UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Potential of South African medicinal plants as treatments for Alzheimer's disease through Aβ42 protein reduction and identification of their active compounds

by

ANURADHA THAKUR

Submitted in partial fulfilment of the requirements for the degree

PHILOSOPHIAE DOCTOR CHEMISTRY

In the Faculty of Natural & Agricultural Sciences

UNIVERSITY OF PRETORIA

PRETORIA

Supervisor: Prof. V. J. Maharaj
Co-supervisor: Dr. N. October

December 2019

Submission declaration

I, Anuradha Thakur, declare that the thesis, which I hereby submit for the degree of *Philosophiae Doctor* in the Department of Chemistry, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

Date: 12th December 2019

Plagiarism declaration

Full names of student: Anuradha Thakur

Student number: 16397003

Title of work: Potential of South African medicinal plants as treatments for Alzheimer's disease through Aβ42 protein reduction and identification of their active compounds

Declaration

1. I understand what plagiarism is and I am aware of the University's policy in this regard.
2. I declare that this thesis is my own original work. Where other people's work has been used (either from a printed source, internet or any other source), due acknowledgement was given, and reference was made according to departmental requirements.
3. I did not make use of another student's previous work and submit it as my own.
4. I did not allow and will not allow anyone to copy my work with the intention of presenting it as his or her own work.

Signature: Date: 12th December 2019

Acknowledgements

I would like to express my sincere gratitude to my supervisors Prof. V.J.Maharaj (main supervisor) and Dr N.October (co-supervisor) for their support, guidance, time, ideas and significant scientific contribution to this research project.

I would also like to thank Dr H.O.Yang and Dr Y.S.Chun, A.Park and Dr J.Kim of Korean Institute of Science and Technology (KIST) for the training provided me on Aβ42 and bioassaying at KIST.

Special thanks to Dr. M.Selepe for her help with NMR and LC-MS SPE-NMR and providing me with the necessary training on the use of equipment. I would also like to thank M.Wooding for running samples on UPLC-QTOF-MS.

I am grateful for the help from J.Sampson the curator at University of Pretoria for the collection of plant material from University of Pretoria gardens. I also thank M.Nel for identifying plant species at plant herbarium at University of Pretoria and proving me voucher specimen numbers.

I express my gratitude for the support from bioprospecting group members (S. Muyisa, M.Sephora, B. Tembeni, B. Mzondo, N.K Khorommbi, L. Kruger, C. Ezeofor, F. Katele) in the department of chemistry at University of Pretoria.

This work would not have been successful without funding from University of Pretoria as well as Korean Institute of Science and Technology (KIST).

I am also thankful for emotional support, love and encouragement from my husband P. Thakur, my little boy Yohan and my parents as well. Above all I want to thank God for the opportunity to pursue my PhD studies and the strength to bring this work to completion.

Summary

Alzheimer's disease is a slow and progressive neurodegenerative disease that destroys memory and other important mental functions. APP (Amyloid precursor protein) is an integral membrane protein expressed in higher levels in brain and its abnormal cleavage produces beta amyloid (Aβ42). Accumulation of Aβ42 in the brain produces amyloid plaques, the hallmark of the Alzheimer's disease.

Most of the research for the treatment of Alzheimer's disease on medicinal plants in South Africa, focuses on acetyl cholinesterase inhibition, however this does not play a significant role against amyloid plaques. The goal of this study is to identify, characterize and develop new natural ingredients from the South African medicinal plants for the treatment of Alzheimer's disease by targeting the reduction of Aβ42 resulting in decreased levels of beta amyloid plaques.

A literature survey was done for the selection of plants by using the keywords memory loss, aging, epilepsy, depression, mental health, dementia, forgetfulness, Alzheimer's disease, African medicinal plants, acetylcholinesterase. The plants were prioritized by a scoring system following the key criteria- plant part use, strength of the traditional use in relative to memory loss, plant toxicity, plant availability, published information against acetylcholinesterase inhibition. A total of 21 plant species were identified for collection, extraction and screening.

Different plant parts (leaves, bark, stem, roots and fruits) of the selected plants were collected from the University of Pretoria gardens and extracted with DCM:MeOH (1:1) after drying and grinding. The 34 plant extracts were screened to determine their inhibitory properties for Aβ42 production in HeLa cells stably transfected with APPsw. We found that of 34 extracts, 11 extracts (32.3%) were found significantly decrease the Aβ42 level. Amongst them, the extracts of the leaves of *Xysmalobium undulatum*, leaves of *Cussonia paniculata*, leaves of *Schotia brachypetala* and stems of *Heteromorha arborescens* potently reduced the secreted level of Aβ42 by 77.3 ± 0.5%, 57.5 ± 1.3%, 44.8 ± 0.1% and 68.6 ± 0.2%, respectively at 50 µg/ml.

These extracts were selected for further studies and UPLC- QTOF-MS was used to identify the active ingredients in the extracts. A total of five compounds were tentatively identified including four flavonoids, isoquercetin **(25)**, myricetin-3-*O*-alpha-L-rhamnopyranoside **(26)**, quercetin-3-*O*-rhamnoside **(27)**, quercetin **(28)** from leaf extract of *S. brachypetala*. The quercetin derivatives were previously studied for their potential to treat Alzheimer's disease through Aβ reduction in different bioassays, in all likelihood the compounds would be responsible for the Aβ42 reduction in *S. bracypetala*.

Using ESI negative mode of UPLC-QTOF-MS, a total of nine compounds were tentatively identified as quinic acid **(29)**, 3,5-dicaffeoylquinic acid **(30)**, rutin **(31)**, valerianannoid E **(32)**, acuminoside **(33)**, dictamnoside D **(34)**, 2''-O-β-D-glucopyranosylsaikosaponin B2 **(35)**, clinoposaponin C **(36)**, spinasaponin C **(37)** from the leaf extract of *C. paniculata*. Rutin is one of the most researched compound for the Alzheimer's disease in previous studies. 3,5-dicaffeoylquinic acid was previously reported for AChE inhibition. Rutin or 3,5-dicaffeoylquinic acid are in all likelihood the active ingredients in *C. paniculata* leaf extract.

Using ESI negative mode, a total of four compounds were tentatively identified named cynarin (1,5-dicaffeoylquinic acid) **(38)** alternoside IX **(39)**, alternoside I **(40)** and saikogenin B_4 **(41)** the stem extract of *H. arborescens*. Cynarin was previously reported for the reduction of Aβ cytotoxicity while the other identified compounds, triterpenoid saponins were not examined for Alzheimer's disease, but found to exhibit neuroprotective properties. These compounds may in all likelihood contribute as the active ingredients from *H. arborescens* leaf extract for the reduction of Aβ42.

The DCM:MeOH leaf extract of *X. undulatum* was fractionated using silica gel column chromatography to produce 15 fractions which were screened for Aβ42 production. Of these two fractions, were found to be active as they significantly reduced Aβ42 levels. Five pure compounds were successfully isolated from active fractions using preparative HPLC. Using NMR and UPLC-QTOF-MS, the structure of these compounds were elucidated as acetylated glycosydated crotoxigenin **(56)**, xysmalogenin-3, β-D-glucopyranoside **(57)**, crotoxigenin-3-*O*-

β-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**, crotoxigenin 3-*O*-glucopyranoside **(59)** and desglucouzarin **(60)** isolated from the leaves of *X. undulatum*. The present study reports for the first time, the evaluation of the compounds for their potential to reduce Aβ42 levels. The four cardenolide glycosides, acetylated glycosydated crotoxogenin **(56)**, xysmalogenin-3, β-D-glucopyranoside **(57)**, crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**, desglucouzarin **(60)** were found to significantly decreased the Aβ42 levels at 10μM by 75.04 ± 0.85%, 74.66 ± 0.1%, 65.55 ± 0.19% and 69.15 ± 0.25%, respectively as compared to the negative control. These results revealed that all the pure compounds including the inactive compound (crotoxigenin 3-*O*-glucopyranoside **(59)**) isolated from *X. undulatum* leaf extract were cardiac glycosides and contain the characteristic lactone ring which is known to be responsible for the cardiotoxicity. Since this was present in both the inactive and active cardenolides in all probability the lactone ring is the active site of the molecules contributing towards activity as such any analogues synthetically prepared lacking the lactone ring may still retain Aβ42 reduction while also reducing cardiotoxicity. Further the results of dose response studies of the 4 cardenolides indicated that crotoxigenin-3-*O*-β-digitalopyranosyl-(1-4)-*O*-β-digitoxopyanoside **(58)** was the most potent compound as it significantly reduced Aβ42 levels at the low concentration of 0.025 μM. These results revealed that this is the most active compound responsible for Aβ42 reduction in the plant. The bioassay-guided approach used for identifying the active ingredient in *X. undulatum* responsible for the reduction of Aβ42 resulted in identification of 4 cardenolides active ingredient, with one being the most active at nanomolar levels, which could be develop further into pharmaceutical drugs.

Table of Contents

List of Figures

List of Tables

Abbreviations

^{1}H NMR	Proton NMR
^{13}C NMR	Carbon-13 nuclear magnetic resonance
5-HT	5- hydroxytryptamine
6-OHDA	6-hydroxydopamine
ABTS	2,2′-azino-bis-3-ethylbenzotiazolin-6-sulfonic acid
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibition
AD	Alzheimer's disease
ANOVA	Analysis of variance
APOE	Apolipoprotein E
APP	Amyloid precursor protein
APPsw	APP carrying Swedish mutation
Aβ	Amyloid beta
B. natalensis	*Bulbine natalensis*
B. salvifolia	*Buddleja salvifolia*
BPI	Base peak ion
BuChE	Butyrylcholinesterase
C. africana	*Comelina Africana*
C. asiatica	*Centella asiatica*
C. edulis	*Catha edulis*
C. orbiculata	*Cotyledon orbiculate*
C. paniculata	*Cussonia paniculate*
C. spicata	*Cussonia spicata*
ChEBI	Chemical Entities of Biological Interest
CNS	Central nervous system
COSY	Correlation spectroscopy
COX	Cyclooxygenase
DCM	Dichloromethane
DEPT	Distirtionless enhancement by polarisation transfer
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Effective concentration 50
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Electron spray ionisation
FDA	Food and drug administration
FTIR	Fourier-transform infrared spectroscopy
GLIDE	Grid based Ligand Docking with Energetics
H. arborescens	*Heteromorpha arborescens*
H. trifoliate	*Heteromorpha trifoliate*
HEK	Human embryonic kidney
Hep2	Human epithelial type 2
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum correlation
IC_{50}	Inhibitory concentration 50
iFit	Isotopic fit value

KIST	Korean Institute of Science and Technology
LPS	Lipopolysaccharides
M. whitei	*Mondia whitei*
m/z	Mass to charge ratio
MAO	Monoamine oxidase
MAP	Microtubule-associated protein
MeOD	Deuterated methanol
MeOH	Methanol
min	Minute
ml	Millilitre
MS	Mass spectrophotometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ng	Nanogram
NMDA	N-methyl-D-aspartate receptor
NMDA	*N*-methyl-D-aspartate
NMR	Nuclear magnetic resonance
Nrf2	Nuclear factor erythroid 2–related factor 2
OGD/R	oxygen-glucose deprivation followed by reoxygenation
P. auriculata	*Plumbago auriculata*
PBS	Phosphate-buffered saline
PDE-4	phosphodiesterase-4
PHF	Paired-helical filament
Prep HPLC-MS	Preparatory High-performance- liquid- chromatography
R. graveolens	*Ruta graveolens*
S. brachypetala	*Schotia brachypetala*
SANBI	South African National Biodiversity Institute
sAPPα	soluble alpha-amyloid precursor protein
sAPPβ	Soluble beta-amyloid precursor protein
SASH	South Africa Stress and Health
SEM	Scanning electron microscope
SERT	Serotonin transporter
SSRI	Serotonin-specific re-uptake inhibitor
T.dregeana	*Trichilia dregeana*
T.riparia	*Tetradenia riparia*
TLC	Thin layer chromatography
TMT	Treadmill Stress Test
TPA	Tetradecanoylphorbol
UPLC-QTOF-MS	Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry
UPLC-QTOF-MS	The ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry
VCR	Vincristine
VLB	Vinblastine
WHO	World Health Organization
X. undulatum	*Xysmalobium undulatum*
Z. capense	*Zanthoxylum capense*
Z. mucronata	*Ziziphus mucronata*

Supplementary data

Chapter 1
General introduction

1.1. Natural products and their value in drug discovery

Natural products have historically demonstrated their value as a powerful source of bioactive compounds with therapeutic potential, and nowadays still represent an important tool for the identification of novel drug leads. This field of study forms a vital part of drug discovery as there is a consistent need to identify and develop novel pharmacophores to fight diseases that include Alzheimer's, cancer, HIV-AIDS and many infectious diseases. In the developing countries, medicinal plants are continued to be used as the primary source of medicine by about 80% of the population for health care, due to their easy accessibility and low costing. The analysis of secondary metabolites derived from these medicinal plants contain several bioactive compounds which may potentially lead to the development of novel drugs for the pharmaceuticals [1]. In addition, particularly in developing countries, there is a long history of the use of herbal medicine because of its cultural and spiritual point of views [2]. In the early 19th century, the isolation of active compounds from medicinal plants began with the isolation of morphine from opium. Furthermore, the isolation and identification of many earlier discovered potent drugs such as cocaine, codeine, digitoxin, and quinine from medicinal plants pathed the way for continuous interest in the field [3].

The rational use of traditional medicines was encouraged by the member states of the World Health Assembly (WHO, 1978), aiming for providing affordable and accessible health care to the global population. In order to facilitate this process, WHO in 1993 and 1998 has designed some guidelines for the assessment of the quality, safety and efficacy of herbal medicines [4]. An analysis based on the *Annual Reports of Medicinal Chemistry* from 1983 to 1994, indicated that 60-70% of anti-cancer and anti-infective approved drugs were shown to be of natural origin [5]. There are a number of plant derived compounds which include vinblastine, vincristine, topotecan and irinotecan, etoposide, camptothecin derivatives, and paclitaxel

(taxol®) have been used clinically as anticancer agents. While some other natural products including flavopiridol and combretastin A4 phosphate are considered as promising potent anticancer agents [6]. Over 60% of currently using anticancer agents are derived from natural sources including plant species, micro-organisms and marine organisms. Vinca alkaloids, vinblastine (VLB) and vincristine (VCR) are the first anticancer agents isolated from Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae). These agents are used in combination with other cancer chemotherapeutic drugs for the treatment of various types of cancers and also diabetes [6].

It was found historically that quinoline based antimalarials and artemisinin derivatives derived from the medicinal plants are most commonly used for the treatment of malaria and its symptoms [7]. Artemisinin isolated from an ancient Chinese herb named Qing Hao (*Artemisia annua*) is used as remedy in traditional Chinese medicine for the treatment of malaria. [8].

A variety of secondary metabolites derived from plant species including alkaloids, coumarins, flavonoids, lignans, phenolics, quinones, saponins, terpenes, xanthones and some other natural products showed moderate to good anti- HIV activity. Currently, there is no plant-derived drug in clinical use for the treatment of HIV-AIDS. But three natural products, calanolide A, 3-hydroxymethyl-4-methyl DCK (PA-334B) and DSB (PA-457) have shown promising activities to develop into a potent drug for HIV-AIDS [9].

Salvia officinalis and Melissa officinalis plant extract showed efficacy towards the treatment of mild to moderate Alzheimer's. Both plant extracts exhibited CNS acetylcholine receptor activity [10].

Natural products and their derivatives contribute more than one-third of all FDA-approved new molecular entities (NMEs). It is also revealed that one-quarter of these NMEs are derived from plants [11].

1.2. Background and history of Alzheimer's disease (AD)

Alzheimer's disease, is a multi-factorial neurodegenerative disorder that progresses slowly and destroys human memory, important mental functions and interrupt the daily activities in the ageing population [12]. It is a heterogenous group of dementia, also known as senile dementia that share common clinical symptoms, involving progressive cognitive impairment [13]. Alzheimer's is one of the most common form of dementia and primarily affects the people after age 65. The disease was named after the German Doctor Alois Alzheimer who was the first person to describe Alzheimer's in a patient named Auguste D. The patient, 51 years old woman who had shown some symptoms of progressive cognitive impairment, reduced comprehension and memory, aphasia, disorientation, unpredictable behavior, paranoia, hallucinations, delusions and psychosocial incompetence [14].

1.2.1. Epidemiology, facts and figures and risk factors of Alzheimer's disease

Alzheimer's patients have been predicted to grow from 47 million to 130 million by [15]. Global prevalence of dementia, a Delphi consensus study, in 2001, estimated that 24.3 million people have dementia with 4.6 million new cases reported every year and is expected to be doubled every 20 years and will reach up to 81.1 million by 2040 [16].

1.2.2. Causes and risk factors of Alzheimer's disease

Like all types of dementia, Alzheimer's is caused by brain cell death. It is a neurodegenerative disease which means there is progressive brain cell death that happens over a course of time. The total brain size shrinks with Alzheimer's – the tissue has progressively fewer nerve cells and connections [17].

1.2.2.1. Structural abnormalities

Although cerebral atrophy is a typical manifestation of Alzheimer's disease, it does not distinguish normal aging from Alzheimer's accurately enough to be diagnostic; this applies to

neuroimaging as well as gross inspection at post-mortem. However, microscopic examination reveals the critical features of the disease – a cerebral cortex peppered with the deposits of amyloid plaques or senile plaques and neurofibrillary tangles [18]. Alzheimer's disease is characterised by two deposits of abnormalities, senile plaques and neurofibrillary tangles and neural cell death [13]. Amyloid plaques contain mainly amyloid beta (Aβ) peptide generated by the proteolysis of a large β-amyloid precursor protein (APP) [19]. Accumulation of amyloid β in the brain is the primary factor causing AD [20]. Neurofibrillary tangles consist of an abnormal form of hyperphosphorylated tau aggregates [21]. Tau is one of the major microtubule-associated protein (MAP) in the brain, abundant in neurons present in any part of the body and performs as a stabilizer of microtubules in neurons and other cells, and also plays an important role in cell differentiation and polarization [22]. These neurofibrillary tangles make neuron unable to properly maintain its cytoskeleton and also to perform numerous processes [18]. The microscopic examination reveals the comparison of a healthy brain and Alzheimer's brain and also reveals the presence of senile plaques and neurofibrillary tangles in Alzheimer's brain as shown in Figure 1.1.

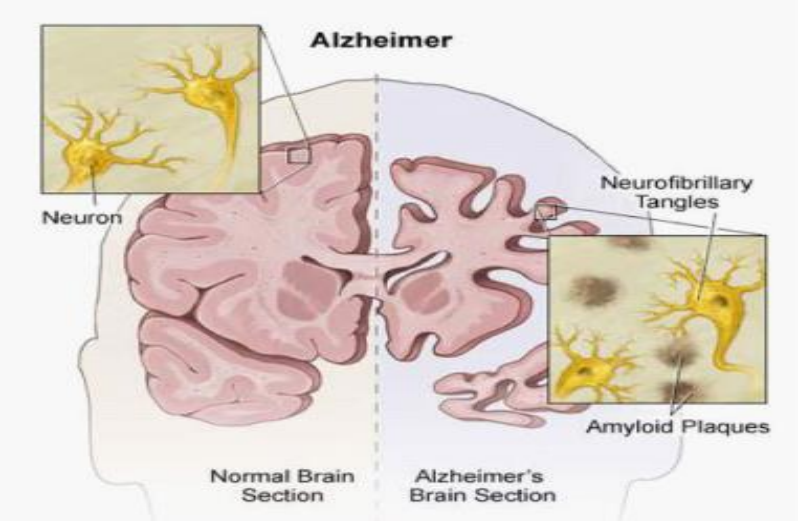


Figure 1.1: Comparison of normal brain and Alzheimer's brain[1]

[1] The picture of normal brain and Alzheimer's brain was adapted from an online resource: https://www.hopkinsmedicine.org/healthlibrary/conditions/adult/nervous_system_disorders/alzheimers_disease_85,p00772

1.2.2.2. The role of the genes

Genetic studies identified specific mutations in three different genes which are responsible for the early - onset of Alzheimer's disease, although these genetic mutations account for less than 1% of cases [23]. These gene mutations are APP (on chromosome 21), PSEN-1 (on chromosome 14), PSEN-2 (on chromosome 1). These are proposed to increase the production of less soluble and more toxic Aβ42 [24]. The gene responsible for the late-onset Alzheimer's disease has been identified as *APOE* located on chromosome 19. *APOE* is involved in the process of synaptic repair and also plays an important role in the maintenance of neuronal structure and cholinergic functions [18]. *APOE* has three allelic variants (ε2, ε3 and ε4) with *APOE*- ε4 is the one which is generally associated with the late-onset Alzheimer's disease while *APOE*- ε2/*APOE*- ε3 appears to lower the Alzheimer's risk [25]. Everyone inherits one of these genes from each parent and the people who inherit one copy of *APOE*- ε4 develop four-fold risk as compared with the people having two copies of *APOE*- ε3 who have 12-fold risk of developing Alzheimer's. Also *APOE*- ε4 is also responsible for developing Alzheimer's at a younger age than those with the other two allelic variants (ε2, ε3) of *APOE* gene [26].

1.2.2.3. Family history and Aging

Family history, age, environmental factors, cardiovascular risk factors and education are some other risk factors which can contribute to the Alzheimer's disease. 25% of people with age 55 or older suffering from dementia have a family history of dementia involving a first-degree relative. However a family history does not necessarily means that an individual will develop the disease [27]. Aging is one of the most important risk factor for Alzheimer's disease and thought to affect 4-8% of the population over 65 years [28].

1.2.3. [2]Signs and Sympyoms of Alzheimer's disease

The common symptoms include difficulty in retaining information and expressing thoughts. Patient may start forgetting recently learned information, important dates or events and relying on the memory aids or on the family members for the daily activities. The Alzheimer's patients become furious, confused, depressed or anxious and may become angry for no apparent reason. Alzheimer's patients may struggle with the loss of speech and language. Patient may start showing some personality changes. A previously sociable person may withdraw from conversation and activities. People with Alzheimer's may have confusion with time and place. They can lose the track of dates, seasons, and passage of time.

1.3. Effective Alzheimer targets

1.3.1. Amyloid beta

Amyloid beta peptide is a sticky peptide generated by the cleavage of APP by β-secretase followed by an accumulation of γ-secretase in the brain, which finally leads to the formation of amyloid plaques, a key peptide responsible for Alzheimer's disease [28]. The entire process of production of Aβ42 is explained by the amyloid cascade hypothesis [20] shown in Fig.1.3.

[2] https://www.alz.org/alzheimers-dementia/10_signs

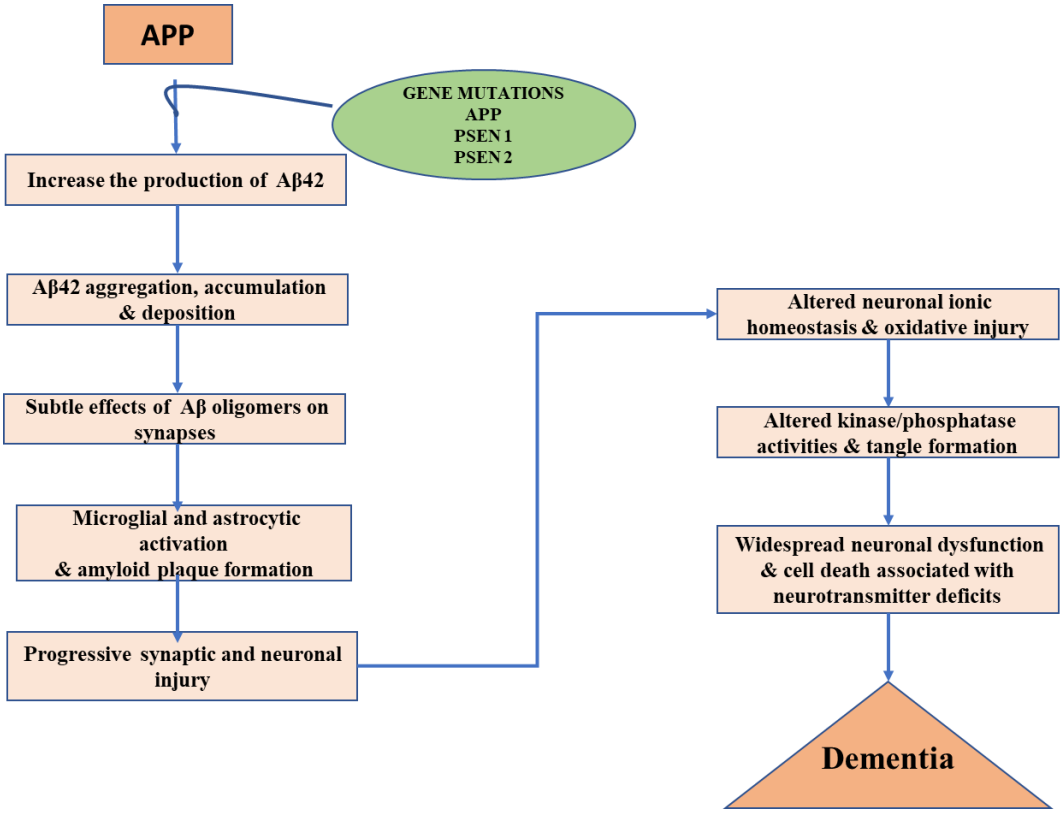


Figure 1.2: Amyloid Cascade Hypothesis

The Aβ protein is the main target and the core focus of Alzheimer's disease drug development research, and it can be achieved by reducing the Aβ production which involves either the inhibition of β- or γ- secretase activities, or by enhancing the α- secretase activity [29]. As mentioned earlier, amyloid plaques comprise mainly of amyloid β (Aβ) peptide generated by the proteolysis of a large β-amyloid precursor protein (APP) [19]. APP can be processed by the proteolytic enzymes called secretases (α, β and γ) in two different pathways [20]. The processing of APP by α-secretase in a non-amyloidogenic pathway produces a soluble fragment sAPPα which has neuroprotective activities. The other pathway is an amyloidogenic pathway in which APP is processed by β-secretase and γ-secretase, resulting in the production of neurotoxic Aβ peptide (39-43 amino acids) [30], [31]. Aβ40 (amyloid peptide containing 40 amino acids) and Aβ42 (amyloid peptide containing 42 amino acids) are the major isoforms, but Aβ42 is the major contributor to the Alzheimer's disease [32]. Certain gene mutations increase the production of Aβ42, and thus its accumulation in the brain induces aggregation to form amyloid plaques [33].

