ACETYLCHOLINESTERASE INHIBITION AND CYTOTOXIC ACTIVITY OF EXTRACTS AND A NOVEL COMPOUND ISOLATED FROM FLOWERS OF THE WILD EVERLASTING (*HELICHRYSUM ARGYROSPHAERUM DC*)

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN INDUSTRIAL BIOCHEMISTRY

OF

THE UNIVERSITY OF NAMIBIA

BY

ETUHOLE MTULENI

218203417

OCTOBER 2024

MAIN SUPERVISOR: PROF STEFAN LOUW (UNIVERSITY OF NAMIBIA)

CO-SUPERVISOR: PROF PETRINA KAPEWANGOLO (UNIVERSITY OF NAMIBIA)

Abstract

Helichrysum argyrosphaerum is a poisonous plant responsible for livestock losses in southern Africa. *Helichrysum* species primarily affect the central nervous system when ingested by livestock. However, previous attempts to identify the toxins produced by these plants resulted in only tentative identification of some chemical constituents. This study aimed to isolate and characterize compounds from the flowers of *H. argyrosphaerum* and to determine the *in vitro* acetylcholinesterase inhibitory activity and cytotoxicity of the methanol (MeOH) and dichloromethane (DCM) flower extracts and isolated compounds. Several flavonoid glycosides (tentatively identified as apigenin-O-glycosides) as well as an unknown chlorogenic acid analogue were isolated from the methanol extract of *H. argyrosphaerum* flowers using semi-preparative high-performance liquid chromatography (HPLC). The chemical structures of three isolated compounds were elucidated as apigenin, chamaemeloside, and a novel compound, 14-acetyl chamaemeloside, based on their high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance spectroscopy (NMR) data. Interestingly, both the MeOH and DCM *H. argyrosphaerum* flower extracts as well as 14-acetyl chamaemeloside induced acetylcholinesterase activity. In the cytotoxicity assay, neither the MeOH and DCM extracts nor 14-acetyl-chamaemeloside exhibited toxicity against HeLa cells. This research marks the initial report of acetylcholinesterase activity and cytotoxicity in both *H. argyrosphaerum* MeOH and DCM flower extracts, as well as the isolated compound, 14-acetyl-chamaemeloside, against HeLa cells. Consequently, these findings will contribute to future toxicological studies and aid in developing preventative agents that can be used in the treatment of animals poisoned by *H. argyrosphaerum*.

Keywords: *Helichrysum argyrosphaerum*, livestock losses, neurotoxins, acetylcholinesterase, chamaemeloside.

CONFERENCE PRESENTATIONS

E. Mtuleni, P. Kapewangolo, S. Louw, Acetylcholinesterase inhibition, cytotoxic activity, and chemical characterisation of potential toxic compounds isolated from the wild everlasting (*Helichrysum argyrosphaerum* DC), **National Students' Research Symposium**, Windhoek, Namibia, 21-22 August 2024 (oral presentation).

Table of contents

List of Figures

List of Tables

List of abbreviations and acronyms

$[M+H]^+$	precursor ion or pseudo-molecular ion λ – wavelength
1D NMR	one dimensional nuclear magnetic resonance
2D NMR	two-dimensional nuclear magnetic resonance
2D55-AChE	oncolytic adenoviral vector
ACh	acetylcholine
AChE	acetylcholinesterase
ACN	acetonitrile
AD	Alzheimer's disease
AF	ammonium formate
APCI	atmospheric pressure chemical ionisation
APT	Attached proton test
CGA	chlorogenic acid
ChAT	Choline Acetyltransferase
COSY	correlation spectroscopy
Da	Dalton
DAD	diode array detector
DCM	dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EI - MS	electron impact mass spectrometry
ERK1/2	The extracellular signal-regulated kinase 1/2 cascade
ESI	electro-spray ionisation
F1	initial flow rate
F2	final flow rate

FA	formic acid
FQA	feruloylquinic acid
G1	initial gradient time
G2	final gradient time
GC	gas chromatography
HILIC	hydrophilic interaction liquid chromatography
HMBC	Heteronuclear multiple bond correlation
HRMS	high resolution mass spectrometry
HSQC	heteronuclear single quantum correlation
IC50	50% inhibitory concentrations
IL-1	inflammatory cytokine interleukin
JNK	the c-Jun N-terminal kinase pathway
LC-MS	HPLC coupled with MS
m/z	mass to charge ratio
MALDI-TOF MS	matrix-assisted laser desorption or ionisation-time-of-light mass spectrometry
MeOH	methanol
MIC	minimum inhibition concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	tandem mass spectrometry involving multiple fragmentation steps
NAChR	nicotinic acetylcholine receptors
NIST	National Institute of Standards and Technology (U.S)

NMR	nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
PA	pyrrolizidine alkaloids
PBMC	healthy peripheral blood mono nuclear cells
PDA	photo diode array
ROS	reactive oxidative species
RPLC	reverse phase liquid chromatography TIC - total ion chromatogram
SAR	structural activity relations
TOF	time of flight
UPLC	ultra performance liquid chromatography UV - ultraviolet
UV-vis	ultraviolet to visible range

Acknowledgements

I would firstly like to acknowledge God, for being my guide and strength throughout this research. My gratitude also goes out to the following people and institutions:

- Prof. Stefan Louw, my main supervisor, for your encouragement and guidance throughout this research.
- Prof. Petrina Kapewangolo, my co-supervisor, for your dedication and support to seeing this project through.
- The Seeding Labs Instrumental Access 2018-19 grant for providing our laboratory with the Agilent 1100 HPLC used in this study.
- Dr Celestine Raidron, for allowing access to a rotary evaporator used in this study.
- Dr Jaco Brandt of the Central Analytical Facility of Stellenbosch University, for performing the NMR experiments.
- My family, friends and colleagues, for the much-needed prayers, love and support throughout this research.

Dedication

I dedicate this research to my wonderful family, whose unwavering belief in me has been a constant source of strength. Thank you for your steadfast support throughout this journey, especially my sister, Eveste Mtuleni—this work is for you.

Declaration

I, Etuhole Mtuleni, declare hereby that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

Etuhole Mtuleni October 2024

Name of Student Signature

CHAPTER 1

Introduction

1.1 Background

Southern Africa has a diverse and abundant plant life which consists of a variety of plants that could potentially poison animals and humans (1). Poisonous plants are one of the major causes of livestock health problems worldwide and therefore, cause significant economic losses (2). Plant toxins are naturally occurring secondary metabolites that serve as defense mechanisms for plants against a variety of pathogens and herbivores (3).

Helichrysum argyrosphaerum belongs to the Asteraceae family and is widely distributed in Namibia (4). The plant has been reported to be toxic to farm animals like cattle and sheep, causing encephalopathy (alteration of brain function or structure) when eaten in large amounts (5). Symptoms in sheep include blindness, ataxia, and paresis/paralysis while in cattle, it manifests as stiffness and paralysis (5). In a recent study, several potentially toxic compounds were tentatively identified in the plant, including the compound, chamaemeloside (6). It has also been reported that *H. argyrosphaerum* has antibacterial and antifungal activities (7).

1.2 Statement of the problem

Although *H. argyrosphaerum* is known to be poisonous to livestock, its toxic components are still unknown. Although several compounds produced by *H. argyrosphaerum* have previously been tentatively identified, there are no reported

studies on the isolation and characterization of its potentially toxic compounds. In addition, there are no reports of the *in vitro* acetylcholinesterase inhibitory activity and cytotoxicity determination of *H. argyrosphaerum* extracts or isolated compounds.

1.3 Objectives

The aim of this study was to isolate and characterize compounds from *H. argyrosphaerum*, as well as to determine the acetylcholinesterase and cytotoxic activity of the methanol (MeOH) and dichloromethane (DCM) extracts, as well as the isolated compounds.

The objectives of this study were:

a) To isolate and characterize compounds from *H. argyrosphaerum* that were previously tentatively identified as potentially toxic.
b) To determine the effects of the flower extracts of *H. argyrosphaerum* and isolated compounds on the *in vitro* acetylcholinesterase activity.
c) To determine the *in vitro* cytotoxicity of *H. argyrosphaerum* flower extracts and isolated compounds against HeLa cells.

1.4 Significance of study

Identifying potentially toxic compounds from *H. argyrosphaerum* is crucial for understanding the poisoning mechanisms. This knowledge will pave the way for developing strategies to overcome poisoning incidents. Acetylcholinesterase (AChE) is an enzyme that is crucial for the proper functioning of the nervous system, and abnormalities in this enzyme, such as excessive activity or inhibition of the enzyme may lead to a range of neurotoxic effects. Investigating the AChE activity and cytotoxicity of *H. argyrosphaerum* will enhance our understanding of the mechanisms

by which poisoning occurs in livestock. Additionally, examining the potentially toxic compounds isolated from *H. argyrosphaerum* will provide further insights into these mechanisms. This will be the first step towards developing preventative agents for livestock poisoning by *H. argyrosphaerum*.

1.5 Limitations of the study

The samples had to be sent to laboratories at Stellenbosch University for nuclear magnetic resonance (NMR) analysis, since there are no such instruments in Namibia. This caused some delays in generating data.

1.6 Delimitations of the study

The research study was only based on the extracts of *H. argyrosphaerum* collected from Windhoek (Döbra Farm Plot 46) in Namibia, and only focused on the *in vitro* acetylcholinesterase and cytotoxic activity of extracts and one isolated compound. Cytotoxicity studies were only carried out against the HeLa cell line.

CHAPTER 2

LITERATURE REVIEW

2.1 Plants toxins and effect on livestock

There are about 600 indigenous poisonous plant species in southern Africa and about 80 of those are found in Namibia. During extreme climate conditions, animals are forced to feed on poisonous plants which would otherwise be avoided in favourable conditions (8,9). For example, when there is plenty of grass to graze on, farmers keep their livestock on guard against these poisonous plants and monitor them closely after grazing. Monitoring is done by inspecting animals that are lagging or have any unusual behaviour. Animals that are suspected of plant poisoning are then isolated from others and treated accordingly (10). Providing the animals with the appropriate vitamin supplements and feeds will strengthen their defences against potential infections from dangerous plants.

Once farmers are familiar with specific toxic plants, it is easier to identify symptoms and relate them to a specific plant. There are many factors involved in plant toxicity which may range from the growth stage (in some plants, toxicity is higher in mature plants than in younger plants), the plant part (some toxic agents are usually in higher concentrations in some plant parts compared to other plant parts), and the amount ingested by the animal over a certain period of time (11).

The research involving the characterization of specific compounds responsible for the toxicity in plants is important in finding specific ways to reverse toxicity caused by specific plant species. For example, the plant *Delphinium exaltatum* is poisonous to

cattle and the toxicity was found to be due to norditerpene alkaloids present in the plant (12). After much research, it was found that administering physostigmine to animals poisoned by this plant was highly effective (13).

The plant toxins block nicotinic acetylcholine receptors (nAChR), which stop the neurotransmitter from activating muscle and nerve cells. Cattle then exhibit clinical symptoms of poisoning when the larkspur toxins start to affect muscles and nerves (14). Therefore, cholinergic drugs such as physostigmine or neostigmine are used in the treatment of poisoned cattle (12,14). Neostigmine blocks acetylcholinesterase, the enzymes responsible for neutralizing the larkspur toxins in nerves and muscles. This approach maintains the animal's muscle function and allows sufficient time for the toxic alkaloids to be eliminated from its body (14).

Overall, the plant genus *Delphinium* (larkspur) is known to contain high alkaloid levels, making them particularly poisonous (15). Active ingredients such as Picloram and metsulfuron-methyl are used in herbicides against larkspur (16,17). Picloram herbicides are labelled for geyer, prairie, and tall larkspur, while metsulfuron-methyl containing herbicides were tested and used against *Delphinium occidentale* and tall larkspur (16).

2.2 Biological activities of the Genus *Helichrysum*

Plants belonging to the genus *Helichrysum* are usually scented perennial shrubs with dense leaves and durable flower heads (18). These shrubs are members of the asteroid family, which has roughly 600 species of flowering plants (19). The genus is distributed in Europe, Asia and Africa, with the highest diversity in South Africa, where about 500 recognised species exist. Plants from the genus *Helichrysum* have been used for several ailments, like stomach pain, gall bladder problems, jaundice,

colds, wound healing, diabetes, mellitus, skin infections and asthma (20). Most of these plants are sources of biologically active biochemicals like flavonoids, acetophenones, phloroglucinols, pyrones, diterpenes and sesquiterpenes that have antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory and anti-diabetic properties (19). For example, flavonoids isolated from *H. gymnocephalum* were found to have antiplasmodium properties (21).

In another study, flavonoids from *H. chasmolycicum* were reported to have antimicrobial and antioxidant activity (22). Furthermore, it was found that *H. italicum* extracts are primarily composed of non-volatile polyphenolic compounds with antioxidative, anti-inflammatory, antimicrobial and anticarcinogenic properties, as well as cytoprotective activity towards normal cells and cytotoxic activity against cancer cells (23). Other activities include strong cytotoxic activity of *H. zivojinii* tested against HeLa, em-x and $K_5$62 cells. In addition to the plant extracts' remarkable selectivity in their antitumor effects on cancer cells compared to healthy peripheral blood mono nuclear cells (PBMC) (24).

2.3 Helichrysum argyrosphaerum

Helichrysum argyrosphaerum commonly known as wild everlasting, is an annual shrub that grows flat on the ground up to 150 mm high (25). It has simple dark green leaves covered in fine hair (4). As illustrated in **Figure 2.1**, The flowers appear round like a bowl with transparent brats, of which the outer rows are shiny and slippery, while the outer centres rose can be pink, yellow or white, depending on the growth stage (25). This shrub grows in hot, dry and sandy areas in open woodland and grassland. It is widespread in Namibia, Angola, Botswana, Malawi, Zambia,

Zimbabwe, Lesotho, and South Africa (4,25). *Helichrysum argyrosphaerum* has been reported to be toxic to livestock such as sheep and cattle (5). Animals that graze on pastures with a prevalence of *H. argyrosphaerum* exhibit signs of encephalopathy. In sheep, it causes blindness, ataxia, paresis, and paralysis, while in cattle, it manifests a stiffness and posterior paralysis (1). In a different study, plant samples were fed to sheep to better understand the plant's toxicity to animals (26). The results from this experiment showed symptoms in sheep that are consistent with the literature. According to the feeding experiment results, paralysis occurs from a status spongiosis of the brain, spinal cord, ocular fascicles, and nerves, which occurred by consuming significant amounts of *H. argyrosphaerum*. The lack of overt blindness was likely due to the lesion not progressing to the point where this symptom could occur, as the overall pathological picture resembled normal amaurosis and paresis. Additionally, it was demonstrated that while fresh and dry plant material were hazardous during the flowering stage, they were most likely not toxic during a later growth phase (26).

Figure 2.1: *Helichrysum argyrosphaerum* (27).

2.4 Flavonoids with AChE activity

Flavonoids are the most abundant polyphenols present in plant foods and are characterised by a 15-carbon skeleton consisting of a benzopyrone ring with phenolic or poly-phenolic groups at various positions (28,29), as illustrated in **Figure 2.2**. In plants, they are responsible for colour, fragrance, and flavour, therefore attracting pollinators to plants. Flavonoids also regulate cell growth, and can operate as signal molecules, UV filters and reactive oxygen species (ROS) scavengers (30). In addition, flavonoids have been found to have anticancer, antimicrobial, antiviral and antiangiogenic, antimalarial, antioxidant, neuroprotective, antitumor, antiproliferative properties, as well as AChE inhibition activity (27,30).

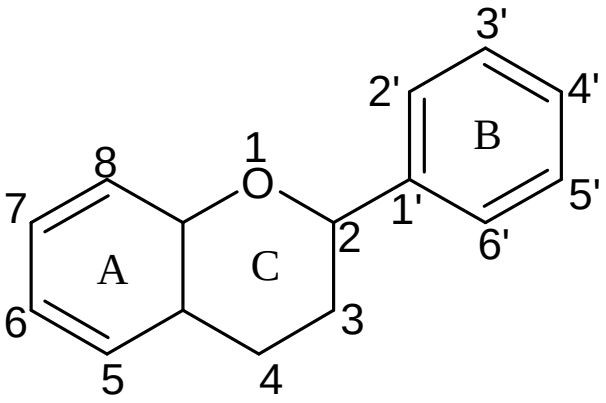


Figure 2.2: Basic structure of flavonoids.

Flavonoids have been studied for their AChE activity. Over the years, flavonoids that inhibit AChE have been promising natural chemicals for the treatment of degenerative Alzheimer's disease (AD) (32). This is because inhibition of AChE is a new therapeutic strategy for the management of neurogenerative disorders such as AD (33). Other flavonoids with AChE inhibitory activity include apigenin, baicalein, naringin, silymarin, rutin and naringenin (34).

Flavonoids have also been studied for their ability to increase AChE activity. For example, a group of flavonoids having osteogenic effects in bones were examined for their ability to induce AChE expression. Five flavonoids, including baicalin, calycosin, genistin, hyperin, and pratensein, significantly enhanced AChE expression at the mRNA, protein, and enzymatic levels (35). In a different study, the induction of AChE was completely blocked by pre-treatment with G15 (a selective G protein-coupled receptor 30 [GPR 30] antagonist), and five of twenty-four commonly found flavonoids tested for their induction on AChE activity had a strong effect, namely daidzin, alpinetin, irisflorentin, cardamonin, and lysionotin (36). Another study reported that –)-Epigallocatechin-3- gallate, a flavonoid, was found to increase AChE activity while also maintaining acetylcholine (ACh) levels in synaptic neurons by enhancing Choline Acetyltransferase (ChAT) activity (37). In rats, co-administration of caffeic acid with malathion boosted AChE activity (38). Moreover, In vitro experiments on rats demonstrated that caffeic acid increased AChE activity in the cerebral cortex, cerebellum, hypothalamus, whole blood, and lymphocytes (40).

Research was done on the structural activity relationship (SAR) between flavonoids and AChE activity. It was found that hydroxylation of the A and B rings of flavonoids increases inhibitory activity against AChE, while methoxylation may increase or decrease activity depending on the class of flavonoids (40).

2.5 Apigenin glycosides

Apigenin is a dietary flavonoid that belongs to the subgroup of flavones. It is commonly found in plants like parsley, chamomile, celery, artichokes, and oregano (41). There have been several studies that have reported apigenin to have strong therapeutic potential against several diseases (42,43). This is attributed to its antioxidant, anti-inflammatory, chemotherapeutic, hepatotoxicity, antigenotic and anticarcinogenic activity amongst other effects (44). Apigenin is considered to have low toxicity as it is commonly associated with health benefits. However, there is no direct evidence on the effects of apigenin glycosides on livestock (44,45).

The flavonoid apigenin **(Figure 2.3)** exists in its glycosylated form in nature (46). Apigenin glycosides are formed by a combination of apigenin with sugars (30). These flavonoids are first metabolised to the free form of apigenin prior to intestinal absorption (46). Glycosylation facilitates transport across the membrane, improving solubility, distribution, and metabolism in flavonoids (30).

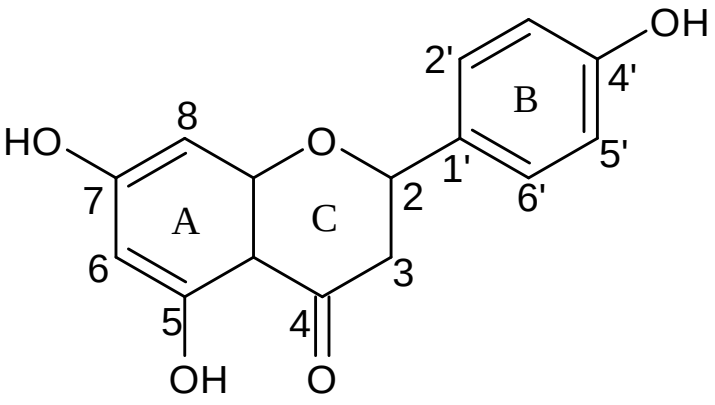


Figure 2.3: The structure of apigenin.

