0.5	0.5	0.5	0.5	0.5	0.5
Ascorbic acid	1	1	1	1	0.1	0.1
I. bolusiana						
I.B $CHCL_3$/EtOAc/MeOH	50	50	50	50	50	50
I.B MeOH	50	10	10	10	10	10
I.B Aqueous MeOH (80%)	10	10	10	10	10	10

The photographs of the TLC chromatograms taken at given time intervals for the compounds and plant extracts of *B. plurijuga* and *I. bolusiana* are shown in Figure 4.2 below.

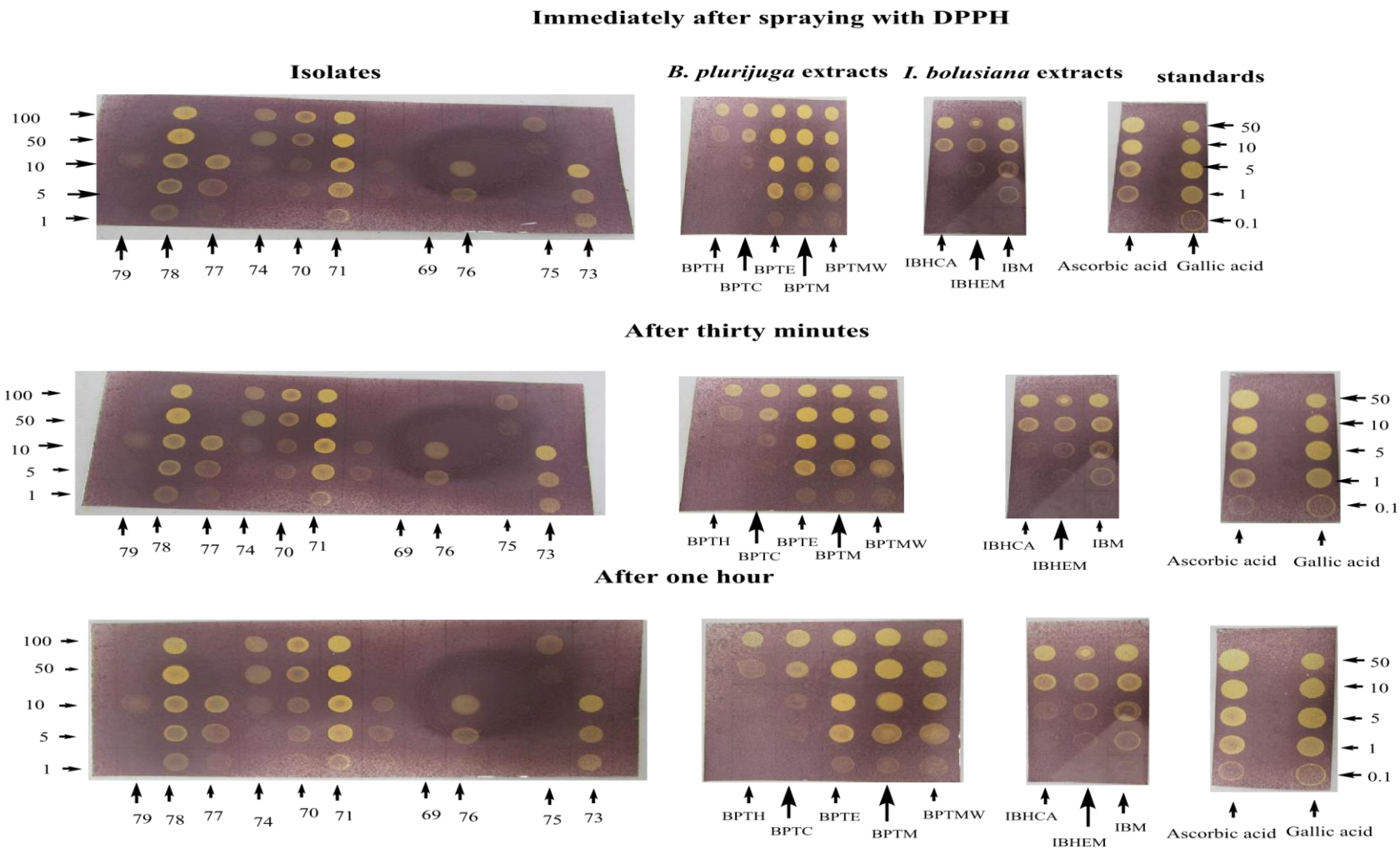


Figure 4.2: TLC autographs showing radical scavenging activity of the isolated compounds and extracts from *Ipomoea bolusiana* and *Baikiaea plurijuga*.

4.3 Spectrophotometric DPPH-RSA Assay

Although the TLC DPPH-RSA assay is an excellent method for the determination of antioxidant ability to both plants extracts and the isolated compounds, it has its limitations in that it cannot be used to tell of the test samples will be active in a living organism since it is *in vivo*. The spectrophotometric DPPH-RSA however has its advantages over the TLC DPPH method in that it is more quantitative even though it is also *in vivo*.

In the spectrophotometric DPPH-RSA assay, a plot of % DPPH scavenging versus concentration of the sample gives the IC_{50} in µg/mL upon extrapolation at 50 %. As the sample concentration increases, the % DPPH radical scavenging activity also increases but slightly with increase of time. From the curve, one can note that the lower the IC_{50} value the more active the sample. The experiment was done over a 24-hour period and the average IC_{50} values of the isolates as well as the crude extracts was determined over this time period calculated from the equations of the inhibition curves. Compounds **73**, **76**, and **79** were not subjected to spectrophotometric DPPH due to their minute quantities although they were effective free radical scavengers as revealed by semi quantitative TLC-DPPH method (Figure 4.2).

Compound **71** was the most active of all the test cinnamic acid derivatives isolated with an IC_{50} value of 0.023 ± 0.006 µM. Caffeic acid and its derivatives are excellent free radical scavengers due to the presence of adjacent hydroxyl groups in the benzene ring (Kurek-Gorecka *et al*., 2013; Pontiki *et al*., 2014). Compound **70** on the other hand showed a lower IC_{50} (0.47 ± 0.06 µM) potency as compared to the latter compound (Table 4.5). The value is comparable to that of dodecyl-*p*-coumaric acid isolated from *Ipomoea sepiaria* (IC_{50} = 0.53 ± 0.006 µM) (Singh and Singh, 2014). The lower potency of this compound may be due to mono-hydroxylation in the benzene ring. Benzoic acid derivatives with a single hydroxyl group in the *para*- or *ortho*- positions are not known to display antioxidant properties (Kurek-Gorecka *et al*., 2013). Although the two compounds are similar in structure in their basic skeletal structure, the caffeic acid derivative has an extra hydroxyl group adjacent to the para-hydroxyl group. This might explain the enhanced potency of compound **71** as compared to compound **70**.

Table 4.5: Average IC_{50} values for the pure isolates of *I. bolusiana*.

Compound/Extract	Average IC_{50} ± Standard Deviation (μM)
Ascorbic acid	0.090 ± 0.01 μM
Compound **70**	0.47 ± 0.06 μM
Compound **71**	0.023 ± 0.006 μM
Compound **74**	0.031 ± 0.007 μM
Compound **75**	0.25 ± 0.02 μM

The values are expressed as mean ± standard deviation.

The combined $CHCl_3$/MeOH/EtOAc extract of the tubers of *Ipomoea Bolusiana* was the least active of *I. Bolusiana* extracts with an average IC_{50} value of 47.42 ± 1.94 μg /mL (Table 4.6). The most active extract of this plant was the aqueous methanol (80%) extract with an IC_{50} value of 37.58 ± 8.37 μg /mL followed by the methanol extract (40.63 ± 8.19 μg /mL). Compounds **71, 70** and sucrose were isolated from the combined $CHCl_3$/MeOH/EtOAc extract while compounds **73**, **74** and **75** were isolated from the methanol extract. This extracts showed promising TLC profiles under UV lamp as compared to the aqueous methanol (80%) extract hence work was done on them. Generally, the extracts of *I. Bolusiana* showed better scavenging ability as compared to the extracts of *B. Plurijuga.*

Table 4.6: Average IC_{50} values for the extracts of *I. bolusiana*.

Name of extract	AverageIC_{50} ± Standard Deviation (μg /mL)
Ascorbic acid	15.84 ± 1.68 μg /mL
Ipomoea Bolusiana (Tubers)	
I.B $CHCL_3$/EtOAc/MeOH (1:1:1 v/v/v)	47.42 ± 1.94 μg /mL
I.B MeOH	40.63 ± 8.19 μg /mL
I.B Aqueous MeOH (80%)	37.58 ± 8.37 μg /mL

The values are expressed as mean ± standard deviateon.

Of the flavonoids isolated, Compound **78** showed the best DPPH radical scavenging activity with an IC_{50} value of 0.047 ± 0.007 μM. The value is comparable to that of the standard ascorbic acid (IC_{50} = 0.090 ± 0.001 μM) over a 24-hour period and a bit lower that the IC_{50} of the Eriodictyol (**78**) isolated from the ethyl acetate extract of *Limonium bonduelli* by Benayache *et al*, 2013 (IC_{50} = 0.02 μM) as shown in Table 4.7. Eriodictyol (**78**) is known for its anti-inflammatory as well as

its antioxidant activity (Murti and Mishra, 2014). Taxifolin (**77**) on the other hand, had an average IC_{50} value of 0.20 ± 0.02 μM over a 24-hour period. The scavenging activity is a bit higher but comparable to that reported by Topal (2015) with an IC_{50} value of 0.25 ± 0.00001 μM. Taxifolin (**77**) scavenges the DPPH radical on its phenolic groups and the antioxidant potency of this molecule lies in its conjugation structures and resonance stabilization of ring A and ring B of the flavanone skeleton (Topal *et al*., 2015). The activity of these compounds in general could be attributed to the arrangement of the two aromatic rings and the two phenolic groups at the *meta*- and *para*- positions with respect to each other (Topal *et al*., 2016). This arrangement seems to be the one responsible for the strong antioxidant activity of these compounds (Topal *et al*., 2015). There are also suggestions that hydroxyl groups near C-3 and C5 in the presence of the 4-oxo group in the A and C ring enhance free radical scavenging effects of a compound (Kurek-Gorecka *et al*., 2013). In general, the study supports the findings that phenolic compounds for example, flavonoids, are excellent scavengers of free radicals and this could be due to their hydrogen donating ability (John *et al*., 2012).

Table 4.7: Average IC_{50} values for the isolates.

Compound/Extract	Average IC_{50} ± Standard Deviation (μM)
Ascorbic acid	0.090 ± 0.01 μM
Compound **78**	0.047 ± 0.007 μM
Compound **77**	0.20 ± 0.02 μM

The values are expressed as mean ± standard deviation.

The n-hexane and the chloroform extracts of *B. plurijuga* showed poor or weak anti-oxidant activity as revealed by the TLC semi-quantitative investigation and therefore were not subjected to DPPH analysis by spectrophotometric method. Generally, % DPPH scavenged increased with increase in concentration of the extract for both plants (Figures 4.3 and 4.4). None of the extracts examined scavenged the DPPH radicals by more than 10 % in the first 30 minutes of the experiment, however, only the ethyl acetate extract of *B. plurijuga* was able to scavenge the DPPH radicals by more than 50 % within the first hour of the experiment at a concentration of 50 μg/mL. No compounds were isolated from this extract because of its small quantity. The 80 % aqueous methanol extract of the *B. plurijuga* was the most active with an average IC_{50} value of 60.86 ± 4.83 μg /mL, followed by the methanol extract (IC_{50} = 62.85 ± 4.40 μg /mL) and lastly the ethyl acetate

extract (IC_{50} = 76.61 ± 6.27 µg /mL) as shown in Table 4.8 below. Compounds **77**, **78** and **79** were all isolated from the methanol extract and this could explain the potency of the methanol extract of this plant. All the isolate compounds are also known to be excellent free radical scavengers.

Table 4.8: Average IC_{50} values for the extracts.

Name of extract	AverageIC_{50} ± Standard Deviation (µg /mL)
Ascorbic acid	15.84 ± 1.68 µg /mL
Baikiaea plurijuga (Twigs)	
B.P EtOAc	76.61 ± 6.27 µg /mL
B.P MeOH	62.85 ± 4.40 µg /mL
B.P Aqueous MeOH (80%)	60.86 ± 4.83 µg /mL

The values are expressed as mean ± standard deviation.

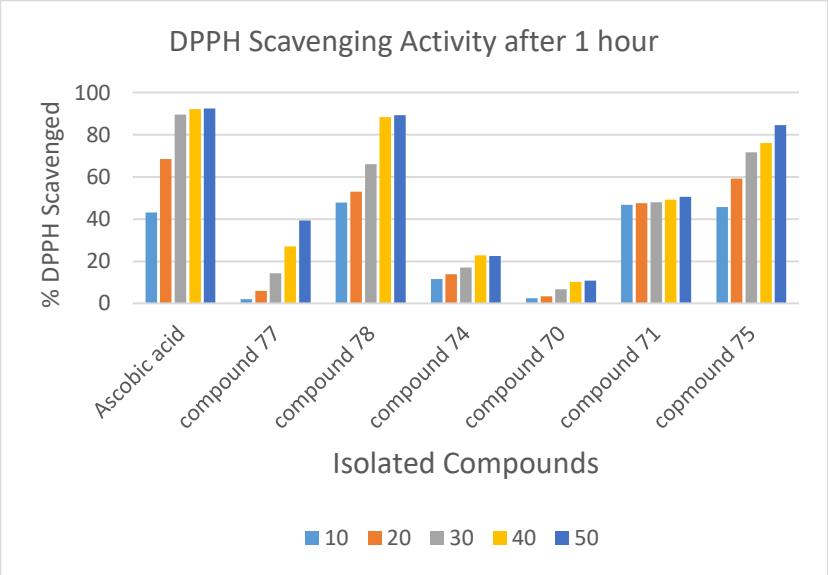


Figure 4.3: DPPH radical scavenging activity measured after 1 hour at different concentrations for the pure compounds.

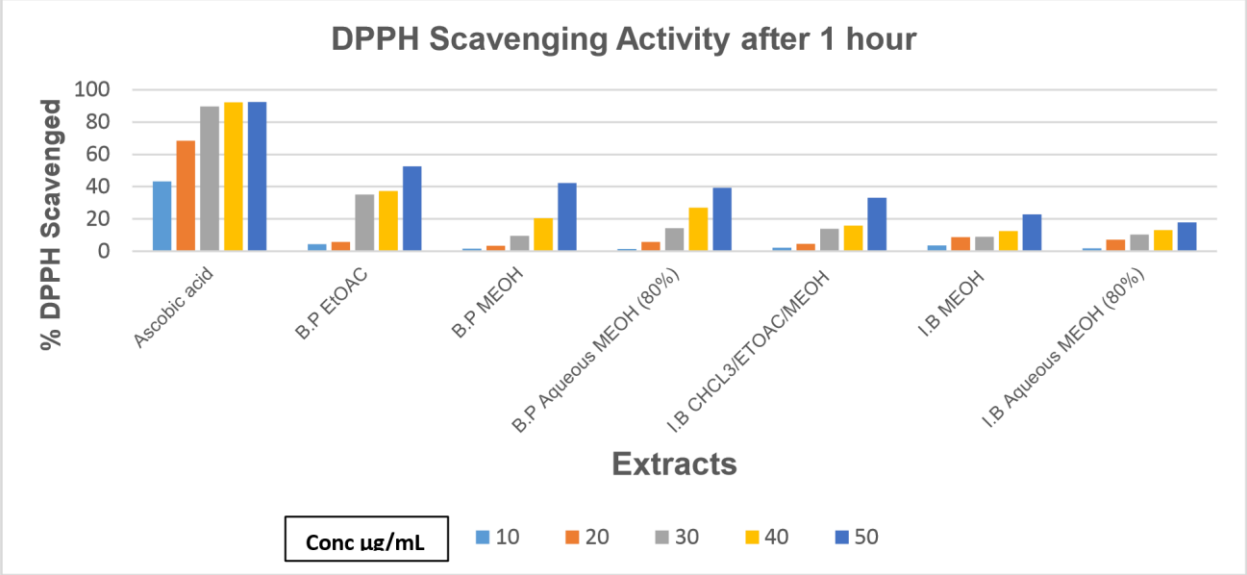


Figure 4.4: DPPH radical scavenging activity measured after 1 hour at different concentrations for the extracts.

4.4 Determination of Total Phenolic Content

Good health in general is closely associated with consumption of fresh vegetables and fruits in addition to proper exercise. Studies have shown that eating of especially raw fruits and vegetables greatly lowers the risk of developing some illnesses such as cancer, cardiovascular diseases, diabetes and age related diseases (Henriquez *et al*., 2010). The benefits of eating these raw food stuffs may in part be attributed to the presence of antioxidants and there is strong evidence that shows that eating these foods have strong protective effects against many ailments including the ones mentioned earlier (Kaur and Kapoor, 2002). The antioxidants are known to delay the oxidation of essential molecules in the body by inhibiting the initiation or propagation of oxidizing chain reactions by free radicals and as a result may reduce oxidative damage to the human body (Ismail *et al*., 2004). Most of the natural antioxidants are phenolic compounds found in plants especially medicinal ones used in local communities.

The use of the Folin Ciocalteu Reagent (FCR) is the adopted method for the determination of the Total Phenolic Content. The reagent is pale-green-yellow in colour and consists of two strong inorganic oxidants, phosphotungstic acid and phosphomolybdic acid (Stavanto *et al*., 2004). Basically, the antioxidants in the extract donate electrons to Mo (IV) ions in the reagent which are yellow in color reducing them to Mo (V) ions which are blue in colour (Eqn 5.3)

Mo (IV) (Yellow solution) + e^- (from phenol) →Mo (V) (Blue solution) ……. (Eqn 5.3)

The antioxidant capacity/content of the extract is followed by observing the intensity of the blue colour and this can be measured spectrophotometrically at 725 nm. The experiment is performed using gallic acid as the standard. The Total Phenolic Content is expressed as mg of gallic acid equivalents per gram of extract (mg GAE/g)

The gallic acid standard curve was linear (r^2 = 0.998, Figure 4.5). The absorbance value (y) of each extract was measured and converted to the equivalent concentration of gallic acid (x mg/mL) by the use of the equation of the line as derived from Microsoft Excel. This concentration was then converted to the Total Phenolic Content (TPC) expressed in mg of gallic acid equivalents per gram of dry extract (mg GAE/g).