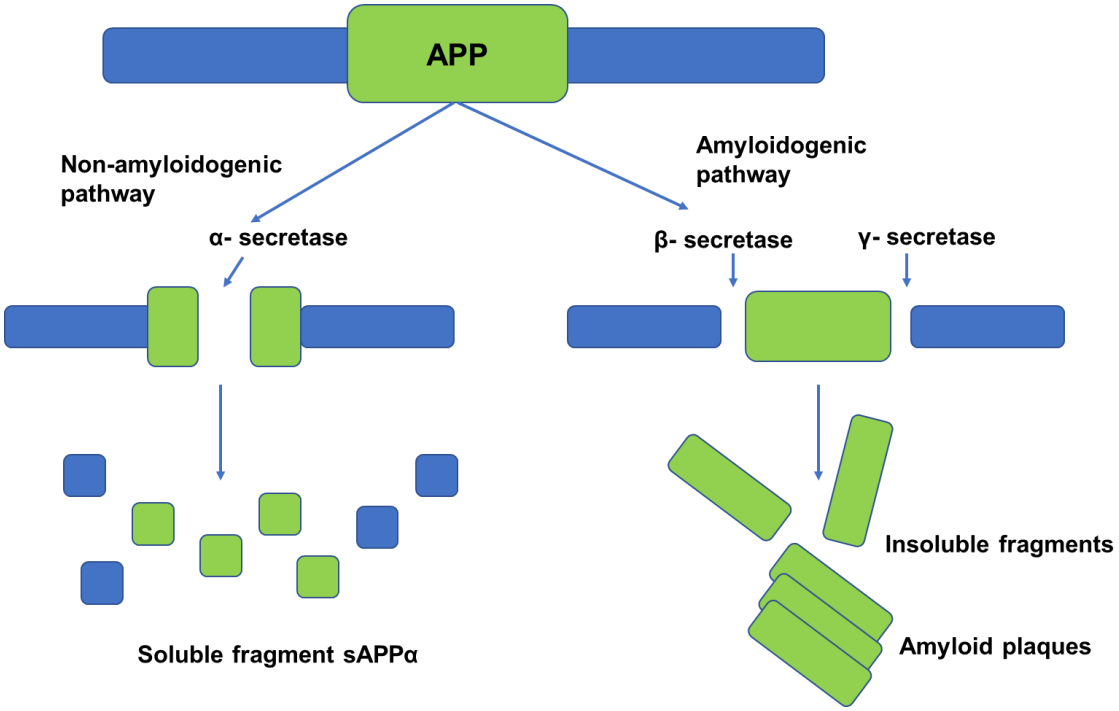


Figure 1.3: Non-amyloidogenic and amyloidogenic pathways for the cleavage of APP

1.3.2. Neurofibrillary tangles

One of the most characteristic neuropathological lesion and the main component of neurofibrillary tangles is the accumulation of paired-helical filaments (PHF) [34]. The PHF consists of hypophosphorylated tau protein, one of the microtubule-associated proteins present in the neurons. The main physiological role of tau protein in the normal neurons is promoting the assemby and stability of axonal microtubules, which play an important role in intraneuronal transport. Tau protein also controls the polymerisation of microtubules [13], [35].

1.3.3. Cholinergic system in AD

The cholinergic system involves the neural pathways in which acetylcholine (Ach) is released for the purpose of neurotransmission. Previous studies identified a pathophysiological significance between cholinergic neurotransmission and amyloidogenesis as well as tau-phosphorylation. This present research supports the hypothesis that the loss of cholinergic innervation is one of the cause of amyloid plaques and neurofibrillary tangles [36].

1.4. Current Alzheimer's treatments

To date there is no cure for Alzheimer's disease, however the drugs currently available in the market have the ability to temporarily slow down and alleviate the symptoms of Alzheimer's and as a result can improve the quality of life for Alzheimer sufferers. The U.S. Food and Drug Administration (FDA) has approved five medications to treat the symptoms of Alzheimer's disease, Table 1.

Table 1.1: FDA-approved drugs

Drug name	Brand name	Approved for	FDA Approved
1. Donepezil	Aricept	All stages	1996
2. Glantamine	Razadyne	Mild to moderate	2001
3. Memantine	Namenda	Moderate to severe	2003
4. Rivastigmine	Exelon	All stages	2000
5. Donepezil and Memantine	Namzaric	Moderate to severe	2014

Unfortunately, the anti-Alzheimer effects of these drugs persist for a limited time, on average 6-12 months. Common side effects are usually mild for these medications and include diarrhea, vomiting, nausea, fatigue, insomnia, loss of appetite, and weight loss.

1.5. Natural products as Potential inhibitors for Alzheimer's disease

Various natural products have shown promise to treat Alzheimer's disease and have contributed towards the search of potential Alzheimer's inhibitors. The phytochemicals derived from these studies could serve as a basis for future research [37]. Some of these promising natural anti-Alzheimer's plants include *Ginkgo biloba, Withania somnifera, Poncirus trifoliate, Hedyotis diffusa, Salvia officinalis, Huperzia serrata, Scrophularia buergeriana, Moringa*

olifera, Bacopa monnieri, Rosmarinus officinalis, Paeonia suffruticosa, uncaria rhynchophylla, Galanthus woronowii, Panax ginseng, Magnolia officinalis, Phyllanthus emblica, Coriandrum sativum [38].

Previous studies showed that phenolic rich extracts from *Schotia brachypetala*, *Camelia sinensis*, *Markhamia platycalyx* exhibited notable anti-Alzheimer's activity, when bioassay tests were conducted. These findings led to the conclusion of a possible relation between the intake of polyphenolic rich foods and the prevention of Alzheimer's disease [39].

Flavonoids containing natural products have also demonstrated the ability to improve memory, learning capacity and cognitive function due to their antioxidant properties [40]. Natural flavonoids were also shown to potently reduce the levels of Aβ deposits either by promoting the α-secretase activity or by inhibiting the β-secretase activity. Another notable inhibitory activity displayed by flavonoids was the reduction of heparin- induced tau formation which is also considered as one of main causes of Alzheimer's disease [40]. The phytochemistry of flavonoid derivatives such as quercetin, myricetin, catechin, epigallocatechin have been extensively studied in the search and hope of finding potential Alzheimer's inhibitors [41].

The *in vitro* and *in vivo* bio-evaluation studies conducted on selected terpenoids such as ginsenosides, gingkolides, cannabinoids, cornel iridoid glycoside, oleanolic acid, tenuifolin, cryptotanshinone and ursolic acid have suggested that these compounds possess excellent neuroprotective effects [42].

It has been shown that a diet rich in certain edible fruits like blue berry, mulberry, strawberry, grape seed, pomegranate, papaya, apple, green tea, walnut, coffee, saffron, curcumin, pepper, cinnamon, garlic, ginger also play an important role in controlling the Alzheimer's disease [38].

Many studies have been conducted involving the isolation of the active subtance responsible for the protective effects against Alzheimer's disease. There are some phytochemicals which have been mostly investigated with a potential of developing drug. These phytochemicals include galanthamine, berberine, curcumin, ginsenoside Rg1, puerarin, silibinin, bilobalide,

quercetin, (-)-epigallocatechin-3-gallate, resveratrol, huperzine A, rosmarinic acid, luteolin, apigenin and chitosan [43], [37].

Galanthamine **(1)**, a natural alkaloid belonging to amaryllidaceae derived from *Galanthus woronowii*, is one of the FDA approved AChE inhibitor drugs currently prescribed for the treatment of Alzheimer's disease. However, this drug is associated with many side effects like frequent nausea, vomiting irregular agitation and sleep disturbance and deprivation [44]. In the early 1960s and 1964, galanthamine was identified as a cholinergic booster and as a reversible inhibitor of acetylcholinesterase (AChE). It was evidently proved to enable its activity in the CNS by penetrating through the blood-brain barrier. [45]

Berberine **(2)** is an isoquinoline alkaloid isolated from *Coptidis rhizoma*. *Coptidis rhizoma* is a major natural product used by the Chinese as a traditional herbal medicine. Previous studies supported that Berberine treatment reduces the reduction of Aβ42 in the brain and stimulates the α-secretase activity [46], [47]. Due to its anti-diarrheal, anti-microbial and anti-inflammatory effects, burberine has been used for the treatment of diarrhea, liver diseases, skin and other disorders [48]. The contribution of berberine's multiple activities such as antioxidant activity, AChE and BChE Inhibitory Activity, MAO inhibitory activity, Aβ level-reducing activity, cholesterol lowering activity and other activities support the fact that berberine may be considered as a potential multipotent natural product to combat Alzheimer's disease [46].

Curcumin **(3)** has many medicinal properties which includes, anti-inflammatory, anti-oxidant, anti-tumour such as chemopreventive and chemotherapeutic properties. Curcumin has been reported for the inhibition of β-secretase and is a promising candidate for the treatment of Alzheimer's disease [49]. Literature has also proposed that curcumin has the ability to inhibit Aβ-aggregation and Aβ-induced inflammation in *in vitro* studies [50]. An *in vitro* study revealed the inhibitory effects of curcumin against fibrillar Aβ aggregation, extension and destabilization at low micro molar concentrations (EC_{50}) between 0.1 and 1.0 μM [51]. The outcome of an *in vivo* study , highlighted the inhibition of Aβ-formtion and extension in the brains of AD model mice, when160-500 ppm (0.43-1.36 *μ*mol/g) of curcumin was administered orally [50]. These

findings propose that curcumin could be a potential phytochemical candidate in the development of Alzheimer's disease drugs therapy.

Ginsenoside Rg1 **(4)**, a pharmacological bioactive component purified from Ginseng. Pre-treatment with the Rg1 helps in the reduction of Aβ42 and also in the improvement of cell viability. It also reduced cerebral Aβ in Tg 2576 mouse model [52], Figure 1.7.

Puerarin **(5)**, is one of the major isoflavonoid isolated from the roots of *Pueraria lobata* (Wild.), a Chinese medicine known as Gegen. It has various medicinal properties for the treatment of cardiovascular diseases, cancer, Alzheimer's disease, Parkinson's disease, cancer, cerebrovascular disorders, hyperlipidemia, osteonecrosis etc [53]. Puerarin treatment decreased Aβ-induced cell death and have a neuroprotective effect in rat hippocampal neurons via various mechanisms [48], Figure 1.8.

Silibinin **(6)**, is a flavonoid isolated from the plant *Silybum marianum* commonly known as milk thistle and is known to exhibit anti-oxidative and anti-inflammatory properties. Silibinin was reported to be an inhibitor of Aβ aggregation and formation in PC12 cells (neuroendocrine in origin), protective against the oxidative damage caused by Aβ42 in SH-SY5Y cells (neuroblastoma cells) by decreasing the H_2O_2 levels and also help in the improvement of behavioural abnormalities in mouse model of Alzheimer's disease [54], Figure 1.9.

Bilobalide **(7)**, is a sesquiterpenoid lactone derived from *Ginkgo biloba*, a plant which exhibits antioxidant, antiplatelet and hemorrheological properties [55], [56]. Earlier research has shown the significant neuroprotective effects of bilobalide against learning and memory impairments induced by $A\beta_{25-35}$ in a Morris water maze test, and it also prevented cerebral alterations of oxidative stress and neuronal apoptosis caused in rats [57].

Quercetin **(8)**, is a natural occuring flavonoid found in various foods, beverages and plants. It has been reported to play an integral role in the prevention of neurodegenerative diseases [58]. Studies suggested that the pre-treatment of quercetin with neurons for 1 h has notably reduced the Aβ42 induced cytotoxicity at lower doses of 5 and 10 μM (showed the best effects

at 5μM), however high doses at 20 and 40 μM did not show any siginificant reduction in Aβ42 and induced neurotoxicity. Quercetin protected HT-22 murine neuroblastoma cells from $A\beta_{25-35}$ oxidative attack by strongly inhibiting the Aβ fibril formation and aggregation [59]. The findings from this work showed that quercetin could be a therapeutic potential for Alzheimer's disease, Figure 1.11.

(-)-Epigallocatechin-3-gallate **(9)**, has been reported to activate α-secretase cleavage which promotes non-amyloidogenic APP processing and prevent the Aβ production [60], Figure 1.12.

Resveratrol **(10)**, is a non-flavonoid polyphenol which occurs in grapes and red wine. Previous reports have demostrated their neuroprotective effects and their potential to reduce amyloid protein concentration due to its allosteric inhibitory activity against the sirtuin proteins [38]. Reports also revealed that resveratrol has the ability to prevent learning impairment, and also reduced the hippocampus neurodegeneration in the p25 transgenic AD mouse model [61], Figure 1.13.

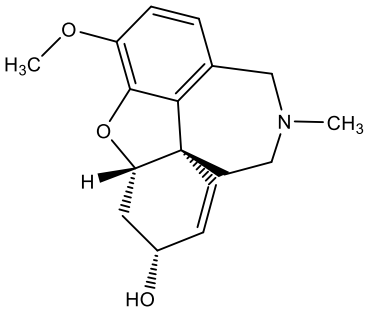
Huperzine A **(11)**, the lycopodium alkaloid derived from the Chinese club moss, *Huperzia serrata* is a potent AChE inhibitor. It is used for the treatment of schizophrenia, swelling and strain. Research has shown that it regulates the amyloid precursor protein (APP) and protein kinase C in human embryonic kidney 293 (HEK-293) cells [38]. In China, Huperzine A is widely used as AD therapy enhance the memory, cognitive skills and daily life activities of AD patients [62], Figure 1.14.

Rosmarinic acid **(12)**, a natural scaeanger of peroxynitrite ($ONOO^-$) has been shown to exhibit protective effects on the memory impairment in a mouse model induced by acute i.c.v. injection of Aβ25–35 [63], Figure 1.15.

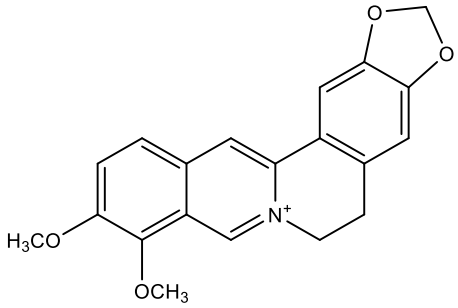
Luteolin **(13)**, a flavonoid derivative is one of the major active ingredients isolated from an edible medicinal plant, *Flos chrysanthemi*. This compound is known to display many pharmacological activities such as anti-oxidant potential, anti-tumorigenic effects and anti-

inflammatory activities [64]. Luteolin has releaved to prevent AD-like symtoms after traumatic brain injury in an amyloid-β depositing mouse model [65], Fogure 1.16.

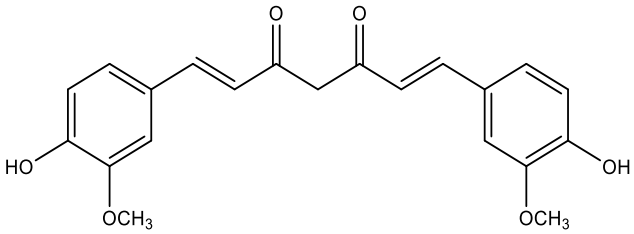
Apigenin (4',5,7-trihydroxyflavone) **(14)**, is a non-mutagenic flavone, found in many fruits, vegetables and medicinal plants including *Elsholtzia Rugulosa* and *Carduus Crispus*. It has been shown to exhibit anxiolytic, sedative and antidepressant effects and also neuroprotective effects against Aβ-induced toxicity in rat cerebral microvascular endothelial cells. Results from studies conducted independently have proven the ability of apigenin to reduce learning and memory deficits by decreasing oxidative damage and improving cholinergic neuronal transmission [66], Figure 1.17.



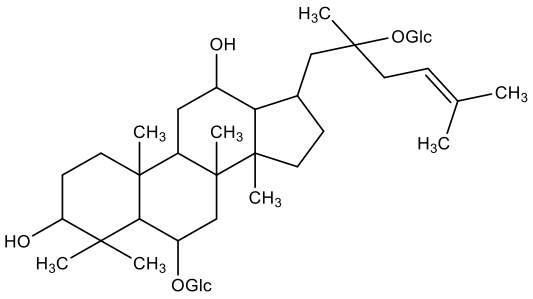
(1)



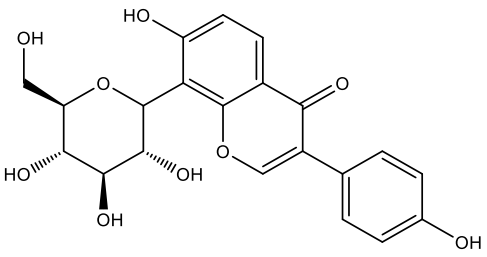
(2)



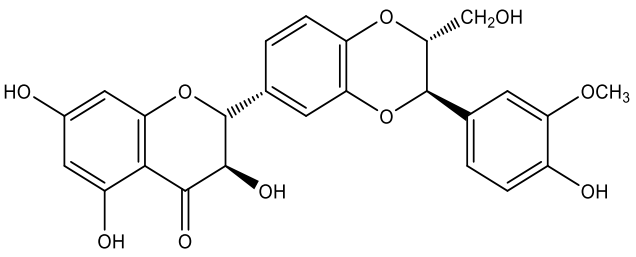
(3)



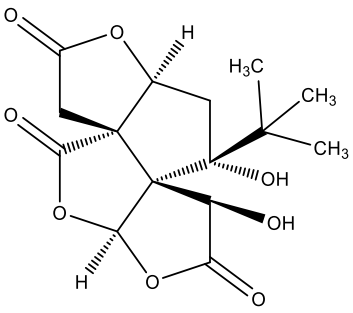
(4)



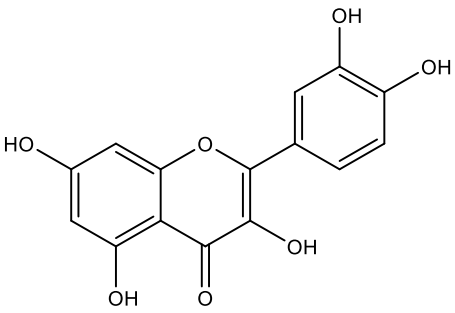
(5)



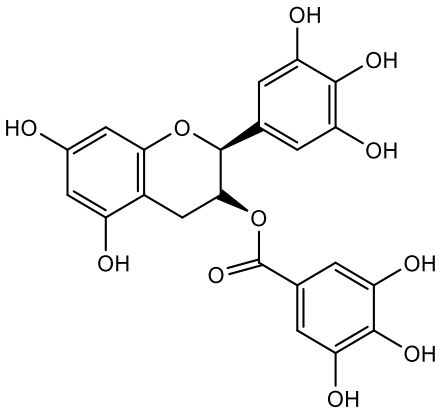
(6)



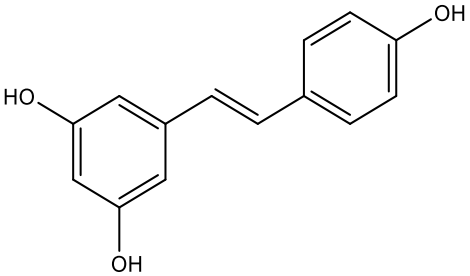
(7)



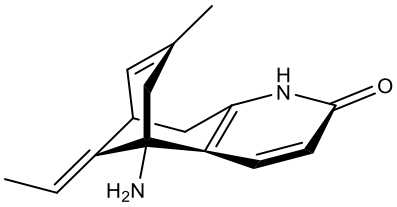
(8)



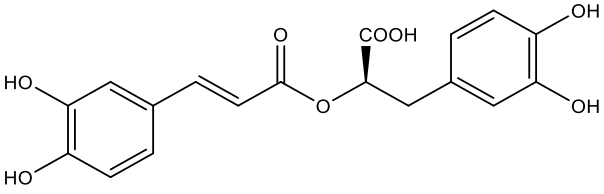
(9)



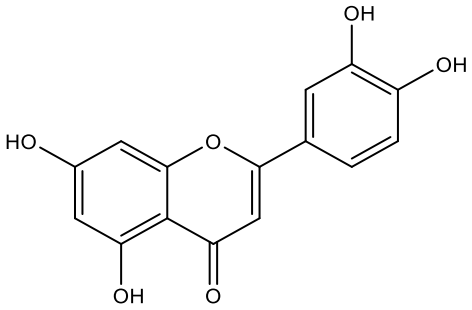
(10)



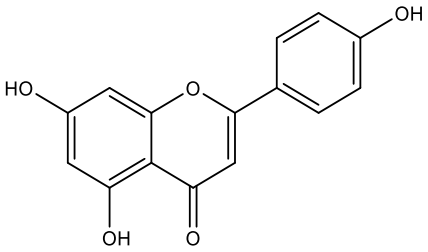
(11)



(12)



(13)



(14)

Figure 1.4: Structure of galanthamine **(1)**, berberine **(2)**, curcumin **(3)**, ginsenoside Rg1 **(4)**, puerarin **(5)**, silibinin **(6)**, bilobalide **(7)**, quercetin **(8)**, (-)-epigallocatechin-3-gallate **(9)**, resveratrol **(10)**, huperzine A **(11)**, rosmarinic acid **(12)**, luteolin **(13)**, apigenin **(14)**

1.6. Problem statement and justification

Regardless of all the research efforts conducted towards understanding the pathology, treatment, and cure of the Alzheimer's disease, to date no cure for this disease has been reported. Consequently, the disease is incurable and irreversible, once an individual start showing distinctive key symptoms related to Alzheimer's disease.

In conclusion, no single treatment has been proven to be significantly effective for the treatment of the Alzheimer's disease. Current FDA drugs used for the treatment of Alzheimer's are overpriced and unaffordable to many or may display undesirable side effects which may be detrimental to human health and well-being.

In South Africa, most of the research is aimed at the diseases such as malaria and HIV-AIDS. However, Alzheimer's disease is also growing slowly and progressively in South Africa and therefore need more attention as such there is a need for developing new drugs in the field of Alzheimer's disease. The outcome of extensive research and studies done in this field, has shown that acetylcholinesterase inhibitors are effective in treatment of cognitive symptoms in

mild to moderate stages of Alzheimer's disease. However these are ineffective against the vascular dementia, suggesting that more attention should be given to other inhibitors, targeting the amyloid plaques and neurofibrillary tangles [67]. The FDA approved acetylcholinesterase drugs also have many side effects. All these approved drugs are temporary treatments for Alzheimer's disease. Hence there is a need for undertaking research for the discovery of new drugs in the field of Alzheimer's disease.

1.7. Aim and objectives

1.7.1. Aim

The aim of this study is to isolate, identify, characterize and develop novel plant-derived anti-Alzheimer's agents from South African's rich, traditional medicinal plant varieties, targeting the amyloid plaques as the precursor enzyme.

1.7.2. Objectives

The overall objectives of the present research are:

- Selection of plants with the help of electronic databases Google scholar, Scopus and Scifinder using the keywords, African traditional medicinal plants in combination with the memory loss, mental illness, depression, Alzheimer's disease, dementia, anxiety, epilepsy, forgetfulness, convulsion, hysteria, sedative effects, acetylcholinesterase inhibition.
- Collection and extraction of different parts of the plant (leaf, stem, root, bark and flowers).
- Fractionation of extracts using silica gel column.
- Identification, isolation and purification of active ingredients using UPLC-MS-QTOF, preparative HPLC.
- Aβ42 bioactivity screening of extracts, fractions and pure compounds.
- Structure elucidation of pure compound using 1D, 2D NMR and mass spectrometry.

Schematic flow chart (Figure 1.18) detailing the overall objectives is as described in Figure 1.5.

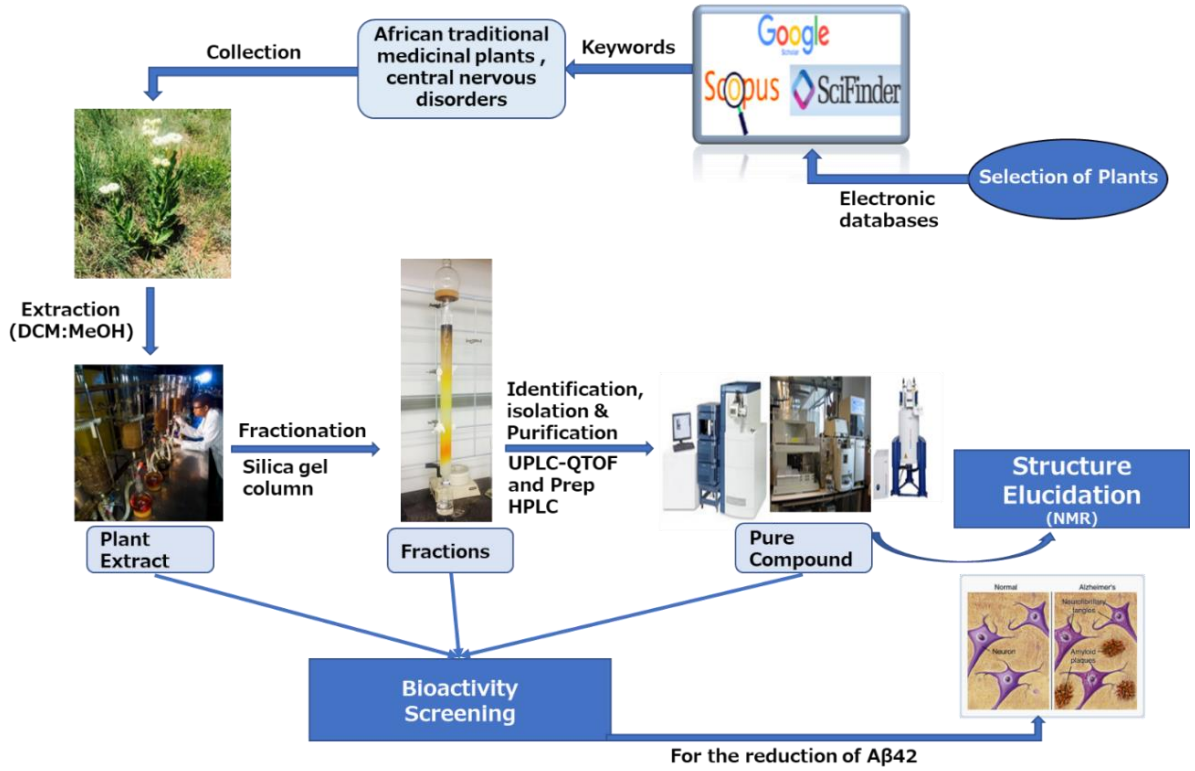


Figure 1.5: Flow chart showing the objectives of present research

The specific objectives of **Chapter 2**:

- Literature survey and selection of plants.
- Plant collection and extraction.
- Bioassay screening of plant extracts for the reduction of Aβ42.
- Selection of the active plant extracts for the further investigation.

The specific objectives for **Chaper 3**:

- Collection and extraction of leaves of *Schotia brachypetala*, *Cussonia paniculata* and *Heteromorpha arborescens*.
- Develop chemical profiles of the leaf extracts using UPLC-QTOF-MS for the identification of the major classes of compounds.
- Confirm the presence of compounds by using standards.

The specific objectives for **Chapter 4**:

- Collection and extraction of leaves of *Xysmalobium.undulatum*.
- Develop chemical profile of the leaf extract using UPLC-QTOF-MS for the identification of the class of compounds.
- Bioassay-guided fractionation to identify the active ingredients from *X.undulatum* leaf extract based on the Aβ42 bioassays.
- Structure elucidation of the active pure compounds using NMR.
- Bioassaying of the pure compounds

The specific objectives for **Chapter 5** include the detailed account of the materials and methods used in the study.

Chaper 6 gives the concluding remarks of the study.