Apigenin glycosides are critical in plant development and physiology, particularly in interactions with other living organisms (47). Apigenin C-glycosides exert strong anti-

inflammatory properties and were found to reduce pulmonary oedema and microvascular permeability. Furthermore, the Ingenuity Pathway Analysis on mice serum showed that apigenin C-glycosides activated networks of protective proteins and pathways that involve inflammatory regulators and elements to apoptosis, such as c-Jun N-terminal kinase (JNK) pathway, the extracellular signal-regulated kinase 1/2 cascade (ERK1/2), and Caspase-3/7 (48).

In particular, Apigenin C-glycoside, well known as vitexin, was found to possess pharmacological effects, such as antioxidant, anti-inflammatory, anticancer, antinociceptive, neuroprotective effects, anti-stress (heat and oxidative stress), and antitumor (49). Another Apigenin glycoside, Apigenin-7-glycoside, protects against LPS-induced acute lung damage by inhibiting MAPK phosphorylation and downregulating oxidative enzyme production (50). Another study reported on the inhibitory activity of apigenin 7-O-glucoside, which was found to have low inhibition of AChE of 30% at 5-50 µg/mL. It was also found to have antibacterial activity against *S. aureus* and *E. faecalis* (51).

2.6 *In vitro* neurotoxicity determination: Acetylcholinesterase (AChE) assay

Since *H. argyrosphaerum* affects the central nervous system (5), an AChE activity assay is one of the methods that can be used to determine neurotoxicity. AChE is an enzyme that helps to maintain normal function of the nervous systemm. The key role of AChE is to terminate impulse signalling at cholinergic synapses by hydrolyzing acetylcholine, a naturally occurring neurotransmitter, into choline and acetic acid (52,53).

During neurotransmission, ACh is released from the nerve into the post synaptic cleft and binds to ACh receptors on the post synaptic membrane, relaying the message (52).

AChE, also embedded on the post synaptic membrane, then terminates the signal by hydrolysing the ACh (52,53). AChE inhibitors inhibit the breakdown of ACh, thus, ACh accumulates at the neuromuscular junctions and synapses, therefore causing symptoms of both muscalinic and nicotinic toxicity (52,54). Some of these symptoms include cramps, increased salivation, muscular weakness, paralysis, diarrhoea, and blurry vision (54). The acetylcholinesterase inhibition assay is used to detect AChE activity. It detects the ability of substances to increase or inhibit AChE. AChE inhibition leads to the accumulation of ACh and subsequently, the inhibition of cholinergic neurotransmission (55). The Ellman's assay method is the most common and measures the rate of thioline production from ACh in the presence of AChE (56). It uses 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to measure the amount of thiocholine formed, which is equivalent to the AChE activity (56).

In some cases, substances increase AChE activity instead of inhibiting it. Cholinergic deficit brought on by increased ACh breakdown due to enhanced AChE activity is associated with common symptoms such as memory loss, disorientation, hypo- or hyperactivity, ataxia, reductions in heart and muscle power and tone, and decreased exocrine secretions (57). Increased levels of AChE are also associated with long term stress responses and as a response the brain initiates compensatory mechanisms that would restore functional balance by increasing the cholinergic state (58). It was found that compensatory elevation of ACHE levels and activity results in a rapid hydrolysis of Ach and a consistent suppression of cholinergic neurotransmission. Suggestions are that stress induced AChE activation reduces ACh levels, which in turn activates brain pro inflammatory cytokine interleukin (1L-1) (59). 1L-1 plays an important role in stress induced activation of the hypothalamus-pituitary-adrenal axis and secretion of

glucocorticoids, that mediates the effects of stress on memory function and neuroplasticity, which at low concentrations have positive effects and negative outcomes at elevated levels (59).

Similar symptoms as stress are also seen in transgenic mice overexpressing AChE, such as abnormal behaviour following circadian light or dark shift, intensified long-term potentiation (LTP), development of neuropathologies, learning and memory decline, progressive muscle weariness and progressive neuromuscular junction degeneration (58,59).

Further research is required as the build-up of ACHE may interact with B amyloid plagues and, therefore, cause more neuro toxicity than B amyloid (58,59). Interestingly, AChE expression was discovered in cells outside the nervous and hematopoietic systems. Cells that do not normally express or express low levels of AChE exhibit elevated levels of the enzyme when undergoing apoptosis (60). This could either be by promoting or suppressing cell death.

Irregular expression of ACHE has been discovered in a variety of tumour forms, implying that AChE is involved in tumorigenesis (61). It can then be theorised that certain AChE inhibitors may be effective in the treatment of cancers that express high AChE activity (62). In contrast, reduced AChE activity has been discovered in squamous cell carcinoma of the head and neck, chronic lymphoid leukaemia, hepatocellular carcinoma, gastric, lung and prostate cancer It can then also be theorised that using AChE enhancers may be an effective treatment approach for treatment of cancers with AChE decrease (62).

2.7 *In vitro* cytotoxicity determination using MTT assay

Cytotoxicity is the ability of a substance to cause cell death or damage to live cells. Cytotoxicity assays assess how toxic a particular substance, such as a plant extract, drug or chemical compound, can be to living cells (63). Researchers use different cytotoxicity assays to determine the substance's cytotoxicity level, and some substances are more cytotoxic than others (64). This assay is usually required for preclinical screening of potential drugs and plant extracts (65). Generally, most plants are traditionally used to treat different ailments, hence the importance of studying how cytotoxic they can be. Cytotoxicity assays are also carried out to discover new chemotherapeutics for the treatment of cancer (66). This is because cytotoxic agents disrupt nucleic acid and protein production in cancer cells (67). Examples of cytotoxic drugs include alkylating drugs such as treosulfan, cytotoxic antibiotics such as mitoxantrone and vinca alkaloids like vinorelbine (68).

For this study, the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay was used to determine the *in vitro* cytotoxicity of extracts and isolated compounds of *H. argyrosphaerum*. The MTT assay is one of the most widely used colorimetric assays for determining cell densities because it is safe, simple, cost effective and animal cell lines (69). This assay is based on the ability of mitochondrial enzymes in live cells to reduce water soluble yellow tetrazolium salt to its insoluble formazan (70). When the formed formazan is dissolved in acidified isopropyl, it turns purple. The healthy cells absorb purple formazan which exhibits a distinctive absorbance at 570 nm. The intensity of the purple color indicates the vitality of the cells and therefore indicates the cell viability (71).

2.8 HPLC screening of toxic plant extracts

Separation techniques such as high-performance liquid chromatography (HPLC) are used to isolate compounds. HPLC separates components in a mixture depending on their relative affinities for the mobile and stationary phases (72). It is a widely used analytical tool in Chemistry and Biochemistry for the separating, identifying, and quantifying components in a mixture (73). This is because it is extremely sensitive and allows the detection of many metabolites in a single analysis (74). A soluble compound is injected into the mobile phase and passed through a column (that is packed with the stationary phase) at a high pressure. As the mobile phase passes through the column, the different components in the sample interact with the stationary phase at varying degrees, leading to their separation (75). Researchers have widely used HPLC for the screening of toxic plants.

In a study by Mroczek et al. (76), a procedure that combined gradient HPLC with diode array (DAD) and thermabeam electron impact mass spectrometry (EI-MS), was used to screen for toxic pyrrolizidine alkaloids (PAs) in plant samples from several selected plants. Since the identified alkaloids are predicted to have carcinogenic and hepatotoxic properties, consumption of all examined plants was advised to be avoided. In a different study, HPLC-MS/MS was used for the detection of selected metabolites produced by *Penicillium spp.*in walnuts, hazelnuts, almonds, and chestnuts. Forty-one Commercial samples were analysed and the results showed that 71% of these were contaminated by *Penicillium* toxins (77).

2.9 Semi preparative HPLC isolation of compounds

Semi-preparative HPLC is used to isolate and purify compounds in a sample mixture on a small scale. The compounds are separated based on their affinities for the

stationary phase and are collected as fractions from the column. These fractions can then be further purified by repeating the same procedure as many times as required. Semi-preparative HPLC aids in the separation, fractionation, and purification of chemical compounds, making it possible to isolate and characterize potentially toxic compounds present in complex mixtures (78–80).

For example, A recent study used column chromatography and preparative HPLC for the chromatographic separation of phenolic compounds from *N. multifida* leaves and sixteen compounds were isolated, including luteolin-7-O-(3″,6″-di-O-acetyl)-β-D-glucopyranoside, a novel flavonoid. O-glycosides of luteolin and apigenin, rosmarinic acid, salvianolic acids A and B, and schizotenuin A were among the compounds that were identified. Then, it was discovered that the extract and a few of the phenolic compounds could inhibit acetylcholinesterase (81).

Another study used several isolation methods, including preparative HPLC to isolate 11 compounds (one of which is novel) from the ethanol extracts of the roots of *Thalictrum cultratum* and *T. baracalense*. The new compound, dehydrothalflavin, was later found to be an isoquinoline alkaloid (82)

2.10 Chemical Structure Elucidation

One dimensional (1D) and two-dimensional (2D) NMR spectroscopy techniques are some of the most powerful tools used for the elucidation of the chemical structures of molecules. 1D NMR spectroscopy is a technique that reveals details about the chemical environment of magnetically active nuclei in molecules and are plotted as signal strength versus applied frequency. This in turn gives information on the chemical structure and the dynamics of the molecule with the magnetically active nuclei (83). Over the years, 1D NMR has been used for various applications including

identification of organic compounds, study of molecular structure, studying the purity and state of molecules, metabolomics, quantitative NMR, and selective excitation of individual peaks (83–86).

NMR spectra generated through 1D NMR are easy to interpret for simple molecules. However, it is limited to resolving complex molecular structures because of overlapping signals in the spectrum (86). 1D NMR include ^{1}H NMR, which measures the properties of hydrogen nuclei, and ^{13}C NMR, which measures the magnetic properties of carbon nuclei (85). ^{13}C NMR provides detailed information about the carbon atoms in a molecule, such as the number and types of carbon atoms present, and how they are connected to other atoms in a molecule (87). In contrast, ^{1}H NMR is more sensitive than ^{13}C NMR. This is because hydrogen is more abundant in organic molecules compared to carbon (88). Therefore, a combination of these techniques has been used to provide more detailed information about the structure of a molecule.

Since using 1D NMR for structure elucidation includes limited resolution in complex cases, a combination of both 1D and 2D and MR is preferred. This is because 2D NMR such as correlation spectroscopy (COSY) and heteronuclear single quantum correlation (HSQC) provides additional information on the coupling between nuclei allowing for the elucidation of more complex molecular structures (89). When several metabolites are present, these approaches are highly essential in metabolomics because they assist in distinguishing which peaks are related or are part of the same spin system on the same molecule (90). 2D NMR also increases signal dispersion, thereby overcoming spectral overlap in 1D NMR. Hence, it enables the structural elucidation of metabolites that are too complex to be determined by 1D NMR (90).

By using 2D NMR, structure and relative configuration can be established by identifying signals that indicate proximity or connectedness between nuclei that are close together in space or scalarly connected by chemical bonds (86). Two varieties of 2D NMR exist: Homonuclear NMR, in which the transfer of magnetization occurs from one nucleus to another of the same type, usually ^{1}H to ^{1}H, and Heteronuclear NMR, in which transfer of magnetization occurs from one nucleus to another of a different type, typically ^{1}H to ^{13}C. Overall, these techniques include COSY, HSQC, NOESY (Nuclear Overhauser effect spectroscopy), HMBC (Heteronuclear multiple bond correlation), and many more (86,90).

In this study, COSY, HMBC and HSQC 2D NMR techniques were used. COSY is a homonuclear method that involves tracing atomic interactions via strongly linked bonds. Magnetization is transferred via *J* coupling with coupling distances ranging from geminal, vicinal and (rarely) long range coupling (90). HSQC is a heteronuclear 2D NMR technique that measures single bond correlation between the spins of two distinct types of nuclei such as ^{1}H to ^{13}C, thus, it provides information about the connectivity of atoms in a molecule. Similar to HSQC, HMBC is also a heteronuclear NMR technique. However, it measures the correlation between the spins of two distinct types of nuclei that are two to three bonds away from each other, such as ^{1}H to ^{13}C over two bonds.

One application of NMR is that structures of purified metabolites can be confirmed by using both 1D and 2D NMR, commonly ^{1}H, ^{13}C, COSY, HMBC and HSQC techniques (91). In a continuous study on *Lamiaceae* family by Topcu G and Ulubelen A (92) diterpenoids isolated from selected plants were structurally elucidated using 1D and

2D animal techniques, namely ^{1}H, ^{13}C, APT (Attached proton test), DEPT (Distortionless Enhancement by Polarization Transfer), NOESY, COSY, HMBC and HSQC. Most recently, NMR analysis in connection with matrix-assisted laser desorption or ionisation-time-of-light mass spectrometry (MALDI-TOF MS), was used for the illustration of lignin interunit linkages and functionalities. This led to insights on fundamental aspects of lignin polymerization processes (93).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Materials

Merk HPLC grade chemicals (acetonitrile (ACN), DCM and MeOH) were used for the preparation of extracts and for preparation of chromatography compounds. Other chemicals used for preparation of extracts such as hexane and formic acid were analytical grade. High purity milli-Q water was used for chromatography and double distilled water was used for the acetylcholinesterase inhibition and cytotoxicity assay. The acetylcholinesterase inhibitor screening kit was purchased from Sigma-Aldrich (Germany) with a substrate, Ellman's reagent (5,5 -Dithiobis(2-nitrobenzoic acid) (DTNB), and 10× Tris-HCL buffer (PH 10). Purified enzyme AChE and physostigmine were kept at -20℃. HeLa cells were purchased from Cellonex (SA) and maintained in 10% DMEM.

3.2 Instrumentation

A Perkin Elmer Flexar HPLC consisting of a quaternary pump, degasser, autosampler and photodiode array detector was used for HPLC screening. A Chromera software (version 3.34.1.5904) was used for instrument control and for acquiring data. For semi-preparative HPLC, an Agilent 1100 series with of a quaternary pump, degasser, autosampler and a variable wavelength detector was used for isolation. An Agilent ChemStation (Rev.A.10.02[1757]) was used for instrument control and data acquisition. Before any chromatography analysis, all the samples were filtered using Millipore vacuum pump (model WP6122050) (Merck, Germany), Whatman® filter

paper (150 mm) and Millipore MillexHV Hydrophilic PVDF 0.45 µm pore size syringe filters that were purchased from Merck (Germany).

The reverse phased liquid chromatography (RPLC) was performed on a Phenomenex Kinetex C_{18} column with dimensions 150 mm × 4.6 mm and packed with 2.6 µm particles, while semi-preparative HPLC separations were performed on a Supelco Ascentic C_{18} column (250 mm × 10 mm) packed with 5.0 µm particles. The absorbance values of the acetylcholinesterase and cytotoxicity activity assays were measured at 412 nm and 550 nm (respectively) using a Spectra Max M_2 Spectrophotometer. The 50% inhibitory concentrations (IC_{50} values) were computed using Graph pad prism 6 software.

3.3 Sample collection and processing

Ethical clearance (Apendix I) was obtained from the University of Namibia Ethics Committee (REC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. The plant material was collected (research/collection certificate number: RCIV00022018 (Appendix II) from Döbra Farm Plot 46. *Helichrysum argyrosphaerum* flowers were already dry when the plant material was collected. The dried plant materials were ground into powder and stored in separate beakers covered in foil.

3.4 Preparation of extracts

The flowers of *H. argyrosphaerum* were extracted using sequential extractions with solvents of increasing polarity: Hexane, dichloromethane, and methanol. Briefly, according to Van Wyk ((94), 200mL of hexane was added to 10 g of the sample in a 250 mL Erlenmeyer flask and, using a magnetic stirrer, stirred for 24 hours. The flasks were covered with foil and shaken at room temperature. Filtration was then carried out

by using a Buchner funnel. Afterwards, filtrates were transferred into 250 mL round bottom flasks and concentrated using a rotary evaporator. The retained dry plant residue was then added to 200 mL of dichloromethane for the next extraction, using the same procedure.

For storage purposes, the dried extracts of hexane, DCM and MeOH were dissolved and transferred into pre-weighed 4 mL vials and dried using nitrogen gas. Subsequently, the masses of the hexane, dichloromethane and methanol extracts were determined and recorded as 0.3024 g, 0.127 g, and 1.2682 g respectively. The DCM and MeOH extracts were then subjected to chemical characterisation using HPLC and semi preparative HPLC, as well as acetylcholinesterase and cytotoxic activity assays.

3.5 HPLC screening of extracts and determination of fraction purity

A Reversed-Phase Liquid Chromatography (RPLC) method was developed to analyse the MeOH and DCM extracts of *H. argyrosphaerum* flowers. The extracts were prepared at a 10 mg/mL concentration and filtered into pre-weighed 1.8 mL HPLC vials using 0.45 µm nylon syringe filters. A flow rate of 1.00 mL/min and an injection volume of 20 µl was used throughout. These experiments were performed on a Phonemenex Kinetex C_{18} column of length 150 mm and a diameter of 4.6 mm packed with 2.6 µm particles. The mobile phase A consisted of either 5% MeOH or 5% ACN with 0.1% formic acid (FA), while solvent B consisted of either 100% mlMeOH or 100% ACN with 0.1% FA. The column pressure was monitored and kept below 400 bars for all the runs. Data was acquired at 210, 254, 280 and 320 nm.

The purity analysis was done on 100 µL volumes pipetted out of the round bottom flasks with isolated fractions. These were then pipetted into 150 µL glass inserts that

were carefully placed into HPLC vials. The method used to isolate fractions on the semi preparative column was translated onto the analytical column using the equations below to yield a 0-30% B run in 11 mins gradient with a flowrate of 0.84mins.

Equation 1 $$F2 = F1 \times \left(\frac{\pi r_2}{\pi r_1}\right)^2$$

Equation 2 $$F2 = G1 \times \frac{L_2}{L_1}$$

F1 = initial flow rate, F2 = final flow rate, G1 = initial gradient, G2 = final gradient, r_1 *= initial column radius,* r_2 *= final column radius,* L_1 *= initial column length,* L_2 *= final column length* (6).

3.6 Semi preparative HPLC method development

The isolation of selected compounds, tentatively identified in the MeOH extract of the flowers of *H. argyrosphaerum,* was carried out according to previously established protocols (6,95,96), with minor modifications. A 100 mg/mL solution of the sample was prepared and 20 µl of the sample was injected to isolate the compounds at a flow rate of 4 mL/min. The mobile phase consisted of 5% MeOH, 0.1% FA for mobile phase A and 100% MeOH, 0.1% FA for mobile phase B. A 20-min gradient was applied from 30-80% B in 18 mins, 100% B for 1 min and back to the starting conditions for 5 mins. As the sample eluted out of the column, targeted constituents of the sample were collected in pre-weighed round bottom flasks according to their respective peaks. The collected fractions were then concentrated using a rotary evaporator and fully dried using a freeze dryer. The fractions were transferred into pre-weighed vials for compound purity analysis, NMR analysis, acetylcholinesterase inhibition, and cytotoxic activity assays.

3.7 Elucidation of the chemical structures of the isolated compounds

The compounds isolated from *H. argyrosphaerum* were sent to the Central Analytical Facility at Stellenbosch University in South Africa (see the APPENDICES section for all the relevant permits) and NMR experiments were carried out on an Agilent 600 MHZ Inova NMR spectrometer fitted with an inverse detection 5 mm dual channel probe and a pulse field gradient coil. The experiment was carried out according to Masike et al (74). Of the isolated compounds, only compounds **5** (purified, 1.2 mg), **6** (11 mg), and **7** (1 mg) were subjected to NMR analysis. The compounds were dissolved in DMSO-d6, and the ^{1}H and ^{13}C NMR experiments were performed at 25°C. Compound **6** was further analysed using correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and Heteronuclear multiple bond correlation (HMBC) NMR experiments. MestReNova (version 10.0.1-14719) software was used to process the NMR data.