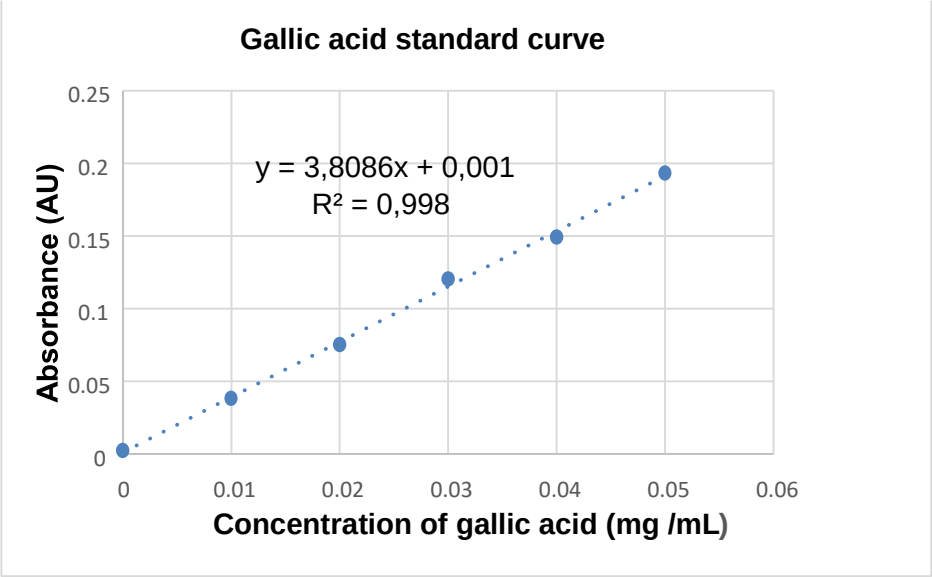


Figure 4.5: Gallic acid standard curve.

The ethyl acetate extract of the twigs of *B. plurijuga* was found to have the highest total phenolic content of 350.0 mg GAE/g followed by the methanol extract (282.0 mg GAE/g) and lastly the methanol water extract with a total phenolic content of 237.0 mg GAE/g (Table 4.9). The non-polar extracts exhibited relatively negligible total phenolic content of 1.0 mg GAE/g for the n-hexane extract and 12 mg GAE/g for the chloroform extract. These results correlate well with the antioxidant activity studies that polar extracts exhibited more radical scavenging activity than non-polar extracts and also most of the isolated compounds were from the polar extracts.

Table 4.9: Total Phenolic Content of extract of *B. plurijuga* expressed in mg/g equivalent to gallic acid.

Name of extract	Avg Absorbance of Extract	Total Phenolic Content mg of gallic acid equivalents (GAE) per g of dry extract
B. P twigs-extracts		
n-Hexane	0.036 ± 0.02	1
$CHCl_3$	0.046 ± 0.03	12
EtOAc	1.336 ± 0.01	350
MeOH	1.075 ± 0.01	282
80 % MeOH (aq)	0.904 ± 0.05	237

The values are expressed as mean ± standard deviation, (n = 3).

The methanol extract of the tubers of *I. bolusiana* was found to have the highest total phenolic content of 150.0mg GAE/g followed by chloroform/ethyl acetate/methanol extract (103.0 mg GAE/g) as shown in Table 4.10 below. The high TPC exhibited by the ethyl acetate extract could be due to the presence of caffeic acid and coumaric acid derivatives present in the extract. The results are given in table 4.6.

Table 4.10: Total Phenolic Content of extract of *I. bolusiana* expressed in mg/g equivalent to gallic acid.

Name of extract	Avg Absorbance of Extract	Total Phenolic Content
		mg of gallic acid equivalents (GAE) per g of dry extract
I.B tubers-extracts		
$CHCL_3$/EtOAc/MeOH (1:1:1 v/v/v)	0.131 ± 0.05	34
MeOH	0.395 ± 0.02	103
80 % MeOH (aq)	0.573 ± 0.06	150

The values are expressed as mean ± standard deviation, (n = 3).

4.4 Antimicrobial Activity

According to Morens *et al*, 2004, infectious diseases have for centuries ranked with wars and famine as a major challenge to human progress and survival. Infectious diseases are mainly caused by pathogens or microorganisms found around us and even in our bodies. There are different types of these pathogens or microorganisms and they include bacteria, parasites and fungi. Bacteria in particular can either be gram positive or gram negative. As an example, malaria, an ailment caused by a parasite affects more than 3.2 billion people worldwide and is directly responsible for more than 1 million deaths per year in Africa alone (World Malaria Report, 2003). This is partly so because of pathogens to resist drugs. This to resistance to drugs is a big problem, for example, in America alone, the unofficial cost of antibiotic resistance is estimated at $100 million annually (Jacoby and Archer, 1991). The pathogens achieve resistance through mutations and as a result reduce the effectiveness of the drug (Jacoby and Archer, 1991).

Gram- negative bacteria are those that do not retain the crystal violet stain used in the gram staining method and examples include *Salmonella*, *Neisseria gonorrhoeae*. The gram-negative bacteria pose the greatest risk to public health as they are highly resistant than their gram-positive counterparts. The high levels of resistance also mean that there are fewer antibacterial drugs available to act against these bacteria (Kumarasamy *et al*., 2010). One bacterium in particular is a major problem in congested areas like cities where close proximity among people is high. *Escherichia coli* are known to cause majority of urinary tract infections. A strain of these bacteria, *E. coli* 0157 was responsible for a bloody outbreak of diarrhea in southern Africa in 1992 (Effler

et al., 2001) and the effects of these strain include morbidity and mortality among children (Muller *et al*., 2001). Antimicrobial agents such as ampicillin, ciprofloxacin and nitrofurantoin are some of the drugs used against *E. coli* although some level of resistance is reported (Karlowsky *et al*., 2002).

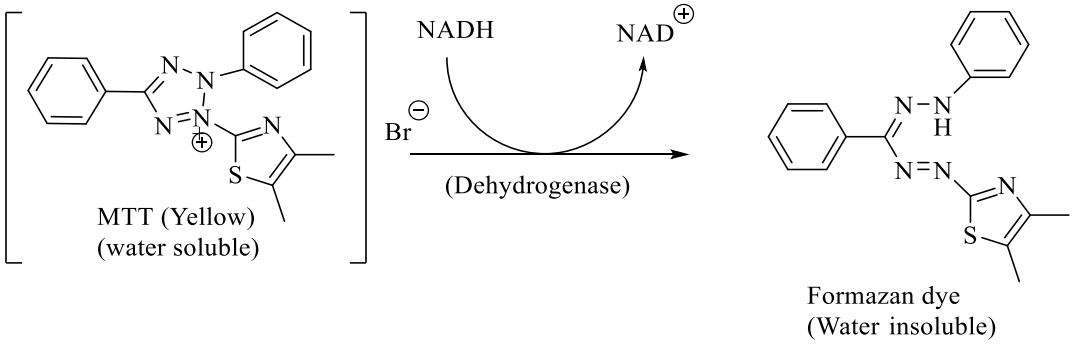
The gram-positive on the other hand take up the crystal violet stain used in the test, and then appear to be purple-colored when seen through a microscope making a positive identification. Examples include *Staphylococcus aureus* which is a frequent contributor to skin and respiratory infections in humans; *Streptococci*, *Pneumococci*, *Bacillus cereus* with some strains known to cause foodborne illness in humans and *Clostridium perfringens* which is the most common cause of food poisoning. Antimicrobial agents like rifampin**,** and trimethoprim-sulfamethoxazole, showed in vitro activity compared with other antibiotics against the methicillin-resistant *S. aureus* (MRSA) (Kim *et al*., 2004).

To add to the already serious problems associated with bacteria, fungi are also agents of serious diseases. Invasive fungal infections at one point were known to occur with increasing frequency in immuno-compromised patients, particularly among those acquired immunodeficiency syndrome (Anaissie *et al*., 1989). Fungi are known to cause diseases that affect the throat, genitals, mouth and the skin surveillance of nosocomial blood stream infections (BSI) in the USA between 1995 and 1996 revealed that *Candida* was the fourth leading cause of nosocomial BSI, accounting for 8% of all infections (Pfaller *et al*., 1998).

In the olden days, people have depended on traditional medicine as a way to remedy situations like the ones mentioned above through prolonged usage and experience. They have learned to correlate the therapeutic effects of plants species to particular diseases or its symptoms. This knowledge has somewhat resulted in increased usage of plants based pharmaceutical remedies. *Baikiaea plurijuga* as well as *Ipomoea bolusiana* are some of the medicinal plants acclaimed with the management/treatment of some of the diseases associated with some of these pathogens.

There are a number of simple and inexpensive bench top methods to investigate activity of extracts/pure isolates against microorganisms such as agar diffusion method (disk or agar well diffusion method), agar-overlay and direct bio-autograph (Marston, 2011). Of these methods, agar-overlay bio-autography described by Rahalison *et al.*, 1991 and Marston, 2011 the agar-overlay bio-autography was selected mainly because of its simplicity and cost effectiveness. It involves

sporting varying concentrations of isolates, extracts and standards on TLC sheets and drying them. Broth of a particular microorganism is then evenly spread over the TLC sheets (Marston, 2011). Inhibition zones are observed as yellow or clear spots against a purple background. The purple background is due to the tetrazolium salt being converted to formazan dye as depicted in Scheme 4.2 below (Corrado and Rodrigues, 2004) while the zones of inhibition are visible because of dehydrogenase activity with the tetrazolium salt (MTT, methylthiazolyldiphenyl-tetrazolium bromide).



Scheme 4.2: Reduction of MTT by the enzyme dehydrogenase to form a formazan dye.

Crude extracts and pure isolates from *B. plurijuga* and *I. bolusiana* were subjected to both antibacterial and antifungal inhibition tests against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. Crude extracts from the twigs of *B. plurijuga* generally showed activity against the entire test bacteria with an MIQ of 50 µg but the results were even promising against the fungi for the chloroform extract, ethyl acetate and other polar extracts at 10 µg loading (Table 4.11).

Table 4.11: Antimicrobial activities of the extracts from *B. plurijuga*.

Name of extract	Microorganism and minimum inhibitory quantity (MIQ) in µg				
	Gram-negative bacteria		Gram-positive bacteria		Fungus
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*C. albicans*
B. P- twigs					
n-Hexane	50	NA	50	50	100
$CHCl_3$	50	NA	50	50	10
EtOAc	50	50	50	50	10
MeOH	50	50	50	50	10
80 % MeOH (aq)	50	50	50	50	50

NA: Not Active at 100 µg loading, NT: Not Tested.

Of all the test compounds, the flavonoids derivatives exhibited the best activity against the test microorganisms. Compound **79** showed the best activity against gram-positive bacteria, *S. aureus* at 5 µg loading. The compound did not show activity against all other microorganisms. Compound **78** on the other hand showed activity against all gram-negative bacteria *E. coli* and *P. aeruginosa* and also against the fungi *C. albicans* at 50 µg loading (Table 4.12). The activity of flavonoids in general is attributed to the presence and arrangement of hydroxyl groups (Murti and Mishra, 2014). Hydroxylation at positions 7, 3' and 4' is known to exhibit activity against gram-negative bacteria and yeast (Taleb-Contini *et al*., 2003). Compound **79** however exhibited the same substitution pattern but showed activity against gram-positive bacteria *S. aureus*. This could be due to the presence of a hydroxyl group at position 3. Catechin is a known antimicrobial agent against a wide array of microorganisms (Manimozhi *et al*., 2012). Cinnamic acid derivatives **70**, **71** and **73** showed poor activity against all the test organisms except the activity of compound **71** against *S. aureus* at 50 µg loading. The antimicrobial activity of cinnamic acid derivatives is attributed to the presence of the ester and amide groups although compounds **70** and **71** do not have amide groups (Sharma, 2011).

Table 4.12: Antimicrobial activities of the isolated compounds from *B. plurijuga.*

Extract or compound	Microorganism and minimum inhibitory quantity (MIQ) in µg				
	Gram-negative bacteria		Gram-positive bacteria		Fungus
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*C. albicans*
Compound **79**	NA	NA	NA	5 µg	NA
Compound **78**	50 µg	50 µg	NA	NA	50 µg
Compound **77**	NA	NA	NA	NA	5 µg
Chloramphenicol	0.5 µg	0.5 µg	0.5 µg	0.5 µg	
Miconazole					0.5 µg

NA: Not Active at 100 µg, NT: Not Tested.

Crude extracts from the tubers of *I. bolusiana* showed a general activity at 50 µg against all the test organisms except that the crude extracts did not show any activity against the fungi *Candida albicans* (Table 4.13). Presence of coumaric acid derivatives isolated from the tubers of *I. bolusiana* could in part explain the antimicrobial activities of the extracts of this plant as they exhibited stronger activity against some of the test microorganisms.

Table 4.13: Antimicrobial activities of the extracts from *I. bolusiana.*

Name of extract	Microorganism and minimum inhibitory quantity (MIQ) in µg				
	Gram-negative bacteria		Gram-positive bacteria		Fungus
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*C. albicans*
I. B-tubers					
$CHCL_3$/EtOAc/MeOH (1:1:1 v/v/v)	50	100	50	50	50
MeOH	50	50	50	50	NA
80 % MeOH (aq)	50	50	50	50	NA
Chloramphenicol	0.5	0.5	0.5	0.5	
Miconazole					0.5

NA: Not Active at 100 µg loading, NT: Not Tested.

Compounds **76** and **74** showed activity at 100 against gram-negative bacteria *E. coli* and *P. aeruginosa*. The two compounds were however not active against other test organisms. Compound **76** showed activity against gram-negative *P. aeruginosa* at 10 µg loading. Other compound isolated did not show any activity against all the test organisms even at 100 µg loading. None of the test compounds showed activity close to the standards used (see Table 4.8 below).

Table 4.14: Antimicrobial activities of the isolated compounds from *I. bolusiana*.

Extract or compound	Microorganism and minimum inhibitory quantity (MIQ) in µg				
	Gram-negative bacteria		Gram-positive bacteria		Fungus
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*C. albicans*
Compound **74**	NA	100	NA	NA	NA
Compound **70**	NA	NA	NA	NA	NA
Compound **71**	NA	NA	NA	50	NA
Compound **69**	NA	NA	NA	NA	NA
Compound **76**	10 µg	5 µg	NA	NA	NA
Compound **74**	NA	NA	NA	NA	NA
Compound **73**	5 µg	5 µg	NA	NA	NA
Chloramphenicol	0.5 µg	0.5 µg	0.5 µg	0.5 µg	
Miconazole					0.5 µg

NA: Not Active at 100 µg, NT: Not Tested.

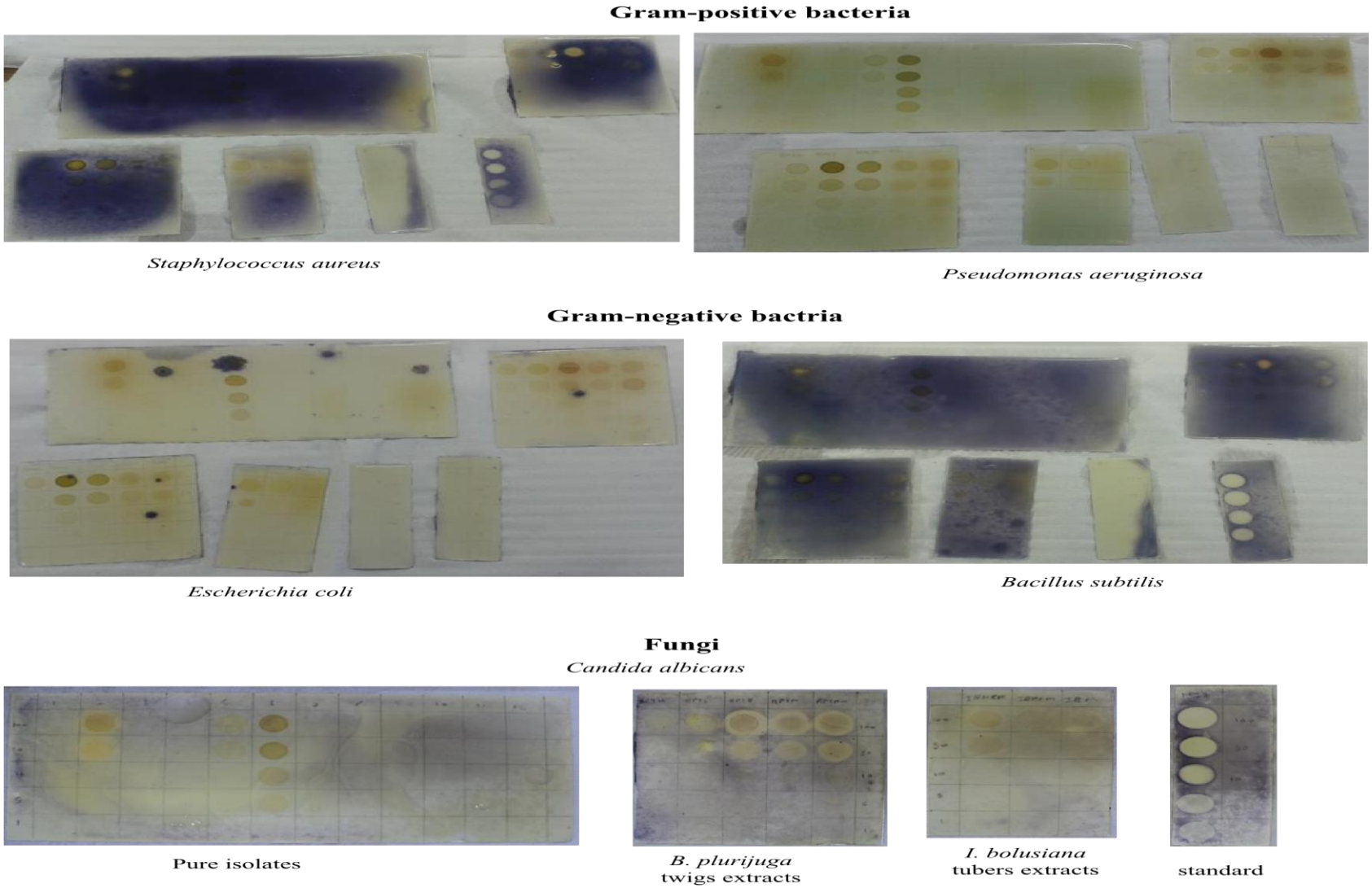


Figure 4.6: Bioautograms for the antimicrobial activities against *S. aureus*, *P. aeruginosa*, *E. coli*, *B. subtilis* and *C. albicans*.

4.5 Experimental Procedures

4.5.1 Procedure for The Antioxidant Activity Tests

4.5.1.1 TLC Method

Each sample and standard (Ascorbic acid and Gallic acid) were dissolved in their respective solvents making stock solutions of 1mg/mL. Different concentrations (100 µg/mL, 50 µg/mL, 10 µg/mL, 5 µg/mL, 1 µg/mL and 0.1 µg/mL for the standards were initially prepared by serial dilution method from the stock solutions. The activity of more active compounds was further tested for lower concentrations (0.1 µg/mL, 0.2 µg/mL, 0.4 µg/mL and 0.5 µg/mL) with gallic acid as the standard at a later stage. A solution of DPPH was prepared by dissolving 2 mg of DPPH in 50 mL of methanol. The solution was shaken well and kept in the refrigerator until it was needed. The dry TLC plates were then sprayed with the DPPH solution to give a purple background. The antioxidant activities of the compound as well as the extracts were examined after 30 minutes, 1 hour, 2 hours, 3 hours and 24 hours. Zones of inhibition were observed as yellow sports against a purple background.