References

1. Palombo EA: **Traditional medicinal plant extracts and natural products with activity against oral bacteria: potential application in the prevention and treatment of oral diseases**. *Evidence-Based Complementary and Alternative Medicine* 2011, **2011**.
2. Koduru S, Grierson D, Afolayan A: **Ethnobotanical information of medicinal plants used for treatment of cancer in the Eastern Cape Province, South Africa**. *Current Science (00113891)* 2007, **92**(7).
3. Balunas MJ, Kinghorn AD: **Drug discovery from medicinal plants**. *Life sciences* 2005, **78**(5):431-441.
4. Scott G, Springfield E, Coldrey N: **A pharmacognostical study of 26 South African plant species used as traditional medicines**. *Pharmaceutical Biology* 2004, **42**(3):186-213.
5. Newman DJ, Cragg GM, Snader KM: **Natural products as sources of new drugs over the period 1981− 2002**. *Journal of natural products* 2003, **66**(7):1022-1037.
6. Cragg GM, Newman DJ: **Plants as a source of anti-cancer agents**. *Journal of ethnopharmacology* 2005, **100**(1-2):72-79.
7. Clarkson C, Maharaj VJ, Crouch NR, Grace OM, Pillay P, Matsabisa MG, Bhagwandin N, Smith PJ, Folb PI: **In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa**. *Journal of ethnopharmacology* 2004, **92**(2-3):177-191.
8. Brown GD: **The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of Artemisia annua L.(Qinghao)**. *Molecules* 2010, **15**(11):7603-7698.
9. Singh IP, Bharate SB, Bhutani K: **Anti-HIV natural products**. *CURRENT SCIENCE-BANGALORE-* 2005, **89**(2):269.
10. Akhondzadeh S, Noroozian M, Mohammadi M, Ohadinia S, Jamshidi A, Khani M: **Salvia officinalis extract in the treatment of patients with mild to moderate Alzheimer's disease: a double blind, randomized and placebo‐controlled trial**. *Journal of clinical pharmacy and therapeutics* 2003, **28**(1):53-59.
11. Patridge E, Gareiss P, Kinch MS, Hoyer D: **An analysis of FDA-approved drugs: natural products and their derivatives**. *Drug discovery today* 2016, **21**(2):204-207.
12. Carmo Carreiras M, Mendes E, Jesus Perry M, Paula Francisco A, Marco-Contelles J: **The multifactorial nature of Alzheimer's disease for developing potential therapeutics**. *Current topics in medicinal chemistry* 2013, **13**(15):1745-1770.
13. Imahori K, Uchida T: **Physiology and pathology of tau protein kinases in relation to Alzheimer's disease**. *The Journal of Biochemistry* 1997, **121**(2):179-188.
14. Maurer K, Volk S, Gerbaldo H: **Auguste D and Alzheimer's disease**. *The Lancet* 1997, **349**(9064):1546-1549.
15. Nguyen TT, Van Giau V, Vo TK: **Current advances in transdermal delivery of drugs for alzheimer's disease**. *Indian journal of pharmacology* 2017, **49**(2):145.
16. Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, Hall K, Hasegawa K, Hendrie H, Huang Y: **Global prevalence of dementia: a Delphi consensus study**. *The lancet* 2005, **366**(9503):2112-2117.
17. Nussbaum RL, Ellis CE: **Alzheimer's disease and Parkinson's disease**. *New england journal of medicine* 2003, **348**(14):1356-1364.
18. Munoz DG, Feldman H: **Causes of Alzheimer's disease**. *Canadian Medical Association Journal* 2000, **162**(1):65-72.
19. Haass C, Kaether C, Thinakaran G, Sisodia S: **Trafficking and proteolytic processing of APP**. *Cold Spring Harbor perspectives in medicine* 2012:a006270.
20. Hardy JA, Higgins GA: **Alzheimer's disease: the amyloid cascade hypothesis**. *Science* 1992, **256**(5054):184.

21. Oddo S, Caccamo A, Kitazawa M, Tseng BP, LaFerla FM: **Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease**. *Neurobiology of aging* 2003, **24**(8):1063-1070.
22. Mandelkow E-M, Mandelkow E: **Biochemistry and cell biology of tau protein in neurofibrillary degeneration**. *Cold Spring Harbor perspectives in medicine* 2012:a006247.
23. Bekris LM, Yu C-E, Bird TD, Tsuang DW: **Genetics of Alzheimer disease**. *Journal of geriatric psychiatry and neurology* 2010, **23**(4):213-227.
24. O'Brien RJ, Wong PC: **Amyloid precursor protein processing and Alzheimer's disease**. *Annual review of neuroscience* 2011, **34**:185-204.
25. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small G, Roses AD, Haines J, Pericak-Vance MA: **Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families**. *Science* 1993, **261**(5123):921-923.
26. Spinney L: **The forgetting gene**. *Nature* 2014, **510**(7503):26.
27. Loy CT, Schofield PR, Turner AM, Kwok JB: **Genetics of dementia**. *The Lancet* 2014, **383**(9919):828-840.
28. Venugopal C, Demos CM, Jagannatha Rao K, Pappolla MA, Sambamurti K: **Beta-secretase: structure, function, and evolution**. *CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders)* 2008, **7**(3):278-294.
29. Chow VW, Mattson MP, Wong PC, Gleichmann M: **An overview of APP processing enzymes and products**. *Neuromolecular medicine* 2010, **12**(1):1-12.
30. Vardy ER, Catto AJ, Hooper NM: **Proteolytic mechanisms in amyloid-β metabolism: therapeutic implications for Alzheimer's disease**. *Trends in molecular medicine* 2005, **11**(10):464-472.
31. Sadigh-Eteghad S, Sabermarouf B, Majdi A, Talebi M, Farhoudi M, Mahmoudi J: **Amyloid-beta: a crucial factor in Alzheimer's disease**. *Medical principles and practice* 2015, **24**(1):1-10.
32. Sun X, Chen W-D, Wang Y-D: **β-Amyloid: the key peptide in the pathogenesis of Alzheimer's disease**. *Frontiers in pharmacology* 2015, **6**:221.
33. Annaert W, De Strooper B: **A cell biological perspective on Alzheimer's disease**. *Annual review of cell and developmental biology* 2002, **18**(1):25-51.
34. Grundke-Iqbal I, Iqbal K, Tung Y-C, Quinlan M, Wisniewski HM, Binder LI: **Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology**. *Proceedings of the National Academy of Sciences* 1986, **83**(13):4913-4917.
35. Weingarten MD, Lockwood AH, Hwo S-Y, Kirschner MW: **A protein factor essential for microtubule assembly**. *Proceedings of the National Academy of Sciences* 1975, **72**(5):1858-1862.
36. Mesulam M: **The cholinergic lesion of Alzheimer's disease: pivotal factor or side show?** *Learning & memory* 2004, **11**(1):43-49.
37. Ansari N, Khodagholi F: **Natural products as promising drug candidates for the treatment of Alzheimer's disease: molecular mechanism aspect**. *Current neuropharmacology* 2013, **11**(4):414-429.
38. Essa MM, Vijayan RK, Castellano-Gonzalez G, Memon MA, Braidy N, Guillemin GJ: **Neuroprotective effect of natural products against Alzheimer's disease**. *Neurochemical research* 2012, **37**(9):1829-1842.
39. Hassaan Y, Handoussa H, El-Khatib AH, Linscheid MW, El Sayed N, Ayoub N: **Evaluation of plant phenolic metabolites as a source of Alzheimer's drug leads**. *BioMed research international* 2014, **2014**.
40. Baptista FI, Henriques AG, Silva AM, Wiltfang J, da Cruz e Silva OA: **Flavonoids as therapeutic compounds targeting key proteins involved in Alzheimer's disease**. *ACS Chemical Neuroscience* 2014, **5**(2):83-92.

41. Ono K, Yoshiike Y, Takashima A, Hasegawa K, Naiki H, Yamada M: **Potent anti‐amyloidogenic and fibril‐destabilizing effects of polyphenols in vitro: implications for the prevention and therapeutics of Alzheimer's disease**. *Journal of neurochemistry* 2003, **87**(1):172-181.
42. Yoo K-Y, Park S-Y: **Terpenoids as potential anti-Alzheimer's disease therapeutics**. *Molecules* 2012, **17**(3):3524-3538.
43. Kim MH, Kim S-H, Yang WM: **Mechanisms of action of phytochemicals from medicinal herbs in the treatment of Alzheimer's disease**. *Planta medica* 2014, **80**(15):1249-1258.
44. Fulton B, Benfield P: **Galanthamine**. *Drugs & aging* 1996, **9**(1):60-65; discussion 66-67.
45. Rainer M: **Galanthamine in Alzheimer's disease**. *CNS drugs* 1997, **7**(2):89-97.
46. Ji H-F, Shen L: **Berberine: a potential multipotent natural product to combat Alzheimer's disease**. *Molecules* 2011, **16**(8):6732-6740.
47. Asai M, Iwata N, Yoshikawa A, Aizaki Y, Ishiura S, Saido TC, Maruyama K: **Berberine alters the processing of Alzheimer's amyloid precursor protein to decrease Aβ secretion**. *Biochemical and Biophysical Research Communications* 2007, **352**(2):498-502.
48. Kim MH, Kim S-H, Yang WM: **Mechanisms of action of phytochemicals from medicinal herbs in the treatment of Alzheimer's disease**. *Planta Med* 2014, **80**(15):1249-1258.
49. Ringman JM, Frautschy SA, Teng E, Begum AN, Bardens J, Beigi M, Gylys KH, Badmaev V, Heath DD, Apostolova LG: **Oral curcumin for Alzheimer's disease: tolerability and efficacy in a 24-week randomized, double blind, placebo-controlled study**. *Alzheimer's research & therapy* 2012, **4**(5):43.
50. Hamaguchi T, Ono K, Yamada M: **Curcumin and Alzheimer's disease**. *CNS neuroscience & therapeutics* 2010, **16**(5):285-297.
51. Ono K, Hasegawa K, Naiki H, Yamada M: **Curcumin has potent anti‐amyloidogenic effects for Alzheimer's β‐amyloid fibrils in vitro**. *Journal of neuroscience research* 2004, **75**(6):742-750.
52. Fang F, Chen X, Huang T, Lue L-F, Luddy JS, Yan SS: **Multi-faced neuroprotective effects of Ginsenoside Rg1 in an Alzheimer mouse model**. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 2012, **1822**(2):286-292.
53. Zhou YX, Zhang H, Peng C: **Puerarin: a review of pharmacological effects**. *Phytotherapy Research* 2014, **28**(7):961-975.
54. Yin F, Liu J, Ji X, Wang Y, Zidichouski J, Zhang J: **Silibinin: A novel inhibitor of Aβ aggregation**. *Neurochemistry international* 2011, **58**(3):399-403.
55. Johnston G, Chebib M, Duke R, Fernandez S, Hanrahan J, Hinton T, Mewett K: **Herbal products and GABA receptors**. *inEncyclopedia of Neuroscience, LR Squire, Ed* 2009:1095-1101.
56. Gornik HL, Creager MA: **Medical treatment of peripheral artery disease**. In: *Vascular Medicine: A Companion to Braunwald's Heart Disease.* edn.: Elsevier; 2013: 242-258.
57. Yin Y, Ren Y, Wu W, Wang Y, Cao M, Zhu Z, Wang M, Li W: **Protective effects of bilobalide on Aβ25–35 induced learning and memory impairments in male rats**. *Pharmacology Biochemistry and Behavior* 2013, **106**:77-84.
58. Dajas F, Abin-Carriquiry JA, Arredondo F, Blasina F, Echeverry C, Martínez M, Rivera F, Vaamonde L: **Quercetin in brain diseases: Potential and limits**. *Neurochemistry international* 2015, **89**:140-148.
59. Ansari MA, Abdul HM, Joshi G, Opii WO, Butterfield DA: **Protective effect of quercetin in primary neurons against Aβ (1–42): relevance to Alzheimer's disease**. *The Journal of nutritional biochemistry* 2009, **20**(4):269-275.

60. Reznichenko L, Amit T, Zheng H, Avramovich‐Tirosh Y, Youdim M, Weinreb O, Mandel S: **Reduction of iron‐regulated amyloid precursor protein and β‐amyloid peptide by (–)‐epigallocatechin‐3‐gallate in cell cultures: implications for iron chelation in Alzheimer's disease**. *Journal of neurochemistry* 2006, **97**(2):527-536.
61. Vingtdeux V, Dreses-Werringloer U, Zhao H, Davies P, Marambaud P: **Therapeutic potential of resveratrol in Alzheimer's disease**. *BMC neuroscience* 2008, **9**(2):S6.
62. Zhang HY, Zheng CY, Yan H, Wang ZF, Tang LL, Gao X, Tang XC: **Potential therapeutic targets of huperzine A for Alzheimer's disease and vascular dementia**. *Chemico-Biological Interactions* 2008, **175**(1-3):396-402.
63. Alkam T, Nitta A, Mizoguchi H, Itoh A, Nabeshima T: **A natural scavenger of peroxynitrites, rosmarinic acid, protects against impairment of memory induced by Aβ25–35**. *Behavioural brain research* 2007, **180**(2):139-145.
64. Wang H, Wang H, Cheng H, Che Z: **Ameliorating effect of luteolin on memory impairment in an Alzheimer's disease model**. *Molecular medicine reports* 2016, **13**(5):4215-4220.
65. Sawmiller D, Li S, Shahaduzzaman M, Smith A, Obregon D, Giunta B, Borlongan C, Sanberg P, Tan J: **Luteolin reduces Alzheimer's disease pathologies induced by traumatic brain injury**. *International journal of molecular sciences* 2014, **15**(1):895-904.
66. Zhao L, Wang J-L, Liu R, Li X-X, Li J-F, Zhang L: **Neuroprotective, anti-amyloidogenic and neurotrophic effects of apigenin in an Alzheimer's disease mouse model**. *Molecules* 2013, **18**(8):9949-9965.
67. Parnetti L, Mignini F, Tomassoni D, Traini E, Amenta F: **Cholinergic precursors in the treatment of cognitive impairment of vascular origin: ineffective approaches or need for re-evaluation?** *Journal of the neurological sciences* 2007, **257**(1-2):264-269.

Chapter 2
In vitro Anti-Alzheimer's activity of medicinal plants native to or naturalised to South Africa

2.1. Introduction

2.1.1. The potential of South African medicinal plants in drug discovery

According to South African National Biodiversity Institute (SANBI), South Africa is the third most diverse country in the world. South Africa owns such a rich plant diversity because of variety of climate and altitude. It exhibits 24,000 plant taxa reported to belong to 368 families. About 15% of these plant species are used for medicinal purposes. Approximately 3000 plant species are used as the traditional medicines [1] and about 80% of South African people use traditional medicines for their health care [2]. Of 3000 plant species, 350 species are traded as medicinal plants and 132 plant species are included on the basis of well-known and fully understood herbal medicines [3].

According to WHO, African traditional medicine is the sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or societal imbalance, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing [4]. African traditional medicine also known as 'Cradle of Mankind', is the oldest medicinal system and South Africa has a cultural diversity marked by traditional healing practices [5]. Traditional healers are the major source of health services for a large population in South Africa. In South Africa, the traditional doctor is usually a man also known as inyanga who uses herbal medicinal preparations for treating disease [4]. They are also called herbalists with a great knowledge of herbs and medicines of animal origin [6].

In South Africa, approximately 200 000 traditional healers practice herbal medicines [7]. The use of the medicinal plants by South African people has a long history. For example, native

hottentots used the willow species for the treatment of rheumatic fever in the Cape of Good Hope [8]. In addition a Basuto tribal healer used a root decoction prepared from *Pelargonium sidoides* for the cure of tuberculosis of an Englishman, named Charles Stevens [8] which is now commonly traded in Europe as a herbal medicine. In Sub-Saharan Africa, traditional healer practitioners together with the modern health practitioners play a significant role in the treatment of sexually transmitted diseases including HIV/AIDS by integrating the biomedical information of HIV/AIDS in their practices [9]. Bapedi traditional healers from Limpopo province, South Africa used 35 exotic plant species belonging to 21 different families for the treatment of different ailments such as hypertension, diabetes mellitus, erectile dysfunction and gonorrhoea, diarrhoea and tuberculosis. The families, Fabaceae, Solanaceae, Apocynaceae and Asteraceae contain largest number of exotic plant species with medicinal value in traditional healing [10]. In 2002, Steenkamp conducted a survey in which he found that about 10% of the 156 plant species were used for treating gynaecological problems including infertility, menopausal complaints, breast and uterus cancer and menstrual disorders; amenorrhoea, dysmenorrhoea, menorrhagia and irregular menstruation [8], [11]. Two alkaloids, vincristine and vinblastine isolated from *Catharanthus roseus* (Apocynaceae) are well known for their antitumor activity. This plant has been reported for its use as a treatment of breast and uterine cancer [11].

An ethnobotanical survey conducted in Eastern Cape Province; South Africa revealed that 17 plant species from 13 different families were used by traditional healers for the treatment of cancer [12]. From the 13 families, two named Hyacinthaceae and Hypoxidaceae were used prominently and *Solanum aculeastrum*, belonging to family Solanaceae is the most commonly used plant species for cancer treatment [12]. The combretastatin, isolated from *Combretum caffrum*, commonly known as bush willow act as anti-angiogenic agents and played an important role in the treatment of cancer. Six South African plants *Bidens pilosa*, *Centella asiatica*, *Cnicus benedictus*, *Dicoma capensis*, *Hypoxis hemerocallidea* and *Sutherlandia frutescens* were found to exhibit cytotoxicity against two cancer cell line MCF-7 and a non-

malignant breast cell line MCF-12A [13]. *Pelargonium reniforme*, a South African plant species was reported to exhibit antioxidant properties and used for the treatment of liver disorders [14].

The anti-plasmodial activity of 134 plant taxa native to or naturalised in South Africa, belonging to 54 families were tested *in vitro* the results of which showed that 17% were found to exhibit high anti-plasmodial activity [15].

The acetone and aqueous extracts of *Cryptocarya latifolia*, *Helichrysum melanacme*, *Euclea natalensis*, *Nidorella anomala* and *Thymus vulgaris* have been reported to inhibit the growth of *M. tuberculosis* when screened against a drug-sensitive strain,H37Rv by using the agar plate method [16].

The Sceletium species are the well-researched and commercially promising plant species among the South African plants from a pharmaceutical point of view [17]. *Sceletium tortuosum* has been reported to reduce anxiety, stress or depression and has also been reported to elevate mood in a human clinical trial [18]. The standardised *S. tortuosum* extract, Zembrin®, has been shown to inhibit phosphodiesterase-4 (PDE-4) with an IC_{50} value of 8.5 µg/ml and also act as a potent blocker in 5-HT transporter binding assay with an IC_{50} value of 4.3 µg/ml [19]. Three major alkaloids mesembrine, mesembrenone and mesembrenol isolated from *S. tortuosum* are responsible for the potent activity of the plant and affect many CNS disorder targets [18].

There are few African plants which have been recognised for their commercialization and role in drug discovery on a global scale. These plants are *Xysmalobium undulatum, Agathosma betulina, Aloe ferox, Aspalathus linearis, Artemisia afra, Cyclopia genistoides, Hoodia gordonii, Harpagophytum procumbens, Hypoxis hemerocallidea, Lippia javanica, Warburgia salutaris, Pelargonium sidoides* and *Sutherlandia frutescens* [20].

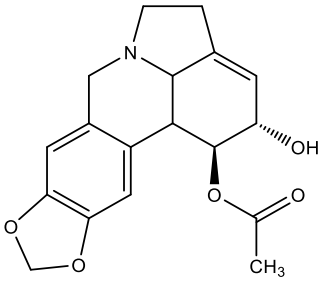
South Africa with a rich biodiversity has resulted in the isolation and discovery of several potent bioactive compounds. Thus, in this dissertation, finding novel potential plant-derived anti-Alzheimer compounds from the South African medicinal plants, through conducting bioassay

guided fractionation, employing standard isolation and modern spectroscopic techniques, underpins the main focus and aim of this study.

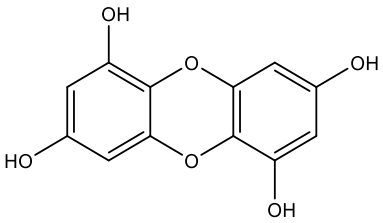
2.1.2. South African medicinal plants with acetylcholinesterase inhibition and reduction of Aβ42 for treating Alzheimer's disease

Acetylcholinesterase inhibitors are considered as having one of the promising therapeutic potential in the field of Alzheimer's disease [21]. The majority of research done on South African medicinal plants for treating Alzheimer's disease has focused on the inhibition of acetylcholinesterase. Alkaloids isolated plant extracts *Boophone disticha*, *Crinum campanulatum*, *Crinum moorei*, *Crinum macowanii*, *Crinum bulbispermum* and *Cyrtanthus falcatus* belonging to family *Amaryllidaceae* have been reported for AChE inhibition activity [22]. One of the alkaloid, 1-*O*-acetyllycorine **(15)** was found to be potently active against AChE inhibitory with an IC_{50} value of 0.96 ± 0.04μM while galanthamine exhibited IC_{50} value 1.9 ± 0.16μM in the same assay indicating that the compound was twice as potent as galanthamine [23]. Withanoloids isolated from *Withania somnifera* (L.) Dun belonging to family Solanaceae, inhibited AChE and BuChE dose dependently. It also significantly regenerated axons and dendrites in memory- deficient mice [24]. The DCM:MeOH root extracts of *Chamaecrista mimosoides* potently inhibited the AChE activity with IC_{50} value 0.03 ± 0.08 μg/ml [25]. Aqueous and ethanol extracts of *Crinum moorei* and *Boophane disticha* showed promising results for the treatment of Alzheimer's disease by potently inhibiting AChE activity in the TLC bio-autography assay. Both species belong to *Amaryllidaceae* family, known for containing alkaloids which are responsible for the inhibition of AChE [26]. Phenolic compounds isolated from *Croton gratissimus* exhibited good acetylcholinesterase inhibitory activity [27]. Phlorotannins, dibenzo [1,4] dioxine-2,4,7,9-tetraol **(16)** and eckol **(17)** isolated from *Ecklonia maxima* have been reported to exhibit AChE inhibitory activity with an IC_{50} value of 84.48 ± 0.26 and 76.70 ± 0.35, respectively as compared to the galanthamine [28]. A crinine alkaloid, 6-hydroxycrinamine **(18)** isolated from methanol extract of the bulbs of *B. disticha* was found to exhibit AChE inhibitory activity with an IC_{50} value of 445 μM. Although the

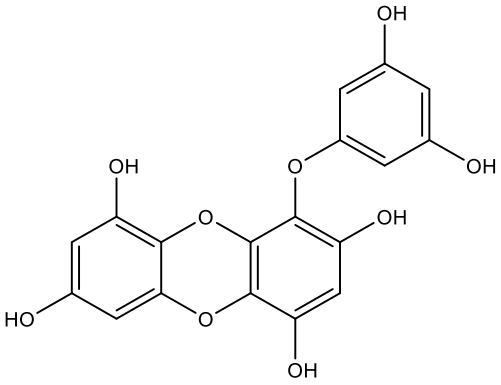
compound was active, it showed cytotoxicity towards neuroblastoma cells [29]. The three compounds, hydroxyphenyl ethyl vanillate **(19)**, acteoside **(20)** and quercetin **(21)** isolated from *Buddleja salviifolia* showed AChE inhibition activity with an IC_{50} values of 259.5±0.00, 123.1±1.23, 66.8±0.09, respectively as compared to galanthamine [30]. Assoanion **(22)**, one of the alkaloids isolated from *Cyrtanthus contractus* showed very good AChE activity with an IC_{50} value of 3.87 ± 0.24 µM as compared to the control galanthamine with an IC_{50} value of 1.9 ± 0.16 µM [31].



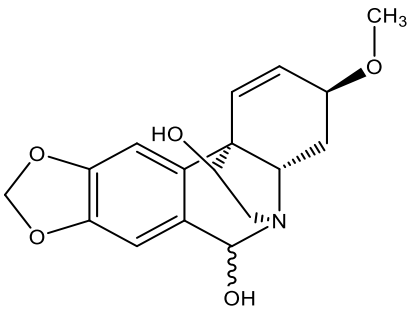
(15)



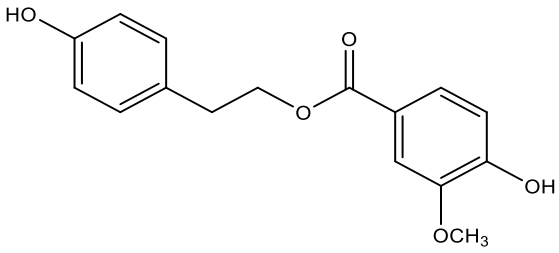
(16)



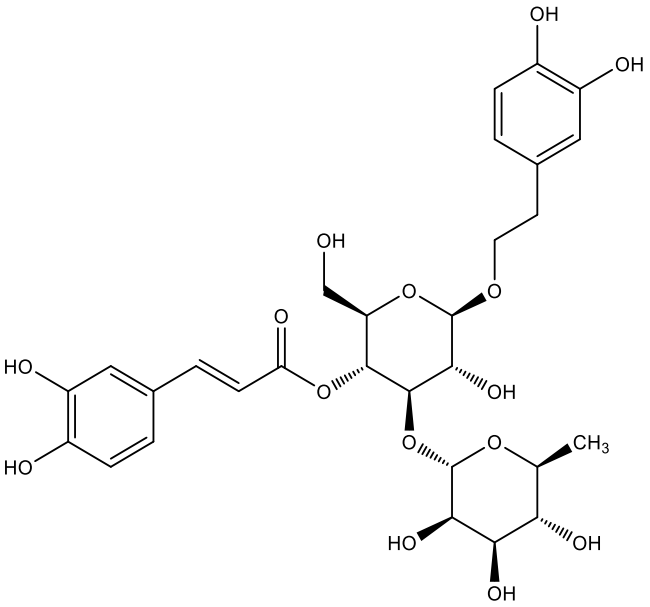
(17)



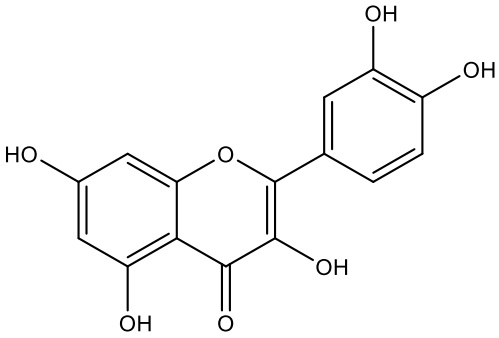
(18)



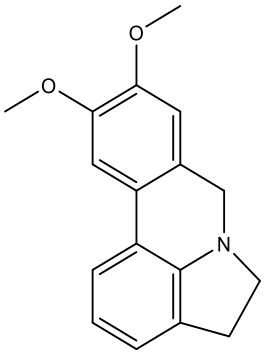
(19)



(20)



(21)

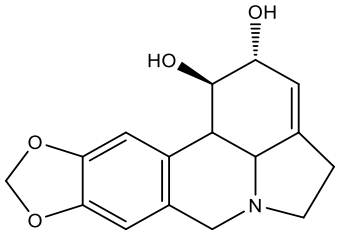


(22)

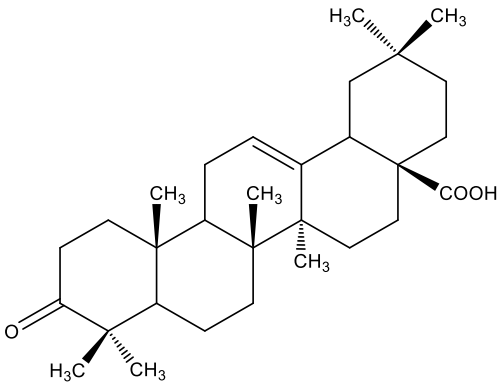
Figure 2.1: Structures of the compounds exhibited acetylcholinesterase inhibition activity, 1-O-acetyllycorine **(15)**, dibenzo [1,4] dioxine-2,4,7,9-tetraol **(16)**, eckol **(17)**, 6-hydroxycrinamine **(18)**, hydroxyphenyl ethyl vanillate **(19)**, acteoside **(20)**, quercetin **(21)** and assoanion **(22)**

One of the previous studies showed that phenolic rich extracts of three plants *Schotia brachypetala*, *Camellia sinensis*, *Markhamia platycalyx* were able to significantly reduce Aβ42 and could lead to the discovery of anti-Alzheimer's drugs [32]. The ethyl acetate extracts of *Xysmalobium undulatum* also exhibited acetylcholinesterase inhibition activity in *in vitro* studies [33]. Lycorine **(23)** isolated from *Euclea crispa* and 3-Oxo-oleanolic acid **(24)** isolated

from *Crinum macowanii* exhibited the significant Aβ42 reduction from APPsw-transfected HeLa cells in a dose dependent manner [34]. The extracts of *Monsonia* species belong to family Geraniaceae were found very active in suppressing the Aβ42 production in the brain [35].