3.8 Acetylcholinesterase assay

The effect of *H. argyrosphaerum* MeOH and DCM flower extracts, and an isolated compound against the AChE enzyme was carried out according to the AChE assay kit provided by Sigma-Aldrich (Germany). Stock solutions of the samples were prepared in dimethyl sulphoxide (DMSO) and diluted to a final concentration of 1 mg/mL for flower extracts and 200µg /mL for the isolated compound. In short, 45 µL of AChE Reference Enzyme was introduced into individual wells. The Assay Buffer and background control wells served as the negative control, while physostigmine, a recognized inhibitor of AChE, functioned as the positive control. DMSO (5 µL) was added to the control wells and one AChE well. Afterwards, 5 µL of the samples were

added to the remaining AChE wells, followed by a 15-min incubation period. Subsequently, 150 µL of a reaction mixture comprising DTNB, substrate, and assay buffer was administered to each well and thoroughly mixed. The absorbance was then measured at 412 nm after 10 min using a SpectraMax M2 spectrophotometer (Molecular devices, USA). This experimental procedure was conducted thrice, with each trial performed in triplicate.

3.9 Cell culture

HeLa (human cervical cancer cells) were obtained from Cellonex (SA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Cellonex (SA)) and supplemented with 10% fetal bovine serum (FBS). The cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

3.10 *In vitro* cytotoxicity assay

The cytotoxicity of the *H. argyrosphaerum* extracts and an isolated compound was assessed using tetrazolium dye (MTT) as described by Liu et al. (97) with minor modifications. In short, HeLa cells were seeded in triplicates in 96 well tissue culture treated plates at 1×10^4 cells per well and incubated with 200 µg/mL of extract and compound concentrations for 48 hours at 37 ℃ in a humidified incubator with 5% carbon dioxide (CO_2). Auranofin was used as a positive control for cytotoxicity, whereas wells with untreated cells, background control, and media only were used as controls for the samples. Acid propanol was used to dissolve formed formazan crystals and absorbance readings were done at 570 nm after 40 mins. Each experiment was repeated four times, with the trials conducted in triplicate. The 50% cytotoxic concentrations (IC_{50} values) of the DCM extract and auranofin were computed using Graph Pad prism 6 software.

CHAPTER 4

Results and Discussion

4.1 Semi preparative HPLC fractionation of the methanol extract of the flowers of *H. argyrosphaerum*

The main aim was to develop a method that provided a good separation of compound peaks. A 30-min gradient (0-100% B in 30 mins) was used and this method resulted in a pressure buildup of up to 400 bars and therefore the run was automatically terminated. This method was then repeated using ACN instead of methanol (5%ACN and 0.1% FA) as mobile phase A, and solvent B consisted of 100% ACN with 0.1% FA. This run was a success (**Figure 4.1**) with well resolved peaks. Therefore, ACN was the mobile solvent of choice. The chromatogram of *H. argyrosphaerum* with peaks tentatively identified by Namate (6) was similar to that obtained in this research by HPLC screening. Therefore, a comparison of the UV data, retention times and overview of the chromatograms lead to identifying which peaks are similar. The peaks were then also numbered according to the 8 major compounds tentatively identified by Namate (6). Subsequently the compounds of interest were isolated from the extract using semi-preparative HPLC. This was done by translating the method used from the analytical column to the semi preparative column.

The 30-min gradient used to screen the extracts was modified to reduce the analysis time and use less solvent during semi preparative HPLC separation. The semi-preparative HPLC column has a larger diameter compared to the analytical column. Therefore, the flow rate used was 4 mL/min, with MeOH as the solvent of choice and the pressure did not exceed 400 bar.

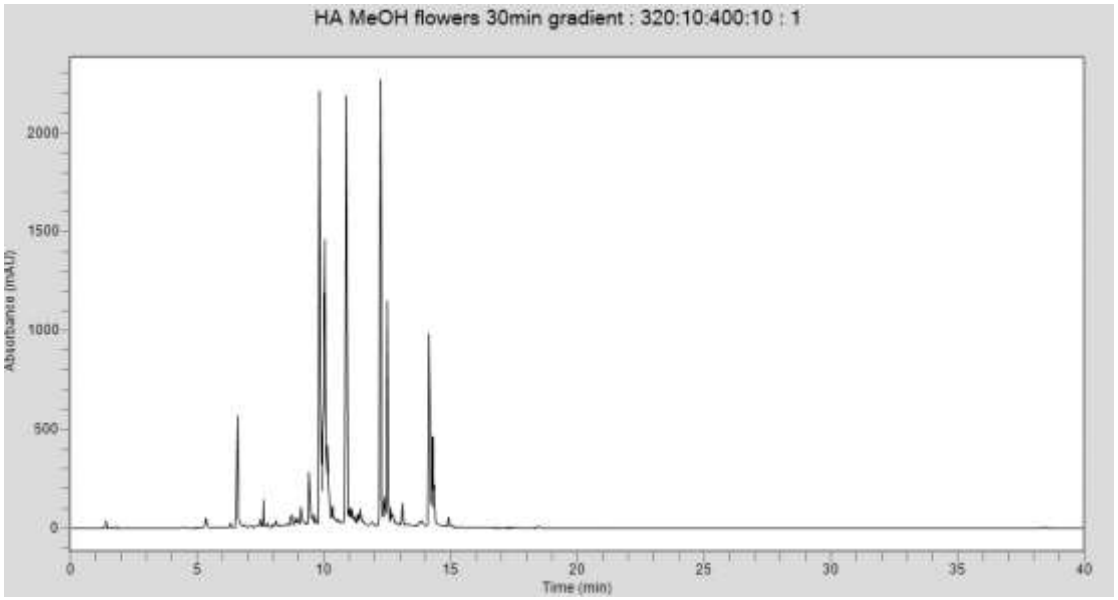


Figure 4.1: HPLC chromatogram obtained at 320 nm for the RPLC analysis of the MeOH extract of the flowers of using a gradient of 0-100% B in 30 mins and a mobile phase containing 0.1 % FA in 5 % ACN (phase A) / 0.1 % FA in 100% ACN (phase B).

In the first analysis, the 30-min gradient (0-100%B) was used with a high injection volume of 100 µL. This analysis yielded extremely broad peaks; therefore, a 20 µL injection volume was used after that. To get the peaks to elute early and reduce analysis time, the gradient was changed to 20-100% B (**Figure 4.2(A)**). The results showed peaks that still eluted from 10 mins. Following these results, the gradient was changed to 30-100% B in 25 mins, and the results were promising, with peaks eluting from 5 mins (**Figure 4.2 (B)**).

Since the isolation of compounds requires repeating the runs multiple times to collect the fractions, there was a need to reduce the analysis time further. This was done by changing the gradient to 30-80% B in 18 mins, and to 100% B from 19-20 mins to remove the rest of the sample, and finally going back to the starting conditions for 5 mins. This method yielded promising results, with the peaks starting to elute from

about 2 mins, and a gradient time of 18 mins (**Figure 4.2 (C)**). Subsequently, 50 portions of a solution of the MeOH flower extract were analysed using the optimized method and the relevant fractions were collected. Since compound **1** was already positively identified as chlorogenic acid by Namate it was not deemed necessary to isolate it. In addition, compound **8** was not clearly visible in the semi-preparative separations **(Figure 4.2)** and therefore only compounds **2** – **7** were isolated. The masses of the resulting isolated fractions are displayed in **Table 4.1**.

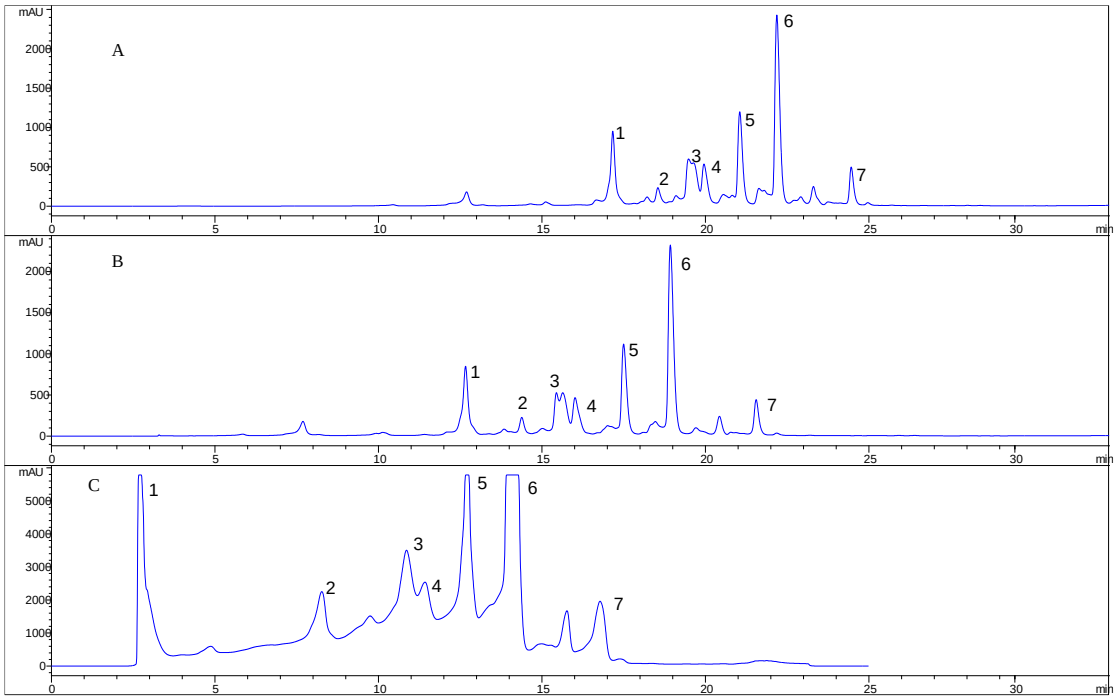


Figure 4.2: Stacked Stacked HPLC-UV chromatograms of the method development from semi preparative isolation of compounds from the MeOH flower extracts of *H. argyrosphaerum.* A gradient of 20-100% B was initially used (A), Followed by a 30-100% B in 25 mins (B). A gradient of 30-80% B in 18 mins, then to 100% B from 19-20 mins (C), was used to isolate compounds.

Table 4.1: Masses of fractions isolated from MeOH extract of *H. argyrosphaerum* flowers.

Fraction[a]	Mass (mg)
2	5.9
3	7.1
4	6.0
5	9.5
6	16.0
7	2.1
8	2.4

[a]Fractions are named according to the compounds that were targeted for isolation

To make sure that the resulting fractions were pure compounds, purity checks were done using the analytical column. The method used on the semi preparative column was translated onto the analytical column using the following fractions to yield a 0-30% B in 11 mins gradient with a flowrate of 0.84 mL/min. This method was subsequently used to analyze the six different fractions, which are presented in **Figures 4.3 & 4.4**.

Out of the six fractions isolated, only fractions 6 and 7 appeared to have pure peaks. Fraction 5 also looked promising as it only had 2 peaks (of compounds **5** and **6**), therefore a purification of this fraction was considered. The other fractions (fractions 2, 3 and 4) had a mixture of many compounds with very low yield **(Table 4.1)**; and therefore, no further analysis or purification was carried out on them **(Figure 4.3.).**

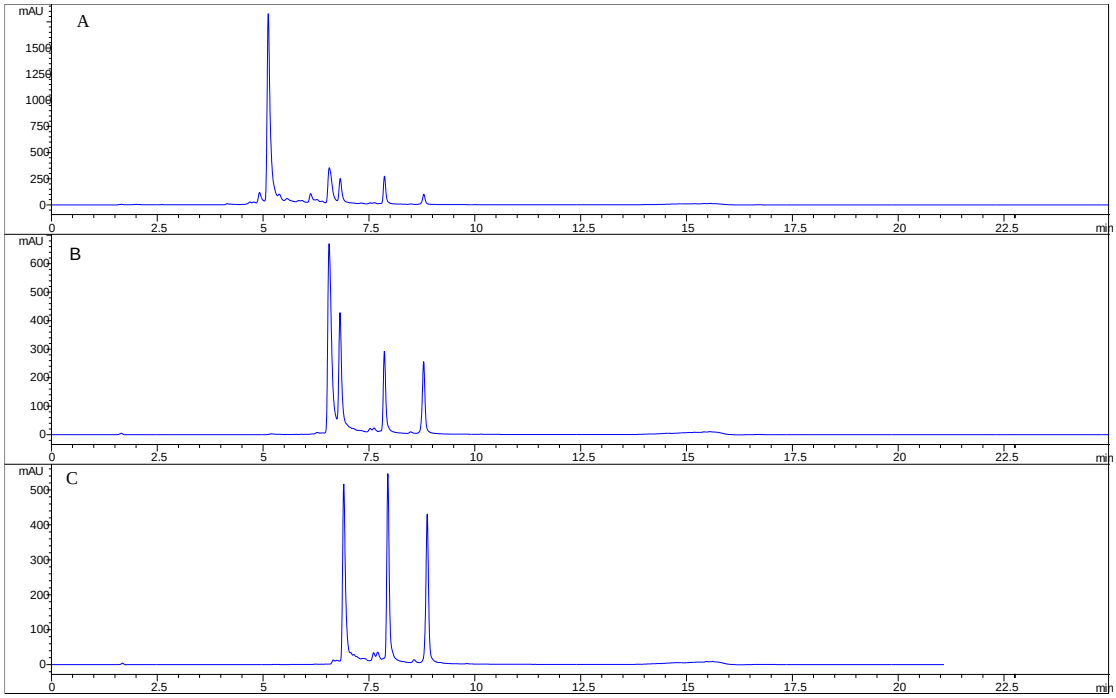


Figure 4.3: Stacked HPLC-UV chromatograms of (A) fraction 2, (B) fraction 3, and (C) fraction 4 isolated from the MeOH flower extract of *H. argyrosphaerum*. A gradient of 30-80% B in 18 mins and changing to 100% B from 19-20 mins was used.

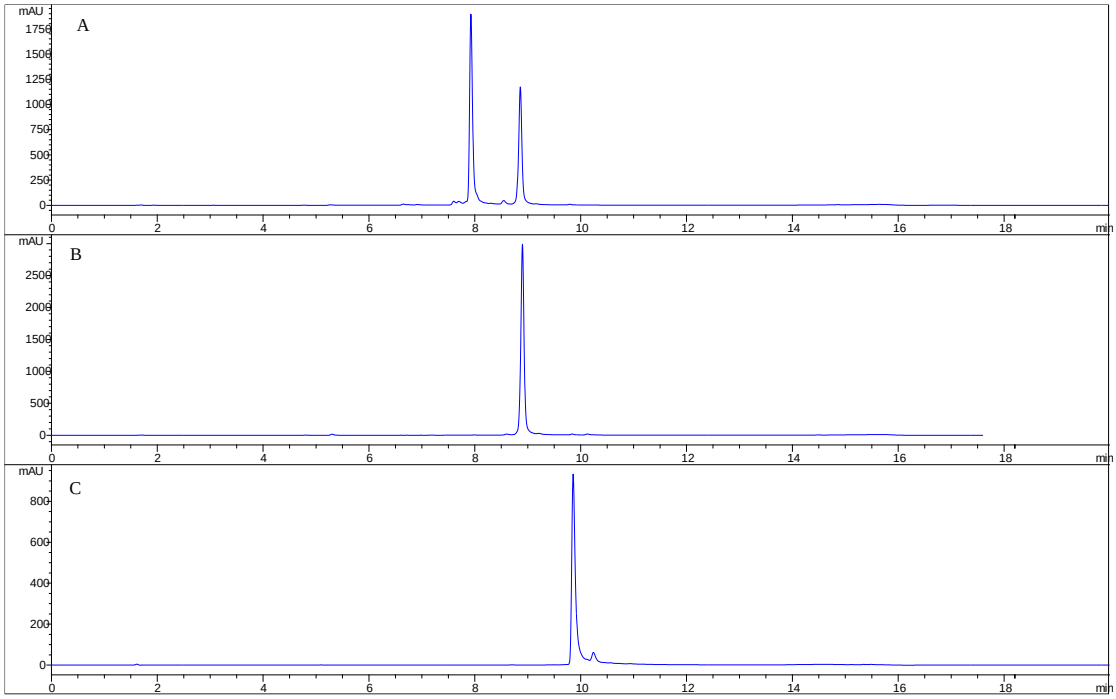


Figure 4.4: Stacked HPLC-UV chromatograms of (A) fraction 5, (B) fraction 6 and (C) fraction 7 isolated from the MeOH flower extract of *H. argyrosphaerum*. A

gradient of 30-80% B in 18 mins and changing to 100% B from 19-20 mins was used.

4.2 Further semipreparative HPLC purification of fraction 5

For further purification of fraction 5, an isocratic method of 50% A and 50% B with an injection volume of 5µL was used to try to get a better separation between compounds **5** and **6**. This resulted in broad peaks that were tailing, as shown in **Figure 4.5 (A).** To resolve this, a gradient method with a relatively flat gradient was employed, i.e., 0-60% B (10 µL injection volume). This method resulted in poorly separated peaks with longer retention times **Figure 4.5 (B)**. With the aim of reducing the analysis time and improving separation, a gradient method with an isocratic step was developed instead, i.e., 0-50% B in 6 mins, and remained there for 17 mins. Due to a pressure spike observed in the trial analysis (results not shown), the flow rate was reduced to 3.5 mL/min. This method yielded a good separation as shown in **Figure 4.5 (C)** and was therefore used to purify fraction 5. Subsequently, 37 portions of a fraction 5 solution were analyzed using the optimized method, resulting in the collection of fractions 5' and 6'. The isolated fractions had masses of 1.2 mg and 1.1 mg, respectively. Utilizing the analytical method, the purity of these fractions was determined to be 78.2% and 72.9% respectively (**Figures 4.6** and **Figure 4.7**).