4.5.1.2 Spectrophotometric Method

The spectroscopic DPPH-RSA assay was done by a method adapted from a procedure by Brand-Williams *et al* (1995) with modifications by Juma and Majinda (2004) and later Yeboah and Majinda (2009). Different concentrations (0.01-0.05 mg/mL) of the pure isolates as well as the extracts together with the standard (ascorbic acid) were prepared by serial dilution using analytical grade methanol as the solvent. A solution of DPPH (0.2 mg/mL) was then prepared and stored in a refrigerator away from direct sunlight until needed. 2 mL of each compound /extract or standard was added to an equal amount of the DPPH solution in screw cap test tube, closed and shaken before being put in a dark place for 30 minutes. For the control, 2 mL of methanol was mixed with an equal amount of DPPH solution. Methanol was used as a blank for baseline correction. The absorbance of each solution was measured using a Shimadzu UV-2101 PC, UV-VIS Scanning Spectrophotometer set at 517 nm. The readings were taken at 30 minutes, 1 hour, 3 hours and after 24 hours. The percentage DPPH, I % was hen calculated using the relation:

I % = [(Absorbance $_{control}$- Absorbance $_{sample}$)/ Absorbance $_{control}$] x 100…….. (Eqn 1)

4.5.2 Procedure for The Determination of Total Phenolic Content

The adapted Folin-Ciocalteau method by Singleton and Rossi, 1965 with modifications by Majinda and Yeboah, 2009 was used to determine the TPC of the crude extracts. A standard solution of each extract (1mg/mL) was prepared in 80 % aqueous methanol. Five different concentrations (0.01-0.05 mg/mL) of the standard Gallic acid were then prepared by serial dilution. A mixture consisting of 5 mL standard/extract, 0.5 mL Folin-Ciocalteau reagent and 5 mL 80 % methanol was put in a screw cap test tube. 1 mL of 20 % sodium carbonate (w/v) was added and the resulting mixture was shaken vigorously for 2 minutes and allowed to stand in a dark place for 2 hours at room temperature. The absorbance of the filtered supernatant solution was then measured at 725 nm using a Thermo Spectronic GENESYS 20 Visible Spectrophotometer in triplicates. Total Phenolic Content was calculated as mg of Gallic acid equivalents per gram of dry extract using the relation $y = 3.8006x + 0.001$, $R^2 = 0.998$ obtained from the standard Gallic acid calibration curve.

4.5.3 Experimental Procedures for The Antimicrobial Studies

4.5.3.1 Preparation of Culture Media

4.5.3.2 Test Microorganisms

Microorganisms used were obtained from the Department of Biology at the Microbiology unit. Chloramphenicol and Miconazole were used as standards for the antibacterial as well as antifungal activities respectively. The minimum detection limits for Miconazole and Chloramphenicol were at 0.01 µg loadings.

Table 4.15: Test microorganisms.

Microorganism	Biological classification
Bacillus subtilis	Gram-positive rod
Staphylococcus aureus	Gram-positive cocci
Pseudomonas aeruginosa	Gram-negative rod
Escherichia coli	Gram-negative rod
Candida albicans	Fungi-yeast

Media (nutrient broth) for the microorganisms was prepared by dissolving the nutrient broth powder (16g, Biolab) in 1000 mL of distilled water in a one litter bottle and stirred until a homogenous solution was obtained. The resulting solution (50 mL) was poured into five 100 mL conical flasks covered with aluminium foil, autoclaved at 121^{o}C for 15 minutes and

allowed to cool to room temperature. Nutrient agar was prepared by dissolving nutrient agar powder (23.0g, Biolab) in 1000 mL of distilled water in a 1 litter bottle, heated to boiling while magnetically stirring on a hot plate to form a homogeneous solution. The solution was autoclaved at 121^{o}C for 15 minutes and stored in an oven set at 45^{o}C until ready for use.

4.5.3.3 Preparation of Standards and Test Samples

A stock solution was initially prepared by dissolving 10 mg of the test sample in 1 mL of the appropriate solvent. The stock solution was then serially diluted to make concentrations of 100, 50, 10, 5, and 1 µg/mL of each test sample. 10 µL of each sample solution was then sported on TLC plates with grids of 1.5 cm by 1.5 cm square grids drawn on them and were allowed to dry. Merck coated silica gel 60 HF_{245} TLC plates were used.

4.5.3.4 Preparation of Microbial Culture Suspensions/Inoculation

A sterilized platinum wire was used to aseptically transfer each microorganism from stock cultures in petri dishes to sterilized 50 mL conical flasks containing 50 mL nutrient broth. The cultures were then incubated for 24 hours at 37 ^{o}C forming a cloudy medium in the process confirming the growth of organisms.

4.5.3.5 TLC Bioautograhy

Into 100 mL of nutrient agar at around 45 ^{o}C, 10 mL aliquots of the culture suspensions of each microorganism was added and the mixture was swirled. Using sterilized 10 mL pipette, the mixture was overlaid over the sported TLC plates and the agar was allowed to solidify. The plates were then put in containers covered with plastic so as not to allow the agar to dry out but with holes punctured into the plastic to aloe for exchange of gases. The TLC plates were then incubated for 24 hours at 37 ^{o}C. Growth of the organisms was identified by a cloudy surface. The plates were then stained with thiazolyl blue solution spray made by dissolving 200 mg of methylthiozolyldiphenyl-tetrazolium bromide powder in 100 mL of distilled water. Inhibition zones were identified as white or yellow sports against a purple background (Corrado and Rodrigues, 2004). The lowest quantity that prevented growth of organisms was taken into account as the minimum inhibition quantity.

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Chapter 5

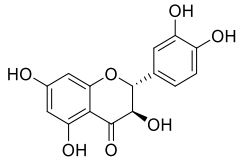
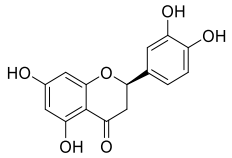
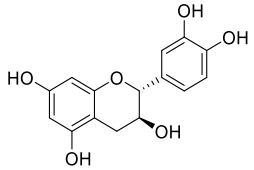
Conclusion

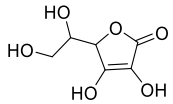
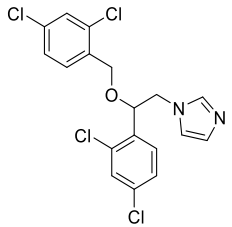
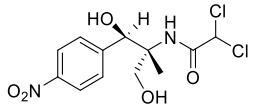
Plants are important sources of medicines and have been used throughout man's history. People depend on plants as sources of medicines and some studies suggest that secondary metabolites from these plants are often used in the discovery of new drugs. The study focused on two uncommon medicinal plants namely *Baikiaea plurijuga* and *Ipomoea bolusiana*.

Baikiaea plurijuga is an uncommon medicinal plant used to treat syphilis, eye diseases, stomach pains and toothache implying that the plant possesses amongst others analgesic, antimicrobial as well as antioxidant properties. Four compounds; (+)-catechin **79**, taxifolin **77**, eriodictyol **78** and sucrose **72**, were isolated from the twigs of *Baikiaea plurijuga* (Table 5.1). Screening of both extracts and isolated compounds from this plant revealed that the extract exhibited moderate antimicrobial and anti-oxidant activities. Amongst the isolated compounds, compound **78** exhibited strong free DPPH radical scavenging activity with an IC_{50} value of 0.047 ± 0.007 μM while compound **77** exhibited moderate DPPH radical scavenging activity with an IC_{50} value of 0.20 ± 0.02 μM. The free radical scavenging activity of compound **77** was comparable to that of the standard ascorbic acid, while compound **78** showed weaker activity as compared to the standard ascorbic acid (0.090 ± 0.01 μM).

All the above mentioned compound exhibited good to moderate anti-microbial activity against the test microorganisms. Compound **79** showed the highest anti-microbial activity against gram-positive bacteria, *S-aureus* with an MIQ of 5 μg while compound **78** showed moderately weak activity against test organisms *E. coli* and *P. aeruginosa* at 50 μg loading. Hydroxylation at positions 5, 7, 3' and 4' may be key in the anti-microbial activity of the test compounds as exemplified by compounds **77**, **78** and **79**. Extracts of the twigs of *B. plurijuga* showed activity against all the test microorganisms with an average MIQ of 50 μg except the chloroform and n-Hexane extracts which did not show any activity against gram-negative bacteria *P. aeruginosa* even at 100 μg loading. The chloroform, n-Hexane and ethyl acetate extracts showed the best activity against the test fungi *C. albicans* with an MIQ of 10 μg. The agar overlay method was used to test the anti-microbial activity of both the extracts and the isolated compounds.

Table 5.1: A summary of compounds isolated from the twigs of *Baikiaea plurijuga.*

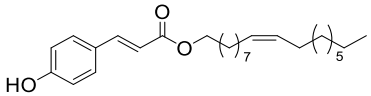
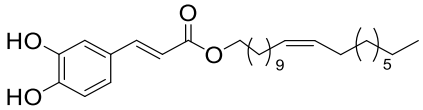
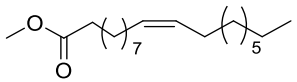
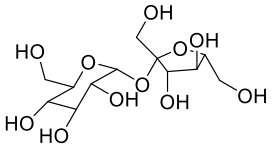
Compound	Class	Antioxidant activity		Antimicrobial activity over a 24-hour period				
		TLC DPPH-RSA Assay over a 24-hour period	Average IC_{50} ± Standard Deviation(μM)	Gram-negative bacteria		Gram-positive bacteria		Fungi
				E. c	*P. a*	*B. s*	*S. a*	*C. a*
Compound **77** 	Flavanol	0.1 μg	0.20 ± 0.02 μM	NA	NA	NA	5 μg	NA
Compound **78** 	Flavanone	0.1 μg	0.047 ± 0.007 μM	50 μg	50 μg	NA	NA	50 μg
Compound **79** 	Flavan-3-ol	5 μg	NT	NA	NA	NA	NA	5 μg

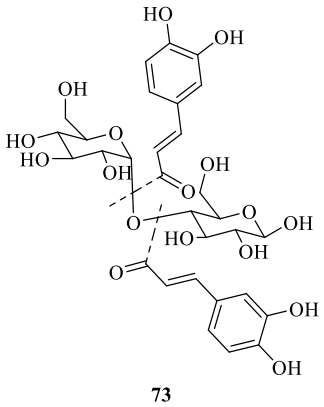
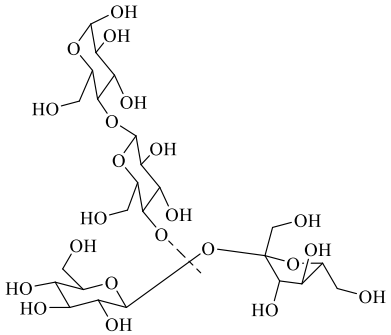
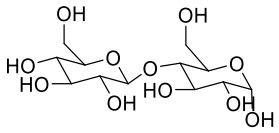
Standards								
Ascorbic acid 		0.5 µg	0.090 ± 0.01 µM					
Miconazole 								0.5 µg
Chloramphenicol 				0.5 µg	0.5 µg	0.5 µg	0.5 µg	

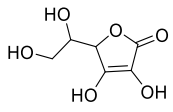
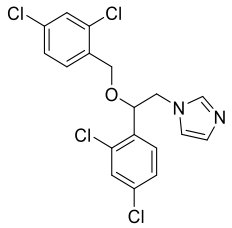
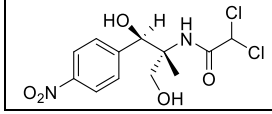
Ipomoea bolusiana is traditionally used in the treatment of sexually transmitted infections, toothache and urinary tract infections. The plant is also eaten by wild animals especially wilder beasts to quench their thirst as large amounts of water (550 mL)) were recovered from 1888.42g of the wet tubers during the extraction of the tubers of this plant. Also, residual isolation of carbohydrates, which are known energy sources may explain why this plants is an energy supplement amongst wild animals. A total of six compounds (Table 5.2) were isolated form the tubers of *Ipomoea bolusiana* and anti-microbial as well as anti-oxidant abilities of *I. bolusiana* are supported by this study.

Screening of extracts as well as the isolated compounds revealed that the plant possesses anti-fungal, anti-bacterial as well as antioxidant activity. Two coumaric acid derivatives **70** and **71,** isolated for the first time, exhibited free radical scavenging activity. Compound **71** however exhibited stronger DPPH radical scavenging activity (0.023 ± 0.006 μM) unlike compound **70** which exhibited weak DPPH radical scavenging activity with an IC_{50} value of 0.47 ± 0.06 μM under this study. The presence of an extra hydroxyl group adjacent to the para hydroxyl group in compound **71** may in part be responsible for this marked difference in activity of these two compounds. Antimicrobial studies on the isolated compounds revealed that compound **74** exhibited the highest activity against gram-negative bacteria *E. coli* and *P. aeruginosa* with an MIQ of 5 μg. Compound **71** on the other hand exhibited moderate activity against gram-positive bacteria with an MIQ of 50 μg. Extracts from the tubers of *I. bolusiana* showed antimicrobial activity against the test microorganisms with an average MIQ of 50 μg. Biological activity studies carried out on both the pure isolate and extracts of this plant may to some extent verify the use of *I. bolusiana* as a medicinal plant. The anti-microbial as well as anti-oxidant potential of the isolated compounds as well as the extracts from the tubers of *I. bolusiana* may to some extent support the folkloric uses of this plant as a traditional medicine as some of the isolated compounds and extracts showed activity in both the antioxidant as well as antimicrobial screening done.

Table 5.2: A summary of isolated compounds from the tubers of *I. bolusiana*.

Compound	Antioxidant activity		Antimicrobial activity over a 24-hour period				
	TLC DPPH-RSA Assay over a 24-hour period	Average IC_{50} ± Standard Deviation(μM)	Gram-negative bacteria		Gram-positive bacteria		Fungi
			E. c	*P. a*	*B. s*	*S. a*	*C. a*
Compound **70*** 	5	0.47 ± 0.06 μM	NA	NA	NA	NA	NA
Compound **71*** 	0.1	0.023 ± 0.06 μM	NA	NA	NA	50	NA
Compound **69** 	NA	NT	NA	NA	NA	NA	5
Compound **72** 	NT						

Compound **73****  73	1	NT	NA	NA	NA	NA	NA
Compound **74**** 	1	0.031 ± 0.007 μM	5	5	NA	NA	NA
Compound **75** 	100	NT	NA	NA	NA	NA	NA

Standards							
Ascorbic acid 	0.5 µg	0.090 ± 0.01 µM					
Miconazole 							0.5 µg
Chloramphenicol 			0.5 µg	0.5 µg	0.5 µg	0.5 µg	

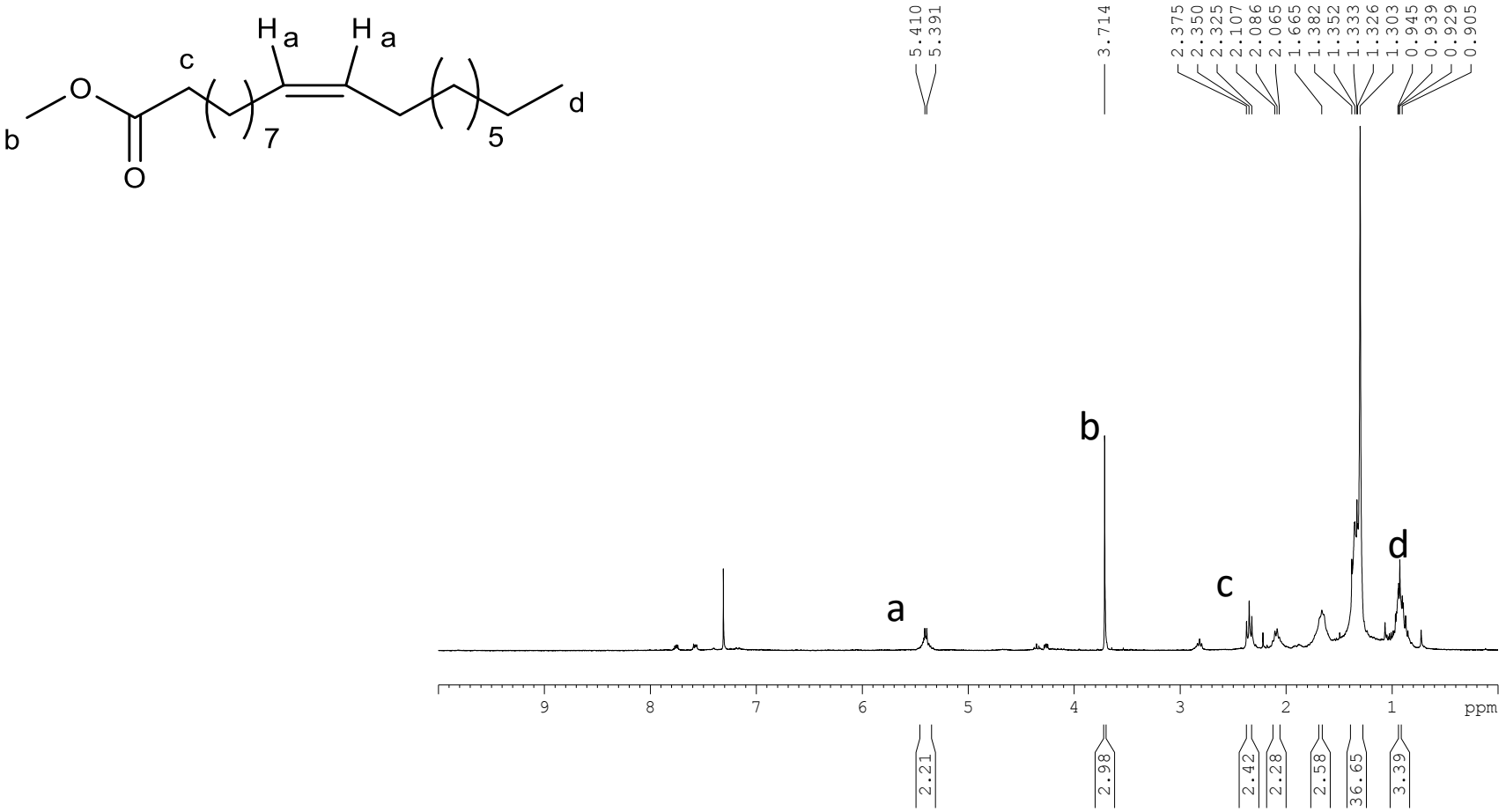
*-New compound.
**- Tentative structure.