(23)



(24)

Figure 2.2: Structure of the compounds exhibited Aβ42 reduction, Lycorine **(23)**, 3-oxo-oleanolic acid **(24).**

Many plants native to or naturalised to South Africa were researched for mental disorders and Alzheimer's disease through Aβ42 and AChE inhibition in *in vitro* and *in vivo* studies. These studies provided evidence that South African plant species could lead to new drug leads for the pharmaceutical industry in the field of Alzheimer's disease.

2.1.3. Need for research on Alzheimer's disease in South Africa

In 2003/4, the South Africa Stress and Health (SASH) conducted a study which estimated that approximately one in three South Africans suffer from some form of mental disorders [36]. In 2014, the Universities of Cape Town and Stellenbosch, further ratified this study and came to conclusion that mental disorders is probably higher than one in three of the South African population [36].

Another report on “sick state of mental health” in South Africa published by the country’s Sunday Times Newspaper stated that “More than 17-million people are dealing with depression, substance abuse, anxiety, bipolar disorder and schizophrenia” [37]. These surveys showed that mental illnesses is becoming a serious threat to South Africa and needs special attention. In South Africa, a database analysis was conducted on the prescription pattern and cost of drugs for Alzheimer’s disease. This study revealed that a total of 588 patients were found who received 2623 medicine items with an average cost per item R596.15 (South African Rand). This cost may be unaffordable for the South African people. This data analysis indicated that either the Alzheimer’s disease is under diagnosed or the medicine for Alzheimer’s disease is under-prescribed in South Africa because of the cost effectiveness [38]. Later it was found that approximately 750 000 people in South Africa are suffering from Alzheimer’s disease [39]. Due to the rapidly growing problem of mental disorders such as Alzheimer’s disease in South Africa, there is a need to focus on it. South African plants have great therapeutic potentials and can also lead to the discovery of novel and affordable drugs in the field of Alzheimer’s disease. This study thus focuses on the medicinal plants of South Africa with traditional uses associated with mental disorders to determine their efficacy by screening the different plant part extracts against Aβ42 as a potential treatment of Alzheimer’s disease.

2.2. Materials and Methods

2.2.1. Selection and collection of plant material

A literature study was undertaken using Google scholar, SciFinder and Scopus for the selection of the plants by using the keywords; African medicinal plants and traditional medicinal plants in combination with the memory loss, mental illness, depression, Alzheimer’s disease, dementia, anxiety, epilepsy, forgetfulness, convulsion, hysteria, sedative effects, acetylcholinesterase inhibition. The identified plants were then prioritized by a scoring system

of 1, 2 and 3 where 1 represents strong, 2 medium and 3 weak. The criteria used for scoring was as follows

a) Strength of the traditional uses for Alzheimer's disease, in which the plants with direct relation with Alzheimer's disease either through Aβ42 reduction or by AChE inhibition were given a score of 1, plants with traditional uses such as memory loss were given a score of 2 and the plants with other traditional uses such as epilepsy, hysteria, headache and sedative effects were given a score of 3.

b) Strength of published use for Alzheimer's disease (peer reviewed) - plants with significant published information for Alzheimer's disease were given a score of 1, plants with less and no published information for Alzheimer's disease were given scores 2 and 3, respectively.

c) Plant toxicity - plants with no or limited toxicity were given a score of 1 and plants with medium and high toxicity were given scores of 2 and 3, respectively.

d) Plant part used - leaves given a score of 1, stems and stalks given a score of 2 and roots given a score of 3. The schematic process of selection of plants is shown in Figure 2.3.

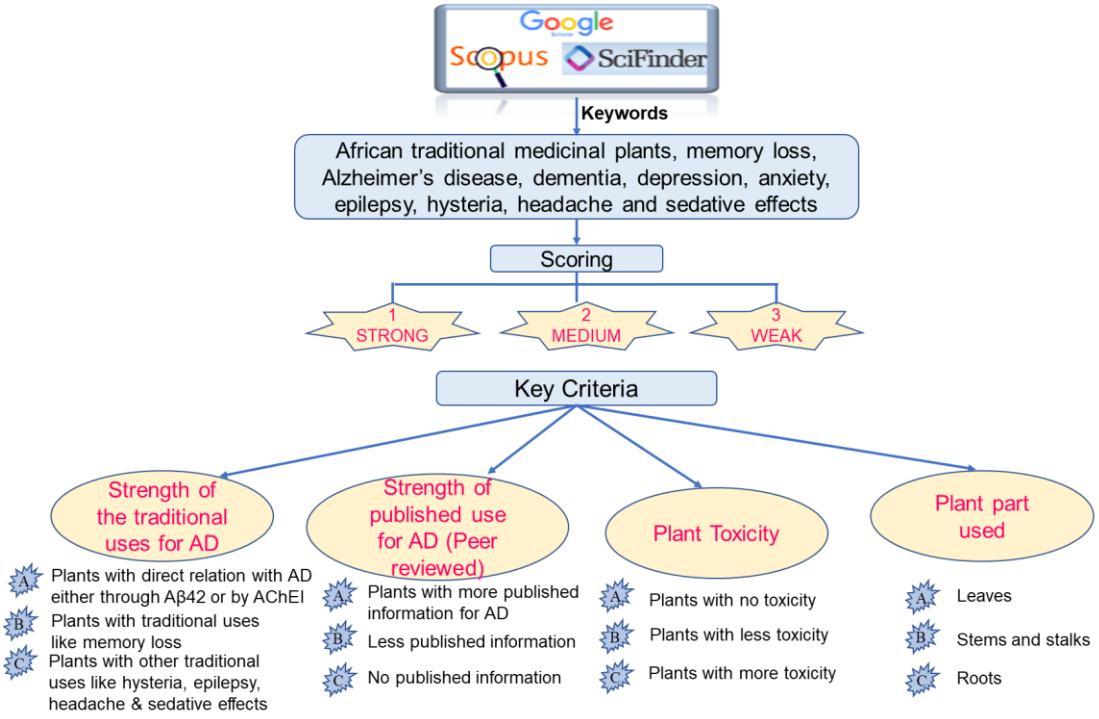


Figure 2.3*:* Criteria for the selection of plants species

2.2.2. Collection and extraction of plants

Plants with their different parts were collected from Maine *vander* Schijff Botanical Garden and experimental farms of University of Pretoria, South Africa with the help of Curator Jason Samuels. Plant identification was established at the H.G.W.J. Schweickerdt Plant Herbarium at of Pretoria University. Plant parts were separately cut into small pieces and then dried in an oven at 60°C. The drying time varied depending on the nature of plant and the plant part. Dried plant material was ground to a coarse or fine powder using a grinder. Each of the powdered plant materials (2g) were extracted repeatedly three times for 8 hours with dichloromethane/methanol (DCM/MeOH) (1:1) with the help of a magnetic stirrer. Extracts were filtered using a Buchner funnel. Plant extracts obtained from three different extractions were combined and then concentrated using a rotary vacuum evaporator and dried under high

vacuum. All the extracts were stored in a cold room prior to screening prior to bioassaying in collaboration with the Korean Institute of Science and Technology (KIST).

2.2.3. Cell culture

HeLa cells stably transfected with APP carrying Swedish mutation (APPsw) were cultured at 37°C, 5% CO_2, in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum containing 100 units/ml penicillin, 100 µg/ml streptomycin, 260 µg/ml Zeocin, 400 µg/ml G418.

2.2.4. Aβ42 inhibition bioassay

APPsw-transfected HeLa cells at 80% confluence in a 35mm dish were cultured with sample solubilized in dimethyl sulphoxide (DMSO) for 8 h in serum free medium. The conditioned medium was analyzed by a sandwich ELISA (Invitrogen) for detection of Aβ42 according to the supplier's instruction.

2.2.5. Statistical analysis

Data was expressed as mean ± SEM. Statistical comparisons between controls and treated experimental groups were performed using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

2.3. Results and discussion

2.3.1. Selection of plant material

Using the selection criteria and the databases searches, at least 52 plant species were identified as candidates for screening their potential for Aβ42 reduction. As this was a broad general search, the additional criteria and scoring system was applied which resulted in 21 plant species being identified for the collection, extraction and screening. A scoring system

which was shown to be useful was adapted from the previous studies [40], [15]. In instances when the criteria could not be applied for the plant species as the information may not have been publicly available, these were excluded from the scoring system. This resulted in the identification of 21 species which had scores between 1 and 9. Table 2.1 shows the plants species identified through the scoring system with the scores assigned for each of the criteria and the total scores per plant. These species represented 15 different families.

Plant species with the lowest scores representing the highest priority based on the criteria used where *Mondia whitei* and *Schotia brachypetala* with scores of 4. Two plants species *Centella asiatica* and *Trichilia dregeana* scored 5; five plants species , *Commelina africana*, *Cussonia paniculata*, *Cussonia spicata*, *Tetradenia riparia* and *Zanthoxylum capense* had a score of 6; seven plants*, Buddleja salvifolia, Bulbine natalensis*, *Catha edulis*, *Heteromorpha arborescens, Ruta graveolens*, *Xysmalobium undulatum* and *Ziziphus mucronata* had a score of 7; three plants *Cissus quadrangularis*, *Cotyledon orbiculata*, and *Tabernaemontana elegans* scored 8 and two plants *Plumbago auriculata* and *Stapelia gigantea* with the lowest score of 9. The published phytochemistry of the plants was also established through literature searches and this was done to guide any further fractionation process to identify the active ingredients and to provide any information on whether some of these compounds may have been published for the treatment of Alzheimer's disease.

Table 2.1: Scoring for selection of plant species

Plant species and Family	**Traditional use (A)**	**Previously reported bioassays (B)**	**Toxicity (C)**	**Phytochemistry**	**Total Score (T) (A)+(B)+(C) = (T)**
Buddleja salvifolia	Traditionally used in the treatment of neurodegenerative disorders. Buddleja species together with *Heteromorpha trifoliate* & *Cussonia*	*B. salvifolia* leaf extracts showed significant antimicrobial and acetylcholinesterase inhibitory properties [30]. The whole plant showed good antioxidant	Highly toxic, DCM and MeOH leaf extract WSI Cell line, IC_{50}>200µg/ml [41], [42]	4′-hydroxyphenyl ethyl vanillate, Acteoside and quercetin [30].	

	paniculata are used for the treatment of early nervous and mental disorders in South Africa [25]. **Score- 2**	activity with ABTS; IC_{50} = 0.14 ± 0.08 mg/ml and DPPH; IC50 = 0.23 ± 0.01 mg/ml and DCM:MeOH (1:1) showed IC_{50} value of 0.05 ± 0.02 mg/ml as compared to the positive control, galanthamine with IC_{50} value of 5.3×10^{-4} mg/ml [25]. **Score- 2**	**Score- 3**		**7**
Bulbine natalensis	Tuber decoctions are used for the treatment of convulsions in South Africa [43].	The biochemical analysis of the excisional wound tissue of *B. natalensis* treated wounds showed a significant increase in the total collagen content compared to that of the untreated wounds [44]. The *in vitro* antimicrobial activity of ethanolic extract, n-butanol and ethyl acetate fractions of 4 Gram positive and 12 Gram negative bacteria as well as 3 fungal species were using agar dilution inhibited 75, 87.5 and 100% respectively and produced growth inhibition at MIC range of 1-10, 3-10 as well as 1 and 5	Low toxic, Leaf extract, Brine shrimp Cell line, IC_{50}= 2210.0µg/ml. [46]; Mixed cytotoxicity at different concentration, Cell line, HEp-2	Tannins, anthraquinones, cardiac glycosides, saponins, and alkaloids [47].	

	Score- 3	mg/ml respectively [45]. **Score- 3**	**Score- 1**		**7**
Catha edulis	Fresh leaves reduce fatigue and relieve sleepiness. It increases mental power and communication skills but also causes hypertension and affects male fertility [48]. **Score- 2**	The khat extracts showed selective effect on spatial learning and memory in CBA mice. The high dose (360 mg/kg body weight) of khat extract improved the memory but impaired learning while the moderate (120 mg/kg body weight) and low dose (40 mg/kg body weight) impaired accuracy for spatial memory of the platform location [49]. **Score- 2**	Highly toxic, leukemia cell line [50]. **Score- 3**	The major alkaloids found in Khat is phenylalkylamines and cathedulins. Phenylalkylamines contain (+)-Cathine; (-)-Cathinone; 3,6-Dimethyl-2,5-diphenyl pyrazine; (-)-Norephedrine; (-)-Norephedrine N-formyl; Merucathine; Merucathinone; 1-Phenylpropane-1,2-dione; Pseudomerucathine. Cathedulins contain Cathedulin E1–E6; Cathedulin K1, 2, 6, 12, 15, 17, 19, 20; Cathedulin Y7–Y10; Cathidine A; Cathidine B; Cathidine D [51].	**7**
Centella asiatica	Dried powdered leaves reportedly produce a calming and sedative effect and in some parts of India it is given with milk to improve memory against dementia [24].	The extracts of *C. asiatica* have shown effective to increase the antioxidant enzymes in corpus striatum and hippocampus in the aged rats and rats with Parkinson's disease and also prevented the	Low cytotoxicity, Whole plant, Cell line L6, IC_{50}= 82.6 μg/ml [42], [53].	Triterpenoid saponins including asiaticosides, madecassoside and madsiatic acid. brahmoside and brahminode, isothankuniside and thankuniside, plant sterols,	

		oxidation of proteins, lipid peroxidation and prooxidant processes in them [52].		flavonoids, centilloside and its derivatives [54]	
	Score- 2	**Score- 2**	**Score- 1**		**5**
Cissus quadrangularis	Boiled with water and used for the treatment of epilepsy in Dar es Salaam, Tanzania [55].	Bone fracture healing activity, analgesic activity, antiosteoporotic activity, antioxidant activity, antiulcer activity, Parasympathomimetic activity, Anabolic and Androgenic activity, Antihemorrhoidal activity, Anti-inflammatory activity and Gastroprotective Activity [56].	Moderately toxic MeOH stem extract Cell line- Brine shrimp IC_{50}= 1300.0µg/ml [57], [42].	Tetracyclic triterpenoids-stilbenes resveratrol, piceatannol, pallidol; stilbene derivatives-quadrangularins A, B, and C, parthenocissin A and the known flavonols-quercetin and kaempferol [58]. Alkaloids, resveratrol, piceatannol, pallidol, parthenocissin, quadrangularins, ascorbic acid, carotene, phytosterol substances, calcium, flavinoids, vitamins, enzymes, nicotinic acid, tyrosin, and triterpenoids [59]	
	Score- 3	**Score- 3**	**Score-2**		**8**
Commelina Africana	In Lesotho plant decoctions with *Tephrosia capensis* are used for heart	*C. africana* reduced fasting blood glucose of experimental diabetic rats by	Low toxic, Vero cell line, IC_{50}= 441.1µg/ml [61].	Alkaloids, cardenolides, saponins and tannins [60].	

	complaints and nervous disorders [24]. **Score- 2**	78.0%. Thus showed a comparable activity with the reference hypoglycaemic agent [60]. **Score- 3**	**Score- 1**		**6**
Cotyledon orbiculata	Leaf juice has been used for the treatment of epilepsy [48]. **Score- 3**	*C. orbiculata* has shown the anticonvulsant activity which supports the fact of its traditional use for the treatment of epilepsy [62]. Water extract of *C. orbiculata* at 7.5 mg/ml prevented nematode eggs from hatching with 82.63% success rate and suppressed nematode larval development with 85.32% at the concentration of 2.5 mg/ml which showed that it possess anthelmintic activity [63]. **Score- 3**	Moderate toxic, Cell line, HEK 293 IC_{50}= 44.63µg/ml) [64]. **Score- 2**	Alkaloids, saponins, tanins, cardiac glycosides, triterpene steroids, reducing sugar, flavonoids and anthraquinones [62]. Four bufadienolides, namely orbicusides A,B and C and tyledoside C [65].	**8**
Cussonia paniculata	Used for the treatment of rheumatism, dysmenorrhea, colic and diseases associated with the nervous system [66]. it is used as a traditional medicine against	The stem bark of *C. paniculata* showed anti-inflammatory and analgesic activities. DCM:MeOH leaf extract of *C. paniculata* screened for the in vitro anticancer activity and showed	Low toxic, APPsw-transfected HeLa cells Toxic only at high concentration 100µg/ml [69].	Triterpenoid glycosides [70], sterol/triterpene, flavonoids such as rutin, quercetin and luteolin [71].	

	pain, inflammation, infections and malaria. The species is also used for the treatment of mental illness, diarrhea and sexually transmitted diseases [67]. The thick tuberous roots are peeled and eaten raw as an emergency food or as a source of water. In South Africa, the mixture of *Buddleja* species with *Heteromorpha trifoliate* and *Cussonia paniculata* is used for the treatment of early nervous and mental disorders [25]. **Score- 2**	an inhibition of 100% cell growth at a concentration of 5.25 (renalTK10), 15.0 (melanoma UACC62) and 55.69 g/ml (breast MCF7) [68] . **Score- 3**	**Score- 1**		**6**
Cussonia spicata	The succulent and edible mashed roots of *C. spicata* are used for the treatment of Malaria. The root decoction is used to treat fever and mental illness. The leaves are used for the treatment of indigestion. It is also used for the	Disc diffusion bioassays for *C. spicata*, the ethanolic and ethyl acetate extracts of the bark and roots showed inhibitory activity with the MIC'S value of 12.5 mg/ml against Staphylococcus aureus [67].	Low toxic, Water extract of root, Brine shrimp Cell line IC_{50}= 2600.0$\mu g/ml$ [72].	Molluscicidal saponins, [α -L-arabinofuranosyl-(1 $\rightarrow$ 4)- β -D-glucuronopyranosyl-(1 $\rightarrow$ 3)]-3 β -hydroxyolean-12-en-28-oic acid and [(α -L-arabinofuranosyl-(1 $\rightarrow$ 4)- β -D-	

	treatment of epilepsy in Dar es Salaam, Tanzania [55]. **Score- 2**	**Score- 3**	**Score- 1**	galactopyranosyl-(1 → 2)- β -D-glucuronopyranosyl-(1 → 3)]-3 β -hydroxyolean-12-en-28-oic acid were isolated from *C. spicata* [73].	**6**
Heteromorpha arborescens	Used to treat headache, pain, fever, dysmenorrhea, nervous and mental disorders [74], [75]. **Score- 2**	The organic extracts of root showed a good COX-1 inhibition activity [74]. The methanolic and the aqueous root extracts showed significant pain inhibition of carrageenan induced inflammatory pain and also the significant reduction of edema induced by albumin and carrageenan [75]. The leaf extract of *H. arborescens* inhibited nitric oxide production by 89.98% at a concentration of 30µg/ml and also showed a good *in vitro* anti-arthritic activity with IC_{50} value of 53.78 µg/ml [76]. **Score- 2**	Highly toxic, Leaf extract, Vero monkey kidney Cell line, IC_{50}= 0.04mg/ml [77] **Score- 3**	Saponins, saikogenin B4 and saikogenin F; Falcarindiol, a polyacetylene and sarisan. [74], [78] .	**7**
Mondia whitei	Root infusions are taken by unspecified groups in South	The plant showed a strong acetylcholinesterase inhibition	Low toxic, Breast cancer cell lines, MDA-MB-231,	Alkaloids, flavonoids, saponins, tanins, polyphenols,	

	Africa to treat stress in adults [24]. It is used as a traditional medicine to treat nervous disorders. Pulverized root is used to treat schistosomiasis and abdominal pain and also taken in water for headache [79]. **Score- 2**	bioactivity and acted as an antibacterial agent. [80]. A compound monoterpene–lactone loliolide isolated from *M. whitei* leaves have shown affinity to SERT in a binding assay. The activity of compound with an IC_{50} value of 1.3 nM (Ki=0.6nM) than the activity of Citalopram [81]. **Score- 1**	MCF-7 and MCF-12A [82]. **Score- 1**	moderate anthranal glycosides and terpenoids [83]. Chlorinated coumarinolignan, 5-chloropropacin [84].	**4**
Plumbago auriculata	Used for headache in Zulu, Xhosa and Sotho medicine [85]. **Score- 3**	*P. auriculata* showed a significant *in vitro* antimalarial activity with different solvent extracts. n-hexane extract showed IC_{50} 45.9 ± 3.3µg/ml, DMC extract showed 40.2 ± 1.6 µg/ml; 53.8 ± 3.1 µg/ml; 80.0 ± 15.1µg/ml [86]. Plumbagin isolated from *P. auriculata* inhibited the seed germination of lettuce and wheat [87]. **Score- 3**	High toxic, HGE-17 cell line, MTT assay, IC_{50}= 278.59µg/ml [88]. **Score- 3**	Plumbagin, epi-isoshinanolone, plumbagic acid, palmitic acid, sitosterol and 3-Oglycosylsitosterol [89].	**9**
Ruta graveolens	Leaf infusions are taken for epilepsy, hysteria, convulsions and fits in children [48]. It has been used as a traditional medicine for the	It has been shown to exhibit antibacterial, analgesic, anti-inflammatory, antidiabetic, insecticidal, anticancer, xanthine oxidase inhibition,	High toxic, Hep2 cell line, IC_{50}= 62µg/ml [92].	Rutin, quercetin, psoralen, methoxypsoralen, rutacridone, rutacridone epoxide and gravacridondiol and the main	

	treatment of pain relief, eye problems, rheumatism and dermatitis. It has been used as an antidote for the snake toxins and snake venoms [90]. **Score- 3**	antihyperlipidemic and antiandrogenic activities [90]. The acridone alkaloid arborinine isolated from *R. graveolens* exhibited the strong AChE inhibition activity with an IC_{50} < 50 [91]. **Score- 1**	**Score- 3**	monoterpenes namely, α-Pinene, limonene and 1,8-cineole [90].	**7**
Schotia brachypetala	Roots and barks are used for nervous conditions [93]. Bark decoction is used for the treatment of heartburn and to calm down the effects of too much drinking [48]. The plant is traditionally used in the treatment of neurodegenerative diseases [25]. **Score- 2**	The DCM:MeOH (1:1) root extract of *S. brachypetala* represented the IC_{50} 0.89±0.01 and water extract represented IC_{50} value 3.40±0.50mg/ml for AChEI activity. The DCM:MeOH bark extract displayed IC_{50} 0.27±0.07 mg/ml and 0.49±0.04mg/ml for water extract. The presence of Stilbenes and phenolics have been indicated the plant to have good radical scavenging activity [25]. **Score- 1**	Low toxicity, hexane extract of leaf, Brine shrimp Cell line IC_{50}= 3300.0µg/ml [72]. **Score- 1**	3,3',4',5,5'-pentahydroxystilbene [94], Linolenic acid (9,12,15-octadecatrienoic) and methyl-5,11,14,17-eicosatetraenoate, two antibacterial fatty acids [95], Flavonolacyl glucosides [96].	**4**
Stapelia gigantea	Zulu healers use hot stem infusions to treat hysteria [24]. **Score- 3**	Not known **Score- 3**	Not known **Score- 3**	Steroidal glycosides [97].	**9**
Tabernae montana elegans	Used for the treatment of heart diseases, cancer, tuberculosis, stomach ache,	Monoterpene indole alkaloid and β-carboline alkaloids isolated from *T. elegans* reported	High toxic, THP-1 Cell line IC_{50} < 4µg/ml [72].	Three β-carboline indole alkaloids and a monoterpene	

	infertility[98]. Treat various neurological disorders [99]. **Score- 2**	apoptosis induction activities in human hepatoma HuH-7 cells [100]. **Score- 3**	**Score- 3**	indole alkaloid [101].	**8**
Tetradenia riparia	Leaf infusions are taken for the treatment of respiratory ailments, mouth ulcers, fever, influenza, diarrhea, stomach ache, swollen legs and crushed leaves are used for headache [48]. **Score- 3**	The diterpene diol isolated from *T. riparia* exhibited significant antimicrobial activity against several bacteria and fungi with a bacterial activity of 12.5 µg/ml against Bacillus subtilis and Streptococcus pyogenes [102]. The ethanolic extract of *T. riparia* showed the AChE inhibition activity with IC50 value of 750 µg/ml [103]. **Score- 1**	Moderate toxic, Vero cell line, IC_{50}= Leaf extract, 124.455µg/ml; Stem extract, 186.2µg/ml; Root extract, 153.1µg/ml [104]. **Score- 2**	Tannins, Saponins, Terpenoids, Flavonoids, Cardiac glycosides, Phenols and Alkaloids [105]. A diterpene diol 8(14),15-Sandaracopimaradiene-7α,18-diol [102]. Ibozol, 7a-hydroxyroyleanone, umuravumbolide, deacetylumuravumbolide, deacetylboronolide and 1',2'dideacetylboronolide [102].	**6**
Trichilia dregeana	Used as wound healing, for the treatment of dysentery, fever, stomach and intestinal complaints [106]. Traditionally it is used for the treatment of inflammatory diseases such as kidney pain, a sore back and backache,	Cycloart-23-ene-3,25-diol isolated from the ethyl acetate leaf extract showed a strong inhibitory activity of 80% against COX-2 at a concentration of 100µM with an IC50 value of 40µM. The compound also showed the weak inhibitory effect against acetylcholinesterase in an anti-	No toxicity, MTT cellular viability assay, Cellular viability (% cell growth at 100 µg/ml ± s.d.) Aqueous extract= 100 ± 0.78. Organic extract= 99±0.87 [109].	Limonoids from which a known crystalline compound rohituka-7 and the other compounds designated dregeana-1to 4 were isolated from the seeds of *T. dregeana* [110]. A compound named cycloart-23-ene-3,25-diol was isolated from ethyl acetate leaf	

	bronchial inflammation, fever and rheumatism [107]. **Score- 3**	cholinesterase test at a concentration of 0.4 mM [107]. Stored plant material showed strong AChE inhibitory activity which confirm its therapeutic value towards Alzheimer's disease [108]. **Score- 1**	**Score- 1**	extract of *T. dregeana* [107].	**5**
Xysmalobium undulatum	Plant is used for headache and abdominal pains in Botswana [85]. Traditionally, it is also used for the treatment of diarrhoea, dysentery, stomach cramps, indigestion [48]. **Score- 3**	*X. undulatum* has been reported to exhibit acetylcholinesterase inhibitory activity (IC_{50} = 0.05–0.5 µg/ml) [111]. It has been reported to exhibit various *in vitro* activities such as antioxidant activity, antibacterial, antifungal, antiplasmodial, antidiarrhoel and serotonin reuptake inhibitors (SSRI) antidepression activity [112]. **Score- 1**	High toxic [33] **Score- 3**	Uzarin, xysmalorin and their isomers allouzarin and alloxysmalorin, allouzarigenin, alloxysmalogenin and other compounds ascleposide, coroglaucigenin, corogluaucigenin-3-O-glucoside, pachygenol, pachygenol-3β-O-glucoside, desglucouzarin, smalogenin, desglucoxysmalorin, uzaroside, pregnenolone and β-sitosterol [33].	**7**
Zanthoxylum capense	A species of *Zanthoxylum* is used for the treatment of mental illness in Gabon. It is also used as an epilepsy remedy among Europeans [24].	A compound named decarine isolated from *Z. capense* showed a strong antimycobacterial activity found, both *in vitro* and *ex vivo* mycobacteria [113].	Low toxic, THP-1 Cell line IC_{50}= 45.7µg/ml [42], [72]	Benzophenanthridine alkaloid. 2-arylbenzofuran neolignans [113], [114].	