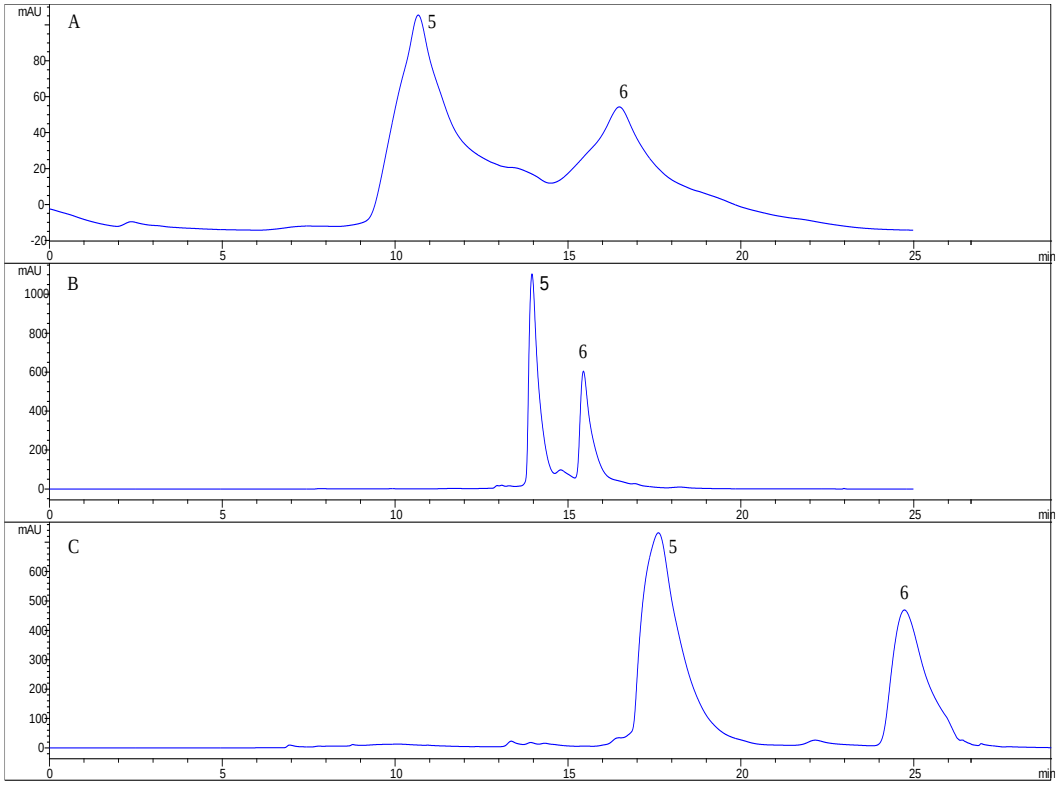


Figure 4.5: HPLC chromatogram of fraction 5 purification method development. In A, an isocratic method of 50% B in 20 mins. The peaks in (B) are a result of a 0-60% B gradient in 20 mins. a combination of an isocratic and gradient method was used in (C), with 0-50% B in 6 to 23 mins and then going back to 0% B for 7 mins, The method used in the purification of compound **5.**

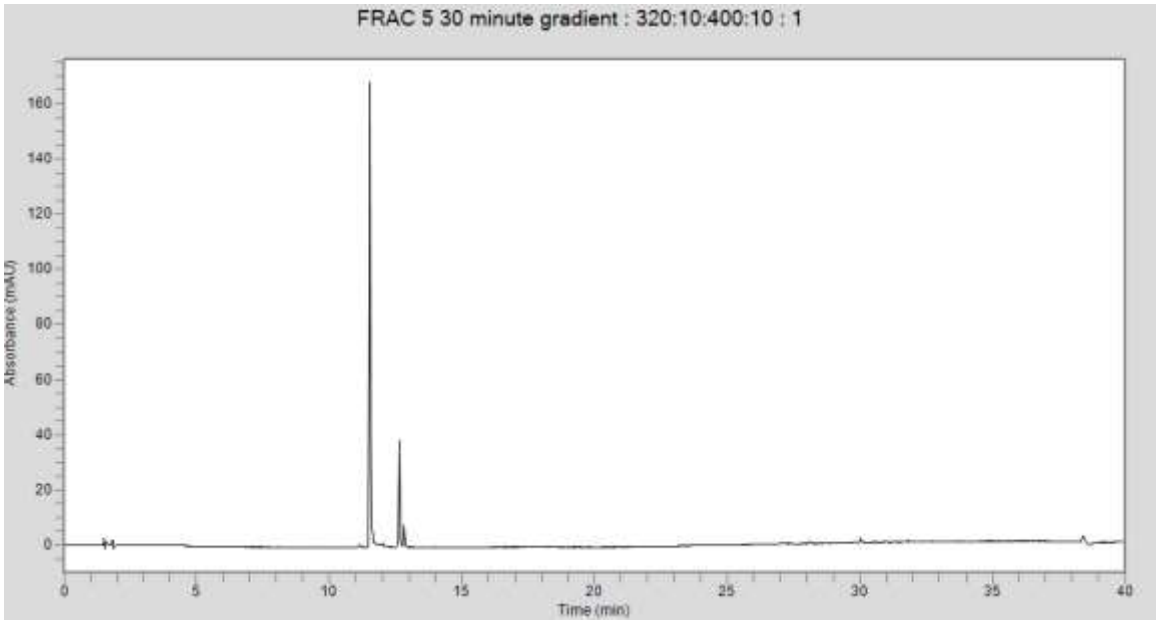


Figure 4.6: HPLC Fraction 5' purity chromatogram after purification of fraction 5 by using a combination of an isocratic and gradient method with 0-60% B in 6 to 23 mins and then going back to 0% B for 7 mins, using a 20µl injection. The purity of this fraction was determined to be 78.2%.

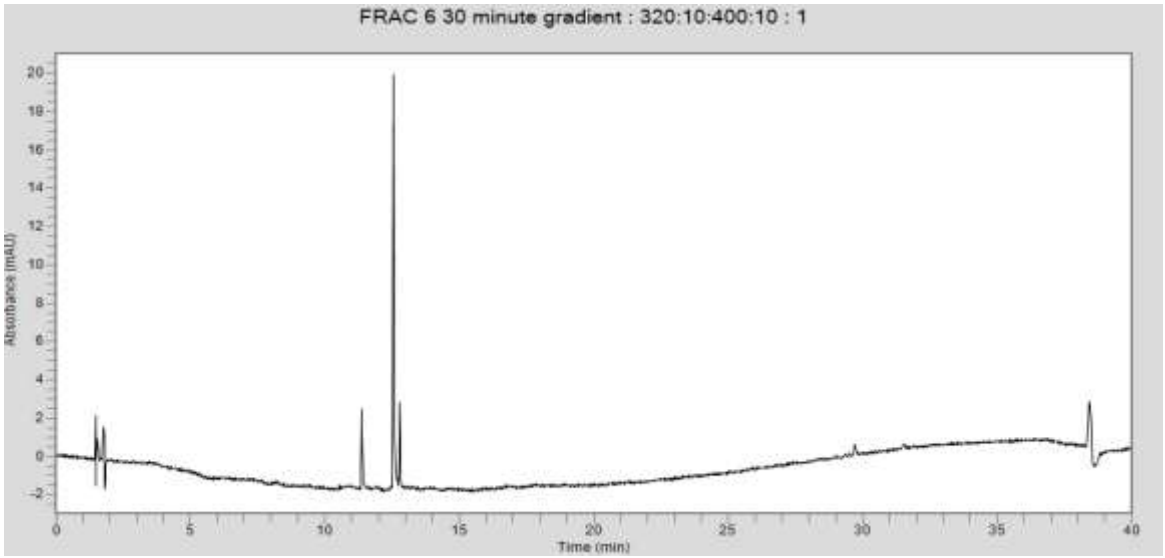


Figure 4.7: HPLC Fraction 6' chromatogram after purification of fraction 5 by using a combination of an isocratic and gradient method with 0-60% B in 6 to 23 mins and then going back to 0% B for 7 mins, using a 20 µL injection. The purity of this fraction was determined to be 72.9%.

4.3 Elucidation of the chemical structures of compounds 5, 6 and 7.

Studies done on the genus *Helichrysum* reported that secondary metabolites from the genus can be categorized structurally as flavonoids and chalcones, phenolic acids, terpenes and essential oils, pyrones, benzofurans (bitalin esters) and phloroglucinols (98). The isolated compounds that were successfully elucidated were found to be flavonoids, with each compound having an apigenin moiety.

Compound **6** was previously tentatively identified as an apigenin 7-O-glycoside, with a molecular formula of $C_{29}H_{30}O_{15}$ (6). The presence of an apigenin moiety is evident in the ^{1}H-NMR spectrum of compound **6 (Figure 4.8)**. Two doublets in the aromatic region, each integrating for 2 protons (indicating the presence of a characteristic *para*-disubstituted ring) could be assigned to H-3', H-5' (δ 6.95, d, J = 8.5) and H-2', H-6' (δ 7.96, d, J=8.9), in the B-ring of apigenin. Hence, the doublets observed in the region below 7 ppm could be assigned to H-8 (δ 6.47, d, J = 4.2) and H-6 (δ 6.82, d, J = 8.5) that are part of the A ring of apigenin. The remaining proton resonance in this region of the spectrum can be assigned to H-3 (δ 6.88, s). In addition, the hydroxyl protons observed at δ 10.38 (s,1H) and δ 13.00 (s,1H) could be assigned to OH groups in the C-4' and C-5 positions, respectively, supporting the suggestion that it is a 7-O substituted apigenin moiety.

In the research article by Tschan G (95), most hydroxyl groups were not identified in the NMR spectra. Similarly, not all hydroxyl groups were identified for compounds 5 and 6 in this study. Difficulties in identifying hydroxyl groups or their absence in ^{1}H NMR data can be attributed to factors such as pH, temperature, and the nature of the solvent, which significantly influence the resolution of phenol OH signals. Under certain conditions, phenol OH signals can be obscured, making it challenging to identify all hydroxyl groups in complex polyphenol natural products (99). Additionally,

some OH groups may be difficult to detect due to overlapping signals, rapid chemical exchange with the solvent (DMSO-d6), or other intermolecular interactions (99,100).

In the literature, the closest analogue to compound **6** is chamaemeloside (**Figure 4.13**), reported by Tschan et al (95). The difference in molecular formula, i.e., C_2H_3O, infers an extra acetyl group to compound **6**. The ^{13}C, ^{1}H NMR, supported by COSY, HSQC and HMBC data, was relatively similar to that of chamaemeloside. This was also verified by comparing the spectra of compound **6** with the ^{1}H NMR and ^{13}C NMR spectra of chamaemeloside predicted using MestReNova. The ^{1}H NMR of compound **6** (**Figure 4.8**), carbons and protons assigned to the apigenin structure are presented in **Figure 4.9** and **Table 4.2.**

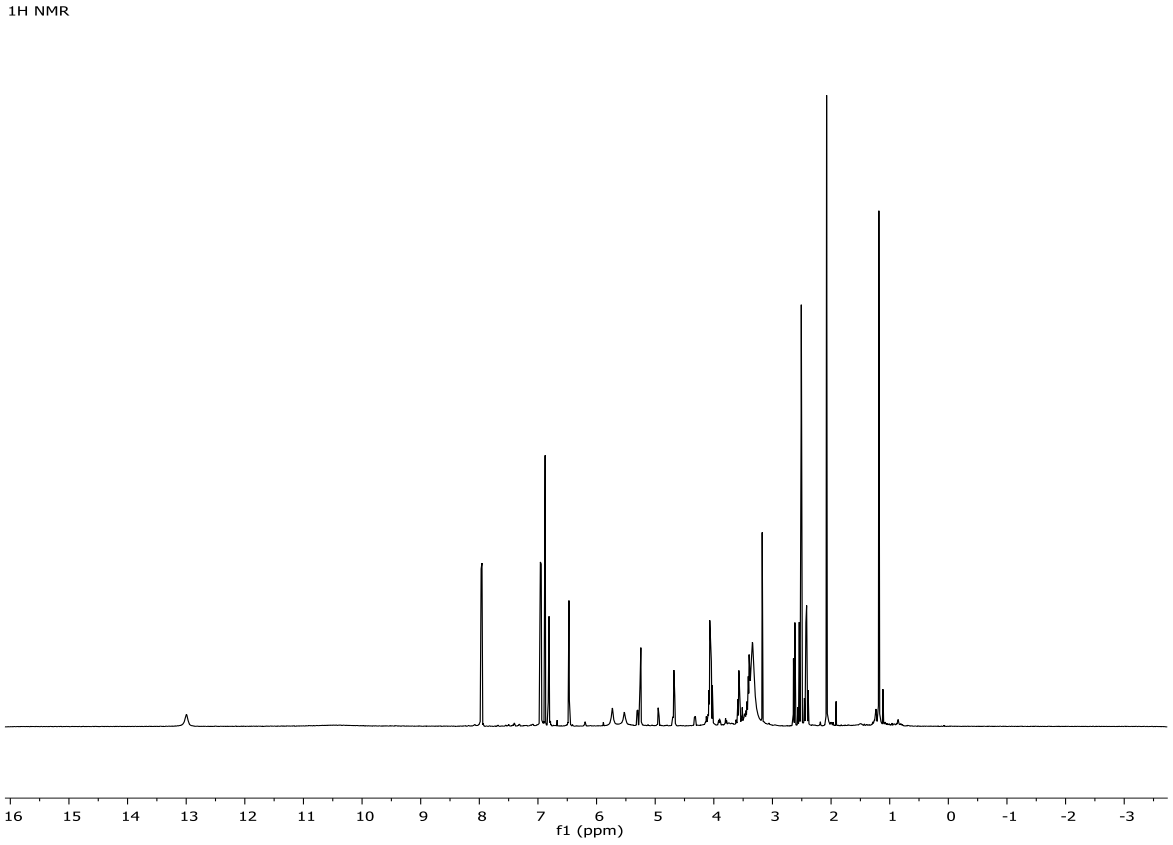


Figure 4.8: ^{1}H NMR of compound **6** isolated from the MeOH flower extracts of *H. argyrosphaerum.*

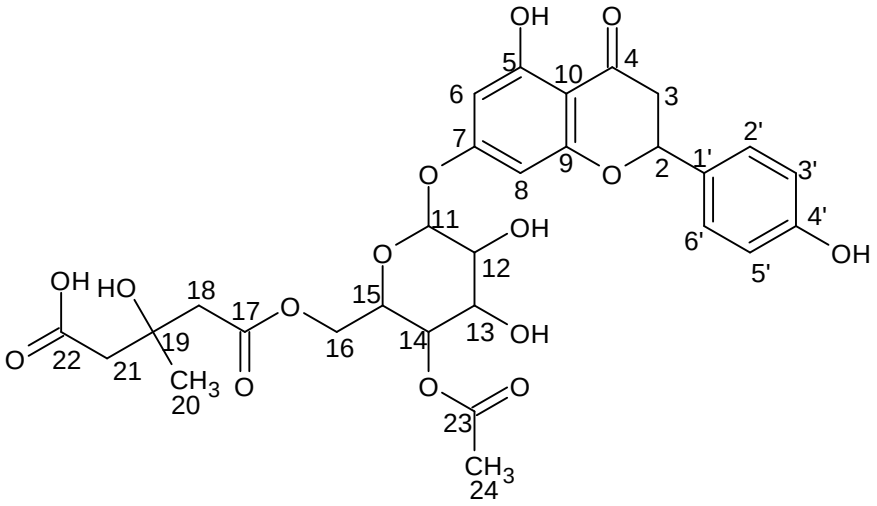


Figure 4.9 Compound **6**, 14-acetyl chamaemeloside.

Table 4.2 : ^{1}H and ^{13}C chemical shifts for compound **6** (δ at 600 MHz) in DMSO-d_6 at 25℃

Position	δ_H	δ_C	OH
1	-	-	
2	-	164.35	
3	6.88	103.16	
4	-	182	
5	-	161.21	13
6	6.82	94.47	
7	-	162.50	
8	6.47	99.34	
9	-	156.94	
10	-	105.52	
11	5.24	99.23	
12	3.39	72.85	5.72
13	3.56	73.28	5.52
14	4.67	70.55	
15	4.05	71.14	
16	4.04	61.96	
17		170.26	
18	2.61, 2.53	45.24	
19		68.66	
20	1.18	27.42	
21	2.42, 2.63	45.09	
22		172	
23		169.72	
24	2.07	20.80	

1’	-	121.48	
2’	7.95	128.56	
3’	6.95	115.92	
4’	-	161.40	10.38
5’	6.95	115.92	
6’	7.95	128.56	

The chemical shifts of the carbons ranging from δ 61.96 – δ 73.28 as well as the resonance at δ 99.23 in the ^{13}C-NMR spectrum of compound **6** are reminiscent of a hexose sugar, with the latter resonance corresponding to the anomeric carbon, C-11. The ^{1}H NMR spectra displayed 6 hydrogen signals between δ 3.39 – 5.24 ppm (δ 5.24 ppm being the resonance of the anomeric proton) that were assigned to carbons C-11 – C-16 through the HSQC data (**Figure 4.12**) of compound **6**. The COSY data also confirmed correlation of δ 4.67 (t, *J* = 9.5 Hz, 1H) with both δ 3.57 (t, *J* = 9.2 Hz, 1H) and δ 4.05 (s, 1H), as well as correlation between δ 3.57 and δ 3.38 (m, 1H) (**Figure 4.10**). This confirms the presence of a glucose moiety, similar to the one present in the structure of chamaemeloside.

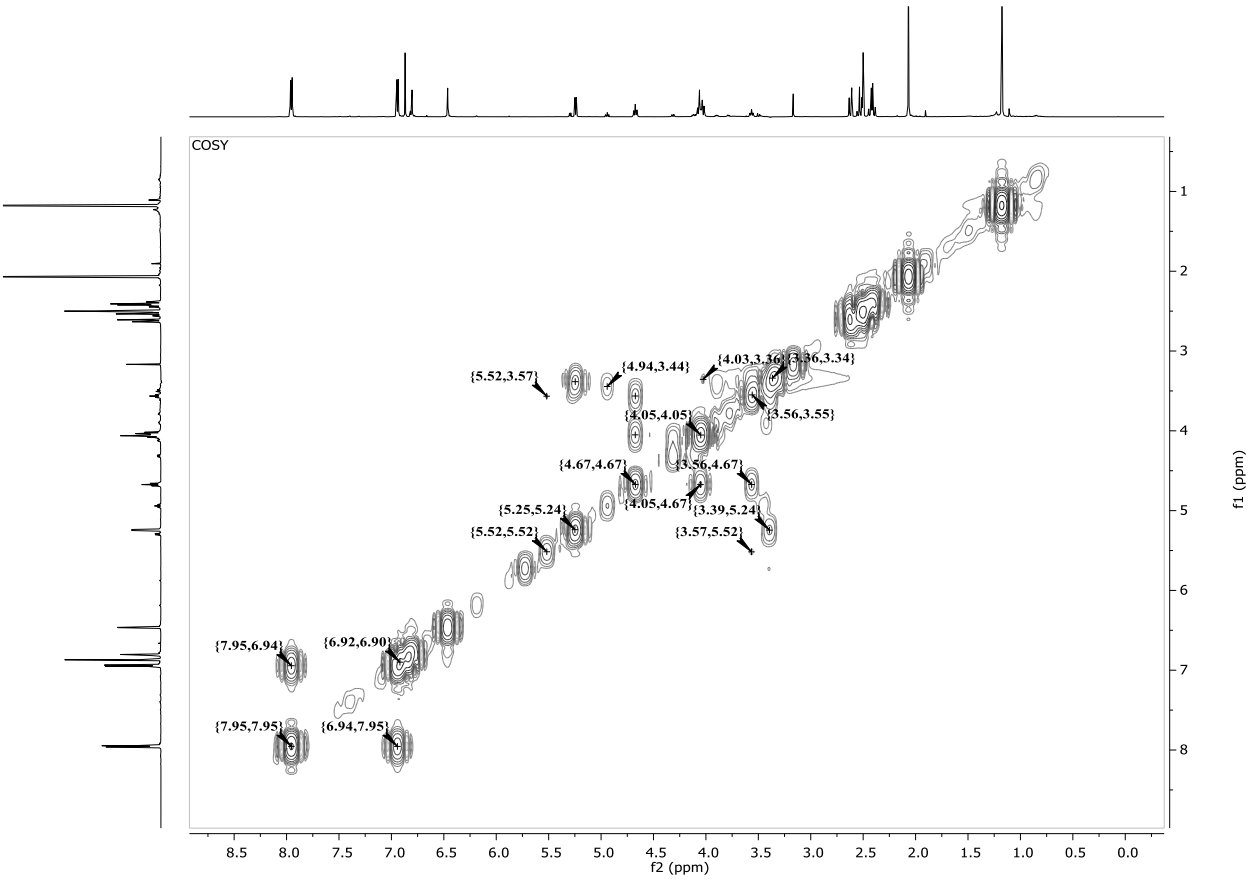


Figure 4.10: COSY correlations for compound **6** in DMSO-d_6 at 25℃

The remaining ^{13}C NMR signals indicated the presence of two carbonyl groups, one for a free acid (172 ppm) and an ester function (170.26 ppm), a tertiary alcohol (68.66 ppm), two methylene groups (45.24, 45.09 ppm) and a methyl group (27.42 ppm). These groups resembled the 3-hydroxy-3-methyl-glutaryl acid moiety that is also part of the structure of chamaemeloside (96). This was also confirmed by the HMBC data (**Figure 4.11**).