Recommendations

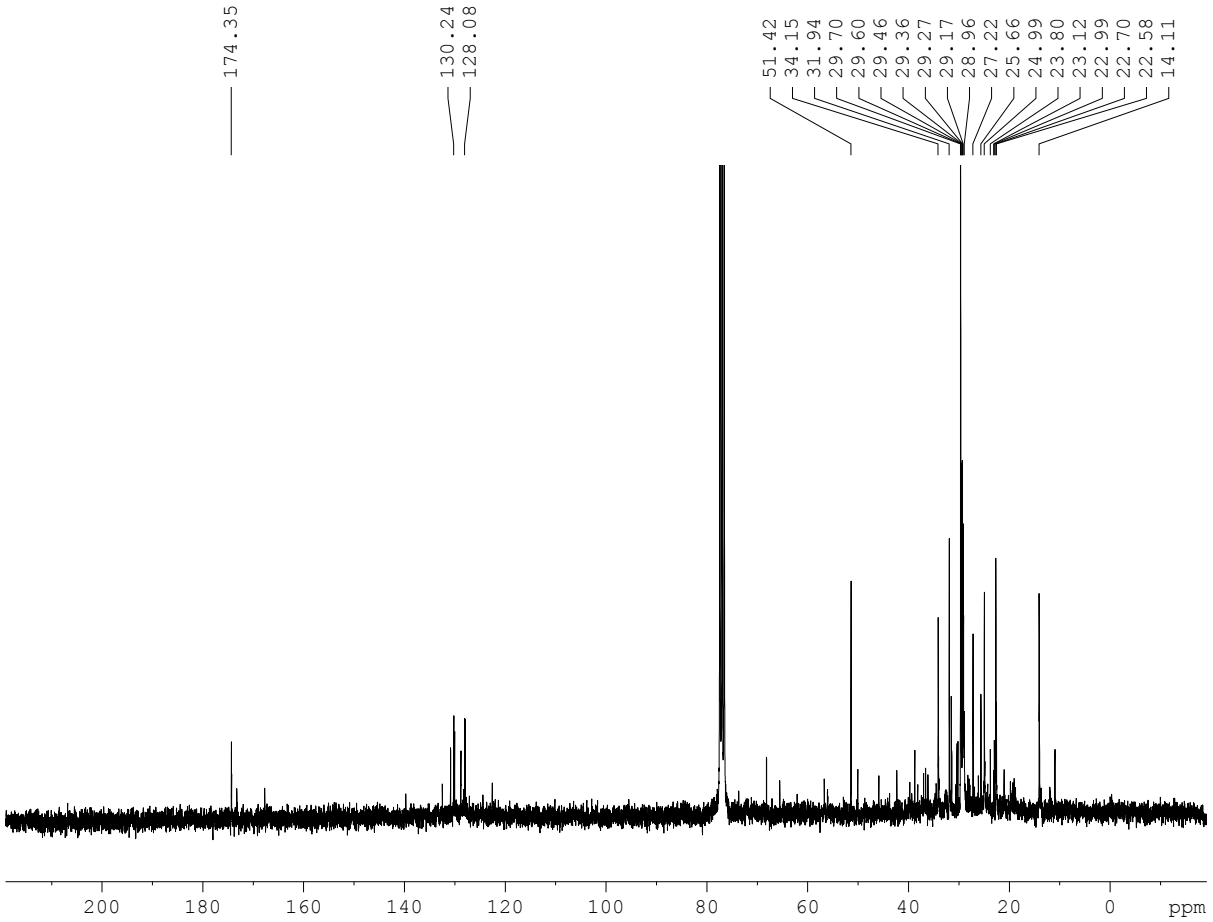
Phytochemical investigation on the twigs of *Baikiaea plurijuga* and *Ipomoea bolusiana* led to the isolation of pure compounds in small quantities, especially the latter plant. There is therefore need to further work on this plants using larger quantities of plant material with the hope of isolating meaningful quantities of pure compounds and even new compounds. Phytochemical work on other parts of the plants could also be done in future with the hope of isolating new compounds as folkloric use of these plants in folklore medicine indicated that they are used in the treatment of a wide variety of ailments. Derivatization of the isolated compounds can also be done to try and enhance both the biological as well as the antioxidant properties of these compounds.

APPENDICES

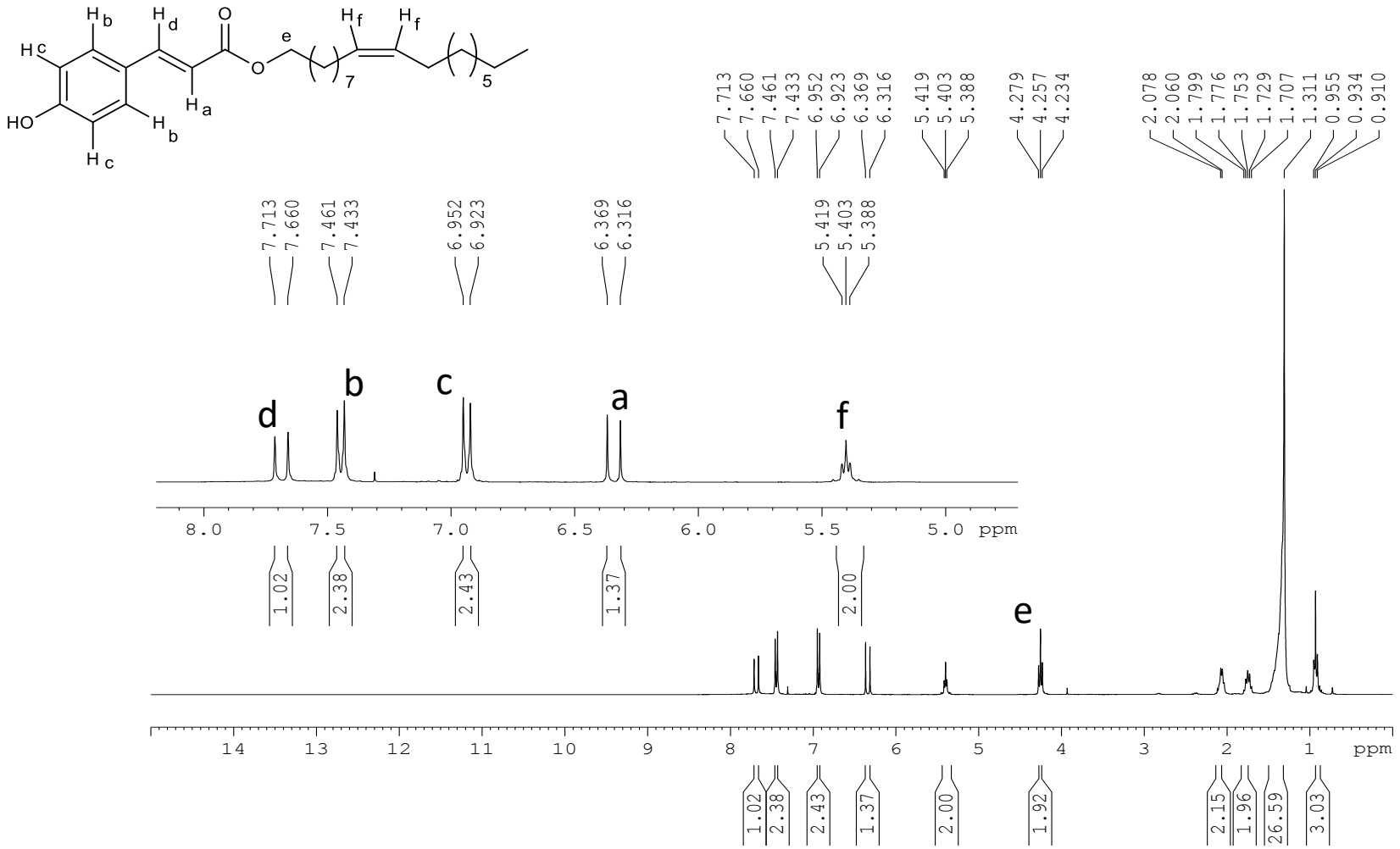
Appendix 1: ^{1}H (300 MHz) NMR data for Compound **69** in chloroform-d



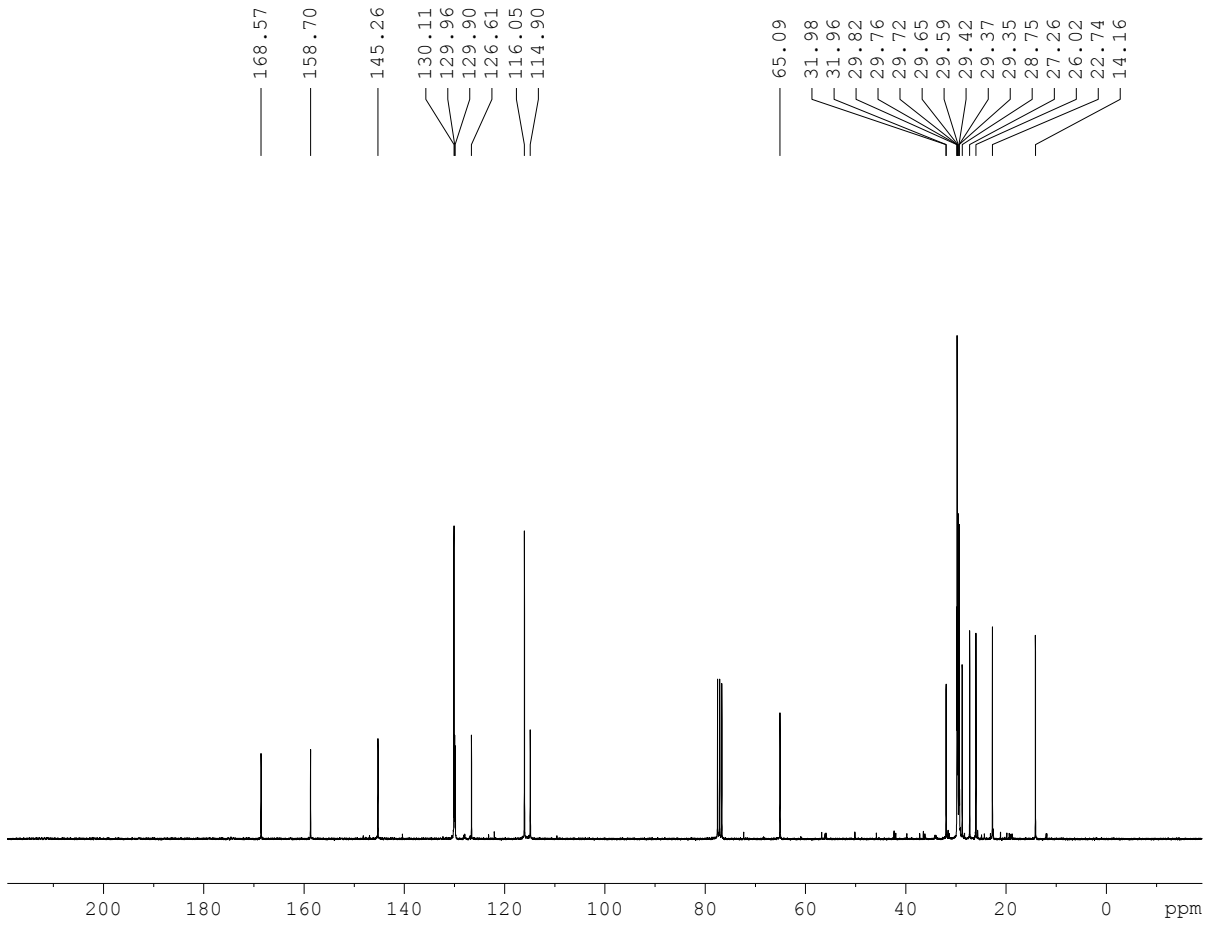
Appendix 2: ^{13}C (75.4) NMR data for Compound **69** in chloroform-d



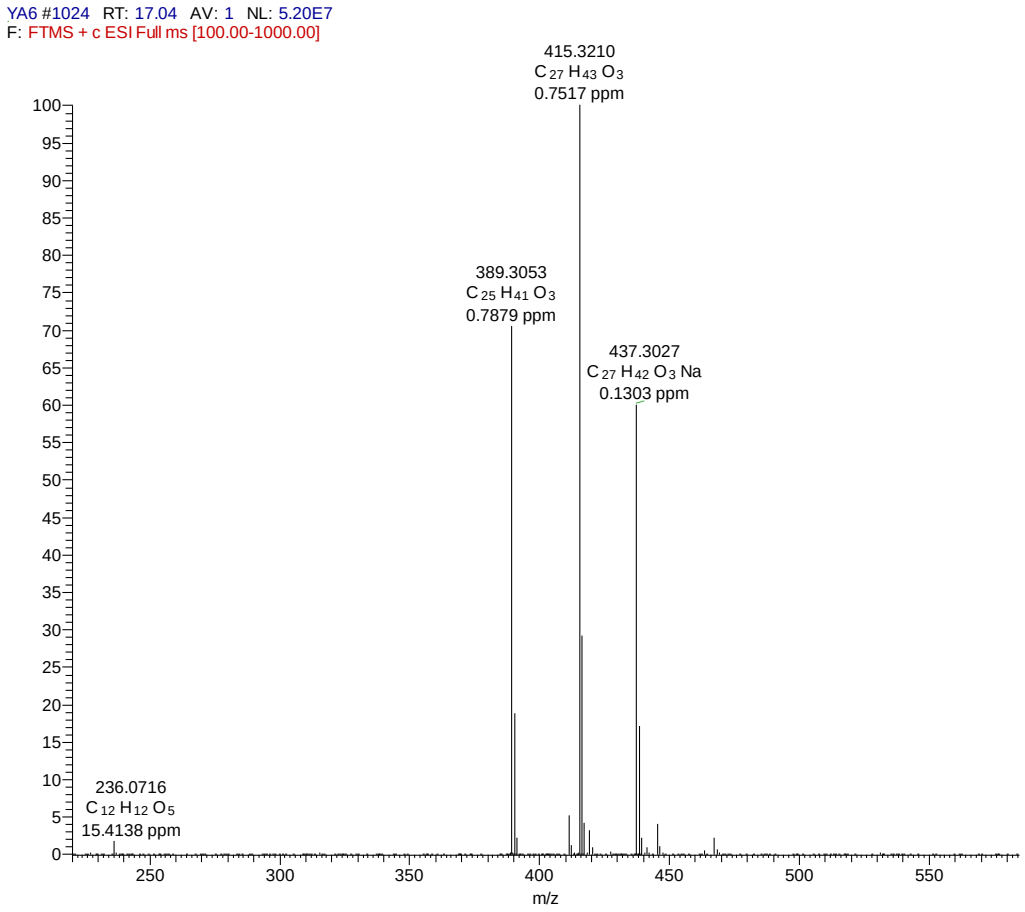
Appendix 3: ^{1}H (300 MHz) NMR data for Compound **70** in chloroform-d



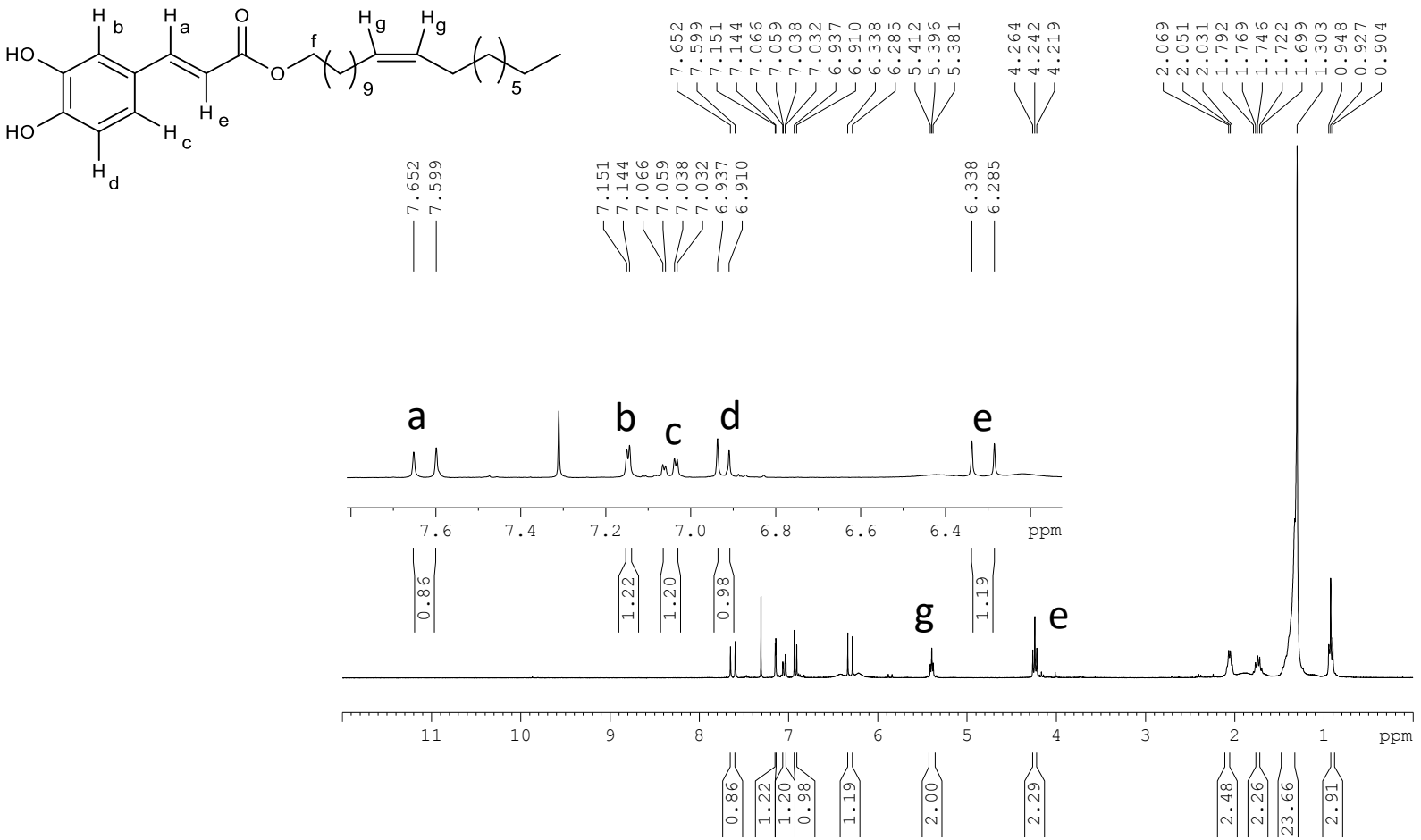
Appendix 4: ^{13}C (75.4) NMR data for Compound **70** in chloroform-d.