	Score- 2	**Score- 3**	**Score- 1**		**6**
Ziziphus mucronata	Decoctions are taken for pneumonia. It is also used for the treatment of the swellings of skin, wounds [115].	The roots of *Z. mucronata* showed good antioxidant and acetylcholinesterase inhibitory activity [99].	High toxic, Brine shrimp Cell line IC_{50} = 900μg/ml [57], [42].	Phenolic compounds, flavonoids and proanthocyanidins [116]. Two cyclopeptide alkaloids were isolated from the roots of *Z. mucronata* [117]. Mucronine D and sanjoinine [118], [3].	
	Score- 3	**Score- 1**	**Score- 3**		**7**

2.3.2. Collection and extraction of plant material

The selected 21 plants species and where possible their different plant parts (e.g. leaves, stems, stalks, flowers) were collected in the spring season during the months October to December 2015. Two plants species, *Commelina africana* and *Ziziphus mucronata* were collected from the Dinokeng Nature Reserve Pretoria; one species, *Xysmalobium undulatum* was collected from experimental farm of University of Pretoria and remaining 18 species were collected from Maine *vander*Schijff Botanical Garden, University of Pretoria. Voucher specimens were deposited and identified at the H.G.W.J. Schweickerdt Plant Herbarium at University of Pretoria. A total of 34 extracts were prepared for the different plants species and their plant parts after extraction with dichloromethane/methanol (DCM:MeOH) (1:1). The extraction yield of 34 plant extracts ranged from 3% to 25%, labelled 1 to 34. The variation in the extraction yields depended on the plants species and the plant part collected. The voucher specimen of the plants and extraction yields are shown in Table 2.2.

Table 2.2: Plant species selected and collected with voucher specimen numbers[3]

Sample No.	Family	Plant species	Plant part extracted	Voucher specimen	Extraction yield (calculated from dry plant material) as *w/w%*
1	Apiaceae	*Centella asiatica* (L.) Urb.	Leaves + Stalks	124298	13.9
2	Apiaceae	*Heteromorpha arborescens* (Spreng.) Cham. & Schltdl.	Leaves	124318	8.7
3	Apocynaceae	*Mondia whitei* (Hook.f.) Skeels	Leaves	124299	10.3
4			Stems		7.6
5	Apocynaceae	*Stapelia gigantea* N.E.Br.	Stems	124308	12.5
6			Seeds		23.3
7			Seed pods		3.1
8	Apocynaceae	*Tabernaemontana elegans* Stapf	Leaves	124300	13.0
9	Apocynaceae	*Xysmalobium undulatum* (L.) W.T.Aiton	Stalks + Flowers	124301	7.6
10	Araliaceae	*Cussonia spicata* Thunb.	Leaves	124302	20.9
11			Stems		6.0
12	Araliaceae	*Cussonia paniculate* Eckl. & Zeyh.	Leaves	124309	5.7
13			Stems		7.7
14	Asphodelaceae	*Bulbine natalensis* Baker	Leaves	124310	24.5
15	Celastraceae	*Catha edulis* (Vahl) Endl.	Leaves	124303	13
16			Stems		6.9
17	Commelinaceae	*Commelina africana* L.	Leaves	124311	11.2
18	Crassulaceae	*Cotyledon orbiculata* L.	Leaves	124312	2.2
19			Stems		6.1
20	Fabaceae	*Schotia brachypetala* Sond.	Leaves	124313	9.2
21	Lamiaceae	*Tetradenia riparia* (Hochst.) Codd	Leaves	124304	12.5
22			Stems		5.4

[3] All plant species name have been confirmed as accepted on http://www.theplantlist.org.

23	Maliaceae	*Trichilia dregeana* Sond.	Leaves	124305	9.9
24			Stems		6.8
25	Plumbaginaceae	*Plumbago auriculata* Lam.	Leaves	124306	19.3
26			Stems		7.1
27	Rhamnaceae	*Ziziphus mucronata* Willd.	Leaves + Stalks	124314	10.5
28	Rutaceae	*Ruta graveolens* L.	Leaves	124315	22.8
29			Stems		10.5
30	Rutaceae	*Zanthoxylum capense* (Thunb.) Harv.	Leaves	124316	7.2
31			Stems + Thorns		9.1
32	Scrophulariaceae	*Buddleja salvifolia* (L.) Lam.	Leaves	124307	10.5
33			Stems		6.0
34	Vitaceae	*Cissus quadrangularis* L.	Stems	124317	9.6

2.3.3. The effect of the plant extracts on Aβ42 production

The bioassaying was made possible with the support of Korean institute of Science and Technology (KIST). Special thanks to the strong collaboration of Dr Yoon Sun Chun, Ansun Park in Dr Hyun OK Yang's laboratory at KIST. I also acknowledge the work done by KIST in the training on running the assays and interpreting of data during my research visit to their laboratories. Cells were incubated with 50 µg/ml extracts for 8 h, and the level of Aβ42 was measured from the conditioned media by using specific ELISA method. Galanthamine, which was reported to inhibit amyloid aggregation [119] and release of Aβ42 was decreased by 37% with 10µM galanthamine in SH-SY5Y cells [120] was used as a positive control (Figure 1). The Aβ42 production of galanthamine was measured by treating cells with varying concentrations for 8 h. However, galanthamine at a high concentration of 10 µM did not significantly affect the Aβ42 production under the experimental conditions, thus, APPsw transfected HeLa cells with same concentrated DMSO (vehicle) was used for negative control.

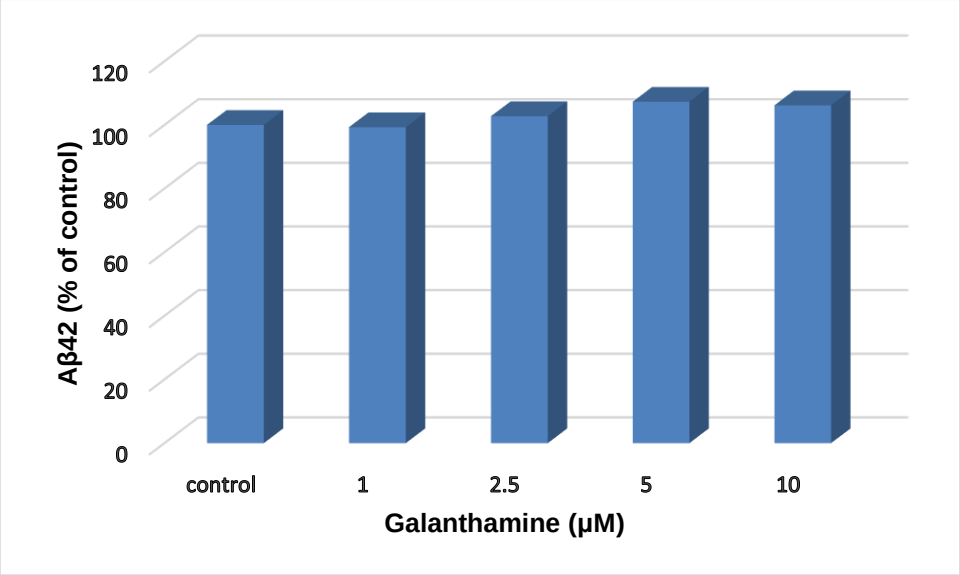


Figure 2.4: Galantamine did not change the level of Aβ42. APPsw-transfected HeLa cells were incubated with indicated concentrations of galantamine for 8 h. The level of Aβ42 was measured from the conditioned media by using specific ELISA methods. The level of Aβ42 was not changed by galantamine (n = 6)

The 34 plant extracts were screened to determine their inhibitory properties for Aβ42 production in HeLa cells stably transfected with APPsw. The results showed that 11 extracts (32.3%) (Extract numbers: 1, 2, 3, 4, 9, 12, 15, 17, 18, 20 and 32) significantly decreased the Aβ42 level as compared to the negative control as shown in Figure 2.5 to Figure 2.8.

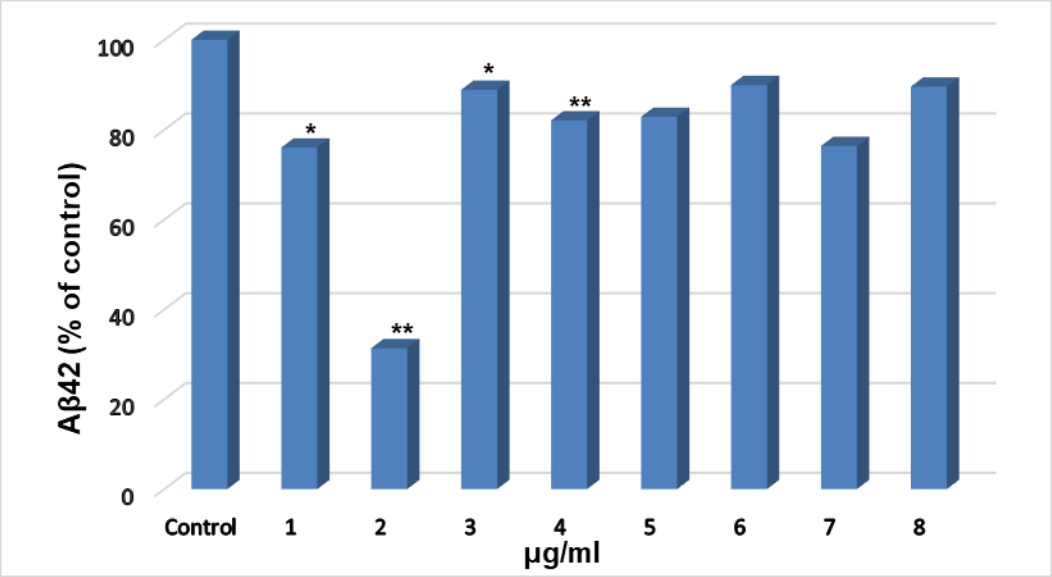


Figure 2.5: Effect of plant extracts on the level of Aβ42 in APPsw-transfected HeLa cells. APPsw-transfected HeLa cells were incubated with 50 µg/ml extracts for 8 h, and the level of Aβ42 was measured from the conditioned media by using specific ELISA methods. *, P<0.05; **, P<0.01. *Centella asiatica* (1), *Heteromorpha arborescens* (2), *Mondia whitei* (3 and 4), *Stapelia gigantea* (5, 6 and 7), *Tabernaemontana elegans* (8)

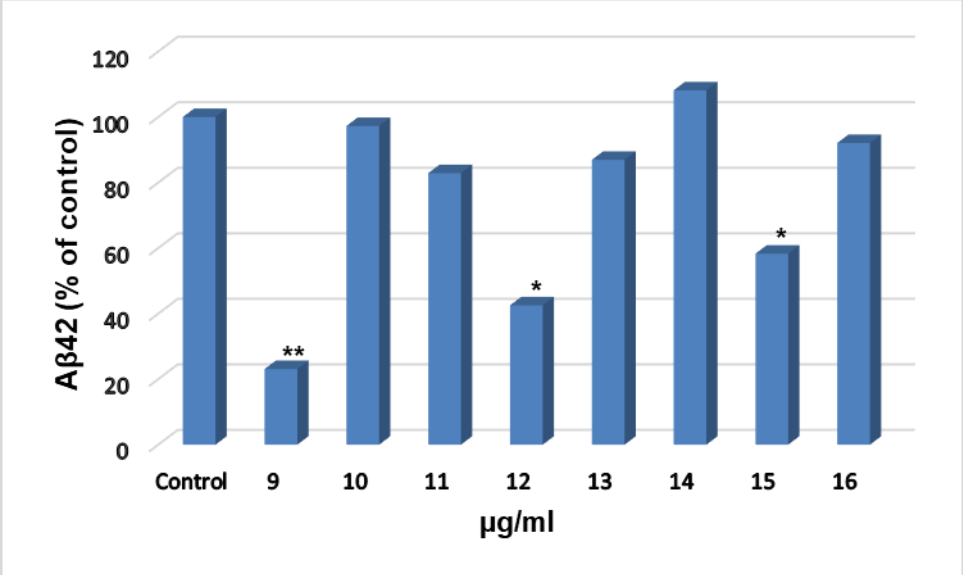


Figure 2.6: Effect of plant extracts on the level of Aβ42 in APPsw-transfected HeLa cells. APPsw-transfected HeLa cells were incubated with 50 µg/ml extracts for 8 h, and the level of Aβ42 was measured from the conditioned media by using specific ELISA methods. *, P<0.05; **, P<0.01. *Xysmalobium undulatum* (9), *Cussonia spicata* (10 and 11), *Cussonia paniculata* (12 and 13), *Bulbine natalensis* (14), *Catha edulis* (15 and 16)

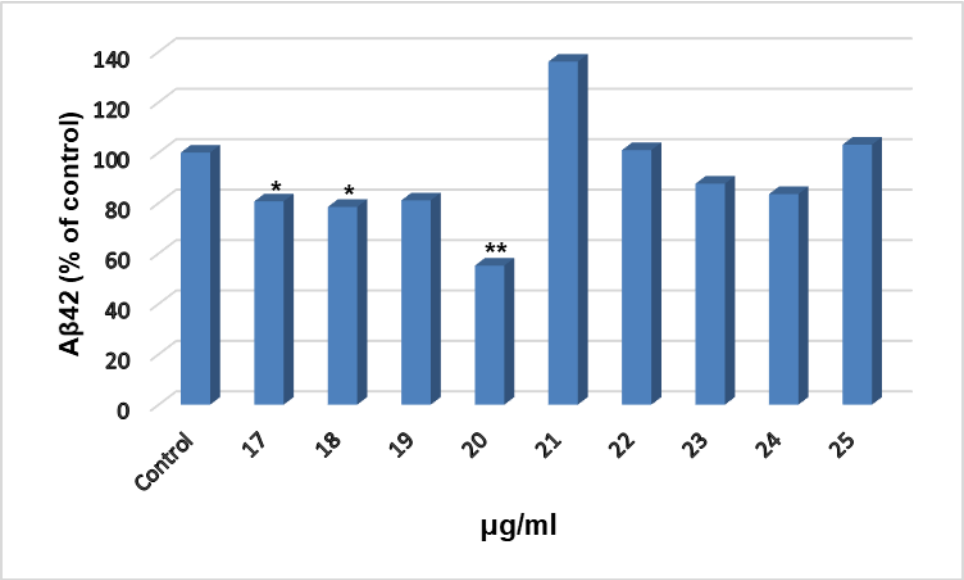


Figure 2.7: Effect of plant extracts on the level of Aβ42 in APPsw-transfected HeLa cells. APPsw-transfected HeLa cells were incubated with 50 µg/ml extracts for 8 h, and the level of Aβ42 was measured from the conditioned media by using specific ELISA methods. *, P<0.05; **, P<0.01. *Comelina africana* (17), *Cotyledon orbiculata* (18 and 19), *Schotia brachypetala* (20), *Tetradenia riperia* (21 and 22), *Trichilia dregeana* (23 and 24), *Plumbago auriculata* (25)

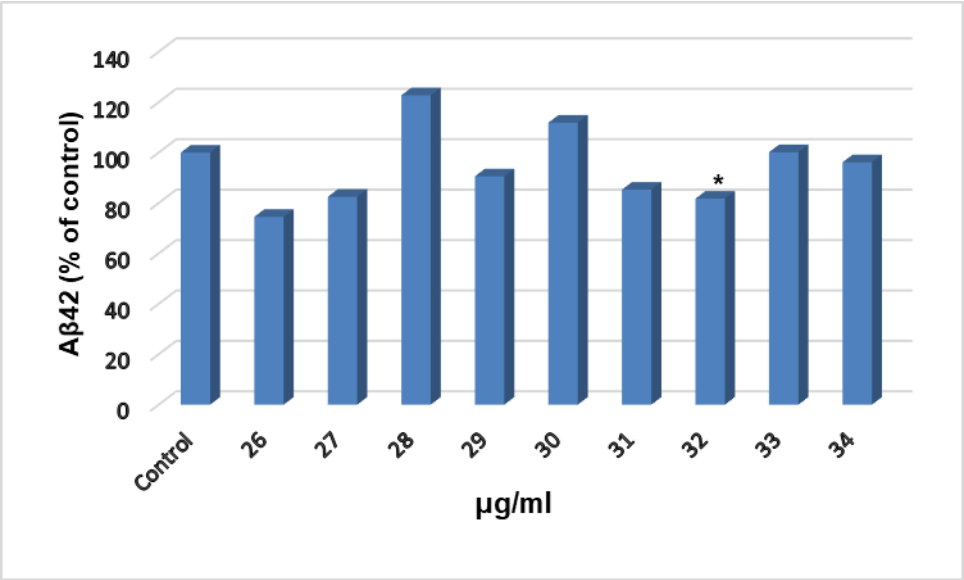


Figure 2.8: Effect of plant extracts on the level of Aβ42 in APPsw-transfected HeLa cells. APPsw-transfected HeLa cells were incubated with 50 µg/ml extracts for 8 h, and the level of Aβ42 was measured from the conditioned media by using specific ELISA methods. *, P<0.05; **, P<0.01. Plumbago auriculata (26), Ziziphus mucronata (27), *Ruta graveolens* (28 and 29), *Zanthoxylum capense* (30 and 31), *Buddleja salvifolia* (32 and 33), *Cissus quadrangularis* (34)

Among them, the extracts of stems of *Heteromorpha arborescens* (No. 2), leaves of *Xysmalobium undulatum* (No. 9), leaves of *Cussonia paniculata* (No. 12) and leaves of *Schotia brachypetala* (No. 20) potently reduced the secreted level of Aβ42 compared to the control by 68.63 ± 0.2, 76.9 ± 1%, 57.5 ± 1.3% and 44.8 ± 0.1%, respectively. These four extracts were selected for further studies due to their ability to inhibit Aβ42 production by 44% and more and their limited toxicity at the same test concentrations. The cytotoxicity of these plants is further discussed in Chapter 3 and Chapter 4.

H. arborescens is traditionally used for the treatment of nervous disorders [121], [74]. *X. undulatum* has been reported to exhibit antidepressant, acetylcholinesterase inhibition activity and Serotonin reuptake inhibitory activity [33], [112], [111]. *C. paniculata* is also used traditionally for the treatment of nervous disorders [29]. In a previous study, phenolic compounds isolated from *S. brachypetala* were reported to inhibit Aβ42 production and proved its efficacy to be the anti-Alzheimer's drug in future [32]. Either the traditional uses or the reported bioassays of these plants also supported their efficacy for the reduction of Aβ42 in the present study.

The active plant extracts were also compared with their scoring system used for the selection of the plants. It was found that, of these four plants only *S. brachypetala* showed very good correlation with the scoring system. It was given a good score **4**, because of its traditional uses related to nervous disorders and a well-researched studies on Aβ42 bioassays [32]. While *M. whitei* with the similar score showed a very low activity towards the reduction of Aβ42, as the levels of Aβ42 was reduced by only 11.1 ± 0.1 and 17.9 ± 0.6 with *M. whitei* leaf and *M. whitei* stem extract, respectively. Some other plant extracts, *C. asiatica*, *C. edulis*, *C. africana*, *C. orbiculata* and *B. salvifolia* also showed some reduction in Aβ42 by 23.9 ± 1.5, 41.7 ± 1.9, 19.3 ± 0.5, 21.5 ± 0.5 and 18.2 ± 0.8, respectively however all these extracts showed low percentage for Aβ42 reduction . Due to the low Aβ42 reduction, these plant extracts were not selected for further studies. The scoring system helped to select the plants

for testing against Aβ42 reduction, but all the selected plant extracts did not show significant reduction towards Aβ42.

2.4. Conclusion

At least 52 plant species were selected using different database searches. Due to the broad search, an additional criterion and a scoring system was used. With the help of scoring system, 21 plant species which were selected for the further experiments including collection, extraction and screening for Aβ42 reduction. A total of 34 extracts were prepared from different plant parts of the 21 plants. Among them, four plant extracts, stems of *H. arborescens*, leaves of *X. undulatum,* leaves of *C. paniculata and* leaves of *S. brachypetala*, potently decreased the levels of Aβ42. The stem extract of *H. arborescens* and leaf extracts of *X. undulatum* and *C. paniculata* were screened first time for Aβ42 reduction. These extracts were scientifically shown to exhibit Aβ42 inhibition activities thus providing some scientific evidence authenticating their traditional uses and also exhibiting their potential to be further developed into anti-Alzheimer's ingredients. The *S. brachypetala* showed its ability for the reduction of Aβ42 but in other assays from the present study. *H. arborescens* and *C. paniculata* were found to be traditionally used for the treatment of mental disorders while *X. undulatum* has been previously reported for acetylcholinesterase inhibition. The traditional uses and preliminary bioassays of these plant extracts also showed their efficacy towards the treatment of mental disorders. These four extracts were selected for further study to identify and isolate the active ingredients responsible for the inhibition of Aβ42 activity.

References

1. Abdalla MA, McGaw LJ: **Bioprospecting of South African plants as a unique resource for bioactive endophytic microbes**. *Frontiers in Pharmacology* 2018, **9**.
2. Street R, Prinsloo G: **Commercially important medicinal plants of South Africa: a review**. *Journal of Chemistry* 2012, **2013**.
3. Van Wyk B-E, Oudtshoorn Bv, Gericke N: **Medicinal Plants of South Africa**: Briza; 1997.
4. Richter M: **Traditional medicines and traditional healers in South Africa**. *Treatment Action Campaign and AIDS Law Project* 2003, **17**:4-29.
5. Van Vuuren S: **Antimicrobial activity of South African medicinal plants**. *Journal of Ethnopharmacology* 2008, **119**(3):462-472.
6. Kale R: **Traditional healers in South Africa: A parallel health care system. South Africa's health**. *British Medical Journal* 1995, **310**(6988):1182-1186.
7. Pretorius E: **Traditional healers**. *South African Health Review* 1999, **249**:256.
8. Lewu F, Afolayan A: **Ethnomedicine in South Africa: The role of weedy species**. *African Journal of Biotechnology* 2009, **8**(6).
9. Homsy J, King R, Balaba D, Kabatesi D: **Traditional health practitioners are key to scaling up comprehensive care for HIV/AIDS in sub-Saharan Africa**. *Aids* 2004, **18**(12):1723-1725.
10. Semenya S, Potgieter M, Tshisikhawe M, Shava S, Maroyi A: **Medicinal utilization of exotic plants by Bapedi traditional healers to treat human ailments in Limpopo province, South Africa**. *Journal of Ethnopharmacology* 2012, **144**(3):646-655.
11. Steenkamp V: **Traditional herbal remedies used by South African women for gynaecological complaints**. *Journal of Ethnopharmacology* 2003, **86**(1):97-108.
12. Koduru S, Grierson D, Afolayan A: **Ethnobotanical information of medicinal plants used for treatment of cancer in the Eastern Cape Province, South Africa**. *Current Science (00113891)* 2007, **92**(7).
13. Steenkamp V, Gouws M: **Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer**. *South African Journal of Botany* 2006, **72**(4):630-633.
14. Atawodi S: **Antioxidant potential of African medicinal plants**. *African Journal of Biotechnology* 2005, **4**(2):128-133.
15. Clarkson C, Maharaj VJ, Crouch NR, Grace OM, Pillay P, Matsabisa MG, Bhagwandin N, Smith PJ, Folb PI: **In vitro antiplasmodial activity of medicinal plants native to or naturalised in South Africa**. *Journal of Ethnopharmacology* 2004, **92**(2-3):177-191.
16. Lall N, Meyer J: **In vitro inhibition of drug-resistant and drug-sensitive strains of Mycobacterium tuberculosis by ethnobotanically selected South African plants**. *Journal of Ethnopharmacology* 1999, **66**(3):347-354.
17. Gericke N, Viljoen A: **Sceletium—a review update**. *Journal of Ethnopharmacology* 2008, **119**(3):653-663.
18. Carpenter JM, Jourdan MK, Fountain EM, Ali Z, Abe N, Khan IA, Sufka KJ: **The effects of *Sceletium tortuosum* (L.) NE Br. extract fraction in the chick anxiety-depression model**. *Journal of Ethnopharmacology* 2016, **193**:329-332.
19. Harvey AL, Young LC, Viljoen AM, Gericke NP: **Pharmacological actions of the South African medicinal and functional food plant *Sceletium tortuosum* and its principal alkaloids**. *Journal of Ethnopharmacology* 2011, **137**(3):1124-1129.
20. Van Wyk B-E: **The potential of South African plants in the development of new medicinal products**. *South African Journal of Botany* 2011, **77**(4):812-829.
21. Mukherjee PK, Kumar V, Mal M, Houghton PJ: **Acetylcholinesterase inhibitors from plants**. *Phytomedicine* 2007, **14**(4):289-300.