The remaining two carbons were found to be an ester carbonyl (169.74 ppm), and a methyl group (20.80 ppm). The HMBC experiment revealed a correlation of both H-14 (δ 4.67, t, J = 9.5 Hz, 1H) and the methyl group protons, H-24 (δ 2.07) coupled with the carbon resonance at δ 169.74 ppm. Therefore, the carbonyl (169.74 ppm), and methyl groups (20.80 ppm) were deduced to be part of an acetyl group at position C-

14. In conclusion, the novel molecular structure of compound **6** was determined as 14-acetyl chamaemeloside (**Figure 4.8**).

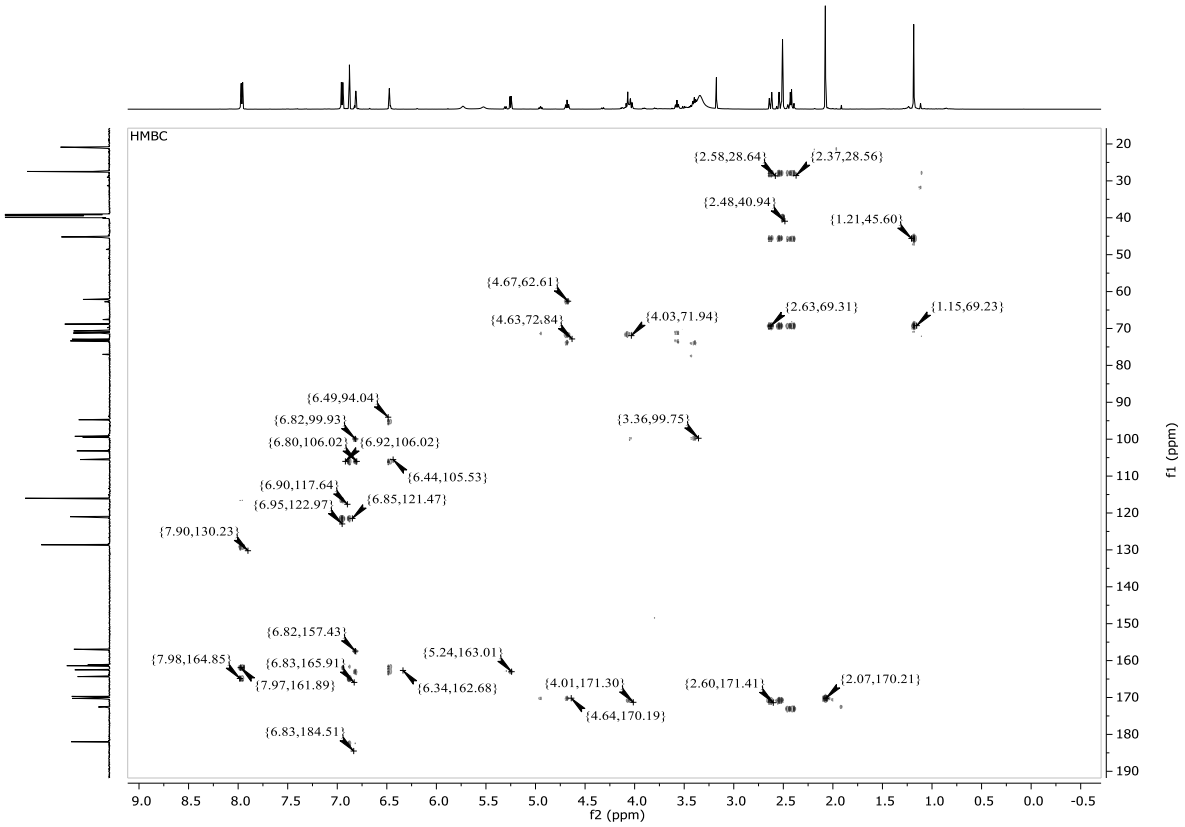


Figure 4.11: HMBC correlations for compound **6** in DMSO-d_6 at 25℃

The HSQC experiment correlated all the proton resonances with those of each corresponding carbon, and their multiplicity (**Figure 4.13**). The experiment displayed 18 correlations that are in line with the proposed structure of compound **6**. The remaining carbons were assigned with the use of long-range proton-carbon correlations in the HMBC experiment.

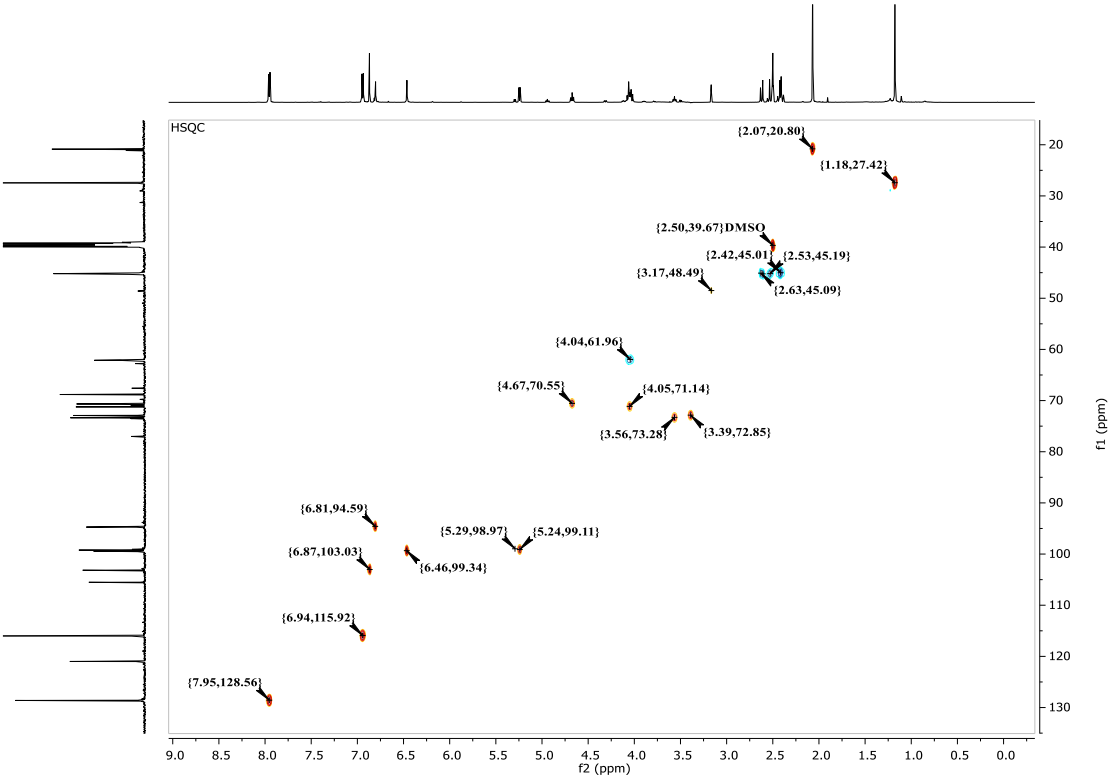


Figure 4.12: HSQC correlations for compound **6** in DMSO-d_6 at 25°C

Compound **5** was previously tentatively identified as chamaemeloside (6). The ^{1}H NMR spectrum (**Table 4.4**) of compound **5** displayed the characteristic signals of apigenin nucleus: aromatic protons at H- 3', H-5' (δ 7.95, d, *J* = 8.4 Hz, 2H), and H-2', H-6' (δ 6.95, d, *J* = 8.4 Hz, 2H, and a hydroxyl group at δ 10.21 that shows the presence of the C ring structure. The two signals at H-6 (δ 6.43, S, 1H) and H-8 (δ 6.85 (S, 1H) indicate the presence of the A ring. The ^{1}H NMR spectrum of compound **5** also revealed the anomeric proton of glucose moiety at 5.09 (s, 1H), showing the configuration of glucose, with the remaining glucose protons appearing in the range of δ 3.52–4.37 ppm. The ^{13}C NMR spectrum showed an ester carbonyl resonance at δ_C 165.7, and a set of signals from 157.58-164.65 ppm that represent carbonyls present in the structure, a set of carbon signals present in the glucose moiety at δ 69.41-76.68 ppm and a methyl group at δ 28.46 ppm. Other ^{1}HNMR and ^{13}C NMR are

assigned as shown in **Table 4.4**. These signals confirm that compound **5** is indeed chamaemeloside.

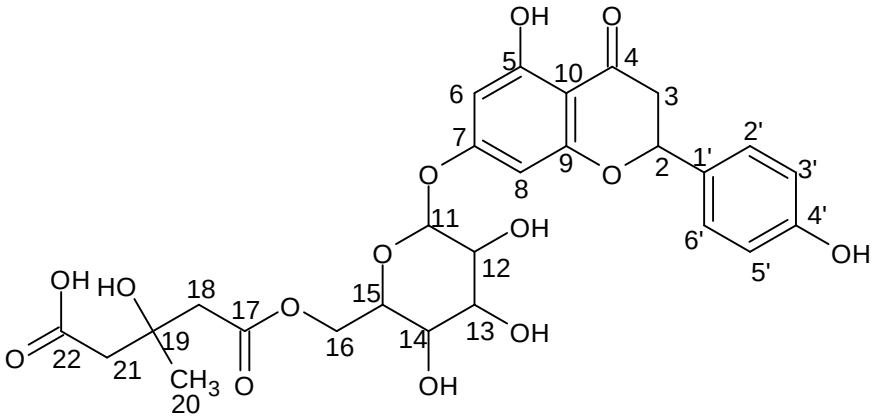


Figure 4.13: Chamaemeloside (compound **5**).

Table 4.3: ^{1}H and ^{13}C chemical shifts for compound **5** (δ at 600 MHz) in DMSO-d_6 at 25℃

Position *δ*H	***δ*H**	***δ*C**	***OH***
2	-	164.58	
3	6.77	100.13	
4	-	182.24	
5	-	(n.d.)	12.99
6	6.17	99.46	
7	-	163.16	
8	6.46	94.54	
9	-	157.58	
10	-	103.71	
11	5.08	76.60	
12	3.13-3.52	69.41	
13	3.52	73.47	
14	4.34	70.18	
15	4.01	74.48	
16		63.56	
17		171.05	
18/21	2.27, 2.18	46.62	
19	-	70.25	
20	1.13	28.46	

22	-	174.64	10.21
1’	-	121.48	
2’	6.94	129.02	
3’	7.94	116.51	
4’	-	161.87	8.42
5’	7.94	116.51	
6’	6.94	129.02	

(n.d) not detected due to low concentration.

Compound **7** was previously tentatively identified by Namate (6) as apigenin. The ^{1}H NMR of compound **7** revealed doublets at δ 7.96 – 7.90 (2H) and δ 7 6.97ppm and a distinct hydroxyl (H-4’) at δ 10.67 ppm. This indicates the presence the aromatic C ring. Furthermore, a hydroxyl (H-5) at δ 12.96 (s), two doublets δH at H-8 (δ 6.46, d, J = 2.1 Hz, 1H) and H-6 (δ 6.17, d, J = 2.1 Hz, 1H) indicates the presence of the A -ring.

The 13C NMR of compound **7** also displayed spectra similar to that of apigenin, and assignments of carbons to protons are based on the chemical shifts and comparison to ^{1}H NMR and ^{13}C NMR of apigenin predicted by MestReNova and from literature (101). The ^{13}C -NMR spectrum exhibited the presence of δC (161.90, 99.46,165.44, and 94.54) for the A -ring, δC (103.21, 182.09, 103.86, 161.90, and 164.) for the B -ring, and δC (116.46, 121.59, 130.56, and 165) for the C -ring of apigenin. These assignments are summarized in **Table 4.5**.

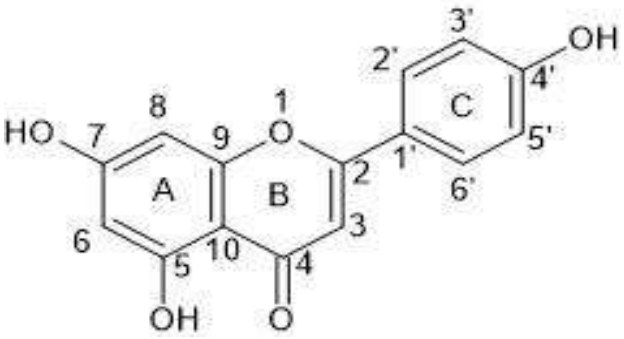


Figure 4.14 The structure of apigenin (compound **7**)

Table 4.4: ^{1}H and ^{13}C chemical shifts for compound **7** (δ at 600 MHz) in DMSO-d_6 at 25℃.

Position *δ*H	***δ*H**	***δ*C**	***OH***
2	-	164	
3	6.77	103.21	
4	-	182.09	
5	-	161.90	12.96
6	6.17	99.46	
7	-	165.44	8.42
8	6.46	94.54	
9	-	103.86	
10	-	161.90	
1’	-	121.59	
2’	6.94	130.56	
3’	7.95	116.46	
4’	-	165	10.67
5’	7.95	116.46	
6’	6.94	130.56	

4.4 Acetylcholinesterase activity

The *in vitro* AChE activity of the MeOH and DCM flower extracts of *H. argyrosphaerum*, as well as isolated compound **6** was assessed and results depicted in

Figures 4.15 & 4.16. Physostigmine, a well-known AChE inhibitor, was included as a standard control. Physostigmine inhibited AChE, having an AChE activity of 0.8% at the highest tested concentration of 1000 µg/mL. Although the DCM extract showed a 91% AChE activity, it was the only sample to show any inhibitory activity against AChE **(Figures 4.15 & 4.16)**. The MeOH extract exhibited AChE activity of 127%, which implies an increase in AChE activity. Compound **6** was tested at 0.2 mg/mL and had an AChE activity of 105%, as shown in **Figure 4.16**. The increase in AChE activity by the MeOH flower extract of *H. argyrosphaerum* could be attributed to compound **6** and other compounds present in the plant.

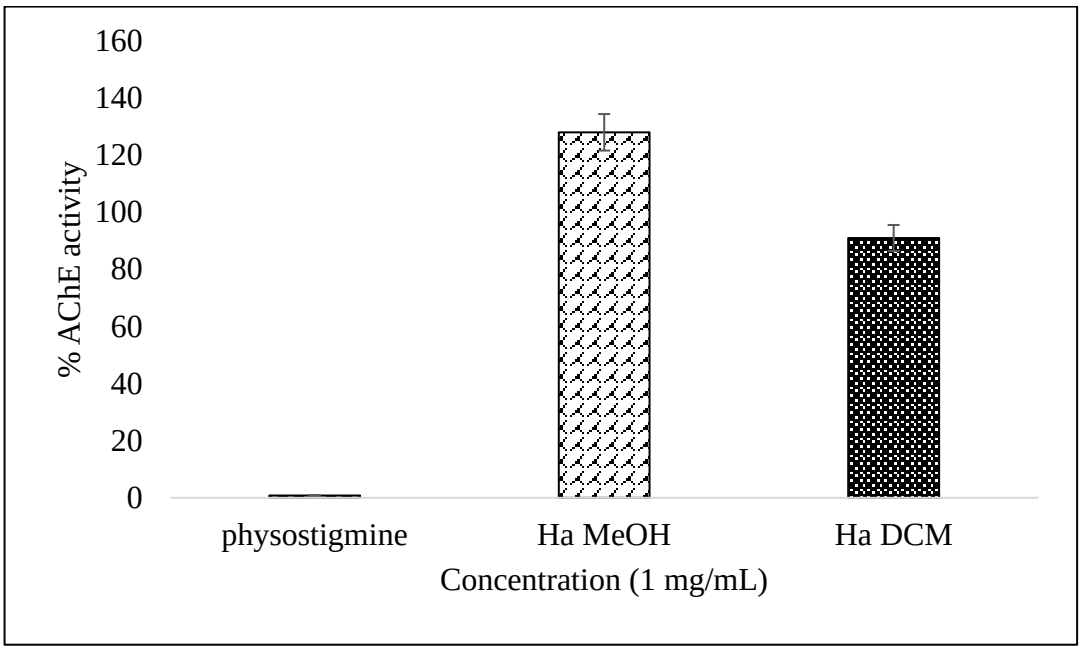


Figure 4.15: % AChE activity of *H. argyrosphaerum* MeOH and DCM flower extracts with physostigmine as a standard control.

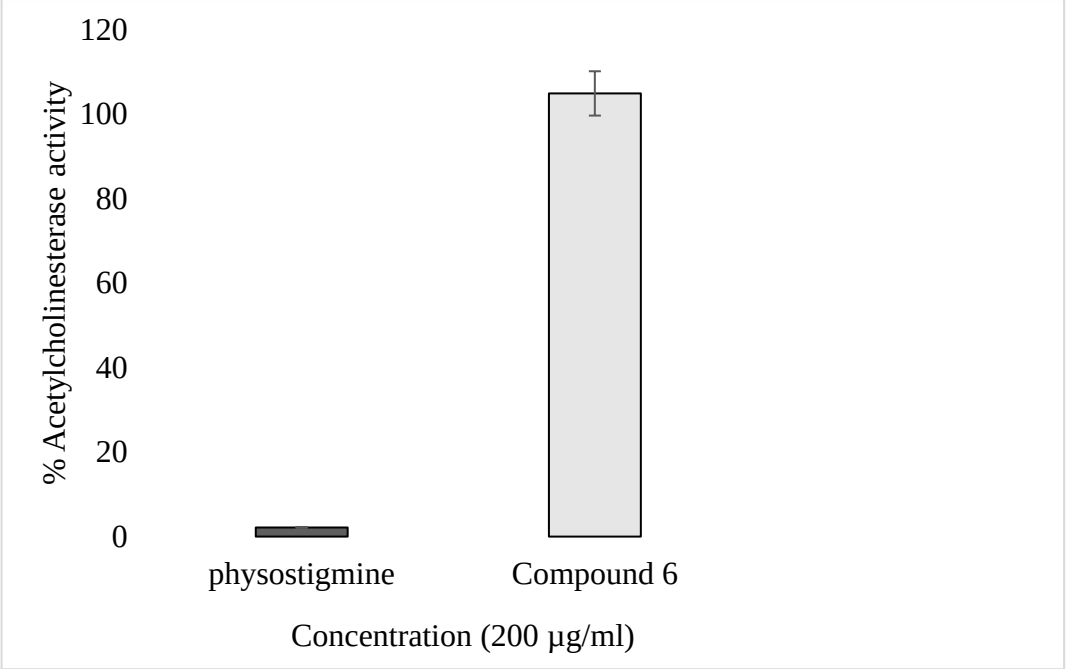


Figure 4.16: % AChE activity of compound **6** isolated from *H. argyrosphaerum* flower extract. Physostigmine was used as a standard control.

Increased AChE activity has been studied and reported in several studies (58,102,103). In this study, the MeOH flower extract from *H. argyrosphaerum* increased acetylcholinesterase (AChE) activity. This enhancement could be due to the presence of allosteric activators in the extract, which bind to AChE at sites other than the active site, inducing conformational changes that boost enzyme activity (104).

Increased ACh breakdown as a result of elevated AChE activity results in a cholinergic deficit, which is linked to typical symptoms including memory impairments, disorientation, hypo- or hyperactivity, ataxia, decreases in heart and muscular power and tone, reduced exocrine secretions (57). Some of the symptoms exhibited by cattle and sheep poisoned by high consumption of *H. argyrosphaerum* are consistent with excess AChE activity, namely ataxia, disorientation, and hypotonus (muscle weakness). ACh deficiency is one of the features of AD, and is responsible for the

majority of the symptoms, including memory loss and as well as a decline in cognition (105).