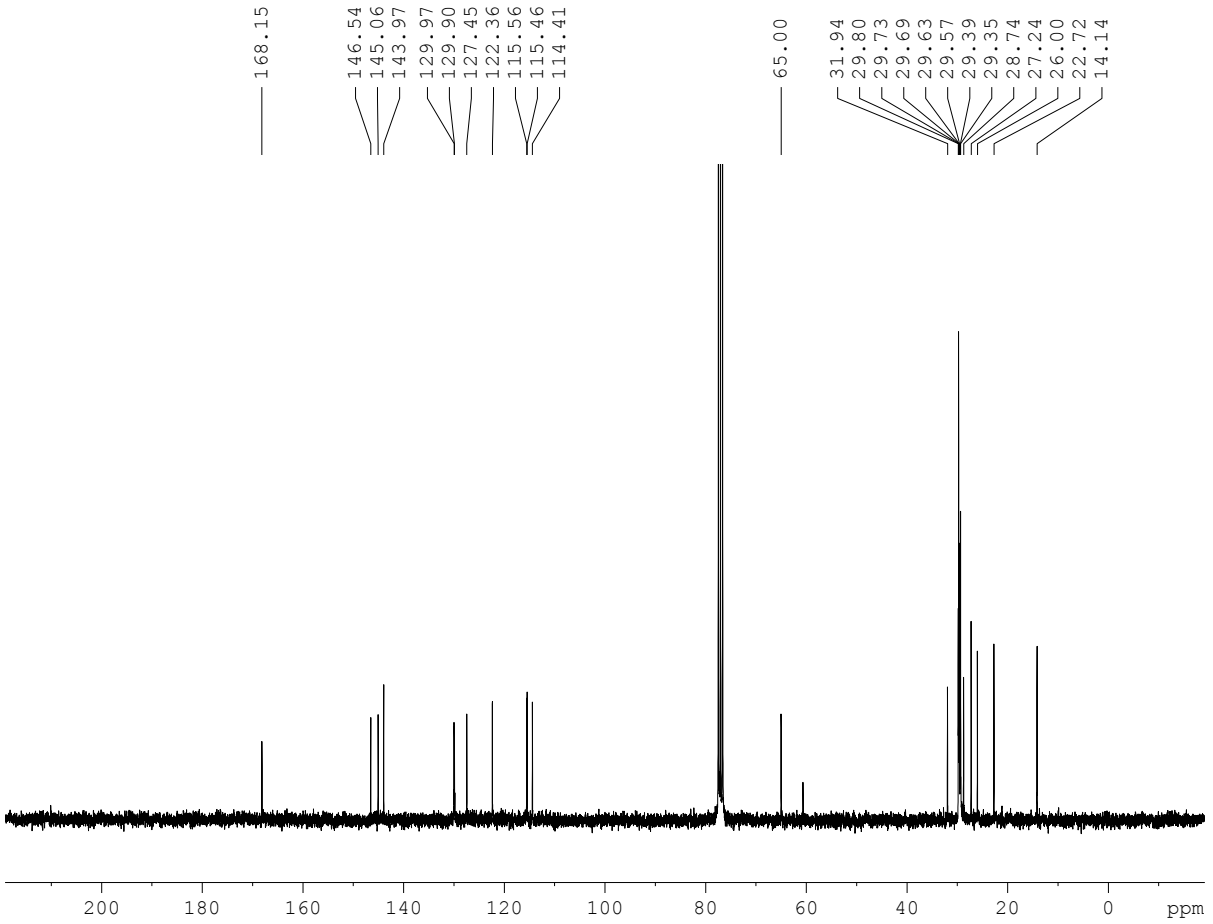
Appendix 5: ESI-HRMS spectrum of compound **70**



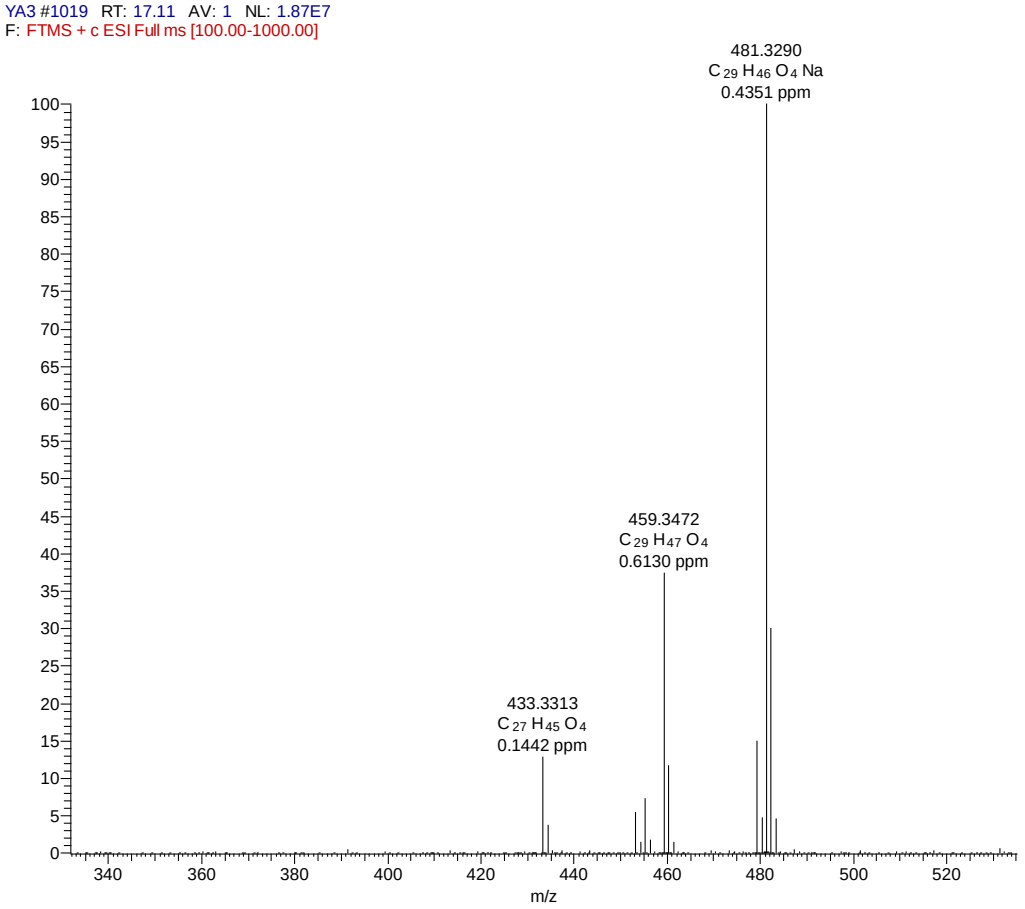
Appendix 6: ^{1}H (300 MHz) NMR data for Compound **71** in chloroform-d



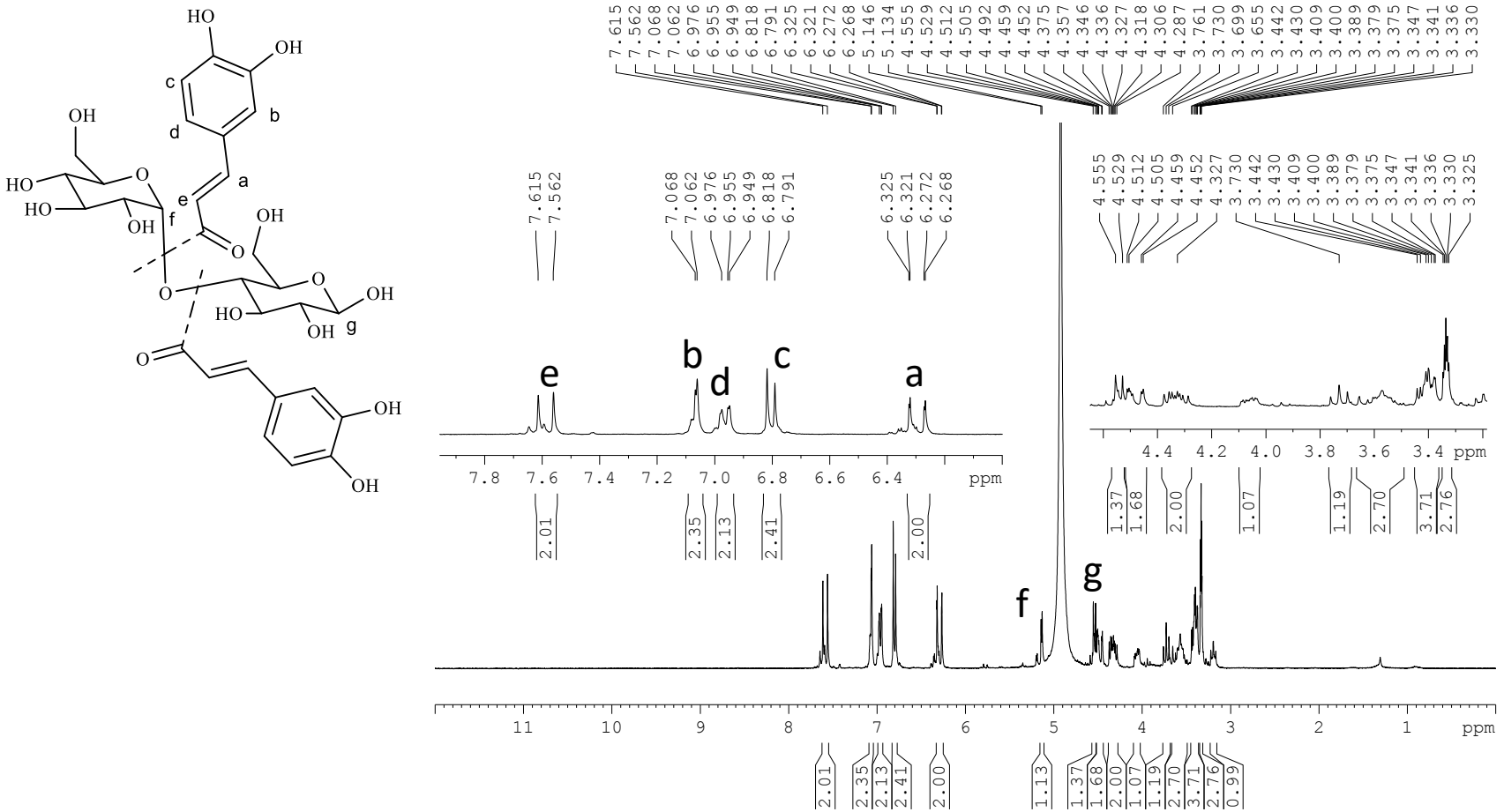
Appendix 7: ^{13}C (75.4) NMR data for Compound **71** in chloroform-d



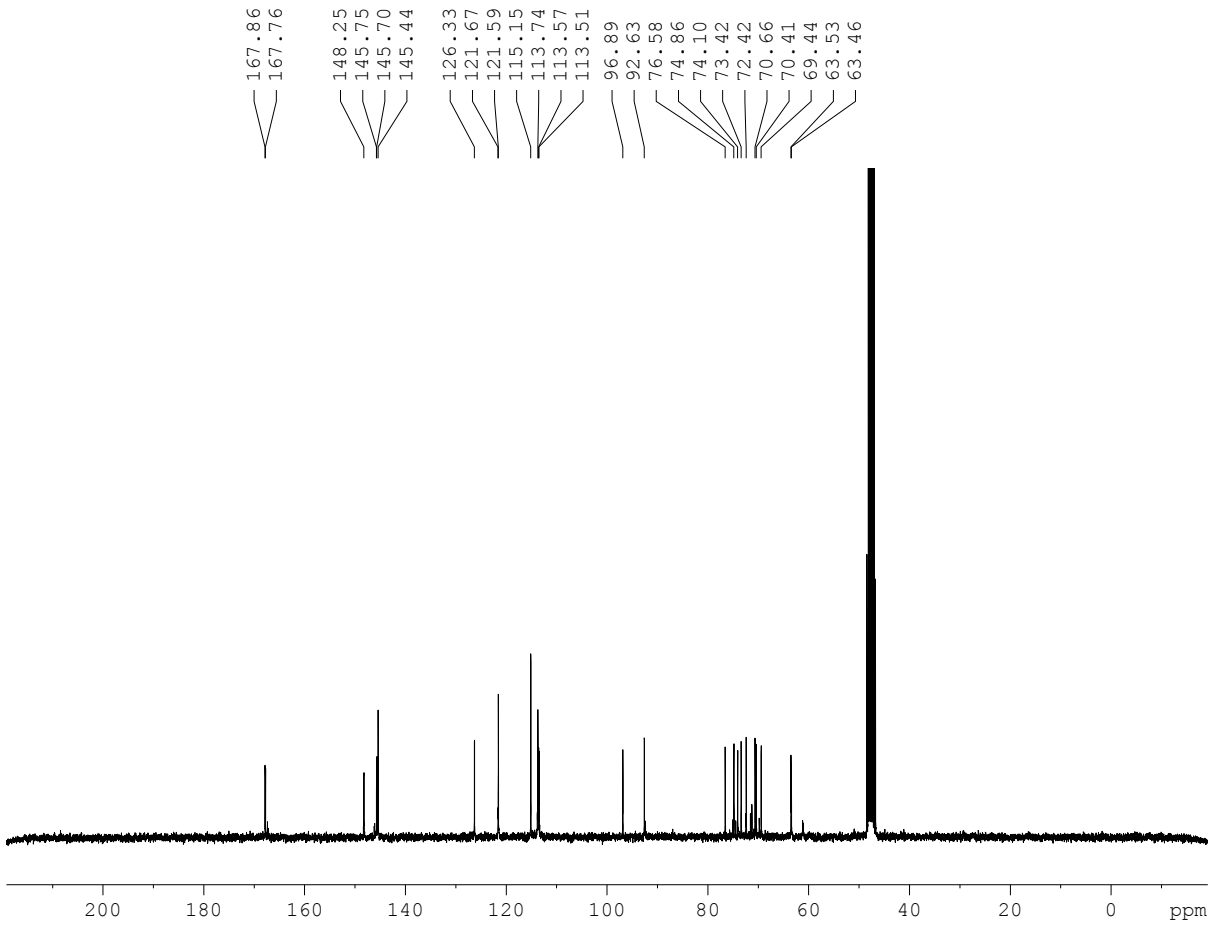
Appendix 8: ESI-HRMS spectrum of compound **71**



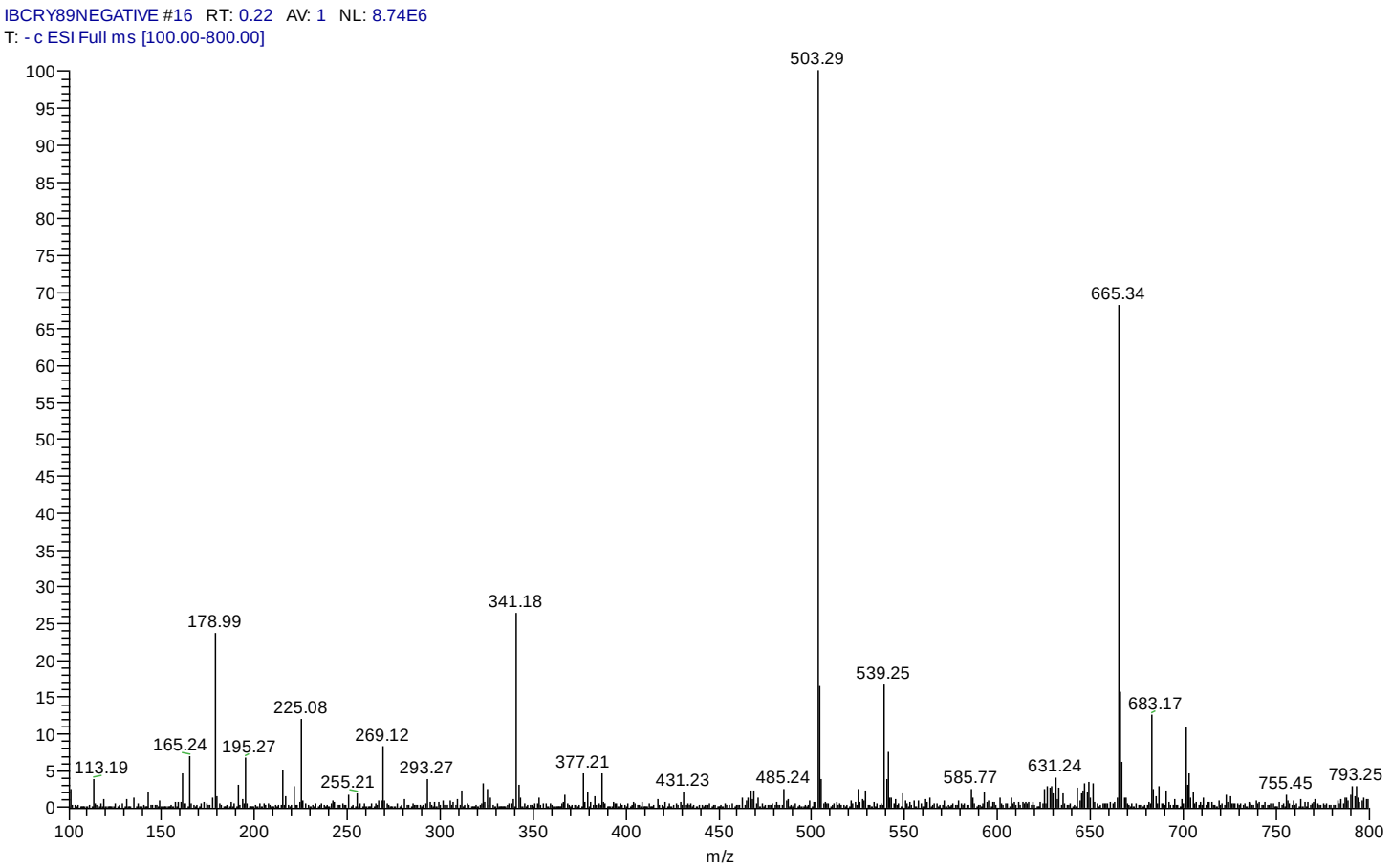
Appendix 9: ^{1}H (300 MHz) NMR data for Compound **73** in methanol-d_4



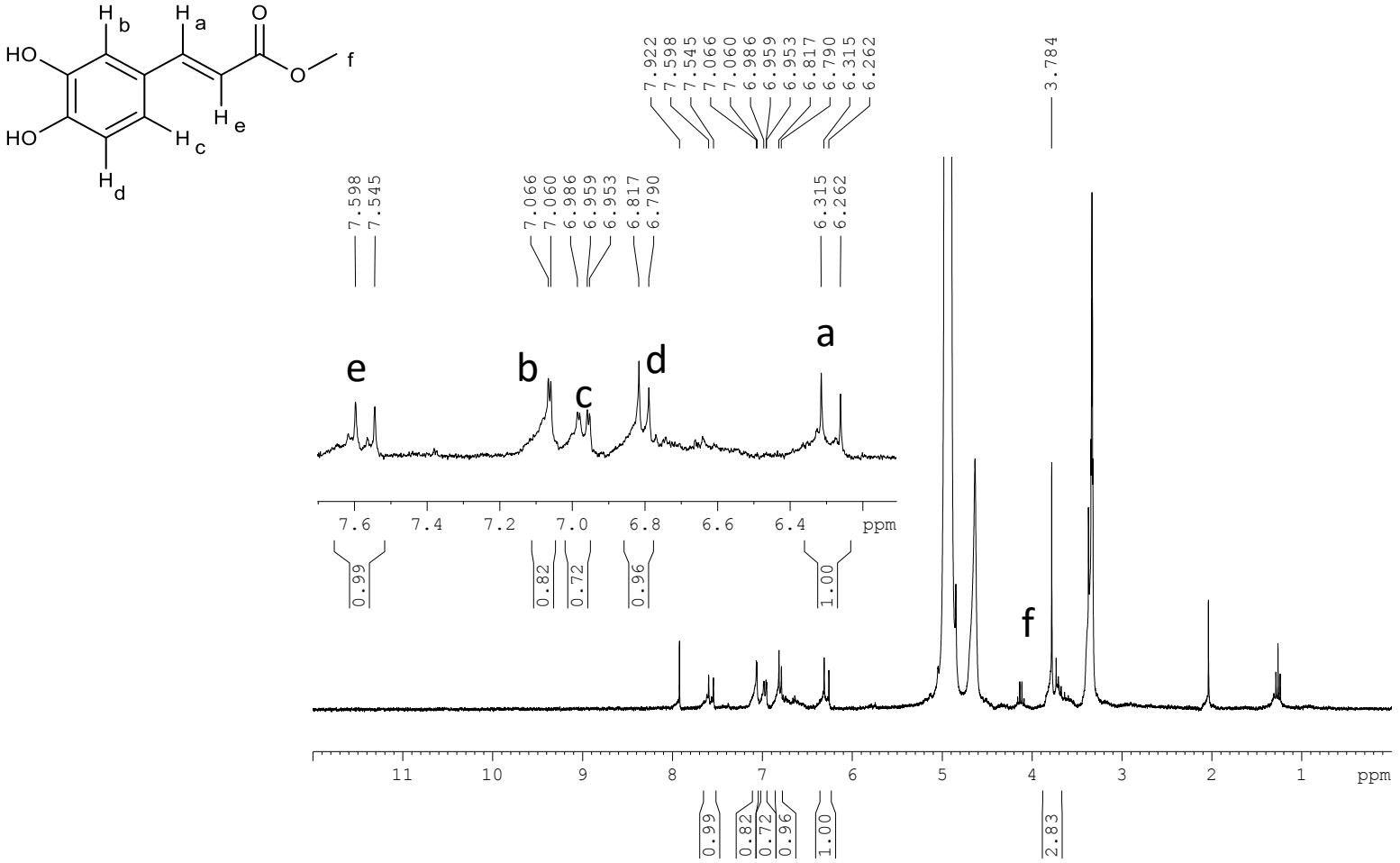
Appendix 10: ^{13}C (75.4) NMR data for Compound **73** in methanol- d_4.