22. Elgorashi EE: **Phytochemistry and Pharmacology of the Family Amaryllidaceae: An Overview of Research at RCPGD**. *Natural Product Communications* 2019, **14**(9):1934578X19872929.
23. Fennell C, Lindsey K, McGaw L, Sparg S, Stafford G, Elgorashi E, Grace O, Van Staden J: **Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology**. *Journal of Ethnopharmacology* 2004, **94**(2-3):205-217.
24. Stafford GI, Pedersen ME, van Staden J, Jäger AK: **Review on plants with CNS-effects used in traditional South African medicine against mental diseases**. *Journal of Ethnopharmacology* 2008, **119**(3):513-537.
25. Adewusi EA, Moodley N, Steenkamp V: **Antioxidant and acetylcholinesterase inhibitory activity of selected southern African medicinal plants**. *South African Journal of Botany* 2011, **77**(3):638-644.
26. Risa A, Risa J, Adsersen A, Stafford G, Van Staden J, Jäger A, Houghton P: **Acetylcholinesterase inhibitory activity of plants used as memory-enhancers in traditional South African medicine**. *South African Journal of Botany* 2004, **70**(4):664-666.
27. Ndhlala A, Aderogba M, Ncube B, Van Staden J: **Anti-oxidative and cholinesterase inhibitory effects of leaf extracts and their isolated compounds from two closely related Croton species**. *Molecules* 2013, **18**(2):1916-1932.
28. Kannan RR, Aderogba MA, Ndhlala AR, Stirk WA, Van Staden J: **Acetylcholinesterase inhibitory activity of phlorotannins isolated from the brown alga, *Ecklonia maxima* (Osbeck) Papenfuss**. *Food Research International* 2013, **54**(1):1250-1254.
29. Adewusi EA, Fouche G, Steenkamp V: **Cytotoxicity and acetylcholinesterase inhibitory activity of an isolated crinine alkaloid from *Boophane disticha* (Amaryllidaceae)**. *Journal of Ethnopharmacology* 2012, **143**(2):572-578.
30. Pendota S, Aderogba M, Ndhlala A, Van Staden J: **Antimicrobial and acetylcholinesterase inhibitory activities of *Buddleja salviifolia* (L.) Lam. leaf extracts and isolated compounds**. *Journal of Ethnopharmacology* 2013, **148**(2):515-520.
31. Nair JJ, Aremu AO, van Staden J: **Isolation of narciprimine from *Cyrtanthus contractus* (Amaryllidaceae) and evaluation of its acetylcholinesterase inhibitory activity**. *Journal of Ethnopharmacology* 2011, **137**(3):1102-1106.
32. Hassaan Y, Handoussa H, El-Khatib AH, Linscheid MW, El Sayed N, Ayoub N: **Evaluation of plant phenolic metabolites as a source of Alzheimer's drug leads**. *BioMed Research International* 2014, **2014**.
33. Vermaak I, Enslin GM, Idowu TO, Viljoen AM: **Xysmalobium undulatum (uzara)–review of an antidiarrhoeal traditional medicine**. *Journal of Ethnopharmacology* 2014, **156**:135-146.
34. Kwon H-C, Cha J-W, Park J-S, Chun Y-S, Moodley N, Maharaj VJ, Youn S-H, Chung S-K, Yang H-O: **Rapid identification of bioactive compounds reducing the production of amyloid β-peptide (Aβ) from South African plants using an automated HPLC/SPE/HPLC coupling system**. *Biomolecules & Therapeutics* 2011, **19**(1):90-96.
35. Yang HO, Chung S-K, Kwon HC, Cha JW, Maharaj VJ, Nthambeleni R, Naidoo D, Kim Y-j, Ham J, Senabe J: **Composition for preventing or treating dementia comprising extracts of monsonia species**. In.: Google Patents; 2016.
36. **Mental health a serious issue in South Africa**
37. Chiumia S, van Wyk A: **Do a third of South Africans really suffer from mental ilnesses**. In.: Available; 2014.
38. Truter I: **Prescribing of drugs for Alzheimer's disease: a South African database analysis**. *International Psychogeriatrics* 2010, **22**(2):264-269.
39. **alzheimer's South Africa facing the challenge of dementia** [www.alzheimers.org.za]

40. Clark T, Appleton C, Drewes S: **A semi-quantitative approach to the selection of appropriate candidate plant molluscicides—a South African application**. *Journal of Ethnopharmacology* 1997, **56**(1):1-13.
41. Jonville M, Kodja H, Strasberg D, Pichette A, Ollivier E, Frederich M, Angenot L, Legault J: **Antiplasmodial, anti-inflammatory and cytotoxic activities of various plant extracts from the *Mascarene archipelago***. *Journal of Ethnopharmacology* 2011, **136**(3):525-531.
42. McGaw LJ, Elgorashi EE, Eloff JN: **Cytotoxicity of African medicinal plants against normal animal and human cells**. In: *Toxicological Survey of African Medicinal Plants.* edn.: Elsevier; 2014: 181-233.
43. Sobiecki J-F: **A preliminary inventory of plants used for psychoactive purposes in southern African healing traditions**. *Transactions of the Royal Society of South Africa* 2002, **57**(1-2):1-24.
44. Pather N, Viljoen AM, Kramer B: **A biochemical comparison of the in vivo effects of *Bulbine frutescens* and *Bulbine natalensis* on cutaneous wound healing**. *Journal of Ethnopharmacology* 2011, **133**(2):364-370.
45. Yakubu M, Mostafa M, Ashafa A, Afolayan A: **Antimicrobial activity of the solvent fractions from Bulbine natalensis tuber**. *African Journal of Traditional, Complementary and Alternative Medicines* 2012, **9**(4):459-464.
46. Mosa R, Lazarus G, Gwala P, Oyedeji A, Opoku A: ***In vitro* anti-platelet aggregation, antioxidant and cytotoxic activity of extracts of some Zulu medicinal plants**. *J Nat Prod* 2011, **4**(2011):136-146.
47. Yakubu M, Afolayan A: **Effect of aqueous extract of *Bulbine natalensis* (Baker) stem on the sexual behaviour of male rats**. *International Journal of Andrology* 2009, **32**(6):629-636.
48. Van Wyk B-E, Van Oudtshoorn B, Gericke N: **Medicinal plants of South Africa**, 2nd ed. edn. Pretoria :: Briza Publications; 2009.
49. Kimani ST, Nyongesa AW: **Effects of single daily khat (*Catha edulis*) extract on spatial learning and memory in CBA mice**. *Behavioural Brain Research* 2008, **195**(1):192-197.
50. Dimba E, Gjertsen BT, Francis GW, Johannessen AC, Vintermyr OK: ***Catha edulis* (Khat) induces cell death by apoptosis in leukemia cell lines**. *Annals of the New York Academy of Sciences* 2003, **1010**(1):384-388.
51. Getasetegn M: **Chemical composition of *Catha edulis* (khat): a review**. *Phytochemistry Reviews* 2016, **15**(5):907-920.
52. Arora R, Sharma J, Selvamurthy W, Shivashankara A, Mathew N, Baliga M: **Asian medicinal remedies for alleviating aging Effects**. *Bioactive Food as Dietary Interventions for the Aging Population: Bioactive Foods in Chronic Disease States* 2012:305.
53. Irungu BN, Rukunga GM, Mungai GM, Muthaura CN: ***In vitro* antiplasmodial and cytotoxicity activities of 14 medicinal plants from Kenya**. *South African Journal of Botany* 2007, **73**(2):204-207.
54. Gohil KJ, Patel JA, Gajjar AK: **Pharmacological review on *Centella asiatica*: a potential herbal cure-all**. *Indian Journal of Pharmaceutical Sciences* 2010, **72**(5):546.
55. Moshi MJ, Kagashe GA, Mbwambo ZH: **Plants used to treat epilepsy by Tanzanian traditional healers**. *Journal of Ethnopharmacology* 2005, **97**(2):327-336.
56. Mishra G, Srivastava S, Nagori B: **Pharmacological and therapeutic activity of *Cissus quadrangularis*: an overview**. *Int J Pharm Tech Res* 2010, **2**(2):1298-1310.
57. McGaw LJ, Van der Merwe D, Eloff JN: **In vitro anthelmintic, antibacterial and cytotoxic effects of extracts from plants used in South African ethnoveterinary medicine**. *The Veterinary Journal* 2007, **173**(2):366-372.
58. Adesanya SA: **Stilbene derivatives from *Cissus quadrangularis***. *Journal of Natural Products* 1999, **62**(12):1694.

59. Baby Joseph* JGaJM: ***Cissus quadrangularis* in the treatment of osteoporosis**. *World Journal of Pharmaceutical research* 2003, **2**(3):596-605.

60. Agunbiade O, Ojezele O, Ojezele J, Ajayi A: **Hypoglycaemic activity of *Commelina africana* and *Ageratum conyzoides* in relation to their mineral composition**. *African Health Sciences* 2012, **12**(2):198-203.
61. Kudumela RG, McGaw LJ, Masoko P: **Antibacterial interactions, anti-inflammatory and cytotoxic effects of four medicinal plant species**. *BMC Complementary and Alternative Medicine* 2018, **18**(1):199.
62. Amabeoku G, Green I, Kabatende J: **Anticonvulsant activity of Cotyledon orbiculata L.(Crassulaceae) leaf extract in mice**. *Journal of Ethnopharmacology* 2007, **112**(1):101-107.
63. Molefe NI: **Anthelmintic, anticancer and phytochemical screening of *Cotyledon orbiculata*; *Hermannia depressa*; *Nicotiana glauca* and potassium permanganate**. University of the Free State (Qwaqwa Campus); 2013.
64. Emamzadeh-Yazdi S: **Antiviral, antibacterial and cytotoxic activities of South African plants containing cardiac glycosides**. University of Pretoria; 2013.
65. Van Wyk B-E, Heerden Fv, Oudtshoorn BV: **Poisonous plants of South Africa**: Briza Publications; 2002.
66. Tetyana P, Prozesky E, Jäger A, Meyer J, Van Staden J, van Wyk B-E: **Some medicinal properties of *Cussonia* and *Schefflera* species used in traditional medicine**. *South African Journal of Botany* 2002, **68**(1):51-54.
67. De Villiers B, Van Vuuren S, Van Zyl R, Van Wyk B-E: **Antimicrobial and antimalarial activity of *Cussonia* species (Araliaceae)**. *Journal of Ethnopharmacology* 2010, **129**(2):189-196.
68. Fouché G, Cragg G, Pillay P, Kolesnikova N, Maharaj V, Senabe J: ***In vitro* anticancer screening of South African plants**. *Journal of Ethnopharmacology* 2008, **119**(3):455-461.
69. Thakur A, Chun YS, October N, Yang HO, Maharaj V: **Potential of South African medicinal plants targeting the reduction of Aβ42 protein as a treatment of Alzheimer's disease**. *Journal of Ethnopharmacology* 2019, **231**:363-373.
70. Grishkovets V, Dovgii I, Kachala V, Shashkov A: **Triterpene glycosides from Cussonia paniculata. II. Acetylated glycosides from leaves**. *Chemistry of Natural Compounds* 2005, **41**(4):436-441.
71. Adedapo AA, Sofidiya MO, Maphosa V, Moyo B, Masika PJ, Afolayan AJ: **Anti-inflammatory and analgesic activities of the aqueous extract of *Cussonia paniculata* stem Bark**. *Records of Natural Products* 2008, **2**(2):46.
72. Luo X, Pires D, Aínsa JA, Gracia B, Mulhovo S, Duarte A, Anes E, Ferreira M-JU: **Antimycobacterial evaluation and preliminary phytochemical investigation of selected medicinal plants traditionally used in Mozambique**. *Journal of Ethnopharmacology* 2011, **137**(1):114-120.
73. Gunzinger J: **Molluscicidal saponins from *Cussonia spicata***. *Phytochemistry* 1986, **25**(11):2501.
74. Lundgaard NH, Prior RM, Light ME, Stafford GI, Van Staden J, Jäger AK: **COX-1 inhibition of *Heteromorpha arborescens***. *South African Journal of Botany* 2008, **74**(2):335-337.
75. Nkomo M, Nkeh-Chungag BN, Kambizi L, Ndebia EJ, Sewani-Rusike C, Iputo JE: **Investigation of the antinociceptive and anti-inflammatory properties of *Heteromorpha arborescens* (Apiaceae)**. *African Journal of Traditional, Complementary and Alternative Medicines* 2011, **8**(4).
76. Elisha IL, Dzoyem J-P, McGaw LJ, Botha FS, Eloff JN: **The anti-arthritic, anti-inflammatory, antioxidant activity and relationships with total phenolics and total flavonoids of nine South African plants used traditionally to treat arthritis**. *BMC Complementary and Alternative Medicine* 2016, **16**(1):307.

77. Adamu M, Bagla V, Eloff J: **Fractionation of Heteromorpha arborescens var abyssinica (Apiaceae) leaf extracts based on polarity leads to a marked change in cytotoxicity that may yield a commercially useful product**. *South African Journal of Botany* 2016, **103**:36-40.
78. McGaw L, Eloff J: **Ethnoveterinary use of southern African plants and scientific evaluation of their medicinal properties**. *Journal of Ethnopharmacology* 2008, **119**(3):559-574.
79. Neuwinger HD: **African traditional medicine: a dictionary of plant use and applications. With supplement: search system for diseases**: Medpharm; 2000.
80. Baskaran P, Kumari A, Ncube B, Van Staden J: **Acetylcholinesterase-Inhibition and Antibacterial Activity of *Mondia whitei* Adventitious Roots and Ex vitro-Grown Somatic Embryogenic-Biomass**. *Frontiers in Pharmacology* 2016, **7**:335.
81. Neergaard JS, Rasmussen HB, Stafford GI, Van Staden J, Jäger AK: **Serotonin transporter affinity of (−)-loliolide, a monoterpene lactone from Mondia whitei**. *South African Journal of Botany* 2010, **76**(3):593-596.
82. Choumessi AT, Loureiro R, Silva AM, Moreira AC, Pieme AC, Tazoacha A, Oliveira PJ, Penlap VB: **Toxicity evaluation of some traditional African spices on breast cancer cells and isolated rat hepatic mitochondria**. *Food and Chemical Toxicology* 2012, **50**(11):4199-4208.
83. Gbadamosi IT, Erinoso SM: **In vitro antioxidant and antimicrobial activities of *Mondia whitei* (Hook. f.) Skeels**. *Journal of Basic and Applied Sciences* 2015, **11**:428-433.
84. Patnam R, Kadali SS, Koumaglo KH, Roy R: **A chlorinated coumarinolignan from the African medicinal plant, *Mondia whitei***. *Phytochemistry* 2005, **66**(6):683-686.
85. Hutchings A, van Staden J: **Plants used for stress-related ailments in traditional Zulu, Xhosa and Sotho medicine. Part 1: Plants used for headaches**. *Journal of Ethnopharmacology* 1994, **43**(2):89-124.
86. Ramalhete C, Lopes D, Mulhovo S, Rosário VE, Ferreira M: **Antimalarial activity of some plants traditionally used in Mozambique**. In: *Workshop Plantas Medicinais e Fitoterapêuticas nos Trópicos IICT/CCCM, 29, 30 e 31 de Outubro de: 2008*; 2008.
87. Choi J-S, Park N-J, Lim H-K, Ko Y-K, Kim Y-S, Ryu S-Y, Hwang I-T: **Plumbagin as a new natural herbicide candidate for Sicyon angulatus control agent with the target 8-amino-7-oxononanoate synthase**. *Pesticide Biochemistry and Physiology* 2012, **103**(3):166-172.
88. Paul AS, Islam A, Yuvaraj P: **Anti-Helicobacter pylori and cytotoxic activity of detoxified root of *Plumbago auriculata*, *Plumbago indica* and *Plumbago zeylanica***. *J PhytoPharm* 2013, **2**:4-8.
89. de Paiva SR, Figueiredo MR, Kaplan MAC: **Isolation of secondary metabolites from roots of *Plumbago auriculata* Lam. by countercurrent chromatography**. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques* 2005, **16**(4):278-281.
90. Asgarpanah J, Khoshkam R: **Phytochemistry and pharmacological properties of *Ruta graveolens* L.** *Journal of Medicinal Plants Research* 2012, **6**(23):3942-3949.
91. Rollinger JM, Schuster D, Danzl B, Schwaiger S, Markt P, Schmidtke M, Gertsch J, Raduner S, Wolber G, Langer T: **In silico target fishing for rationalized ligand discovery exemplified on constituents of *Ruta graveolens***. *Planta Medica* 2009, **75**(03):195-204.
92. Benazir J, Suganthi R, Renjini Devi M, Suganya K, Monisha K, Nizar Ahamed K, Santhi R: **Phytochemical profiling, antimicrobial and cytotoxicity studies of methanolic extracts from *Ruta graveolens***. *Journal of Pharmacy Research* 2011, **4**(5):1407-1409.
93. Wyk BEv, Gericke N: **People's plants: a guide to useful plants of Southern Africa**. Pretoria, South Africa: Briza Publications; 2000.

94. Giacomini E, Rupiani S, Guidotti L, Recanatini M, Roberti M: **The use of stilbene scaffold in medicinal chemistry and multi-target drug design**. *Current Medicinal Chemistry* 2016, **23**(23):2439-2489.
95. McGaw L, Jäger A, Van Staden J: **Isolation of antibacterial fatty acids from *Schotia brachypetala***. *Fitoterapia* 2002, **73**(5):431-433.
96. Du K, Marston A, van Vuuren SF, van Zyl RL, Coleman C, Zietsman PC, Bonnet SL, Ferreira D, van der Westhuizen JH: **Flavonolacyl glucosides from the aril of *Schotia brachypetala* Sond. and their antioxidant, antibacterial and antimalarial activities**. *Phytochemistry Letters* 2014, **10**:cxxiii-cxxviii.
97. Keller WJ: **Hordenine from *Stapelia gigantea***. *Journal of Natural Products* 1981, **44**(3):366-367.
98. **Medicinal Plants, Volume 1**, vol. Volume 11 of Plant resources of tropical Africa. Medicinal Plants, Technical Centre for Agricultural and Rural Cooperation (Ede, Netherlands): PROTA; 2008.
99. Adewusi EA, Steenkamp V: ***In vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from southern Africa**. *Asian Pacific Journal of Tropical Medicine* 2011, **4**(10):829-835.
100. Mansoor TA, Ramalho RM, Mulhovo S, Rodrigues CM, Ferreira MJU: **Induction of apoptosis in HuH-7 cancer cells by monoterpene and β-carboline indole alkaloids isolated from the leaves of *Tabernaemontana elegans***. *Bioorganic & Medicinal Chemistry Letters* 2009, **19**(15):4255-4258.
101. Mansoor TA, Ramalhete C, Molnár J, Mulhovo S, Ferreira MJU: **Tabernines A− C, β-Carbolines from the leaves of *Tabernaemontana elegans***. *Journal of Natural Products* 2009, **72**(6):1147-1150.
102. Van Puyvelde L, Nyirankuliza S, Panebianco R, Boily Y, Geizer I, Sebikali B, De Kimpe N, Schamp N: **Active principles of *Tetradenia riparia*. I. Antimicrobial activity of 8 (14), 15-sandaracopimaradiene-7α, 18-diol**. *Journal of Ethnopharmacology* 1986, **17**(3):269-275.
103. Sadashiva C, Naidoo Y, Naidoo J, Kalicharan B, Naidoo G: **Antioxidant and acetylcholinesterase activities of three species of the family Lamiaceae**. *Bangladesh Journal of Botany* 2014, **43**(3):331-335.
104. Kumari A, Baskaran P, Chukwujekwu JC, de Kock CA, Smith PJ, Van Staden J: **The changes in morphogenesis and bioactivity of *Tetradenia riparia*, *Mondia whitei* and *Cyanoptis speciosa* by an aeroponic system**. *Industrial Crops and Products* 2016, **84**:199-204.
105. Ngule MC, Ndiku MH, Ramesh F, Ngule ME: **Preliminary phytochemical and antibacterial screening of fresh *Tetradenia riparia* leaves water extract against selected pathogenic microorganisms**. *Int J Bioassays* 2014, **3**:3413-3418.
106. Quattrocchi U: **CRC world dictionary of medicinal and poisonous plants: common names, scientific names, eponyms, synonyms, and etymology (5 Volume Set)**: CRC Press; 2012.
107. Eldeen I, Van Heerden F, Van Staden J: **Biological activities of cycloart-23-ene-3, 25-diol isolated from the leaves of *Trichilia dregeana***. *South African Journal of Botany* 2007, **73**(3):366-371.
108. Amoo SO, Aremu AO, Moyo M, Van Staden J: **Antioxidant and acetylcholinesterase-inhibitory properties of long-term stored medicinal plants**. *BMC Complementary and Alternative Medicine* 2012, **12**(1):87.
109. Naidoo D: **Plants traditionally used individually and in combination to treat sexually transmitted infections in northern Maputaland, South Africa: Antimicrobial activity and cytotoxicity**. *Journal of Ethnopharmacology* 2013, **v. 149**(no. 3):pp. 656-667-2013 v.2149 no.2013.
110. Mulholland DA, Taylor DA: **Limonoids from the seed of the natal mahogany, *Trichilia dregeana***. *Phytochemistry* 1980, **19**(11):2421-2425.
111. Masondo N, Stafford G, Aremu A, Makunga N: **Acetylcholinesterase inhibitors from southern African plants: An overview of ethnobotanical, pharmacological**

potential and phytochemical research including and beyond Alzheimer's disease treatment. *South African Journal of Botany* 2018.
112. Balogun FO, Tshabalala NT, Ashafa AOT: **Antidiabetic medicinal plants used by the Basotho tribe of Eastern Free State: a review**. *Journal of Diabetes Research* 2016, **2016**.
113. Luo X, Pires D, Aínsa JA, Gracia B, Duarte N, Mulhovo S, Anes E, Ferreira M-JU: ***Zanthoxylum capense* constituents with antimycobacterial activity against Mycobacterium tuberculosis *in vitro* and *ex vivo* with in human macrophages**. *Journal of Ethnopharmacology* 2013, **146**(1):417-422.
114. Luo X, Pedro L, Milic V, Mulhovo S, Duarte A, Duarte N, Ferreira M-JU: **Antibacterial benzofuran neolignans and benzophenanthridine alkaloids from the roots of *Zanthoxylum capense***. *Planta Medica* 2012, **78**(02):148-153.
115. Rabe T, Van Staden J: **Antibacterial activity of South African plants used for medicinal purposes**. *Journal of Ethnopharmacology* 1997, **56**(1):81-87.
116. Olajuyigbe OO, Afolayan AJ: **Phenolic content and antioxidant property of the bark extracts of *Ziziphus mucronata* Willd. subsp. mucronata Willd**. *BMC Complementary and Alternative Medicine* 2011, **11**(1):130.
117. Barboni L, Gariboldi P, Torregiani E, Verotta L: **Cyclopeptide alkaloids from *Ziziphus mucronata***. *Phytochemistry* 1994, **35**(6):1579-1582.
118. Van Wyk B-E, Gericke N: **People's plants: A guide to useful plants of Southern Africa**: Briza Publications; 2000.
119. Matharu B, Gibson G, Parsons R, Huckerby TN, Moore SA, Cooper LJ, Millichamp R, Allsop D, Austen B: **Galantamine inhibits β-amyloid aggregation and cytotoxicity**. *Journal of the Neurological Sciences* 2009, **280**(1-2):49-58.
120. Li Q, Wu D, Zhang L, Zhang Y: **Effects of galantamine on β-amyloid release and beta-site cleaving enzyme 1 expression in differentiated human neuroblastoma SH-SY5Y cells**. *Experimental Gerontology* 2010, **45**(11):842-847.
121. Nkomo M, Kambizi L: **Antimicrobial activity of *Gunnera perpensa* and *Heteromorpha arborescens* var. abyssinica**. *Journal of Medicinal Plants Research* 2009, **3**(12):1051-1055.

Chapter 3
Chemical profiling of extracts of *Schotia brachypetala*, *Cussonia paniculata* and *Heteromorpha arborescens* using UPLC-QTOF-MS for the purpose of identifying the active compounds

3.1. *Schotia brachypetala*
3.1.1. Botany and geographical distribution

S. brachypetala belongs to family Fabaceae or Leguminosae and is a tree endemic to South Africa. It is an attractive, medium to large deciduous tree with a widely spreading, densely branched round canopy up to 10-16 m tall and 10-15 m wide and occasionally reach up to 22 m. It has a single trunk with branches low down. Leaves are bright red in spring and mature to dark glossy green. The flowers are deep red in color and bloom in spring from August to November. The flowers produce nectar and can also be chewed. The fruit is small, hard and woody pod. It gets split on the tree and releases the seed from inside. The seeds look like domestic broadbean and is the reason why the plant is named as boer-bean while the seeds are edible after roasting. It is commonly known as weeping boer-bean, African walnut in English; huilboerboon in Afrikaans; umfofofo in Xhosa; uvovovo in Zulu and mununzwa, mutanswa in Venda [1].The genus *Schotia* is mainly confined to southern Africa, ranging from southern Zimbabwe and Mozambique to southern South Africa and Namibia. *S. brachypetala* is widely distributed in the Eastern Cape, through KwaZulu-Natal, Swaziland, Mpumalanga, Northern Province and into Mozambique and Zimbabwe. It is found mainly in the sub-tropical regions of the country, growing in warm, bushveld, scrub forests and deciduous woodlands, often found on the banks of rivers [2], [3], [4].

(A) (B)

Figure 3.1: Picture (A) showing *S. brachypetala* tree and (B) shows the *S. brachypetala* leaves (image taken by Anuradha Thakur at the time of collection).

3.1.2. Traditional medicinal uses

The decoction of barks and sometimes the mixture with roots is used for the treatment of heartburn and hangovers, flu symptoms, nervous disorders, heart conditions and diarrhoea. The bark and the root mixtures are also used as a blood purifier and to strengthen the body. The roots and bark are used in South Africa as a traditional remedy for dysentery and diarrhoea [5]. The mixture is also used for the facial saunas. The plant is traditionally used in the treatment of neurodegenerative diseases [6]. Traditionally, it is used to wash body, steam the face as a remedy for pimples and to reduce body swelling. It is also used for the treatment of tropical ulcers and wounds [7]. The seeds are edible and can be eaten after roasting [8].

3.1.3 Phytochemistry

The phytochemistry of *S. brachypetala* is well studied. A 3,3',4',5,5'-pentahydroxystilbene has been isolated in high yields (7.2% on wood weight) from the methanolic extract of the dark red heartwood of *S. brachypetala*. This compound was for the first time obtained as a mixture with 3,3',4',5 tetrahydroxystilbene from the wood of *Vouacapoua macropetala* which was further isolated as the pure pentaacetate and hydrolysed to give free phenol [9]. Stilbene based compounds are known for their remarkable pharmacological activities such as for the treatment of hyperlipidemia, arteriosclerosis and inflammatory diseases [10]. In nature the stilbenes are classified as phytoalexins, a small part of phenylpropanoids, the secondary metabolites synthesized by plants to protect themselves from biotic and abiotic stress. Due to the great potential of stilbene based hybrid compounds in the field of cancer, Alzheimer's and other relevant diseases, these compounds have become a great research interest for the chemists and biologists [11]. Two antibacterial fatty acids named linolenic acid (9,12,15-octadecatrienoic) and methyl-5,11,14,17-eicosatetraenoate were isolated from the ethanol extracts *S. brachypetala* leaves [5]. Flavonol acyl glucosides were isolated from the aerial parts of the plant which exhibit antioxidant, antibacterial and antimalarial activity [8]. Seven quercetin glucoside derivatives, 3-*O*-methylquercetin 7-*O*-β-glucopyranoside, 3,4'-di-*O*-methylquercetin 7-*O*-β-glucopyranoside, 3-*O*-methylquercetin 7-*O*-[β-D-6"(E-p-coumaroyl)glucopyranoside], 3,4'-di-*O*-methylquercetin 7-*O*-[β-D-6"(E-p-coumaroyl)glucopyranoside], quercetin 7-*O*-β-glucopyranoside (E), (2R, 3R)-dihydroquercetin 7-O-β-glucopyranoside and quercetin 3-*O*-[2-*O*-β-xylopyranosyl-6-*O*-α-rhamnopyranosyl]-β-glucopyranoside were isolated from the methanol extract of areal parts of *S. brachypetala* [12]. From the *S. brachypetala* leaf extract, three phenolic compounds gallic acid, myricetin-3-*O*-*α*-L-$^{1}C_{4}$-rhamnoside and quercetin-3-*O*-L-$^{1}C_{4}$-rhamnoside were isolated and identified [13]. Ten other phenolic compounds; daidzein, naringin, procyanidin isomers, procyanidin dimer gallate, quercetin 3-O-rhamnoside, quercetin 3-O-glucuronide, quercetin hexose gallic acid,

quercetin hexose protocatechuic acid, and ellagic acid were isolated from the aqueous alcohol extract of stalks of *S. brachypetala* [14].