4.5 Colorimetric MTT (tetrazolium) assay

The *in vitro* cytotoxic impact of MeOH and DCM flower extracts derived from *H. argyrosphaerum*, as well as the isolated compound **6**, was assessed through the utilization of the MTT assay against the HeLa cell line. Auranofin, a known cytotoxic compound, was used as a positive control against the HeLa cell line and showed CC_{50} value of 2.468 ± 0.0065 µg/mL. The percentage growth inhibition on HeLa cells was found to be increasing as the concentration of samples increased. HeLa cells treated with the MeOH flower extract at the highest tested concentration of 200 µg/mL had a survival rate of more than 50% as indicated in **Figure 4.17**. The results showed that the MeOH extract of *H.argyrosphaerum* flowers were relatively nontoxic towards HeLa cells after an incubation period of 48h.

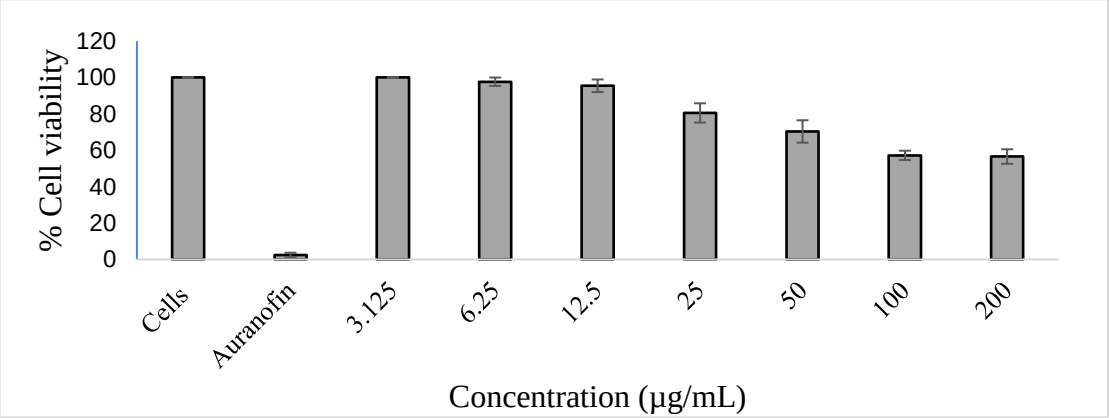


Figure 4.17: *Helichrysum argyrosphaerum* MeOH flower extract cytotoxic effect on HeLa cell line. Cells only wells and Auranofin were used as controls for this experiment.

HeLa cells were exposed to *H. argyrosphaerum* DCM flower extract at a concentration of 200 µg/mL, and the cells displayed low viability of 26.58% as depicted in **Figure**

4.18. At the lowest concentration of 3.125 µg/mL, DCM extract had more than 80% cell viability after 48 hours of exposure. The *H. argyrosphaerum* DCM extract had a CC50 of 60.94 +- 8.05 µg/mL. CC50 values less than 20 µg/mL indicate cytotoxicity, 21-40 µg/mL indicate minimal cytotoxicity, and higher than 41 µg/mL indicate non-cytotoxicity (106). Therefore, the DCM extract is considered to be nontoxic towards HeLa cells. Although the DCM extract exhibited higher cytotoxicity in comparison to the MeOH and DCM extract, it had a CC50 higher than 41 µg/mL.

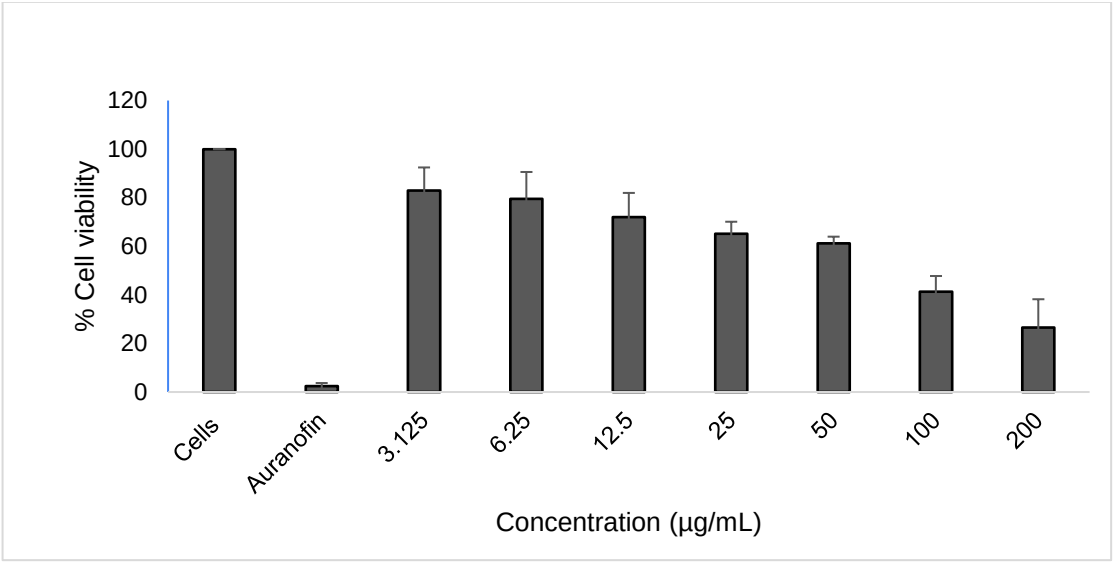


Figure 4.18: *Helichrysum argyrosphaerum* DCM flower extract cytotoxic effect on HeLa cell line at various concentrations from 200 µg/mL. The DCM extract was more cytotoxic in comparison to the MeOH extract and the isolated compound, with a CC50 of 60.94 +- 8.05 µg/mL.

The isolated compound showed the lowest cytotoxicity against the HeLa cell line in comparison to the MeOH and DCM extracts. It was found that at the maximum concentration tested of 100 µg/mL, cell viability was determined to be 86.07% a depicted in **Figure 4.19**. Low cytotoxicity against HeLa cells does not indicate safety

for grazing animals. It suggests that the toxic compounds in the plant may not be specifically harmful to the HeLa cells in vitro. Therefore, the response of animal cells to the plant compound may differ from that of HeLa cell. For instance, Acetate extracts from 16 plants whose leaves are known to be harmful to cattle were tested for cytotoxicity using brine shrimp lethality assay. Thirteen of these plants were found to have no lethal effect against the brine shrimp larvae, although these plants are poisonous to animals.

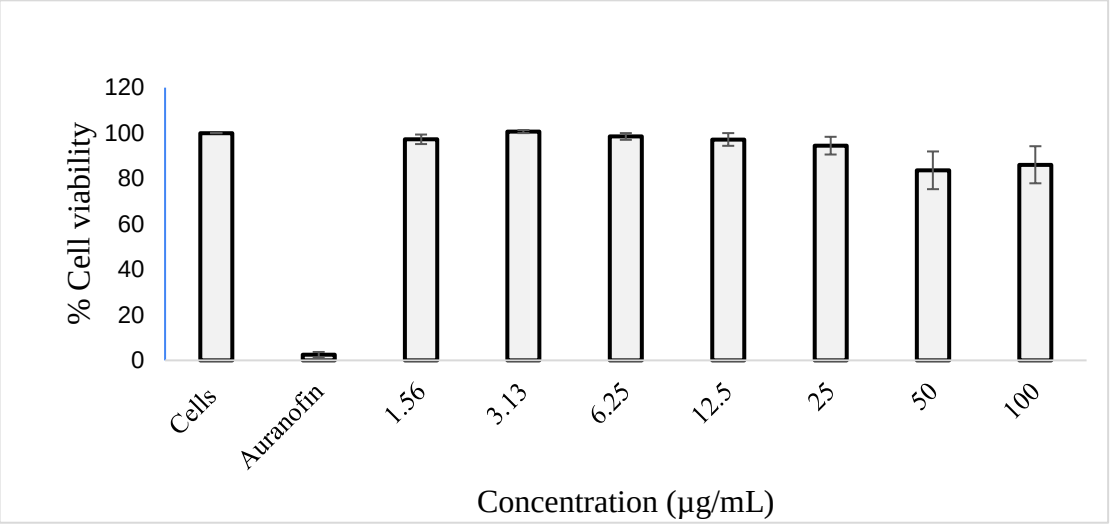


Figure 4.19: The cytotoxic effect of the compound isolated from *H. argyrosphaerum* on HeLa cell line at various concentrations from 100 µg/mL. Unlike the *H.argyrosphaerum* MeOH and DCM flower extracts, the isolated chemical displayed the low cytotoxicity against HeLa cell line.

The current study is the first to report on the cytotoxic activity of *H. argyrosphaerum* MeOH and DCM flower extracts, as well as the isolated compound on HeLa cells. According to Heyman (107), various species of *Helichrysum* (water–methanol extracts), including *H. acutatum, H. callicomum, H. erbaceum,* and *H. psilolepsi*, have been reported to exhibit low cytotoxicity against Vero cells. In a similar study, Oyetunde et al. (108) reported the low cytotoxicity of *H. acutatum* against tumor cell

lines Caco-s and HepG2, and the standard cell line Hek-2-293. Interestingly, another study by Lourens et al (109), reported *H. acutatum* to be cytotoxic against the cancerous cell line, MCF-7. This suggests the cytotoxic effects of samples may vary depending on the cell line used and the type of extracts used. This could be a result of selective cytotoxicity. Therefore, further cytotoxicity assays on different cell lines need to be conducted on the *H. argyrosphaerum* flower extracts as well as isolated compounds.

CHAPTER 5

CONCLUSION

This research delved into the chemical characterization of *H. argyrosphaerum* MeOH and DCM flower extracts, employing HPLC and semi-preparative HPLC to isolate three pure compounds. Through thorough analysis via 1D and 2D NMR, the chemical structures of these compounds were elucidation, namely apigenin, chamaemeloside, and a novel compound, 14-acetyl chamaemeloside. Further exploration of the MeOH and DCM extracts, alongside 14-acetyl chamaemeloside, revealed high acetylcholinesterase activity and noncytotoxic effects towards HeLa cells. Increased acetylcholinesterase (AChE) activity could potentially contribute to the toxicity of *H. argyrosphaerum* in cattle and sheep. The isolated compound also displayed high AChE activity, raising questions about its contribution to the elevated AChE levels in the flower extracts.

This study not only contributes valuable insights into the chemical composition of *H. argyrosphaerum* but also highlights the potential role of AChE in the observed toxic effects, paving the way for future investigations into the intricate relationship between the isolated compounds and the overall toxicity of this plant.

CHAPTER 6

RECOMMENDATIONS

Further research is recommended to expand the investigation into the broader toxicological effects of the identified compounds on various cell lines and animal models. This includes evaluating additional cytotoxic pathways and the overall safety profiles of both the extracts and isolated compounds, as well as assessing other potential cytotoxic pathways to ensure comprehensive safety evaluation. Additional research efforts should prioritize the identification of specific compounds accountable for the observed acetylcholinesterase (AChE) activity in both the methanol (MeOH) and dichloromethane (DCM) extracts. Furthermore, there is a need for a deeper understanding of how elevated AChE activity potentially contributes to animal poisoning induced by *H. argyrosphaerum*. Most research surrounding AChE focuses on its inhibition rather than induction. Extracts and compounds may show an increase in AChE activity, making AChE inhibitor controls unsuitable for interpreting activation levels. Therefore, it is important to have a control that enhances AChE activity in addition to an AChE inhibitor control.

REFERENCES

1. Botha CJ, Penrith ML. Poisonous plants of veterinary and human importance in southern Africa. J Ethnopharmacol. 2008 Oct 28;119(3):549–58.
2. Abdeta D. Identification of Poisonous Plants and Their Toxics Effects on Livestock in Horo Buluk District, Horo Guduru Wollega Zone, Oromia Regional State, Western Ethiopia. Biomed J Sci Tech Res. 2019 Dec 2;23(3).
3. Jaeschke H. Acetaminophen Hepatotoxicity: Not as Simple as One Might Think! Introductory Comments on the Special Issue—Recent Advances in Acetaminophen Hepatotoxicity. Livers. 2022 Jul 1;2(3):105–7.
4. Louise Clapham. Helichrysum argyrosphaerum wild everlasting. Wolwedans Herbarium (NRNR). 2001;
5. Schubert Sonja, Marais Anna, Mannheimer Coleen. Toxic plants of veterinary importance in Namibia. Gamsberg Macmillan Publishers; 2006.
6. Namate TM. Characterisation of major metabolites of wild everlasting, Helichrysum argyrosphaerum. Windhoek; 2017.
7. Mathekga ADM. Antimicrobial activity of *Helichrysum argyrosphaerum* and the isolation of a new phloroglucinol from *Helichrysum caespititium*. Pretoria; 2001.
8. Ortmann JM. Investigation of the toxic compounds in the poisonous plant Dipcadi glaucum, The wild onion. [Windhoek]: University of Namibia; 2020.
9. Van Wyk HM, Schoeman D, Kwembeya E, Hans RH, Pool EJ, Louw S. In vitro acetylcholinesterase inhibitory activity and chemical composition of the dichloromethane extracts of the leaves and inflorescences of the tumbleweed, Trachyandra laxa (N.E.Br.) Oberm. var. rigida (Suesseng.) Roessl. South African Journal of Botany. 2022 Jul 1;147:231–7.

10. Tjatindi C. Farming amidst poisonous plants... Tjijeura rises above gifblaar limitations. The New Era. 2021 Sep 28;
11. Wendt S, Lübbert C, Begemann K, Prasa D, Franke H. Poisoning by Plants. Dtsch Arztebl Int. 2022 May 6;119(Forthcoming):317–24.
12. Green BT, Gardner DR, Pfister JA, Welch KD, Bennett GL, Cook D. The effect of alkaloid composition of larkspur (Delphinium) species on the intoxication of Angus heifers1. J Anim Sci. 2019 Mar 1;97(3):1415–23.
13. Pfister JA, Panter KE, Manners GD, Cheney CD. Reversal of tall larkspur (Delphinium barbeyi) poisoning in cattle with physostigmine. Vet Hum Toxicol. 1994 Dec;36(6):511–4.
14. Green BT, Gardner DR, Stonecipher CA, Lee ST, Pfister JA, Welch KD, et al. Larkspur Poisoning of Cattle: Plant and Animal Factors that Influence Plant Toxicity. Rangelands. 2020 Feb;42(1):1–8.
15. Ehlert K. South Dakota State University Extention. 2022. Poisonous plants on Rangelands : Larkspur and poisonvetch.
16. Lawrence N, Stephenson M. BeefWatch newsletter. 2023. Why larkspur is dangerous for cattle and how to manage it.
17. Ralphs MH, Turner DL, Mickelsen L V, Evans JO, Dewey SA. Herbicides for Control of Tall Larkspur (Delphinium barbeyi). Weed Sci [Internet]. 1990;38(6):573–7. Available from: http://www.jstor.org.ezproxy.unam.edu.na/stable/4045077
18. Abad MJ, Bedoya LM, Bermejo P. Chapter 14 - Essential Oils from the Asteraceae Family Active against Multidrug-Resistant Bacteria. In: Rai MK, Kon KV, editors. Fighting Multidrug Resistance with Herbal Extracts, Essential Oils and Their Components [Internet]. San Diego: Academic Press; 2013. p.

205–21. Available from: https://www.sciencedirect.com/science/article/pii/B9780123985392000148

19. Dinić J, Novaković M, Pešić M. Potential for cancer treatment: natural products from the Balkans. In: Biodiversity and Biomedicine. Elsevier; 2020. p. 137–59.

20. Akinfenwa AO, Sagbo IJ, Makhaba M, Mabusela WT, Hussein AA. Helichrysum Genus and Compound Activities in the Management of Diabetes Mellitus. Plants (Basel). 2022 May 23;11(10).

21. Ranaivoarisoa RH, Ralambonirina STR, Randriamialinoro F, Randrianasolo R, Ratsimbason M, Ranarivelo LR. Antiplasmodial Activity of the Extracts and Flavonoids Isolated from *Helichrysum gymnocephalum* Humbert (Asteraceae) from Madagascar. In 2020. p. 171–8.

22. Süzgeç-Selçuk S, Birteksöz AS. Flavonoids of Helichrysum chasmolycicum and its antioxidant and antimicrobial activities. South African Journal of Botany. 2011 Jan;77(1):170–4.

23. Furlan V, Bren U. Helichrysum italicum: From Extraction, Distillation, and Encapsulation Techniques to Beneficial Health Effects. Foods. 2023 Feb 13;12(4):802.

24. Matić IZ, Aljančić I, Žižak Ž, Vajs V, Jadranin M, Milosavljević S, et al. In vitro antitumor actions of extracts from endemic plant Helichrysum zivojinii. BMC Complement Altern Med. 2013 Dec 18;13(1):36.

25. Botha CJ, Venter E. University of Pretoria, Faculty of Veterinary Science. 2002. Plants poisonous to livestock.

26. Basson PA, Kellerman TS, Albl P, Von Maltitze LJF, Miller ES, Welman WG. BLINDNESS AND ENCEPHALOPATHY CAUSED BY HEL!CHRYSUM

ARGYROSPHAERUMDC. (COMPOSITAE) IN SHEEP AND CATTLE. Vol. 42, Onderstepoort J. vet. R es. 1975.

27. Hyde MA WBTBPCPM. Flora of Caprivi : Species information: Helichrysum argyrosphaerum. 2014.

28. Ullah A, Munir S, Badshah S, Khan N, Ghani L, Poulson B, et al. Important Flavonoids and Their Role as a Therapeutic Agent. Molecules. 2020 Nov 11;25(22).

29. Birt D, Jeffery E. Flavonoids. Adv Nutr. 2013 Sep 1;4(5):576–7.

30. Dias MC, Pinto DCGA, Silva AMS. Plant Flavonoids: Chemical Characteristics and Biological Activity. Molecules. 2021 Sep 4;26(17).

31. Kočevar Glavač N, Glavac I, Kreft S. Flavonoids. Farm Vestn. 2007 Dec 1;58:145–8.

32. Uriarte-Pueyo I, I. Calvo M. Flavonoids as Acetylcholinesterase Inhibitors. Curr Med Chem. 2011 Dec 1;18(34):5289–302.

33. Ademosun AO, Oboh G, Bello F, Ayeni PO. Antioxidative Properties and Effect of Quercetin and Its Glycosylated Form (Rutin) on Acetylcholinesterase and Butyrylcholinesterase Activities. J Evid Based Complementary Altern Med. 2016 Oct 8;21(4):NP11–7.

34. Kim JY, Lee WS, Kim YS, Curtis-Long MJ, Lee BW, Ryu YB, et al. Isolation of Cholinesterase-Inhibiting Flavonoids from Morus lhou. J Agric Food Chem. 2011 May 11;59(9):4589–96.

35. Xu ML, Bi CWC, Kong AYY, Dong TTX, Wong YH, Tsim KWK. Flavonoids induce the expression of acetylcholinesterase in cultured osteoblasts. Chem Biol Interact. 2016 Nov;259:295–300.

36. Liu EYL, Xu ML, Xia Y, Kong X, Wu Q, Dong TTX, et al. Activation of G protein-coupled receptor 30 by flavonoids leads to expression of acetylcholinesterase in cultured PC12 cells. Chem Biol Interact. 2019 Jun;306:147–51.

37. Srividhya R, Gayathri R, Kalaiselvi P. Impact of epigallo catechin-3-gallate on acetylcholine-acetylcholine esterase cycle in aged rat brain. Neurochem Int. 2012 Apr;60(5):517–22.

38. Rezg R, Mornagui B, El-Fazaa S, Gharbi N. Caffeic acid attenuates malathion induced metabolic disruption in rat liver, involvement of acetylcholinesterase activity. Toxicology. 2008 Aug;250(1):27–31.

39. Anwar J, Spanevello RM, Thomé G, Stefanello N, Schmatz R, Gutierres J, et al. Effects of caffeic acid on behavioral parameters and on the activity of acetylcholinesterase in different tissues from adult rats. Pharmacol Biochem Behav. 2012 Dec;103(2):386–94.