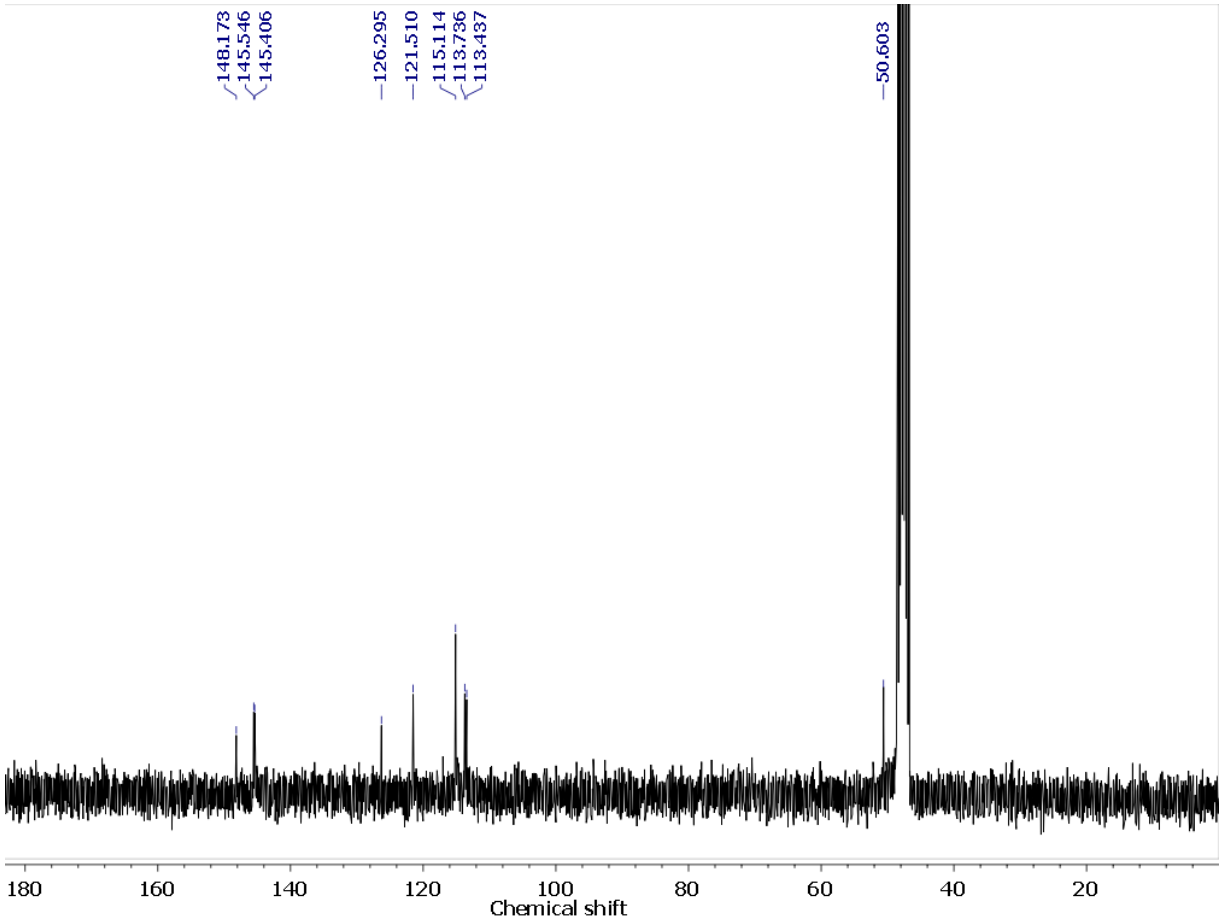
Appendix 11: ESI-HRMS spectrum of compound **73**



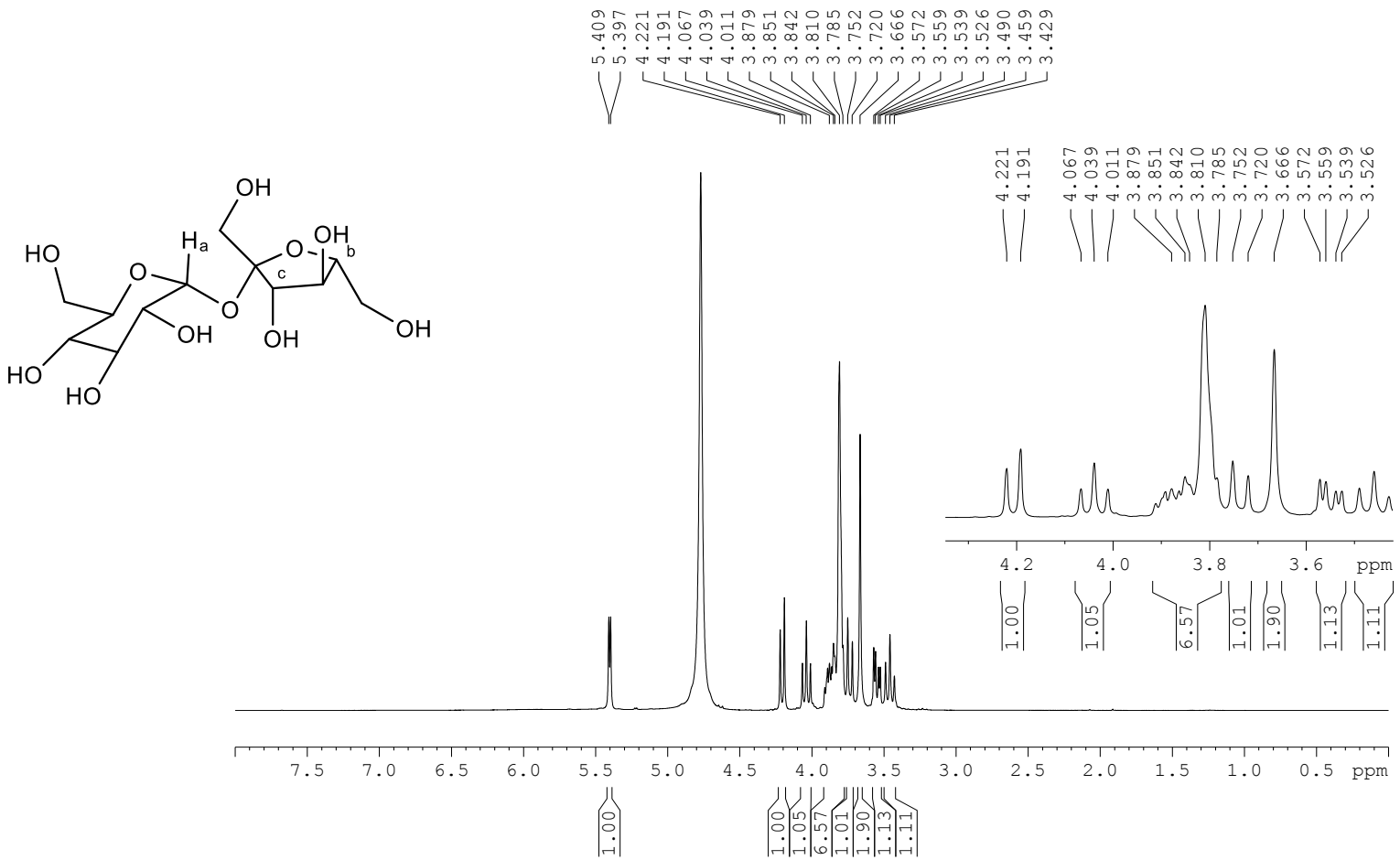
Appendix 12: ^{1}H (300 MHz) NMR data for Compound **76** in methanol- d_4



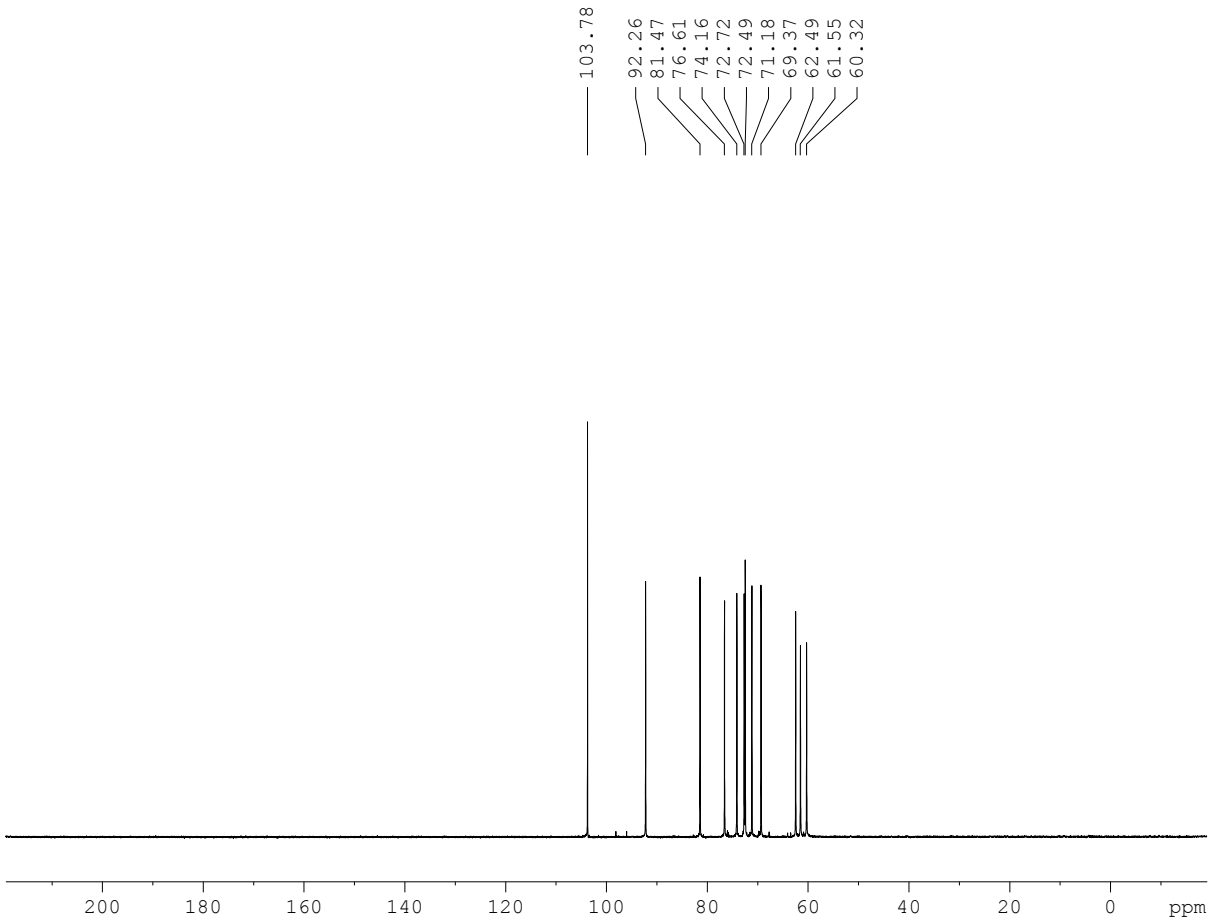
Appendix 13: ^{13}C (75.4) NMR data for Compound **76** in methanol- d_4



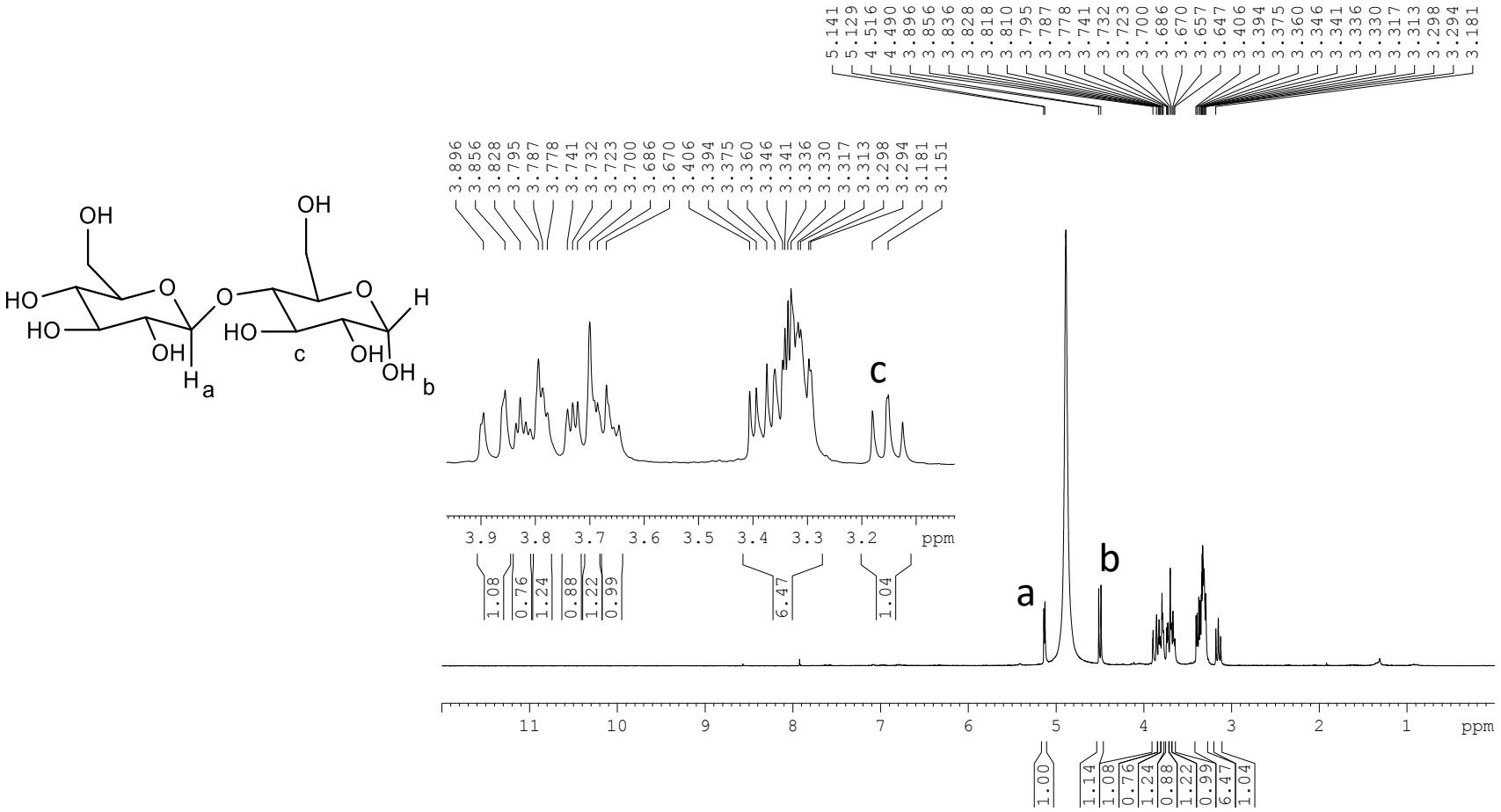
Appendix 14: ^{1}H (300 MHz) NMR data for Compound **72** in deuterium oxide



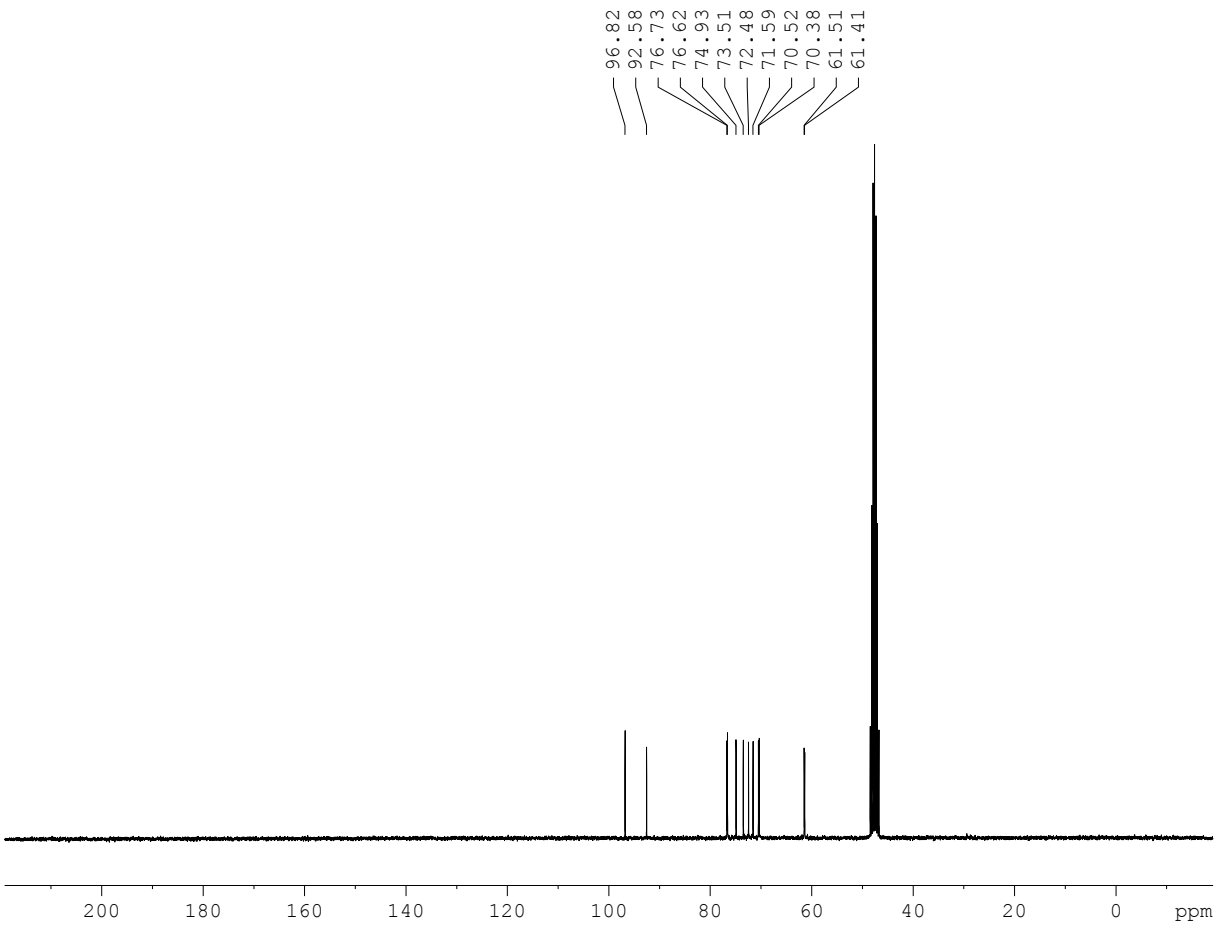
Appendix 15: ^{13}C (75.4) NMR data for Compound **72** in deuterium oxide