3.1.4. Previously reported biological assaying

The wide traditional uses of different parts of *S. brachypetala* by traditional health practitioners have led researchers to seek and explore the opportunities in drug development from this plant. Two antibacterial fatty acids, 9,12,15-octadecatrienoic (linolenic) acid and methyl-5,11,14,17-eicosatetraenoate isolated from the activity-directed fractionation of ethanol extracts of the dry leaves showed strong antibacterial activity against two gram-positive bacteria, *Bacillus subtilis* and *Vibrio parahaemolyticus,* while to a lesser extent, against two gram-negative species, *Escherichia coli* and *Klebsiella pneumoniae* [5]. The presence of stilbenes and phenolics have indicated the plant to have good radical scavenging activity [6]. The *S. brachypetala* bark has been reported to exhibit anti-hyaluronidase activity with an IC_{50} value of 75.13 ± 7.49% which demonstrated its efficacy for anti-aging properties [7]. *S. brachypetala* (root and bark) were found to exhibit antioxidant activities. Antioxidant activity was measured by the DPPH and ABTS radical scavenging tests. The DCM:MeOH extract of *S. brachypetala* roots showed an IC_{50} value of 0.05 ± 0.02 mg/ml while the water extract showed an IC_{50} value of 0.05 ± 0.02 mg/ml measured by DPPH test. For ABTS test, the DCM:MeOH root extract showed an IC_{50} value of $3.26 \times 10^{-7} \pm 0.21X10^{-9}$ mg/ml and the water extract showed an IC_{50} value of $3.7 \times 10^{-7} \pm 0.21 \times 10^{-9}$ mg/ml. The bark of *S. brachypetala* was also tested for DPPH and ABTS scavenging bioassays. It showed an IC_{50} value of 1.90 ± 0.50 mg/ml for the DCM:MeOH extract and an IC_{50} value of 0.13 ± 0.03 mg/ml for the water extract in DPPH test. For ABTS test, and IC_{50} was not determined for the DCM:MeOH extract and for water extract it showed an IC_{50} value of 0.15 ± 0.03 mg/ml. The organic root extract of *S. brachypetala* showed lowest IC_{50} values in both antioxidant assays indicating its good antioxidant potential [6].

3.1.5. Bioassaying of *S. brachyptala* as a potential for Alzheimer's disease

The plant extract was tested for acetylcholinesterase inhibition (AChEI) activity and compared to the positive control galanthamine [15]. The DCM:MeOH root extract of *S. brachypetala* gave an IC_{50} value of 0.89 ± 0.01 mg/ml and the water extract had an IC_{50} value of 3.40 ± 0.50 mg/ml for AChEI activity. The DCM:MeOH bark extract displayed an IC_{50} 0.27 ± 0.07 mg/ml and 0.49 ± 0.04 mg/ml for the water extract [6]. *S. brachypetala* exhibited good monoamine oxidase B (MAO-B) inhibition activity. Recently, MAO-B inhibitors are also taken in consideration for the treatment of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [16]. These bioassays provide evidence of the efficacy of *S. brachypetala* and substantiates further research into the potential of the plant as anti-Alzheimer's drugs.

3.2. *Cussonia paniculata*

3.2.1. Botany and geographical distribution

C. paniculata belongs to the family Araliaceae, commonly known as mountain cabbage tree. It is a short, slow growing tree or a large shrub reaching up to 5 m in height. It turns into an attractive plant in its juvenile stage and become an asset to small gardens. One of the most distinctive features of this plant is cabbage blue leaves which are arranged in whorl pattern. It is considered as succulent pachycaul because of its thick, squat and swollen stem. The roots are also thick and swollen. The plant bears yellowish to greenish colored flowers which blooms in January to April. It is an indigenous plant species which is well distributed in South Africa and very common in KwaZulu-Natal. It is often found in the mountains of Karoo and Eastern Cape through KwaZulu-Natal [17], [18], [19].

Figure 3.2: Picture showing *C. paniculata* tree (image taken by Anuradha Thakur at the time of collection).

3.2.2. Traditional medicinal uses

It is reportedly used as a traditional medicine against pain, inflammation, infections and malaria [17]. The species is also used for the treatment of mental illness, diarrhea and sexually transmitted diseases [20]. The thick tuberous roots are peeled and eaten raw as an emergency food or as a source of water. In South Africa, the mixture of Buddleja species with *Heteromorpha trifoliate* and *C. paniculata* is used for the treatment of early nervous and mental disorders [6].

3.2.3. Phytochemistry

Triterpenoid glycosides, 3-*O*-β-D-glucopyranoside of β-sitosterol, 3-*O*-α-L-arabinopyranosides of oleanolic acids, ursolic acids, hederagenin, 3-*O*-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosideofoleanolic acid, 3-*O*-α-L-arabinopyranosyl-28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosides of oleanolic and ursolic acids and 28-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranoside of 23-hydroxyursolic acid were isolated from *C. paniculata* leaves [21]. Previous studies have shown that the plant contains high sterol/triterpene, flavonoids such as rutin, quercetin and luteolin [22].

3.2.4. Previously reported biological assaying

The stem bark of *C. paniculata* exhibited anti-inflammatory and analgesic activities. In a carrageenan-induced rat paw oedema experimental animal model, 50 mg/kg aqueous extract of *C. paniculata* bark showed potent anti-inflammatory properties at 2 h with percentage inhibition of 0.44 ± 3.1 (96.6) % as compared to 10 mg/kg indomethacin, 3.4 ± 2.1 (73.7) %. The analgesic effect was also recorded for the same extract by conducting a formalin test for 30 min. The extract at 50 mg/kg showed a significant licking time, 5.8 ± 0.8* sec and licking frequency 8.3 ± 0.3*/30 min as compared to indomethacin which exhibited licking time 5.0 ± 0.5* sec and 14.3 ± 1.8*/30 min (* = P<0.05) [22]. The DCM:MeOH leaf extract of *C. paniculata* was screened for its *in vitro* anticancer activity and showed an inhibition of 100% cell growth at a concentration of 5.25 µg/ml (renalTK10), 15.0µg/ml (melanoma UACC62) and 55.69 µg/ml (breast MCF7) [23] . In another study different plant part extracts (leaves, bark, stem and roots) of *C. paniculata* using different solvents (water, ethanol and ethyl acetate) were screened for anti-inflammatory activity in the COX-1 assay as compared to control indomethacin. Indomethacin showed 78% inhibition at a concentration of 5µM. Ethanol and ethyl acetate extracts of *C. paniculata* were more active than water extracts as shown in Table 3.1 [24].

Table 3.1: Inhibition percentage of cyclooxygenase in COX-1 bioassay by different extracts of *C. paniculata*

C. paniculata **(Plant part analysed)**	**% Inhibition at 0.2µg/µl**		
	Water extract	**Ethanol extract**	**Ethyl acetate extract**
Leaf	29	80	58
Bark	0	42	78
Stem	27	85	80
Root	32	15	38

3.2.5. Bioassaying of *C. paniculata* as a potential for Alzheimer's disease

Although traditionally the plant is used for the treatment of diseases associated with nervous system, there is no any scientific study to prove its activity for the reduction of Aβ42 and inhibition of acetylcholinesterase inhibition which are targets for treatment and management of Alzheimer's disease [24].

3.1. *Heteromorpha arborescens*

3.3.1. Botany and geographical distribution

H. arborescens also known as *H. trifoliate* and belongs to the family Apiaceae. It is a medium-sized woody shrub or small tree approximately 15 metres high. The bark is papery, scale–like and is easily peels off. The leaves are green to grey green in colour and variable in shape and size. The leaves turn yellow and red in autumn. The flowers are small, green or yellow in colour with a strong smell and bloom in December and January. Fruits are flattened, heart shaped and pale brown. [2]. The plant is commonly known as "Parsley tree" in English, "wildepitersielie" in Afrikaans and "umBangandlala" in Xhosa. The plant is widely distributed in South Africa throughout the regions Gauteng, Free State, Mpumalanga, KwaZulu-Natal,

Midlands, Limpopo, North West. It is found in wooded grassland, bushveld and on forest margins [25], [2].

(A) (B)

Figure 3.3: Picture (A) showing H. arborescens stem and leaves and (B) shows *H. arborescens* leaves (images taken by Anuradha Thakur at the time of collection).

3.3.2. Traditional medicinal uses

H. arborescens is one of the important medicinal plants of South Africa. It is used to treat abdominal pains, colic, fever, headaches, asthma, coughs, infertility dysmenorrhea, dysentery, nervous and mental disorders as well as a vermicide in children [25]. In Eastern Cape province, South Africa, people take boiled leaves and roots of *H. arborescens* orally for the treatment of diabetes. It is also used traditionally by ethnic groups in central and southern Africa as a remedy for headache, fever and pain [26]. The other major medicinal uses of *H. arborescens*

include the treatment of respiratory problems, shortness of breath, intestinal worms, snakebite, skin infections, an aphrodisiac and malaria [27], [28].

3.3.3. Phytochemistry

Falcarindiol, a polyacetylene was isolated from the roots of the plant while the other compounds, germacrene D, sabinene and α-pinene were isolated from leaves of *H. arborescens* [29]. Two saikosaponins, 16β,23-dihydroxy-13,28-epoxyolean-11-en-3β-yl-[β-D-glucopyranosyl (1→2)]-[β-D-glucopyranosyl (1→3)]-β-D-fucopyranoside named as saikogenin B_4 and 16β,23,28- trihydroxy-11α-methoxyolean-12-en-3β-yl-[β-D-glucopyranosyl (1→2)]-[β-D-glucopyranosyl (1→3)]-β-D-fucopyranoside named saikogenin F were idntified from the leaves of *H. arborescens* [30].

3.3.4. Previously reported biological assying

H. arborescens showed antimicrobial, anti-inflammatory (COX-1) and anthelmintic activities [31]. The leaf and stem methanol extracts of *H. arborescens* has been reported to possess antibacterial activity against gram positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus kristinae* and *Streptococcus faecalis*. The plant extract was found to exhibit antifungal property against *Penicillium notatum*, *Aspergillus flavas* and *Aspergillus niger* with an IC_{50} value ranging from 0.07 to 1.07 mg/ml. [32]. In an *in vitro* α-glucosidase inhibition study, the ethyl acetate leaf extract and methanol bark extract of *H. arborescens* showed 50% α-glucosidase inhibition at concentrations of 3.12 ± 0.62 and 3.10 ± 1.73 mg/ml, respectively [26]. *H. arborescens* extracts also showed *in vitro* anti-arthritic activity with IC_{50} values of 53.78 µg/ml as compared to the positive control diclofenac sodium which had an IC_{50} value of 32.37 µg/ml [33]. Falcarindiol isolated from the roots of *H. arborescens* has been reported to exhibit antifungal, antibacterial and analgesic activity [2]. It has also been found to inhibit COX-1 [29]. The saikosaponins identified from *H. arborescens* leaf extract were reported to have inhibitory action on hepatitis [30]. The methanol leaf extract and ethanol

stem bark extract were observed to possess anti-inflammatory activities when tested against tetradecanoylphorbol acetate (TPA)-induced mouse ear edema with a percentage reduction in edema of 64% and 77%, respectively [34]. The ethanol and methanol extracts of the root bark extract of *H. arborescens* was reported to have antiviral activities against poliovirus at 10 µg/ml to 50 µg/ml conentration range while the DCM extract was active against the rhinovirus at a concentration range of 25 µg/ml to 50 µg/ml [35].

3.3.5. Bioassaying of *H. arborescence* for its potential against Alzheimer's disease

Although the plant is traditionally used for the treatment of nervous disorders, there are no previous studies reported on any bioassaying of *H. arborescens* for the reduction of Aβ42 and acetylcholinesterase inhibition or any other mental disorders.

3.4. Materials and methods

3.4.1 Collection and extraction of plant material

S. brachypetala leaves, *C. paniculata* leaves and *H. arborescens* stems were collected from Maine *vander* Schijff Botanical Garden, University of Pretoria, South Africa with the help of Curator Jason Samuels. Plant identification was established at the H.G.W.J. Schweickerdt Plant Herbarium at Universiy of Pretoria where voucher specimen were kept for further references. The collected leaves and stems were oven dried at 60˚C and the dried plant material was ground to a fine coarse powder. The powdered leaves were extracted repeatedly (3 times) for 8 hours with DCM/MeOH (1:1) at room temperature using a magnetic stirrer. The solvents obtained after filteration of each extraction and were combined and concentrated using a rotary vaccum evaporater at 50-60˚C and then dried under high vaccum for 24 hours. After drying, the extract was stored in the cold room.

3.4.2. Chemical profiling using UPLC-QTOF-MS

The crude extracts were analyzed using a Waters Acquity UPLC system (Waters corp., MAUSA), equipped with a binary solvent delivery system and an autosampler. The sample (1mg/ml) was prepared by dissolving dried extract, fraction, or pure compounds in 100% methanol. The solution was centrifuged at 10 000 g for 10 minutes to remove the particulates. The instrument was centrally operated by MassLynx 4.1 software (Waters Inc., Milford, Massachusetts, USA) for data acquisition. The Separation was achieved on Waters BEH C18 1.7 µm particle size (2.1 mm × 100 mm). The mobile phase consisted of solvent A: water with 0.1% formic acid and solvent B: methanol with 0.1% formic acid). The gradient elution was optimized as follows: 3% B (0-0.1 min), 100% B (0.3-14.00 min), 100% B (14.00-16.00 min), 3% B (16.00-16.50 min), 3% B (16.50-20.00 min). The flow rate was 0.3 ml/min for the entire run, giving a total run time of 20 minute and the injection volume was 5 µl. The instrument was calibrated by direct infusion of 5 nM sodium formate solution at a flow rate of 10 µl/min over a mass range of 50-1200 Da. The following MS source parameter were set for both positive and negative mode: Source temperature 100 ºC, sampling cone 15 V, extraction cone 4.0 V, desolvation temperature 400 ºC, cone gas flow 10.0 L/h, desolvation gas flow 700 L/h, capillary 2.0 kV. Both positive and negative modes were obtained, but the results were analyzed from the negative mode as the higher intensity peaks were obtained using this mode. Compounds were tentatively identified based on their accurate mass generated from MassLynx V 4.1, iFit values and by comparison of MS/MS fragmentation pattern with that of matching compounds from various search databases Reaxys, Scifinder, Scopus, Metlin, Metfusion, Pubchem, Chemspider, Chemical Entities of Biological Interest (ChEBI) and Massbank. The iFit value is an indication of how well the measured isotopic ion ratio compares to the theoretical ion ratio [36]. The acquired accurate masses were also compared with those of known compounds in compound databases.

Compounds were also identified using a novel approach, integrated MassLynx and UNIFI (Waters) informatics software. Raw data was imported to UNIFI software to identify the

compounds which met the match criteria with scientific libraries incorporated in UNIFY software. The UNIFI data analysis automatically matched the compound information with the Chinese traditional medicinal library (Waters UNIFI Scientific Library Component Listing version: 01) and gave potential identifications. The traditional medicinal library contains more than 6000 natural compounds. The compounds with less than 5 mDa were shortlisted for further identification. Each shortlisted compound was provided with the detailed information including compound name, molecular formula, average molecular mass, monoisotopic retention time, exact mass error in mDa and ionization mode [37]. i-Fit confidence % of compounds with good match identified from UNIFI were compared i-Fit confidence % of MassLynx and thus confirmed a positive compound identification.

3.4.3. Cell culture

For cell culture methodology, see section 2.2.3 of chapter 2.

3.4.4. sAPPα, sAPPβ, Aβ peptide assay

APPsw-transfected HeLa cells at 80% confluence in a 35 mm dish were cultured with samples solubilized in dimethyl sulphoxide (DMSO) for 8 h in serum free medium. The conditioned medium was analyzed by a sandwich ELISA (Invitrogen) for detection of Aβ42 and Aβ40 according to the supplier's instruction. The levels of sAPPα and sAPPβ-sw were measured from the conditioned medium using the specific ELISA (IBL). The APPsw-transfected HeLa cells with DMSO (vehicle) as a negative control.

3.4.5. Cell viability measurement

Cell viability was measured by using an EZ-Cytox kit (Daeil Lab Co., Ltd., Republic of Korea) according to the manufacturer's instructions. APPsw-transfected HeLa cells in a 96 well plate were cultured with sample solubilized in DMSO for 8 h, and then incubated with EZ-Cytox solution for 1 h at 37 °C. The absorbance was detected at 450 nm using a microplate reader.

3.4.6. Statistical analysis

For statistical analysis, see section 2.2.5 of chapter 2.

3.5. Results and discussion

3.5.1. Collection and extraction of plant material

S. brachypetala leaves, *C. paniculata* leaves and *H. arborescens* stems (50 g each) were collected from the Maine *vander* Schijff Botanical Garden, University of Pretoria. Voucher specimen (PRU 124313) for *S. brachypetala*, (PRU 124309) for *C. paniculata* and (PRU 124318) for *H. arborescens* were deposited at the H.G.W.J. Schweickerdt Herbarium. After drying in oven at 60ºC, 20 g powdered material of each plant was extracted with DCM:MeOH (1:1) solvent system. Three times repeated extraction using DCM:MeOH gave 9.2g of *S. brachypetala* leaf extract, 5.7g of *C. paniculata* leaf extract and 3.2 g of *H. arborescens* stem extract.

3.5.2. The effect of *S. brachypetala* on APP processing and cytotoxicity

The dose-dependent effect of *S. brachypetala* leaf extract on Aβ42 production at 10, 25, 50, and 100 µg/ml extract for 8 h, showed significant decrease in Aβ42 production only at higher concentrations i.e. 29.69 ± 4% at 50 µg/ml and by 62.17 ± 7% at 100 µg/ml, while the lower concentrations of the leaf extract did not show any significant reduction in Aβ42 i.e. 10 µg/ml, Aβ42 levels were reduced by 6.4 ± 3.2% and by 12.37 ± 4.3% at 25 µg/ml. The decrease in secreted levels of Aβ42 by *S. brachypetala* leaf extract is shown in Figure 3.4.

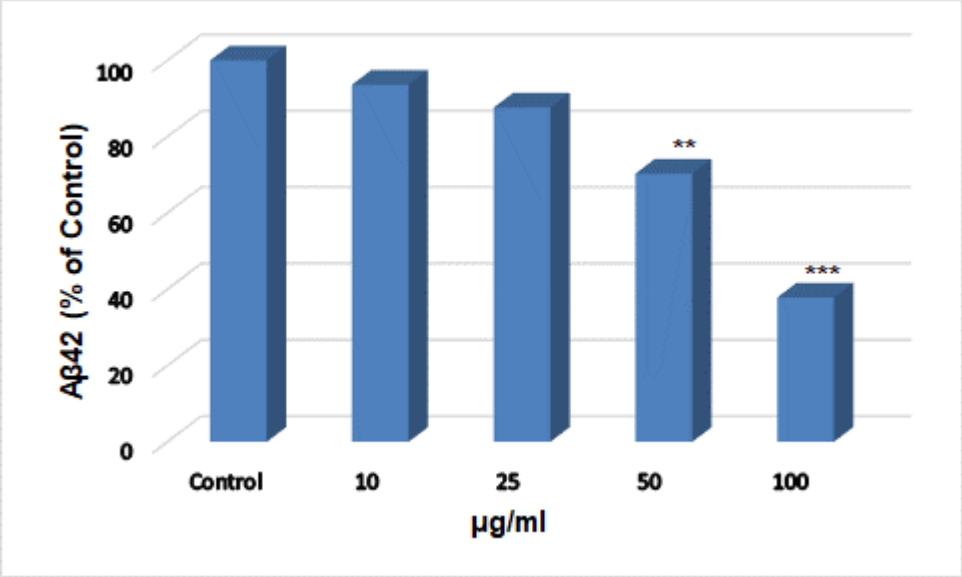


Figure 3.4: Change in the levels of Aβ42. Cells were incubated with indicated concentrations of leaf extract of *S. brachypetala* for 8 h, the levels of Aβ42 were measured from the conditioned media by using specific ELISA methods. The levels of Aβ42 (n = 4) were decreased by the extract at higher concentrations. **, P<0.01; ***, P<0.001.

Leaf extracts of *S. brachypetala* did not influence cell viability but high concentrations of 100 µg/ml induced cytotoxicity (Figure 3.5). At lower concentration, 10, 25 and 50 µg/ml the *S. brachypetala* leaf extract had a cell viability of 98.23, 97.84 and 98.34%, respectively. At the high concentration of 100 µg/ml, the leaf extract induced cytotoxicity with only 70.22% cell viability.

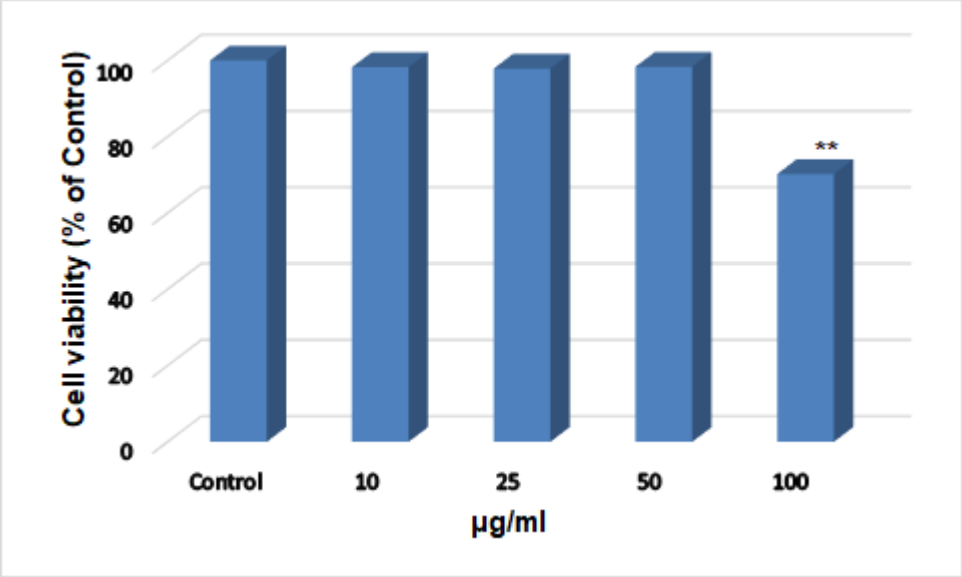


Figure 3.5: Cell viability measurement**.** Cells were incubated with indicated concentrations of extracts of leaves of *S. brachypetala* for 8 h, and then incubated with EZ-Cytox solution for 1 h. The leaf extract of *S. brachypetala* induced decreased cell viability at 100 µg/ml (n = 6). **, P<0.01; ***, P<0.001.

The levels of APP proteolytic products from the conditioned media using specific ELISA kits for Aβ40, sAPPβ-sw and sAPPα was then measured.

The leaf extract of *S. brachypetala* showed significant decrease in Aβ40 levels at the higher test concentrations of 50 µg/ml, for which the levels were reduced by 24.52 ± 4.7% and at 100 µg/ml, the percentage reduction of Aβ40 was 74.65 ± 4.97%. At lower test concentrations, there was no significant reduction in Aβ40 levels. The decrease in secreted levels of Aβ40 by *S. brachypetala* leaf extract is shown in Figure 3.6.

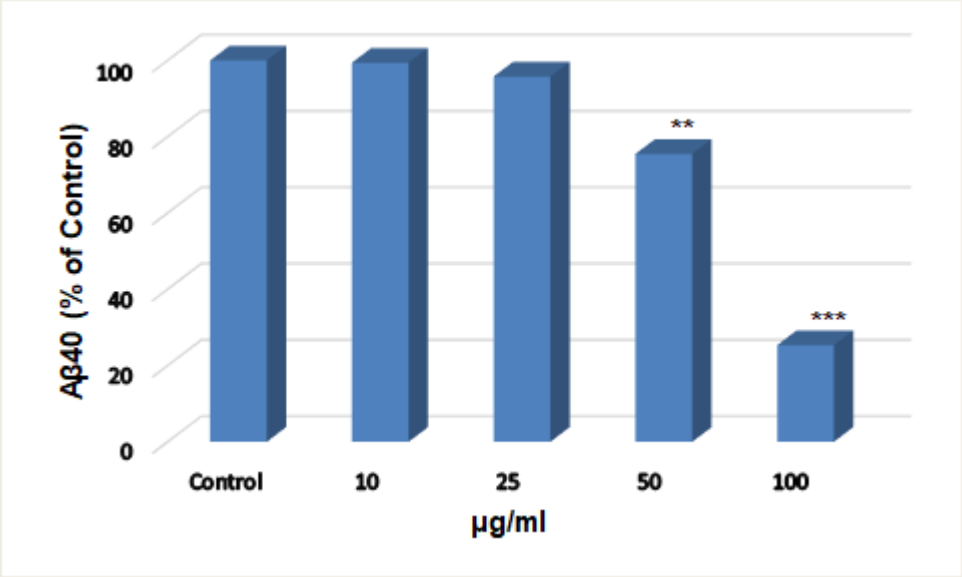


Figure 3.6: Change in the levels of Aβ40. Cells were incubated with indicated concentrations of leaf extract of *S. brachypetala* for 8 h, the levels of Aβ40 were measured from the conditioned media by using specific ELISA methods. The levels of Aβ40 (n = 4) were decreased by the extract at higher test concentrations **, P<0.01; ***, P<0.001.

In addition, this extract induced the decrease of sAPPβ-sw levels (Figure 3.7) and an increase of sAPPα level (Figure 3.8). At 50 µg/ml extract, the sAPPβ-sw level was reduced by 23.7 ± 2.7%, while the sAPPα level was increased by 28 ± 5%. At 100 µg/ml, the extract showed a higher reduction of sAPPβ-sw of 55.24 ± 1.9% while at the same concentration, sAPPα levels were increased by 45.08 ± 11%.

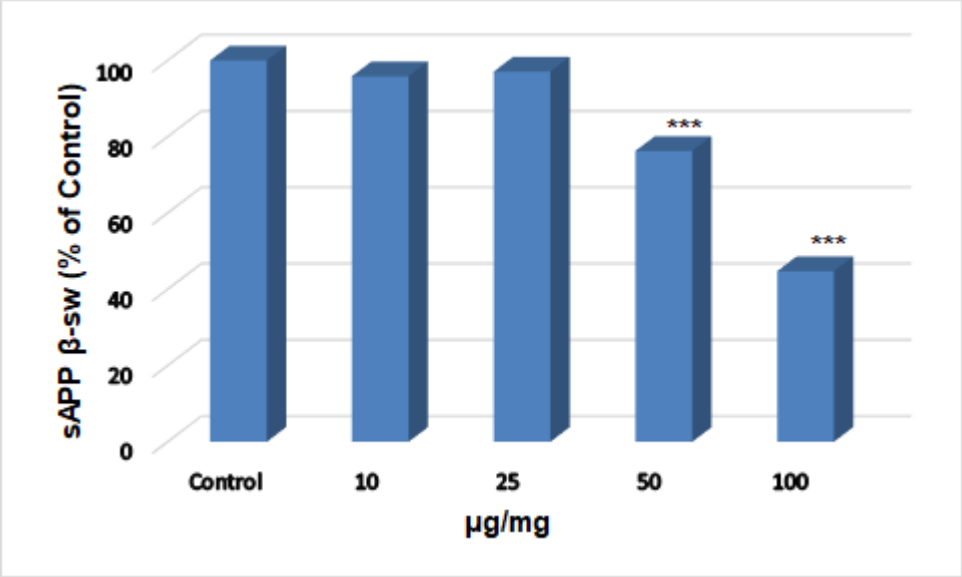


Figure 3.7: Change in the levels of sAPPβ-sw. Cells were incubated with indicated the concentrations of leaf extract of *S. brachypetala* for 8 h, the levels of sAPPβ-sw were measured from the conditioned media by using specific ELISA methods. The levels of sAPPβ-sw (n = 4) were decreased by the extract at higher test concentrations. **, P<0.01; ***, P<0.001.