40. Xie Y, Yang W, Chen X, Xiao J. Inhibition of flavonoids on acetylcholine esterase: binding and structure-activity relationship. Food Funct. 2014 Oct;5(10):2582–9.

41. Kumar V, Kundu S. Studies on Apigenin and Its Biological and Pharmacological Activity in Brain Disorders. Adv Pharm Bull. 2022 Aug;12(4):645–8.

42. Allemailem KS, Almatroudi A, Alharbi HOA, AlSuhaymi N, Alsugoor MH, Aldakheel FM, et al. Apigenin: A Bioflavonoid with a Promising Role in Disease Prevention and Treatment. Biomedicines. 2024 Jun 18;12(6):1353.

43. Kashyap D, Sharma A, Tuli HS, Sak K, Garg VK, Buttar HS, et al. Apigenin: A natural bioactive flavone-type molecule with promising therapeutic function. J Funct Foods. 2018 Sep;48:457–71.

44. Salehi B, Venditti A, Sharifi-Rad M, Kręgiel D, Sharifi-Rad J, Durazzo A, et al. The Therapeutic Potential of Apigenin. Int J Mol Sci. 2019 Mar 15;20(6).

45. Brownie CF. Plants poisonous to animals . MSD Veterinary manual. Rahway: Merck & Co; 2020.

46. DeRango-Adem EF, Blay J. Does Oral Apigenin Have Real Potential for a Therapeutic Effect in the Context of Human Gastrointestinal and Other Cancers? Front Pharmacol. 2021 May 18;12.

47. Sylwia Goławs, Iwona Lukasik, Artur Golawski, Ireneusz Kapusta, Bogdan Janda. Alfalfa (*Medicago sativa* L.) Apigenin Glycosides and Their Effect on the Pea Aphid (*Acyrthosiphon pisum*). Pol J Environ Stud [Internet]. 2010;19(5):913–9. Available from: http://www.pjoes.com/Alfalfa-Medicago-sativa-L-Apigenin-Glycosides-r-nand-Their-Effect-on-the-Pea-Aphid,88465,0,2.html

48. Li K, He Z, Wang X, Pineda M, Chen R, Liu H, et al. Apigenin C-glycosides of Microcos paniculata protects lipopolysaccharide induced apoptosis and inflammation in acute lung injury through TLR4 signaling pathway. Free Radic Biol Med. 2018 Aug;124:163–75.

49. Babaei F, Moafizad A, Darvishvand Z, Mirzababaei M, Hosseinzadeh H, Nassiri-Asl M. Review of the effects of vitexin in oxidative stress-related diseases. Food Sci Nutr. 2020 Jun 14;8(6):2569–80.

50. Ali F, Rahul, Naz F, Jyoti S, Siddique YH. Health functionality of apigenin: A review. Int J Food Prop. 2017 Jun 3;20(6):1197–238.

51. Sezen Karaoğlan E, Hancı H, Koca M, Kazaz C. Some Bioactivities of Isolated Apigenin-7-O-glucoside and Luteolin-7-O-glucoside. Applied Sciences. 2023 Jan 23;13(3):1503.

52. Trang A, Khandhar PB. Physiology, Acetylcholinesterase. 2023.

53. Colović M, Krstić D, Lazarević-Pašti T, Bondžić A, Vasić V. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol. 2013 May;11(3):315–35.

54. Adeyinka A, Kondamudi N. StatPearls. 2023. Cholinergic Crisis.

55. Li S, Li A, Zhao J, Santillo M, Xia M. Acetylcholinesterase Inhibition Assays for High-Throughput Screening. Methods Mol Biol. 2022;2474:47–58.

56. Sigma-Aldrich. Acetylcholinesterase inhibitor screening kit. 2018.

57. Zujalovic B, Mayer B, Hafner S, Balling F, Barth E. AChE-activity in critically ill patients with suspected septic encephalopathy: a prospective, single-centre study. BMC Anesthesiol. 2020 Dec 17;20(1):287.

58. Soreq H, Yirmiya R, Cohen O, Glick D. Acetylcholinesterase as a window onto stress responses. In 2005. p. 585–608.

59. Goshen I, Yirmiya R. Interleukin-1 (IL-1): A central regulator of stress responses. Front Neuroendocrinol. 2009 Jan;30(1):30–45.

60. Zhang XJ, Yang L, Zhao Q, Caen JP, He HY, Jin QH, et al. Induction of acetylcholinesterase expression during apoptosis in various cell types. Cell Death Differ. 2002 Aug 16;9(8):790–800.

61. Liu T, Zhang B, Gao Y, Zhang X, Tong J, Li Z. Identification of ACHE as the hub gene targeting solasonine associated with non-small cell lung cancer (NSCLC) using integrated bioinformatics analysis. PeerJ. 2023 Oct 10;11:e16195.

62. Lazarevic-Pasti T, Leskovac A, Momic T, Petrovic S, Vasic V. Modulators of Acetylcholinesterase Activity: From Alzheimer's Disease to Anti-Cancer Drugs. Curr Med Chem. 2017 Oct 25;24(30).

63. Kuete V, Karaosmanoğlu O, Sivas H. Anticancer Activities of African Medicinal Spices and Vegetables. Medicinal Spices and Vegetables from Africa: Therapeutic Potential Against Metabolic, Inflammatory, Infectious and Systemic Diseases. 2017 Jan 1;271–97.

64. Davey R. News-medical [internet]. 2021. What is cytotoxicity?

65. Cordier W, Steenkamp V. Evaluation of Four Assays to Determine Cytotoxicity of Selected Crude Medicinal Plant Extracts In vitro. Br J Pharm Res. 2015 Jan 10;7(1):16–21.

66. Nemati F, Dehpouri AA, Eslami B, Mahdavi V, Mirzanejad S. Cytotoxic properties of some medicinal plant extracts from mazandaran, iran. Iran Red Crescent Med J. 2013 Nov;15(11):e8871.

67. Frangou EA, Bertsias G, Boumpas DT. Cytotoxic-Immunosuppressive Drug Treatment. In: Systemic Lupus Erythematosus. Elsevier; 2016. p. 533–41.

68. Cytotoxic and immunosuppressant drugs. In: Meyler's Side Effects of Drugs. Elsevier; 2016. p. 800–8.

69. Nga NTH, Ngoc TTB, Trinh NTM, Thuoc TL, Thao DTP. Optimization and application of MTT assay in determining density of suspension cells. Anal Biochem. 2020 Dec;610:113937.

70. Kapewangolo TP. Lamiaceae plant extracts and isolated compounds demonstrate activity against HIV/AIDS. 2013.

71. Bahuguna A, Khan I, Bajpai VK, Kang SC. MTT assay to evaluate the cytotoxic potential of a drug. Bangladesh J Pharmacol. 2017 Apr 8;12(2).

72. Crozier A, Moritz T. Physico-chemical methods of plant hormone analysis. New Comprehensive Biochemistry. 1999 Jan 1;33(C):23–60.

73. Raja PMV BA. Rice University. 2022. High Performance Liquid Chromatography.

74. Masike K, Villiers A, Hoffman E, Brand D, Causon T, Stander M. Detailed Phenolic Characterization of Protea Pure and Hybrid Cultivars by Liquid Chromatography-Ion Mobility-High Resolution Mass Spectrometry (LC-IM-HR-MS). J Agric Food Chem. 2019 Dec 5;68:485–502.

75. Koester V. What is HPLC. European chemical societies publishing. 2016;

76. Mroczek T, Baj S, Chrobok A, Glowniak K. Screening for pyrrolizidine alkaloids in plant materials by electron ionization RP-HPLC-MS with thermabeam interface. Biomedical Chromatography. 2004 Nov;18(9):745–51.

77. Spadaro D, Meloni GR, Siciliano I, Prencipe S, Gullino ML. HPLC-MS/MS Method for the Detection of Selected Toxic Metabolites Produced by Penicillium spp. in Nuts. Toxins (Basel). 2020 May 8;12(5):307.

78. Fornstedt T, Forssén P, Samuelsson J. Modeling of preparative liquid chromatography. In: Liquid Chromatography. Elsevier; 2017. p. 573–92.

79. Pauli GF, Chen SN, Friesen JB, McAlpine JB, Jaki BU. Analysis and Purification of Bioactive Natural Products: The AnaPurNa Study. J Nat Prod. 2012 Jun 22;75(6):1243–55.

80. Surve NS, Thomas AB, Bhole RP, Patil CY. Flash Chromatography and Semi-Preparative HPLC: Review on the Applications and Recent Advancements over the Last Decade. Eurasian Journal of Chemistry. 2023 Mar 27;109(1):20–30.

81. Kashchenko NI, Olennikov DN. FLAVONOIDS AND LIGNANS FROM THE LEAVES OF NEPETA MULTIFIDA (LAMIACEAE) AND THEIR BIOACTIVITY. chemistry of plant raw material. 2022 Jun 10;(2):85–94.

82. Xue JJ, Li JY, Li BJ, Jin YT, Wang CH, Xue CM, et al. [Isoquinoline alkaloids from two species of Thalictrum genus plants]. Zhongguo Zhong Yao Za Zhi. 2022 May;47(10):2676–80.

83. Craik DJ, Horne J, Scanlon MJ. Multidimensional Nuclear Magnetic Resonance of Biomolecules. In: Encyclopedia of Analytical Chemistry. Wiley; 2008.

84. Lipfert M, Rout MK, Berjanskii M, Wishart DS. Automated Tools for the Analysis of 1D-NMR and 2D-NMR Spectra. In 2019. p. 429–49.

85. Rubim de Santana PI, Diz de Almeida JSF, França TCC, Junker J. Quantitative NMR Interpretation without Reference. J Anal Methods Chem. 2022 Nov 10;2022:1–6.

86. Kiraly P, Kern N, Plesniak MP, Nilsson M, Procter DJ, Morris GA, et al. Single-Scan Selective Excitation of Individual NMR Signals in Overlapping Multiplets. Angewandte Chemie International Edition. 2021 Jan 11;60(2):666–9.

87. Tekely P, Reichert D, Zimmermann H, Luz Z. Initial Conditions for Carbon-13 MAS NMR 1D Exchange Involving Chemically Equivalent and Inequivalent Nuclei. Journal of Magnetic Resonance. 2000 Aug;145(2):173–83.

88. Edison AS, Le Guennec A, Delaglio F, Kupče Ē. Practical Guidelines for 13C-Based NMR Metabolomics. In 2019. p. 69–95.

89. Féraud B, Govaerts B, Verleysen M. Assessing the repeatability and statistical advantages of homonuclear 2D-NMR spectra: an innovative clustering approach. In 2013. Available from: https://api.semanticscholar.org/CorpusID:108424409

90. Burgess K, Rankin N, Weidt S. Metabolomics. In: Handbook of Pharmacogenomics and Stratified Medicine. Elsevier; 2014. p. 181–205.

91. Alkhalaf LM, Du YL, Ryan KS. Synthetic Biology Approaches to New Bisindoles. In 2016. p. 21–37.

92. Topcu G, Ulubelen A. Structure elucidation of organic compounds from natural sources using 1D and 2D NMR techniques. J Mol Struct. 2007 May;834–836:57–73.

93. Karlsson M, Romson J, Elder T, Emmer Å, Lawoko M. Lignin Structure and Reactivity in the Organosolv Process Studied by NMR Spectroscopy, Mass Spectrometry, and Density Functional Theory. Biomacromolecules. 2023 May 8;24(5):2314–26.

94. Van Wyk HM, Schoeman D, Kwembeya E, Hans RH, Pool EJ, Louw S. In vitro acetylcholinesterase inhibitory activity and chemical composition of the dichloromethane extracts of the leaves and inflorescences of the tumbleweed, Trachyandra laxa (N.E.Br.) Oberm. var. rigida (Suesseng.) Roessl. South African Journal of Botany. 2022 Jul; 147:231–7.

95. Tschan GM, König GM, Wright AD, Sticher O. Chamaemeloside, a new flavonoid glycoside from Chamaemelum nobile. Phytochemistry. 1996 Feb;41(2):643–6.

96. Xian Q, Chen H, Liu H, Zou H, Yin D. Isolation and Identification of Antialgal Compounds from the Leaves of Vallisneria spiralis L. by Activity-Guided Fractionation (5 pp). Environmental Science and Pollution Research [Internet]. 2006;13(4):233–7. Available from: https://doi.org/10.1065/espr2006.06.314

97. Liu X, Shan K, Shao X, Shi X, He Y, Liu Z, et al. Nanotoxic Effects of Silver Nanoparticles on Normal HEK-293 Cells in Comparison to Cancerous HeLa Cell Line. Int J Nanomedicine. 2021 Feb; Volume 16:753–61.

98. Akaberi M, Sahebkar A, Azizi N, Emami SA. Everlasting flowers: Phytochemistry and pharmacology of the genus Helichrysum. Ind Crops Prod. 2019 Oct; 138:111471.

99. Charisiadis P, Kontogianni V, Tsiafoulis C, Tzakos A, Siskos M, Gerothanassis I. 1H-NMR as a Structural and Analytical Tool of Intra- and Intermolecular Hydrogen Bonds of Phenol-Containing Natural Products and Model Compounds. Molecules. 2014 Sep 2;19(9):13643–82.

100. Mastikhin VM, Nosov A V. 1H NMR studies of the OH groups of anatase. Reaction Kinetics & Catalysis Letters. 1992 Jan;46(1):123–30.

101. Tavakoli S, Khalighi-Sigaroodi F, Khosravi Dehaghi N, Yaghoobi M, Hajiaghaee R, Gholami A, et al. Isolation and purification of apigenin, quercetin and apigenin 7-O-glycoside from *Apium graveolens* L., *Petroselinum crispum* (Mill.) Fuss, *Allium cepa* L., respectively. Journal of Medicinal Plants. 2022 Sep 1;21(83):72–86.

102. Jackson TA, Moorey HC, Sheehan B, Maclullich AMJ, Gladman JR, Lord JM. Acetylcholinesterase Activity Measurement and Clinical Features of Delirium. Dement Geriatr Cogn Disord. 2017;43(1–2):29–37.

103. Sarangle Y, Bamel K, Purty RS. Role of acetylcholine and acetylcholinesterase in improving abiotic stress resistance/tolerance. Commun Integr Biol. 2024;17(1):2353200.

104. Bhagavan NV, Ha CE. Enzymes and Enzyme Regulation. In: Essentials of Medical Biochemistry. Elsevier; 2015. p. 63–84.

105. Saleem H, Ahmad I, Shahid MN, Gill MSA, Nadeem MF, Mahmood W, et al. In vitro acetylcholinesterase and butyrylcholinesterase inhibitory potentials of Jatropha gossypifolia plant extracts. Acta Pol Pharm. 2016;73(2):419–23.

106. Widiyanti P, Prajogo B, Widodo A. EFFECT OF VARYING INCUBATION PERIODS ON CYTOTOXICITY AND VIRUCIDAL ACTIVITIES OF Justicia gendarussa Burm.f. LEAF EXTRACT ON HIV-INFECTED MOLT-4 CELLS. Afr J Infect Dis. 2018;12(1 Suppl):133–9.

107. Heyman HM, Meyer JJM. Metabolomic comparison of selected Helichrysum species in search of antiviral properties. South African Journal of Botany. 2009 Apr;75(2):404–5.

108. Oyetunde Joshua F, Moodley R, Cheniah H, Khan R. Phytochemical and Biological Studies of Helichrysum acutatum DC. Pharmacognosy Journal. 2022 Nov 1;14(5):603–9.

109. Lourens ACU, Van Vuuren SF, Viljoen AM, Davids H, Van Heerden FR. Antimicrobial activity and in vitro cytotoxicity of selected South African Helichrysum species. South African Journal of Botany. 2011 Jan;77(1):229–35.

APPENDIX I: Ethical clearance

UNAM
UNIVERSITY OF NAMIBIA

ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: SOS-0152 **Date: 20 JULY 2023**

This Ethical Clearance Certificate is issued by the University of Namibia Ethics Committee (REC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the ethics committee.

Title of Project:	ACETYLCHOLINESTERASE INHIBITION, CYTOTOXIC ACTIVITY, AND CHEMICAL CHARACTERISATION OF POTENTIAL TOXIC COMPOUNDS ISOLATED FROM THE WILD EVERLASTING (HELICHRYSUM ARGYROSPHAERUM DC) AND THE WILD ONION (DIPCADI GLAUCUM (BURCH. EX KER GAWL.) BAKER)
Student:	ETUHOLE MTULENI
Student Number:	218203417
Supervisor(s):	Prof. STEFAN LOUW Prof. PETRINA KAPEWANGOLO

Centre for Research Services

Take note of the following:

1. Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the ethics committee. An application to make amendments may be necessary.
2. Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the ethics committee.
3. The Principal Researcher must report issues of ethical compliance to the ethics committee (through the Chairperson) at the end of the Project or as may be requested by the ethics committee.
4. The ethics committee retains the right to:

i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,
ii) Request for an ethical compliance report at any point during the course of the research.

The ethics committee wishes you the best in your research.

Dr. Zivayi Chiguvare (Chairperson Ethics Committee)

Prof. Davis Mumbengegwi (Head, Multidisciplinary Research)

APPENDIX II: Research permit

NCRST

AUTHORIZATION OF RESEARCH PROJECTS

Authorization is hereby granted in terms of Section 21 of the RST Act No. 23 of 2004, to:

Name: University of Namibia

Address: Private Bag 13301, Pioneers Park,
Windhoek

Coworkers: Stefan Louw, Petrina Kapewangolo, Celine Mukakalisa, Jonathan M. Ortmann, Kathithileni M. Kalili, Renate H. Hans & Michael G. Knott.

Certificate Number (if applicable): RCIV00022018 **Authorization No:** 202302014

Type of Research:
Non- Commercial research and the use of resources be limited to what is in the proposal.

Title of Research Authorized:
Identification of toxic compounds in indigenous plants responsible for livestock poisoning in southern Africa.

Locality:
Khomas region, Aranos, Gobabis, Aus (based on the specific locations documented for each of the plant species).

Duration: 21 August 2023 - 28 February 2024

Research / Sample Collection Conditions:
Plant species (Dipcadi glaucum (wild onion), Trachyandra laxa (tumbleweed), Helichrysum argyrosphaerum (Wild Everlasting) and Chrysocoma ciliata (Afr: bitterbos).

Yours sincerely,

Prof. Anicia Peters
Chief Executive Officer

National Commission on Research Science & Technology
27 AUG 2023
P/Bag 13253
Windhoek, Namibia

Head Office:

Innovation Hub:

APPENDIX III: Export permit

MINISTRY OF ENVIRONMENT AND TOURISM

Directorate Scientific Services
Private Bag 13306
Windhoek, Namibia
Enquiries: Permit Office
Tel +264-61-284 2541

PERMIT No.:	138224
VALID FROM:	03 October 2023
VALID TO:	03 April 2024
RECEIPT No.:	95387790 N$50-00

GENERAL PERMIT

Name: S. Law

Residential Address:

Box 13301

Postal Address: Windhoek

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975)

To export 2x 1mg of Wild Everlasting and 1x 10mg Wild everlasting (Helichrysum argyrosphaerum) and 2x 20mg of Wild onion (Dipcadi Glaucum) Plant Compound and extracts to Dr J Brand NMR Laboratory Stellenbosch University, P/bag 1, Room 1001/1014 Mike De Vries BLD, De Beer Road Stellenbosch, 7600 RSA

-Samples to be used for research purposes Only.

Remarks

Subject to expat and import regulations

IMPORTANT: This permit is not valid if altered in any way and is not transferable.