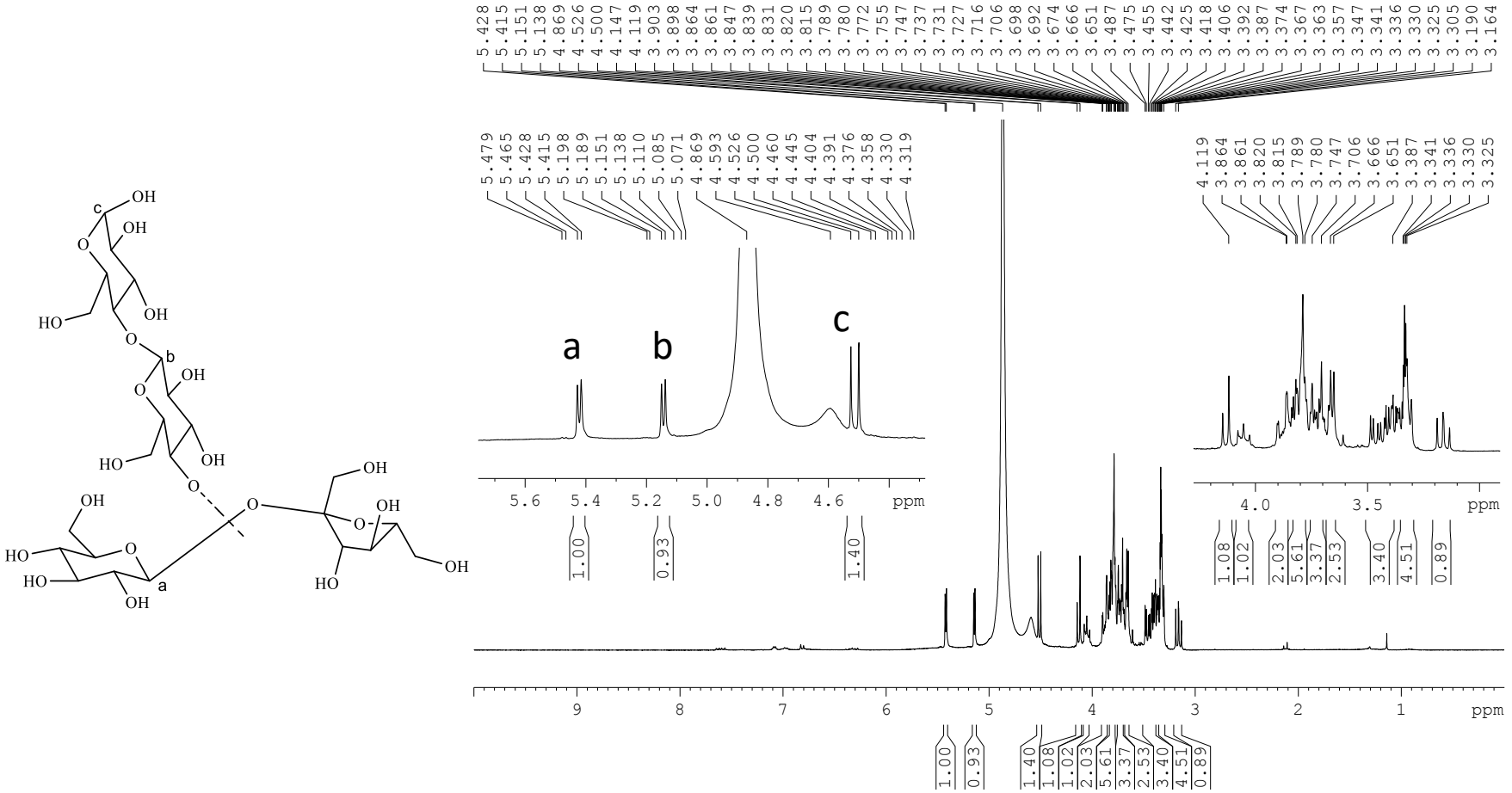
Appendix 16: ^{1}H (300 MHz) NMR data for Compound **75** in methanol-d



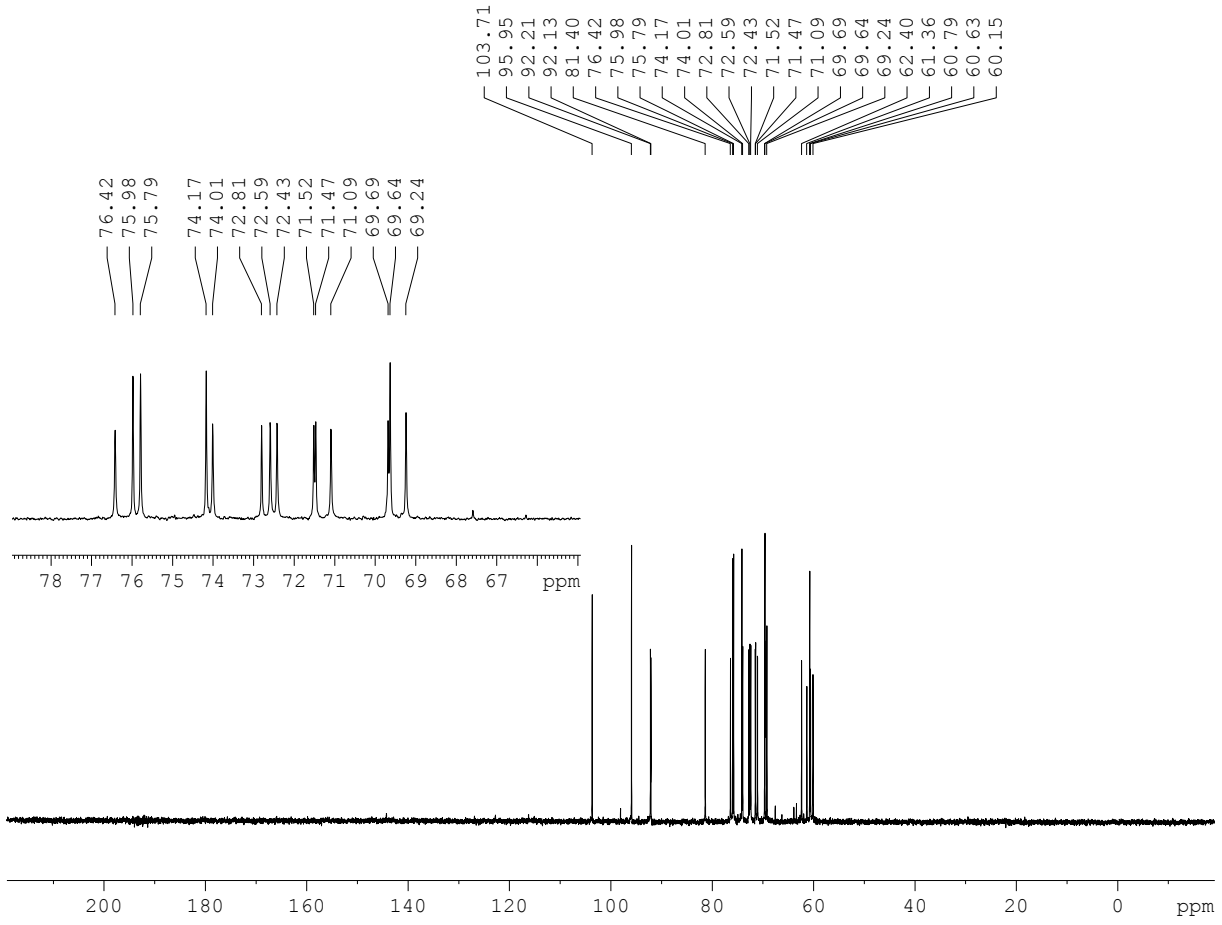
Appendix 17: ^{13}C (75.4) NMR data for Compound **75** in methanol-d



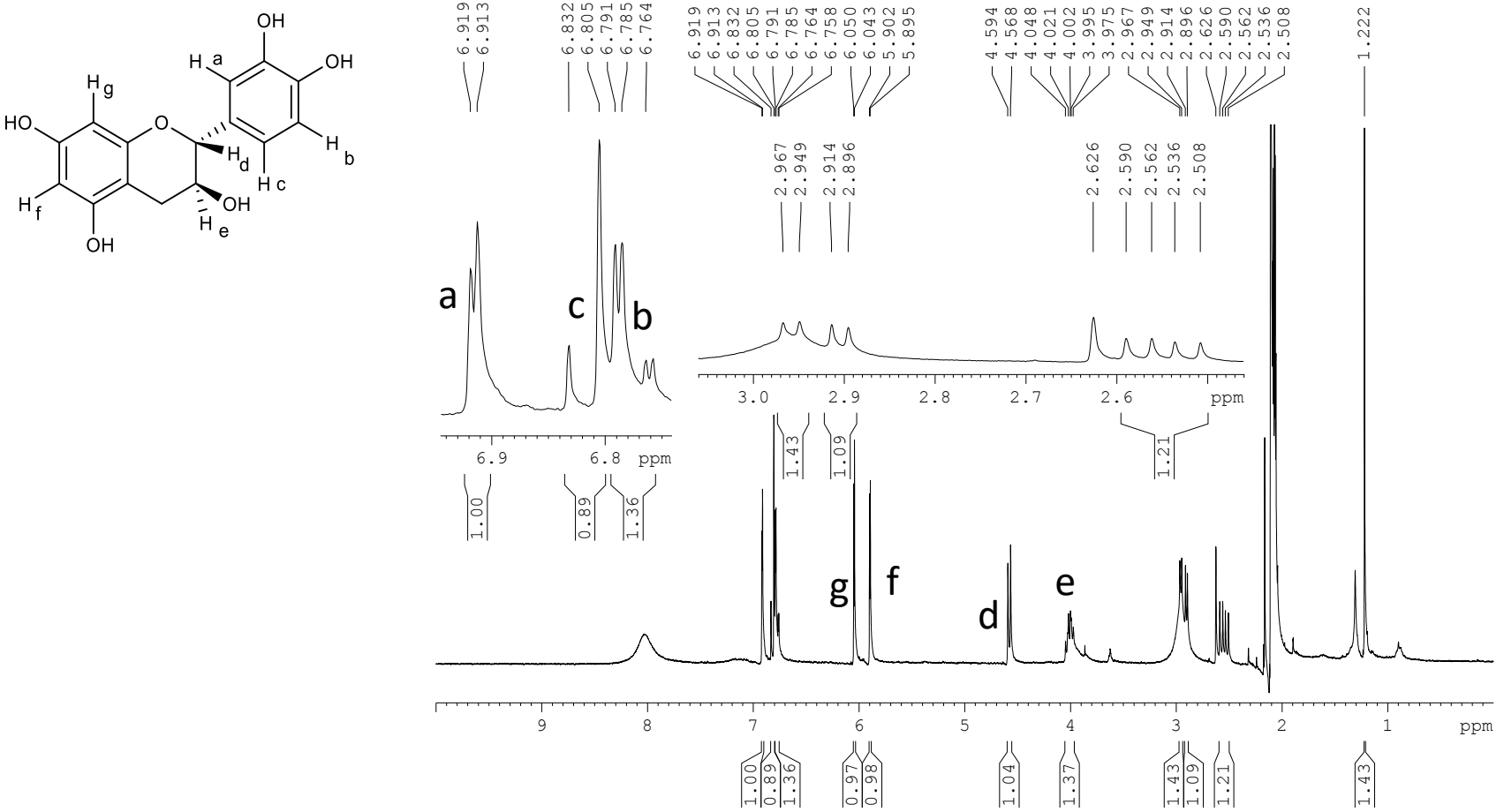
Appendix 18: ^{1}H (300 MHz) NMR data for Compound **74** in methanol- d_4



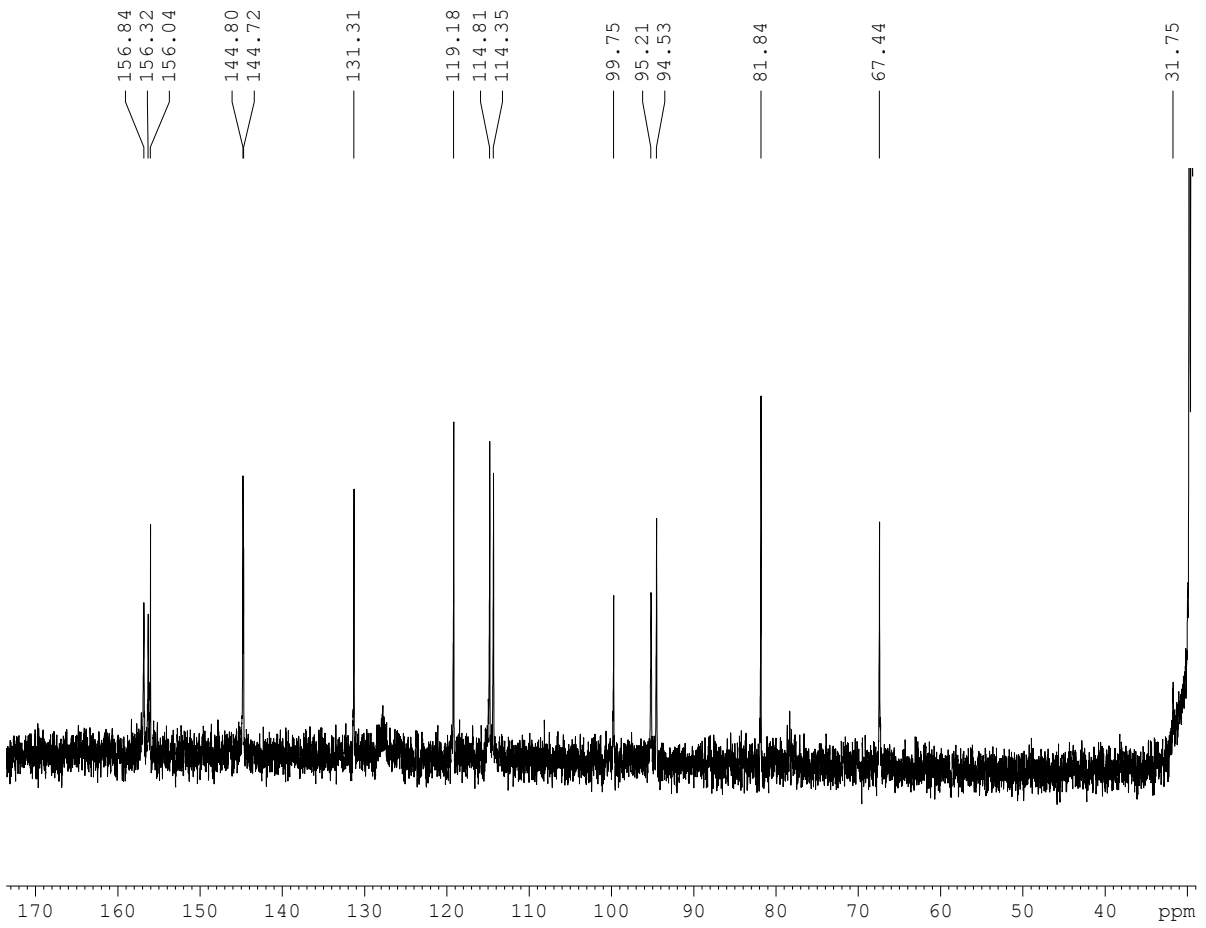
Appendix 19: ^{13}C (75.4) NMR data for Compound **74** in methanol- d_4



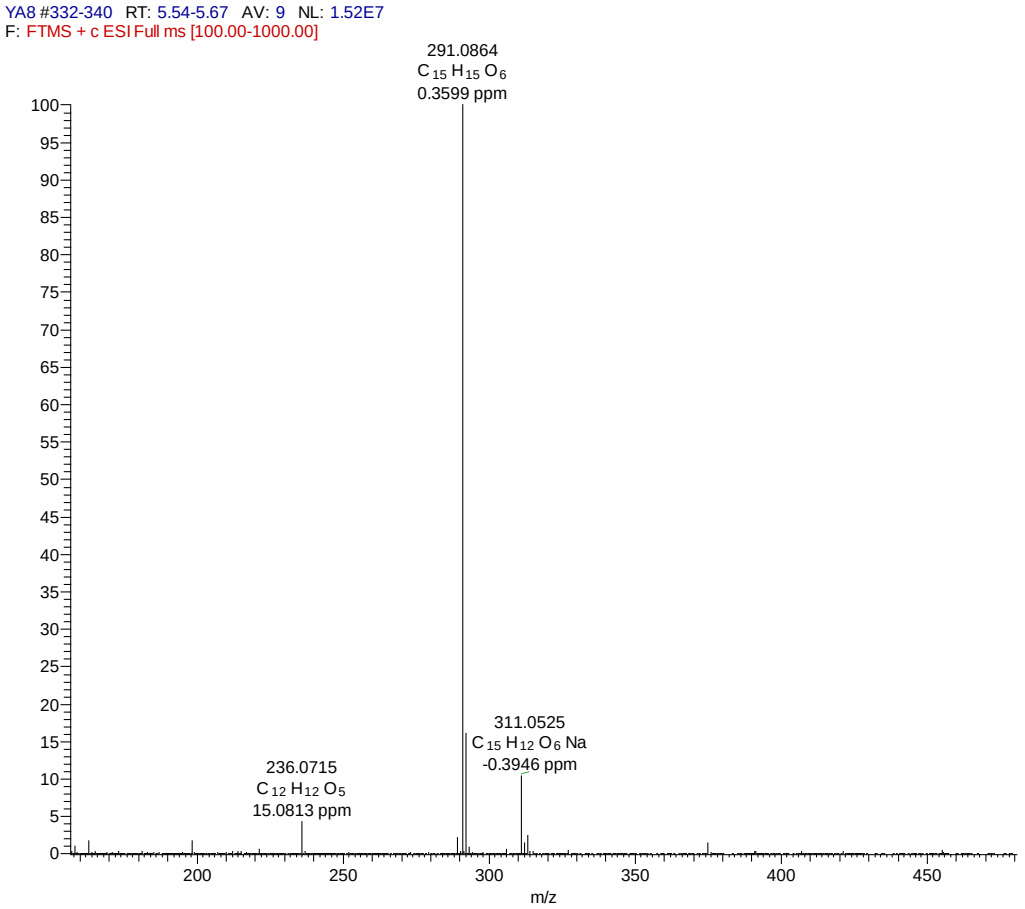
Appendix 20: ^{1}H (300 MHz) NMR data for Compound **79** in acetone-d_6



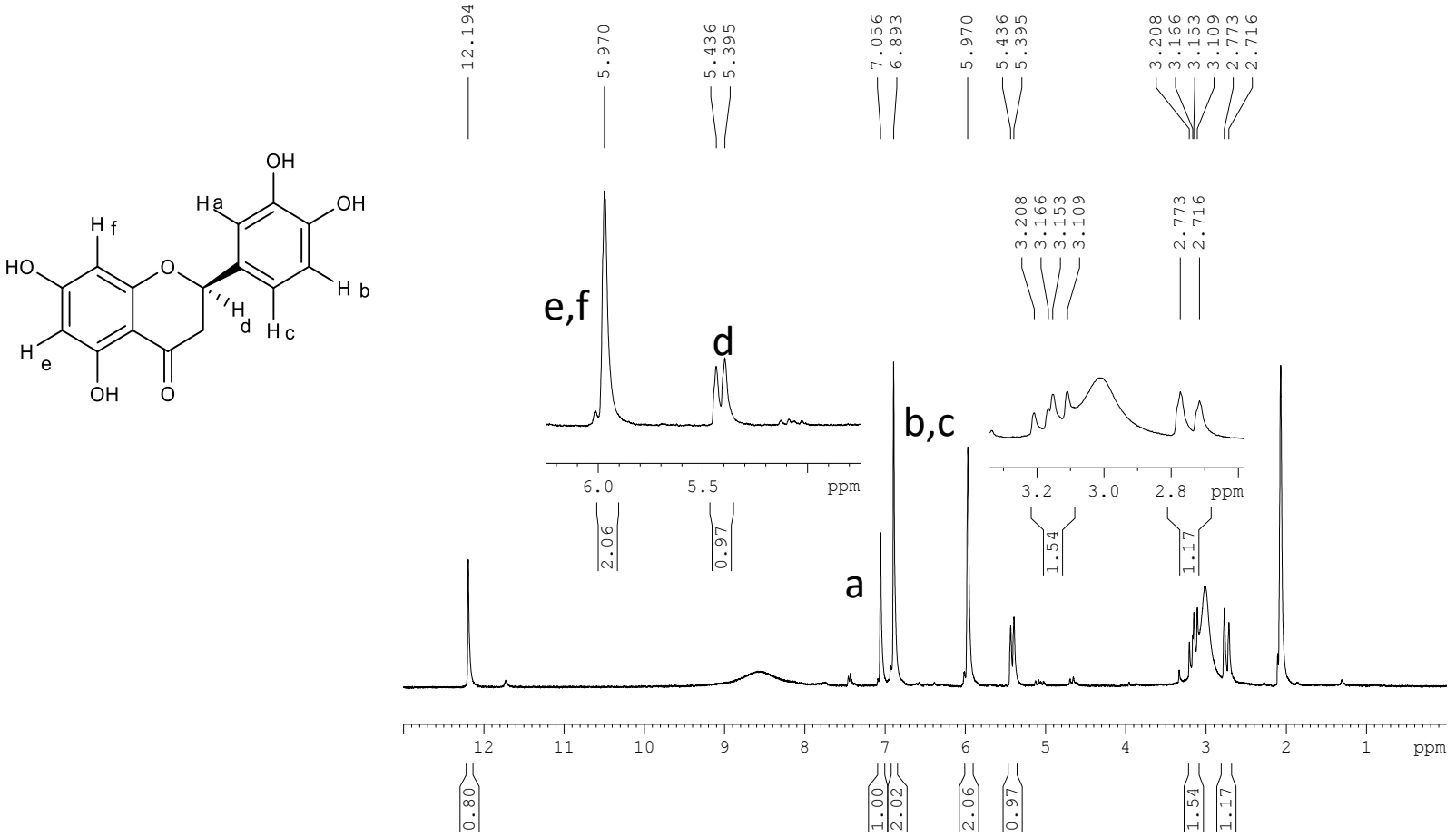
Appendix 21: ^{13}C (75.4) NMR data for Compound **79** in acetone-d_6



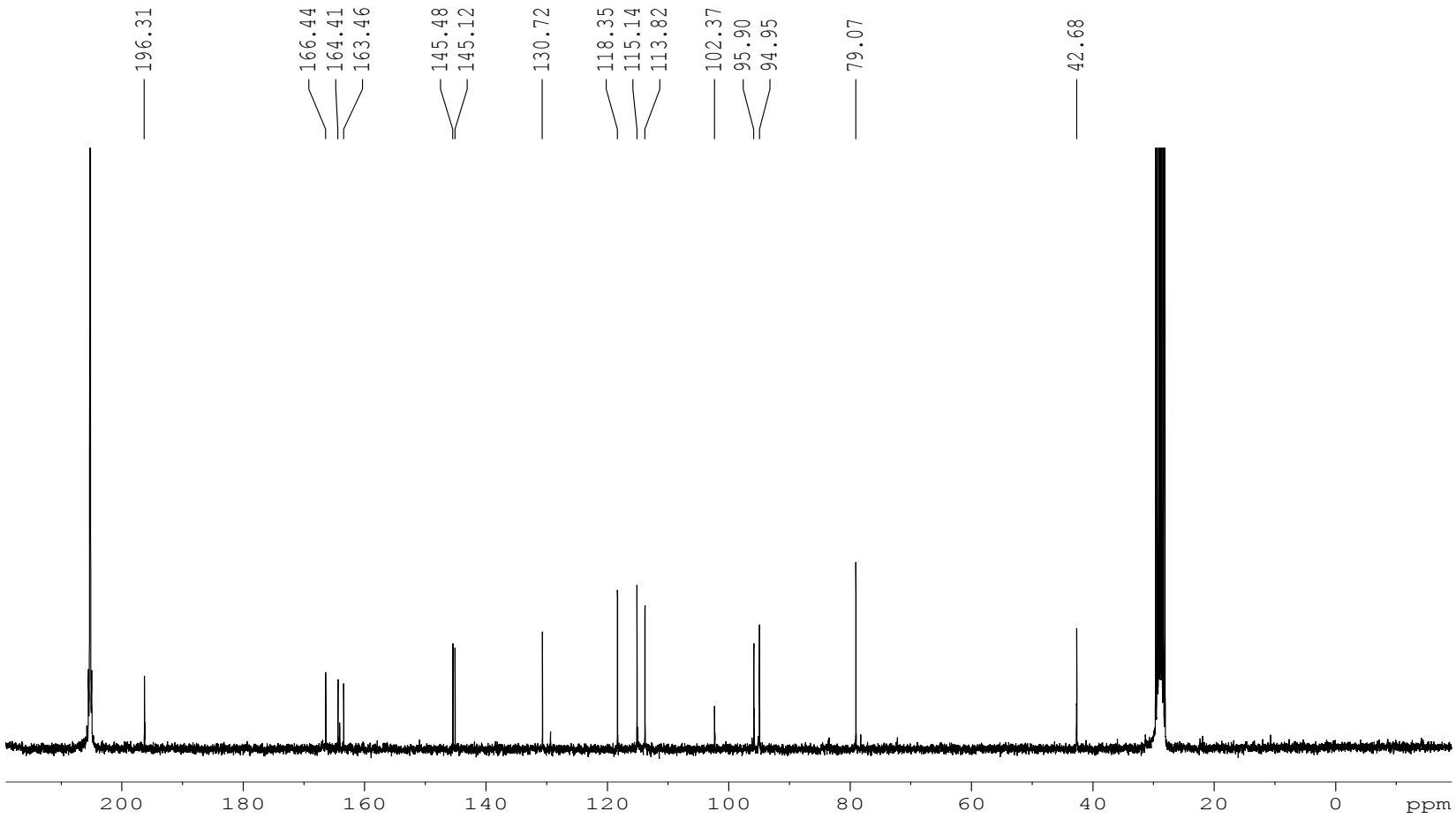
Appendix 22: ESI-HRMS spectrum of compound **79**



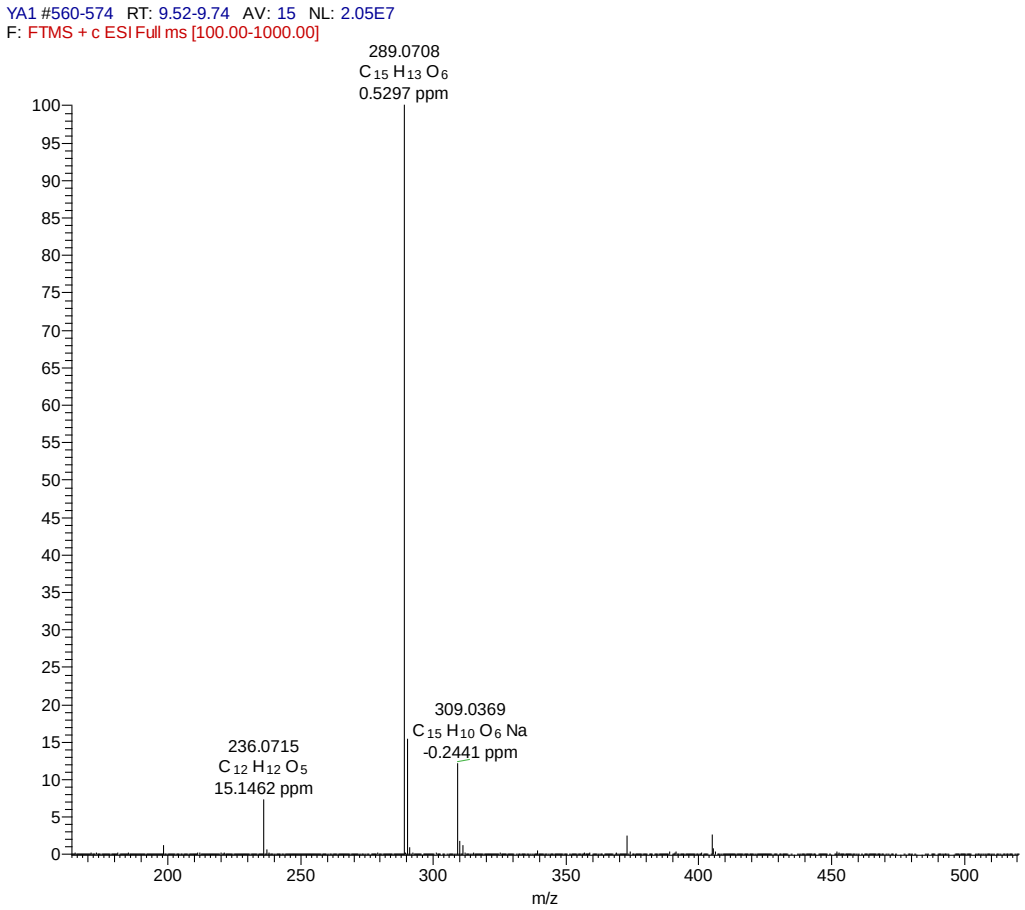
Appendix 23: ^{1}H (300 MHz) NMR data for Compound **78** in acetone-d_6



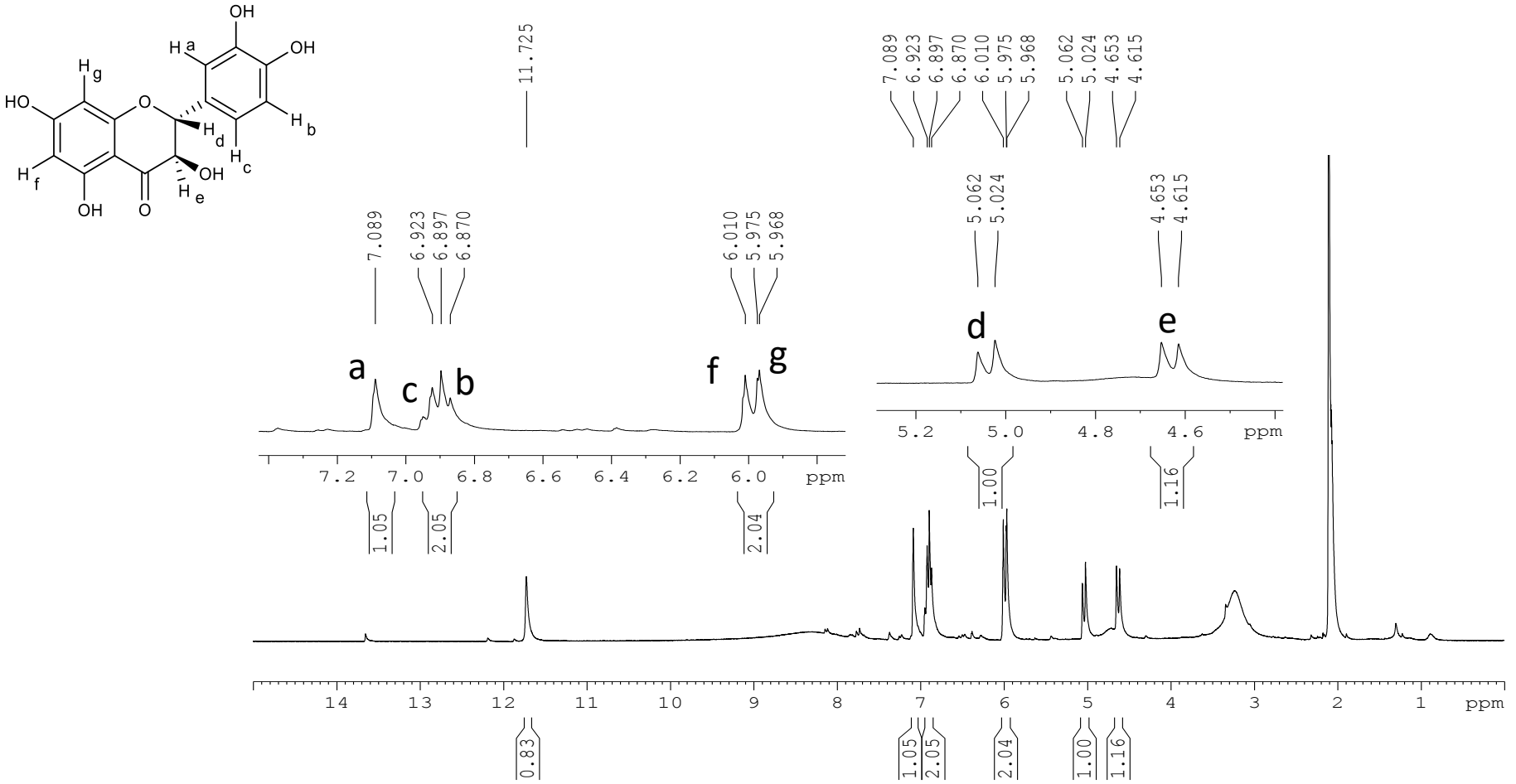
Appendix 24: ^{13}C (75.4) NMR data for Compound **78** in acetone-d_6



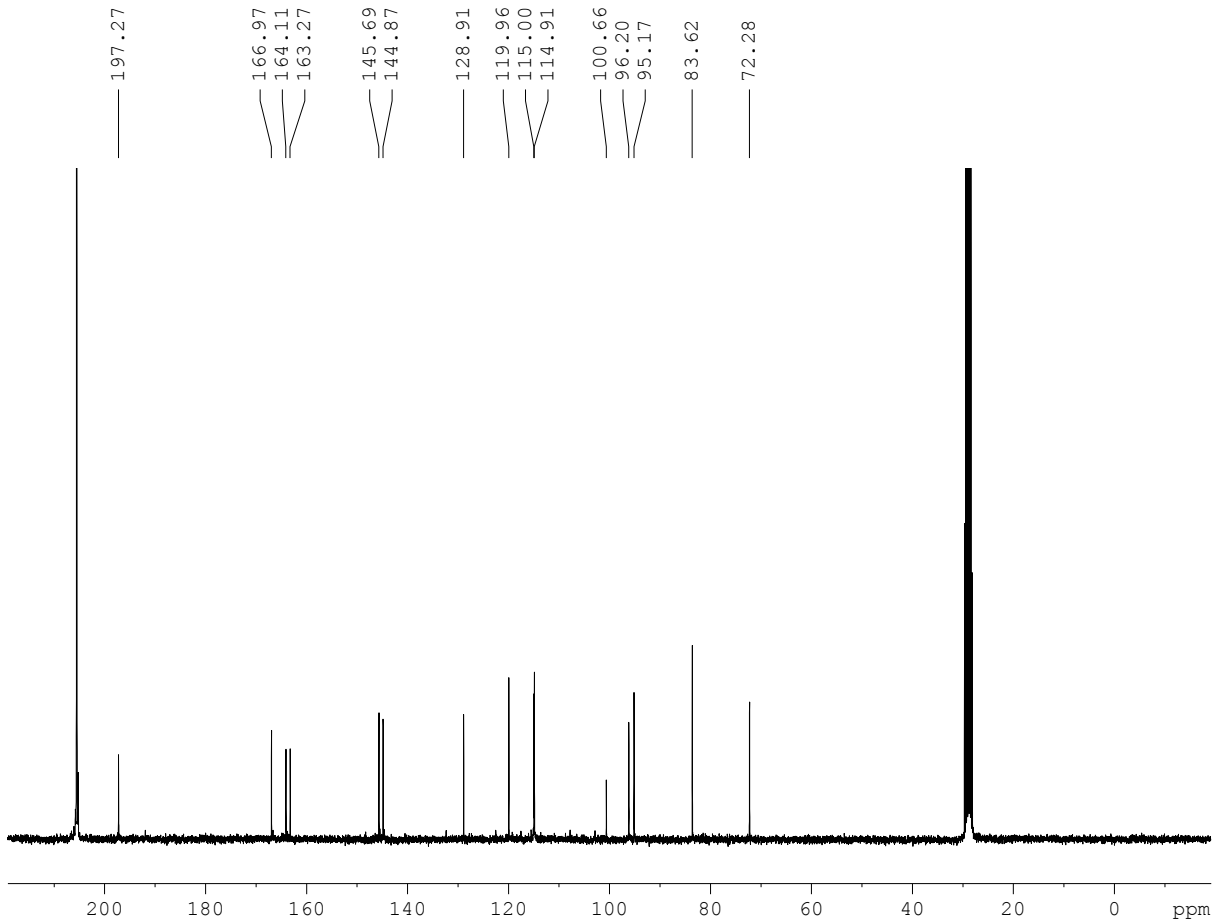
Appendix 25: ESI-HRMS spectrum of compound **78**



Appendix 26: ^{1}H (300 MHz) NMR data for Compound **77** in acetone-d_6



Appendix 27: ^{13}C (75.4) NMR data for Compound **77** in acetone-d_6



Appendix 28: ESI-HRMS spectrum of compound **77**