Official MET date stamp

2023 -10- 03

IMPORTANT

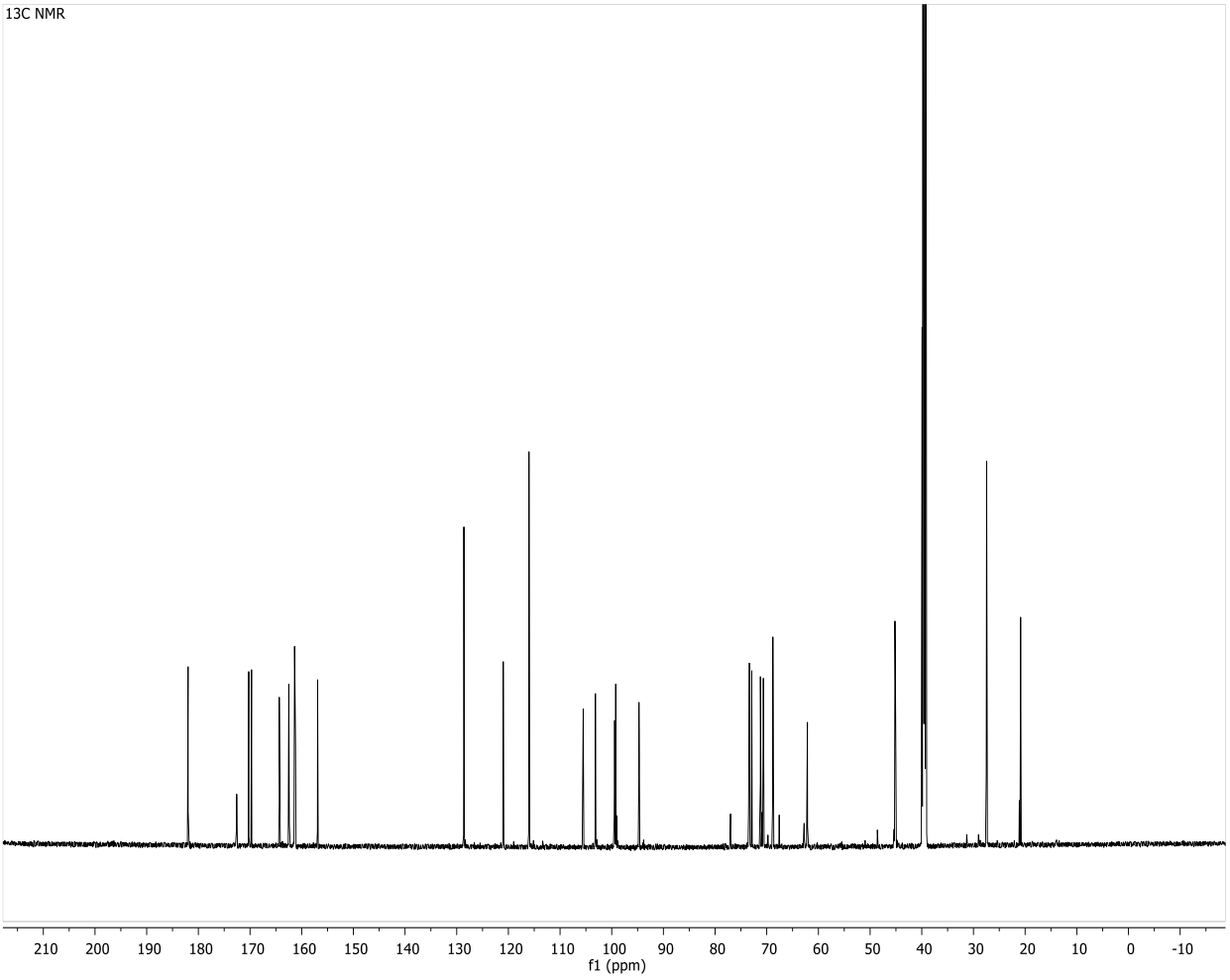
This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all prescribed conditions and regulations.

RESEARCH/SAMPLE COLLECTION CONDITIONS

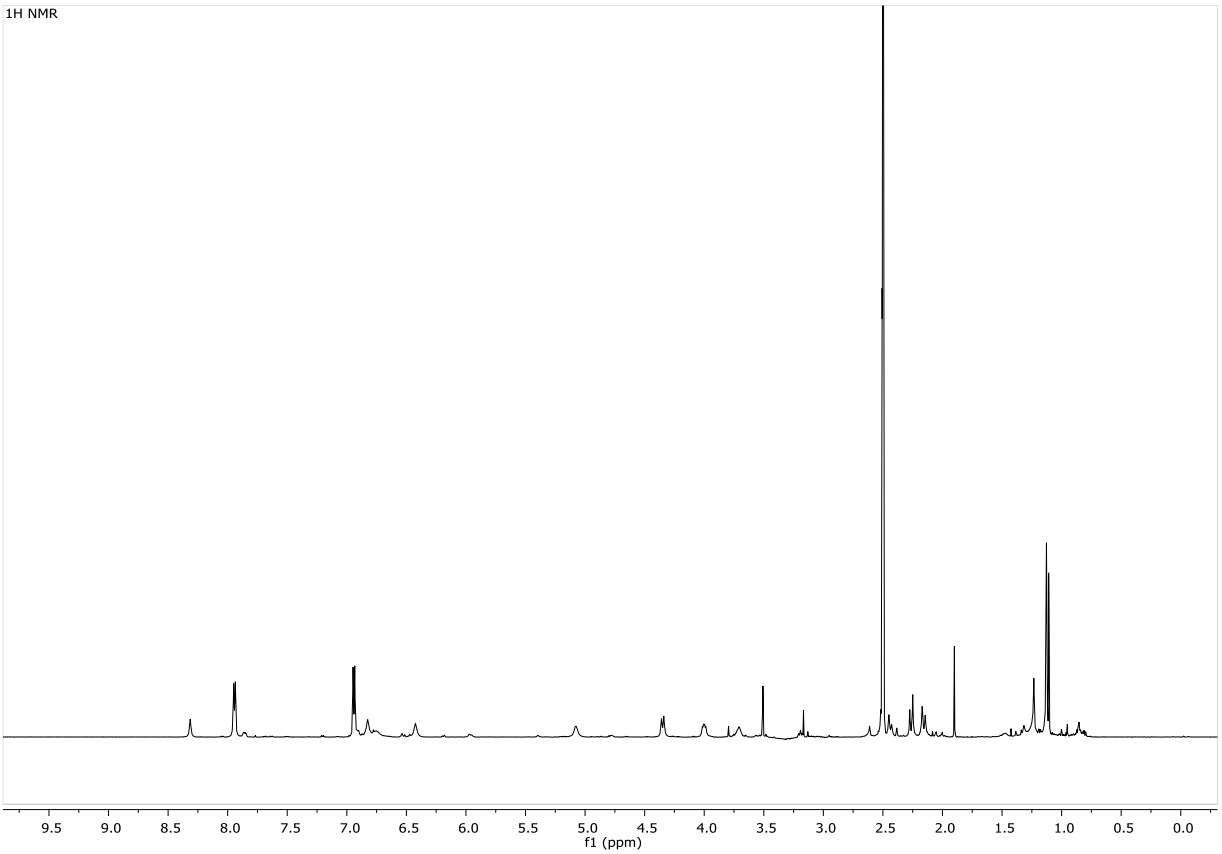
1. You must report to the Park Chief warden and / or Regional Office of the Ministry of Environment and Tourism prior to arrival in fieldwork area, and must present your permit.
2. This permit does NOT entitle the holder to free entry to the protected areas or state land outside protected areas.
3. For Field work in National Parks you have to make arrangement with park management in advance prior to arrival in fieldwork area.
4. Voucher specimens should be deposited with National Museum of Namibia.
5. If you would like to export samples of specimens you must loan them from the National Museum of Namibia.
6. To conduct research work in the rhinos and elephants range all persons listed on the permit must be in possession of a police clearance certificate.
7. The permission of the land owner is required to work/collect on private lands.
8. The permission of the concession holder is required to work/collect in concession areas.
9. The permission of the communal authority is required to work/collect in communal areas.
10. No commercial filming will be permitted without prior approval by the Ministry of Environment and Tourism under this permit.
11. Duplicates of publications and / or final report should be made available to the Ministry of Environment and Tourism and also the final report.
12. The specimens and their derivatives may be used for the purposes of this study only and may not be patented, commercialised, donated or sold to a third party without the written consent of the Ministry of Environment and Tourism.
13. All results (raw materials) or technology derived directly or indirectly from this research must be made available free of charge without reservations to the Ministry of Environment and Tourism.
14. A report on the work conducted under this permit must be submitted to the Ministry of Environment and Tourism not later than one month after the expiry of this permit as well as to regional office in whose area research was conducted.
15. Applications for renewal of this permit must reach this office at least three months prior to the expiry of this permit.
16. Habitat destructive collecting methods must not to be used.
17. Veterinary restriction may apply in the case of movement of samples and it is the applicants' responsibility to obtain such permits.
18. Foreign (or destination) wildlife import, and veterinary import permits may be required.
19. CITES import permit from the country of the destination is required for the application of export permit for CITES –listed species.
20. All field teams must be in possession of the permit and permit copy must accompany the transport of specimens.
21. You are subject to all conditions listed on the entry permit to any of the protected areas, unless specifically exempted.
22. Failure to adhere to the conditions will lead to cancellation of the research permit.
23. It is your responsibility to make the necessary contacts and arrangements as specified above.
24. The researcher should submit a copy of the final report to NBRI and the MEFT library.

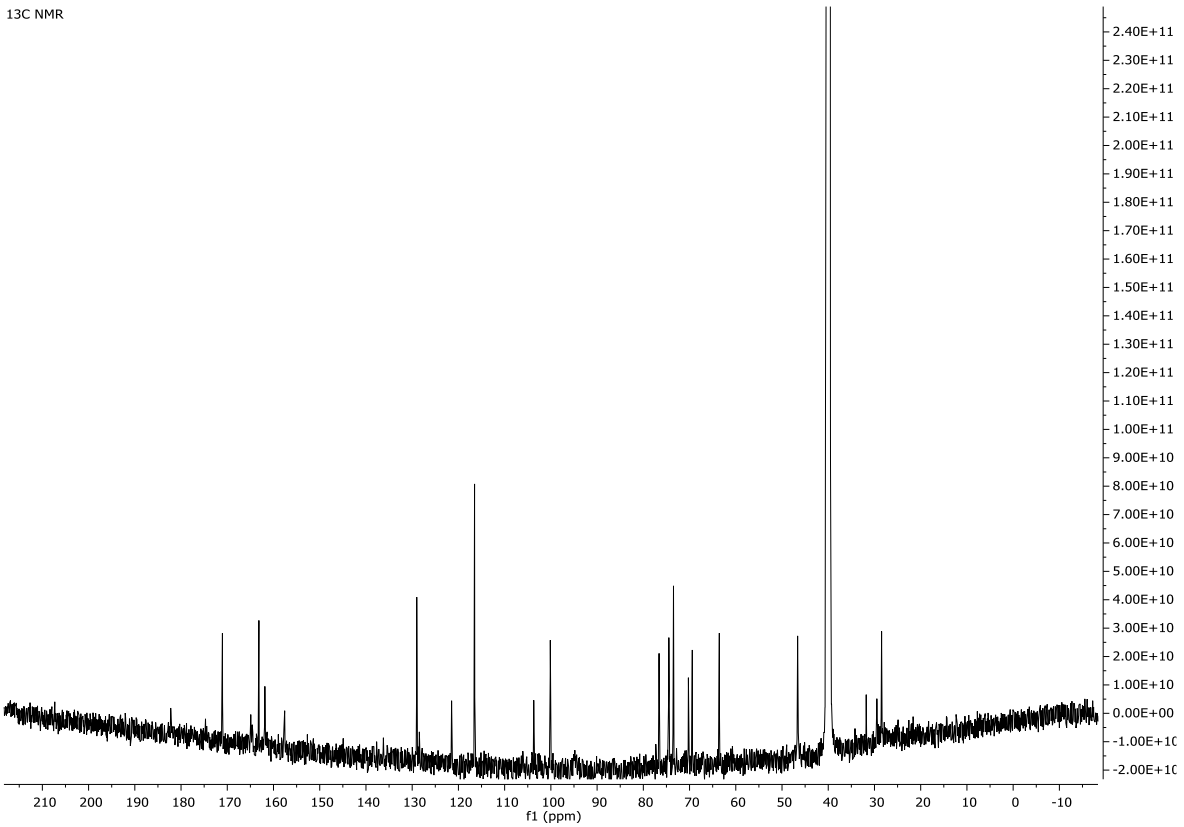
APPENDIX V

Compound **6** ^{13}C NMR

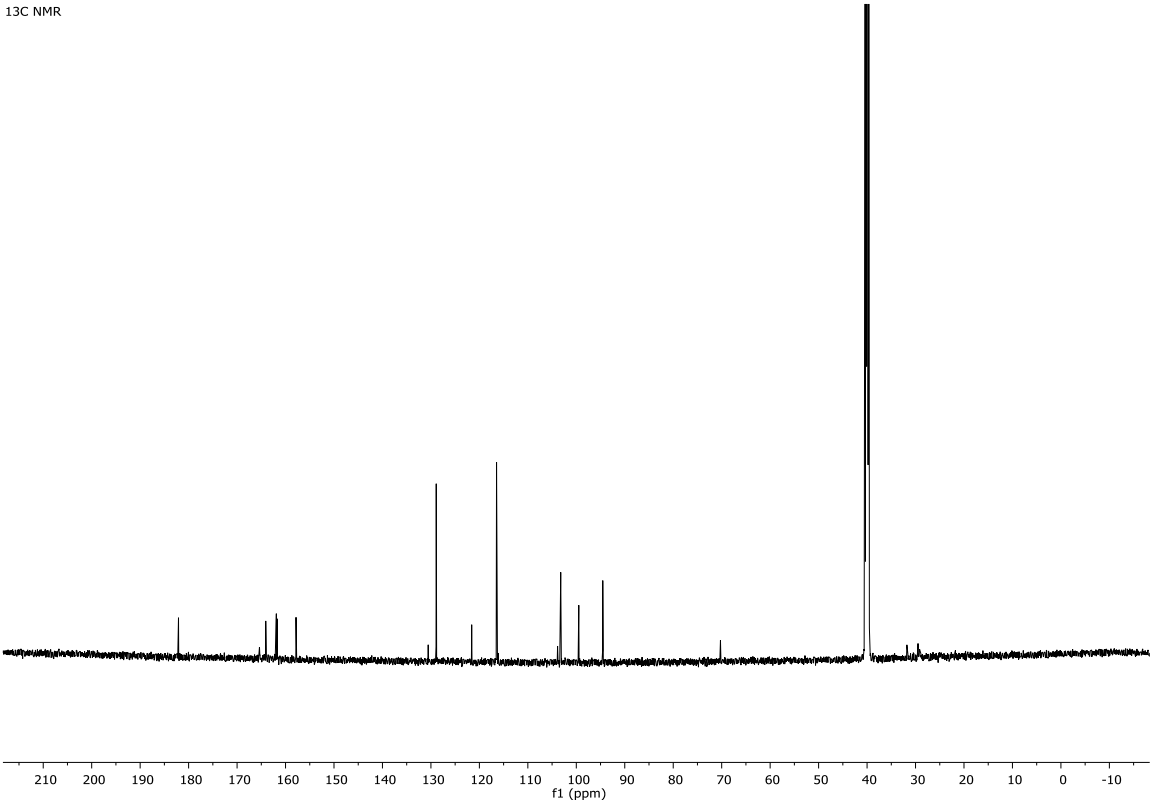


Compound **5** NMR





Compound **7** NMR



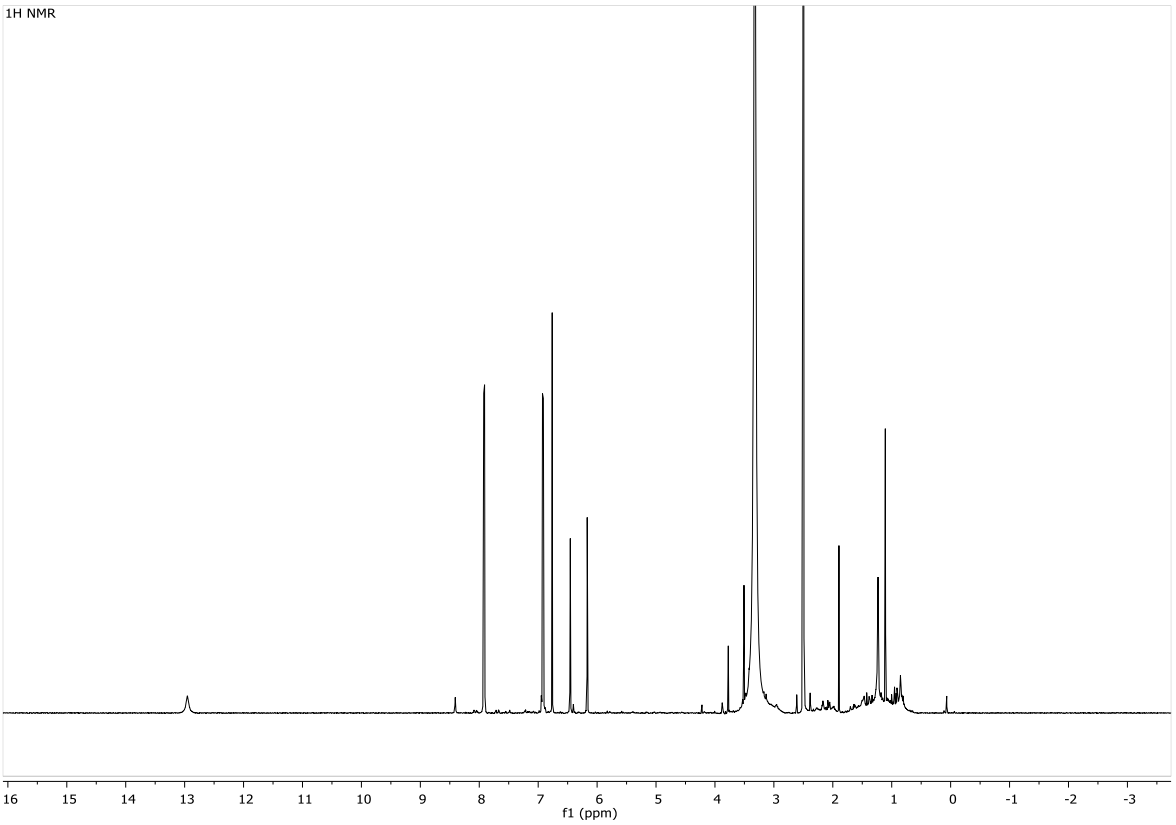


Table 0.1 : Major compounds tentatively identified in the MeOH extract of *H. argyrosphaerum* flowers (6).

Peak number	Retention time	Constituent	Mass of $[M+H]^+$	$[M+H]^+$ ion formula	Mass of major production	Production ion formula
1	7.12	Chlorogenic acid[a]	355.1021	C16H19O9	163.0402	C9H7O3
2	12.01	1'→3-Lactone-4,5-di-O-caffeoylquinic acid	499.1238	C25H23O11	163.0393	C9H7O3
3	13.36	Apigenin-7-O-glucuronopyranoside or Apigenin-7-O-galacturonopyranoside	447.0911	C21H19O11	271.0591	C15H11O5
4	13.68	Apigenin-4'-O-glucopyranoside	433.1134	C21H21O10	271.0595	C15H11O5
5	15.31	Apigenin-7-O-glucoside-[6"-O-(4-carboxyl-3-methylbutanoyl)]	577.1545	C27H29O14	271.0598	C15H11O5
6	16.52	Unknown apigenin-7-O-substituted diglycoside	619.1663	C29H31O15	271.0603	C15H11O5
7	17.56	Apigenin	271.0597	C15H11O5	153.0193	$[^{1,3}A]^+$
8	18.51	Feruloylquinic acid derivative	877.4039	unknown	177.0551	C10H11O4