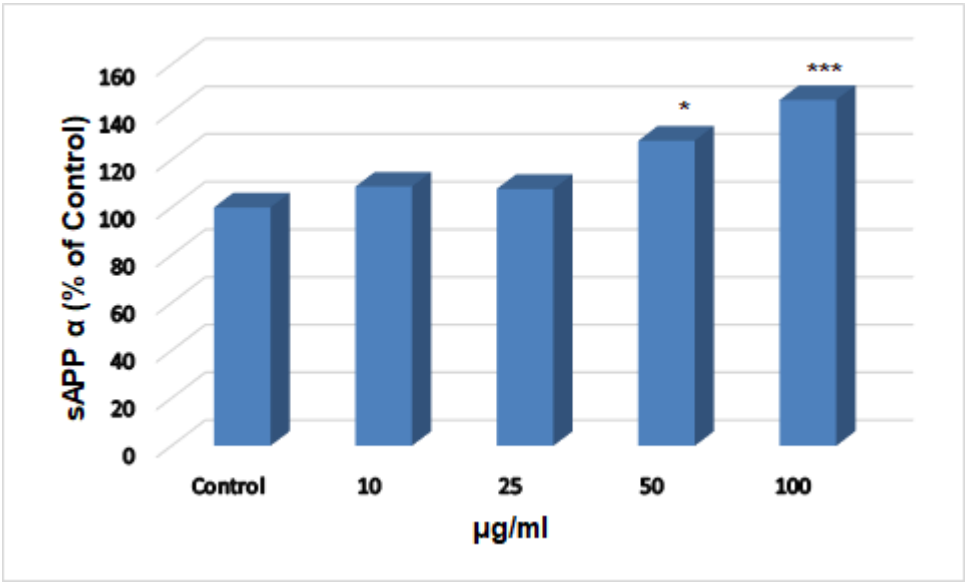


Figure 3.8: Change in the levels of sAPPα. Cells were incubated with the indicated test concentrations of leaf extract of *S. brachypetala* for 8 h, the levels of sAPPα were measured from the conditioned media by using specific ELISA methods. The levels of sAPPα (n = 4) were increased by the extract at higher test concentrations. **, P<0.01; ***, P<0.001.

From the dose dependent studies for the Aβ42, Aβ40, sAPPβ-sw and sAPPα bioassays, it was observed that only at the higher concentrations of 50 µg/ml and 100 µg/ml, *S.*

brachypetala leaf extract showed the significant results. These results suggest that the extract of *S. brachypetala* leaf extract decreased Aβ42 production at non-toxic concentrations through decreasing amyloidogenic processing of APP and increasing the nonamyloidogenic processing of APP.

Two features, one involving the reduction of Aβ42 through amyloidogenic process and other involving increase in the levels of sAPPα through nonamyloidogenic process are proven to exhibit the neuroprotective properties and also known to enhance the memory [38]. Thus these two features make the plant potent for the therapeutic target for Alzheimer's disease. In the present study, *S. brachypetala* leaf extract supported both the features to prove its efficacy to be potent towards the drug discovery. In a previous study, the plant was well researched for its activity towards the reduction of Aβ42. The *S. brachypetala* (leaves and stalks) was found to significantly decrease the Aβ42 peptide in the *in vivo* analysis on the mean mouse Aβ42 concentration using ELISA assay [14]. The present study confirmed the previous findings for the potential of the plant to treat Alzheimer's disease.

3.5.3. Chemical characterization of DCM:MeOH leaf extract of *S. brachypetala*

A chemical analysis of DCM:MeOH leaf extract of *S. brachypetala* using the UPLC-QTOF-MS was undertaken to identify the major peaks and the compounds which could be responsible for the reduction of Aβ42. In ESI negative mode (Figure 3.9), the peaks were mainly distributed within the 5.9 to 8 min range of the 18 min chromatogram. The ionization in ESI positive mode did not produce enough ionization of compounds for analysis and it was not further analysed.

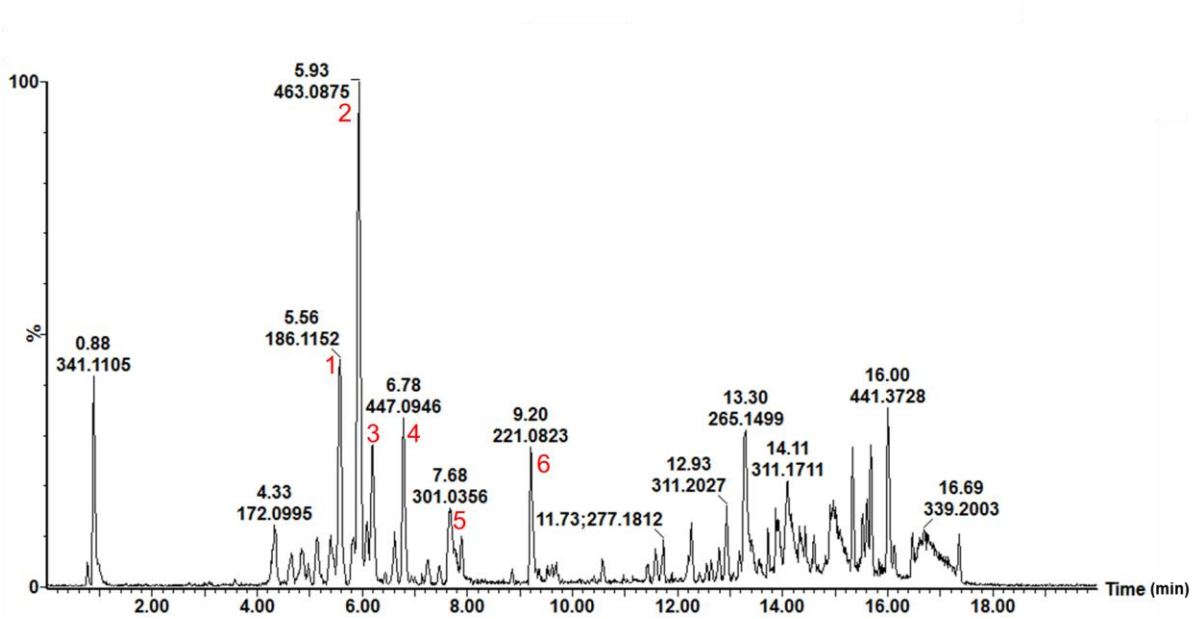


Figure 3.9: ESI negative mode BPI chromatogram of *S. brachypetala* leaf extract.

The chromatogram indicated the general elution region for polar to medium polar compounds. A total of five compounds were tentatively identified shown in Table 3.2, belonging to the class of flavonoids, peak 1 was unidentified; peak 2 identified as isoquercetin **(25)** with *m/z* 463.0875 $[M-H]^-$; peak 3 identified as myricetin-3-*O*-alpha-L-rhamnopyranoside **(26)** with *m/z* 463.0872 $[M-H]^-$; peak 4 identified as quercetin-3-*O*-rhamnoside **(27)** with *m/z* 447.0946 $[M-H]^-$ and peak 5 identified as quercetin **(28)** with *m/z* 301.0356 $[M-H]^-$ and peak 6 was unidentified.

Table 3.2: Tentative identification of compounds obtained from ESI-MS negative mode of DCM:MeOH extract of *Schotia brachypetala* (leaf)

Peak	RT (min)	Acquired $[M-H]^-$ *m/z*	Formula of possible structure	Theoretical $[M-H]^-$ *m/z*	Possible structure	Mass error (ppm)	MS/MS Data (Fragments)	Ref.
1	5.56	186.1152	-	-	unidentified	-	-	-

2	5.93	463.0875	$C_{21}H_{20}O_{12}$	463.0877	isoquercetin	-0.4	317.0262, 301.0377, 271.0243, 179.0014	[39]
3	6.23	463.0877	$C_{21}H_{20}O_{12}$	463.0877	myricetin-3-*O*-alpha-L-rhamnopyranoside	0.0	301.0344, 300.0272, 271.0257	[40], [41]
4	6.78	447.0943	$C_{21}H_{20}O_{11}$	447.0927	quercetin-3-*O*-rhamnoside	3.6	301.0352, 300.0436, 271.0420, 255.0608	[42], [40]
5	7.68	301.0351	$C_{15}H_{10}O_7$	301.0348	Quercetin	1.0	151.0066, 121.0306, 107.0152	[40]
6	9.20	221.0823	$C_{16}H_{13}O$	221.0966	unidentified	64.6	-	-

The most intense peak (labelled as peak 2) in the DCM:MeOH leaf extract of *S. brachypetala* was observed at *m/z* 463.0875 $[M-H]^-$ at retention time 5.93 min. The molecular formula $C_{21}H_{20}O_{12}$ was generated through the MassLynx with a normalized iFit value 0.212 **(supplementary data 1)**. The compound was identified as Isoquercetin **(25)** shown in Figure 3.14.

The compound was further confirmed by using pure standard isoquercetin shown in Figure 3.10. The confirmation of compound was based on the comparison of close retention time and same accurate masses of peak 2 (retention time 5.93 min and *m/z* 463.0872) with that of pure standard isoquercetin (retention time 6.23 minutes and *m/z* 463.0882).

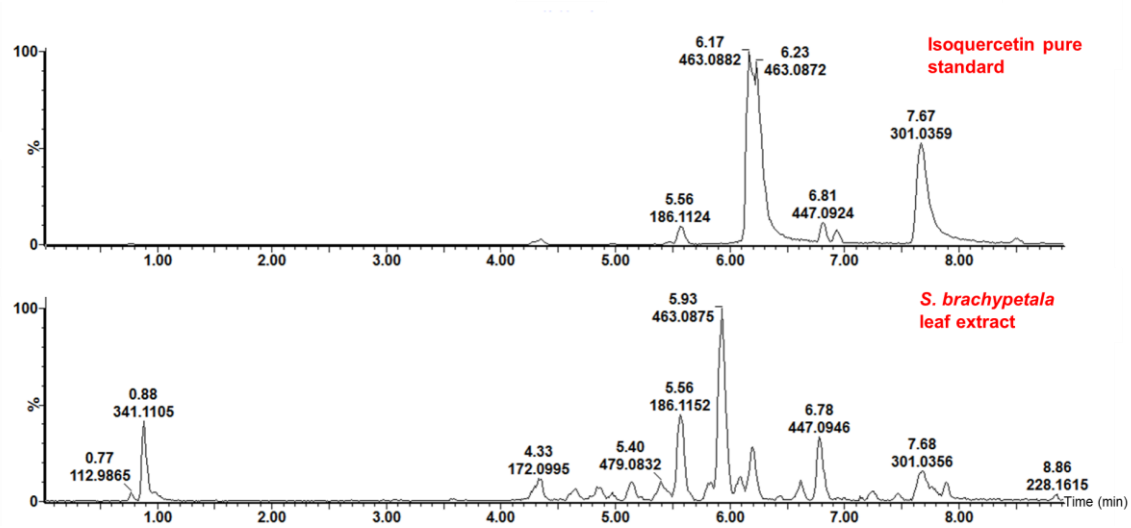


Figure 3.10: ESI negative mode BPI chromatogram of S. brachypetala leaf extract overlaid with isoquercetin pure standard. The pure standard peak was at retention time 6.23 min and m/z 463.0872 which corresponded to the peak 2 at retention time 5.93 min and m/z 463.0875 in the extract.

The MS/MS data analysis **(supplementary data 4)** gave a quercetin fragment at *m/z* 301.0344 $[M-162]^-$ with the loss of a glucoside moiety, *m/z* 271.0257 $[M-162-CO-2H]^-$. Compound was also confirmed by comparing fragmentation pattern with that of pure standard isoquercetin shown in Figure 3.11. The fragmentation pattern was also supported by the literature studies [40], [41].

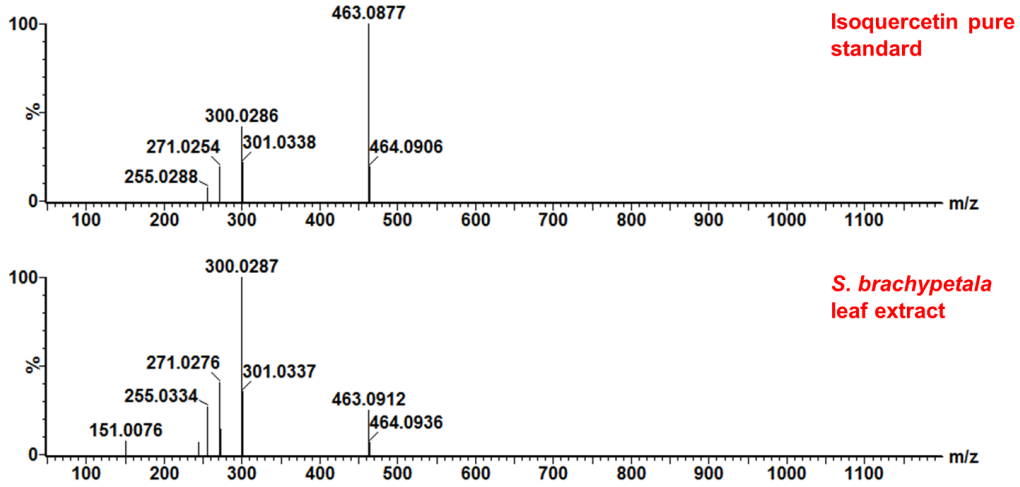


Figure 3.11: MS/MS peak 3 (isoquercetin) in S. brachypetala crude extract overlaid with the MS/MS of isoquercetin pure standard

Isoquercetin has many therapeutic properties including antioxidants, anti-allergic and anti-inflammatory. It was found to exhibit neuroprotective properties by increasing Nrf2 through ERK 1/2 pathways, preventing oxygen-glucose deprivation followed by reoxygenation (OGD/R) -induced increase in apoptosis, lactate dehydrogenase release and reduction in cell viability. These studies evaluated the potency of isoquercetin towards the treatment of ischemia and other associated diseases [43]. Isoquercetin significantly reduced the glutamate-induced oxidative neurotoxicity in HT22 hippocampal cells at a concentration of 100 mM ($p < 0.001$) with an IC_{50} of 88.8 ± 11.1** (**p<0.001) [44]. Isoquercetin also exhibited the protective effects against toxicity induced by 6-hydroxy dopamine (6-OHDA) in rat pheochromocytoma (PC12) cells. It showed a significant increase in cell viability at 10 µM with a value of 73.2 ± 1.01 ($p < 0.001$) as compared to 6-OHDA (50.80 ± 1.66) at 100 µM [45]. There is no previously reported studies which showed direct relation of isoquercetin with Aβ42 reduction and Alzheimer's disease, however its neuroprotective properties may contribute to the fact that it could be an active ingredient in the present study.

Peak 3 was observed at *m/z* 463.0872 $[M-H]^-$ with a retention time of 6.20 min. The molecular formula of compound was generated through MassLynx with a normalised iFit value of 0.176 **(supplementary data 3)**. The structure of the compound corresponding to this peak was tentatively identified as myricetin-3-*O*-alpha-L-rhamnopyranoside **(26)** shown in Figure 3.14.

The second order mass spectrum of the precursor ion showed fragments at *m/z* 316.0229 $[M-146-2H]^-$ with the loss of a rhamnosyl moiety, at *m/z* 271.0243 $[M-146-CO-H_2O-H]^-$ and at *m/z* 179.0014 **(supplementary data 2)**. The observed fragmentation pattern shown was in agreement with the fragmentation pattern obtained from literature and led to the identification of the compound [39].

In a previous study myricetin was found to exhibit an anti-tau effect when tested at 50 µM in HeLa-C3 cells [46]. It has also been found to exhibit oxidative effects towards the Met35 residue in Aβ peptides by interfering in Aβ aggregate formation which was obtained in docking studies [47]. These studies supported the fact that myricetin and its derivatives play an

important role in the neuroprotective activities and could be responsible for the reduction of Aβ42.

Peak 4 represent a precursor ion at *m/z* 447.0946 $[M-H]^-$ with retention time 6.78 min. The molecular formula of compound, $C_{21}H_{20}O_{11}$ was generated through MassLynx with a Normalised iFit value 0.65 **(supplementary data 5)** identified as quercetin-3-*O*-rhamnoside **(27)** shown in Figure 3.14.

The MS/MS spectra **(supplementary data 6)** produced a quercetin fragment ion at *m/z* 301.0352 $[M-146]^-$ which involved the loss of a rhamnosyl moiety, a typical flavon-3-*O*-monoglycoside fragment at *m/z* 271.0420 $[M-146-CO-H]^-$. The same fragment pattern was described in literature [42], [40].

The flavonoid quercetin-3-*O*-rhamnoside was previously isolated from the aqueous extract of *S. brachypetala* leaves and stalks. *S. brachypetala* leaves (100 mg/kg/day) and *S. brachypetala* stalk (100 mg/kg/day) significantly reduced Aβ42 concentration by 76.2% and 72.8% as compared to (Lipopolysaccharide) LPS group, on a treatment for 6 days. This study indicated that identified flavonoids including quercetin-3-*O*-rhamnoside could be responsible for the activity [14]. The same compound isolated from *Salsola grandis* showed no percentage inhibition when tested against AChE [48]. These previous studies suggest that the compound was inactive towards AChE, however based on its reported reduction of Aβ42 concentration points to the compound being one of the active compounds in the plant extract.

The peak labelled as 5 had a precursor molecular ion at *m/z* 301.0356 with retention time 7.68 min and corresponded to the proposed molecular formula $C_{15}H_{10}O_7$ generated through MassLynx with a normalised iFit value of 0.026 **(supplementary data 7)**. The compound was tentatively identified as quercetin **(28)** shown in Figure 3.14.

The retention time of the pure standard of quercetin of 7.65 min (*m/z* 301.0384) with that of peak 5 in the crude extract retention time 7.68 min (*m/z* 609.1465) confirmed the identity of peak 5 as quercetin (Figure 3.12).

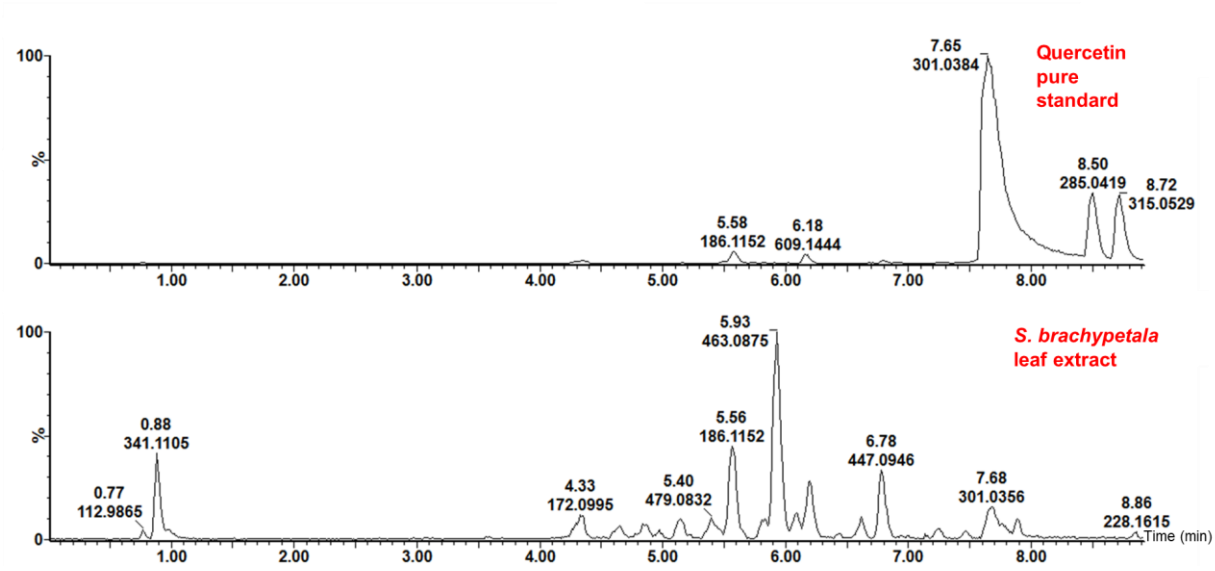


Figure 3.12: ESI negative mode BPI chromatogram of S. brachypetala leaf extract overlaid with isoquercetin pure standard. The pure standard peak was at retention time 7.65 minutes and m/z 301.0384 which corresponded to the peak at retention time 7.68 and m/z 301.0356 in the extract.

A comparison of the MS/MS fragmentation pattern of peak 5 (quercetin) in the crude extract with that of the pure standard of quercetin also confirmed the identity of peak 5 as quercetin (Figure 3.13).

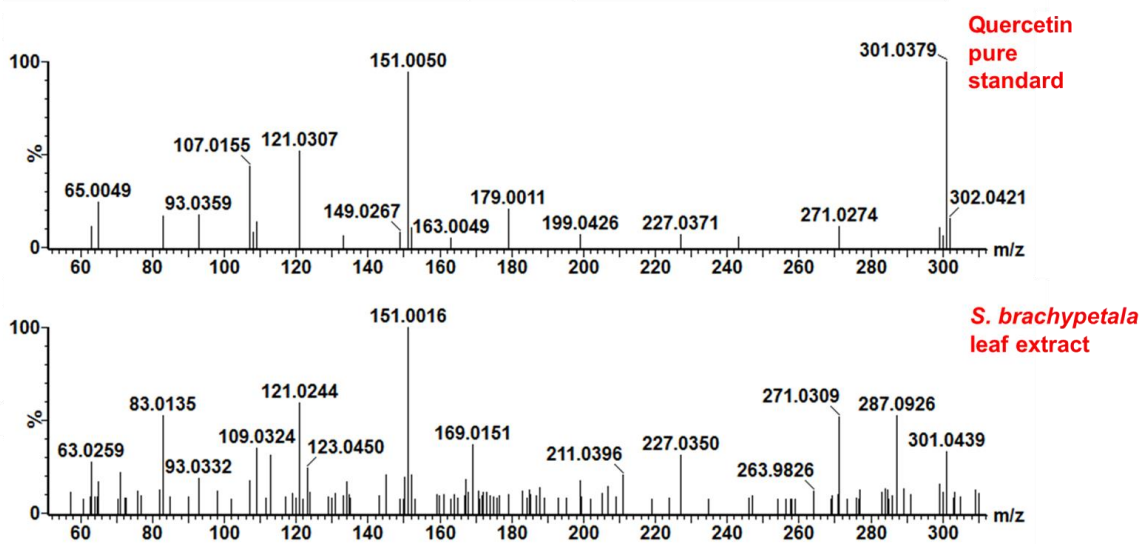
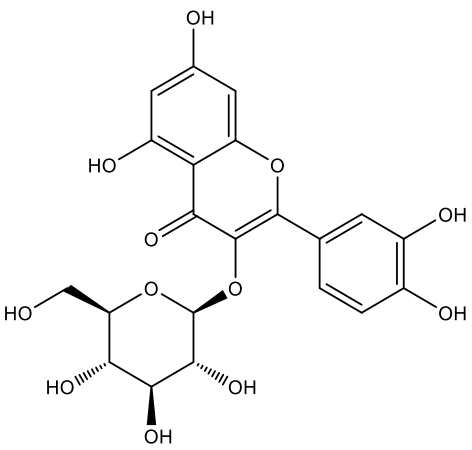


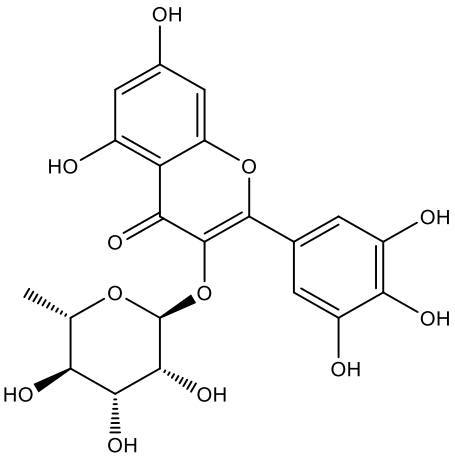
Figure 3.13: MS/MS peak 5 (quercetin) in *S. brachypetala* crude extract overlaid with the MS/MS of quercetin pure standard.

The compound was also confirmed by comparing the fragmentation pattern with literature. The MS/MS spectra **(supplementary data 8)** gave the fragment ions at *m/z* 271.0274 $[M\text{-}CO\text{-}H]^-$, *m/z* 179.0011 and *m/z* 151.0050 which are similar to the fragmentation pattern obtained from literature [40].

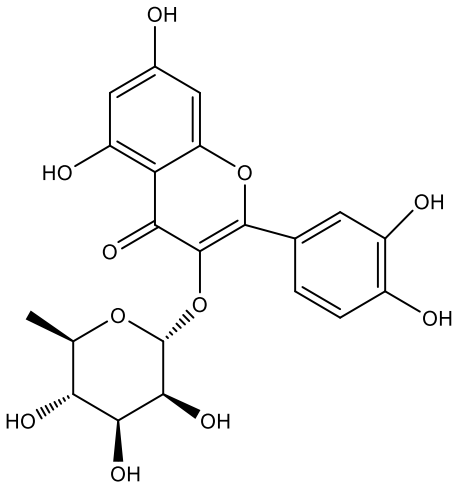
The quercetin flavonoid showed strong antioxidant and free radical scavenging properties in *in vitro* studies [49]. In an *in vivo* study quercetin significantly decreased β-amyloidosis in aged 3xTg-AD mice when treated for three months as compared to the vehicle-treated 3xTg-AD mice. The reduction in the $A\beta_{(1–40)}$ and $A\beta_{(1–42)}$ levels were detected in the hippocampus of the quercetin-treated 3xTg-AD mice compared to the vehicle-treated 3xTg-AD mice [50]. At the lower doses (5 and 10 μM), quercetin on pre-treatment with neurons for 1 h significantly reduced the $A\beta_{(1–42)}$ - induced cytotoxicity [51]. Quercetin showed the neuroprotective effects by inhibiting Lipooxidation, neuronal cell apoptosis, hippocampal neuronal cell damage after exposure to cadmium [52]. Quercetin was also reported to induce NF-E_2-related factor-2 (Nrf_2) which increased the neuroprotection in cerebellar granule neuronal culture [53]. Quercetin protected HT22 murine neuroblastoma cells from $A\beta_{(25\text{-}35)}$ oxidative attack by inhibiting Aβ fibril formation [51]. All these previous studies supported the fact that quercetin could be the active ingredient in the present study because of its ability to reduce the Aβ levels in different bioassays reported in previous studies.



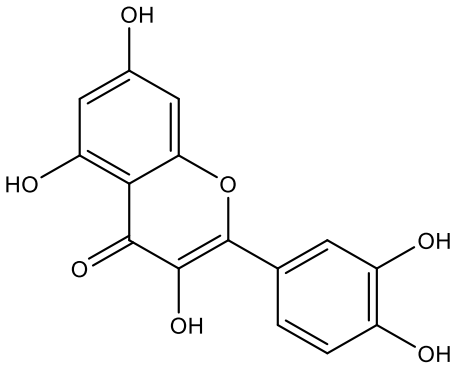
(25)



(26)



(27)



(28)

Figure 3.14: Chemical structures of compounds identified from S. brachypetala leaves, Peak 2: isoquercetin **(25)**, Peak 3: myricetin-3-O-alpha-L-rhamnopyranoside **(26)**, Peak 4: quercetin-3-O-rhamnoside **(27)**, Peak 5: quercetin **(28)**

After LC-MS analysis, it was found that main class of compounds identified in the *S. brachypetala* leaves is flavonoid glycosides. The flavonoid glycosides were identified from *S. brachypetala*, with a typical characteristic fragmentation pattern of flavonol-3-*O*-glycosides produce a fragment ion at *m/z* 301 which revealed the presence of a quercetin moiety. The common secondary fragment ions at *m/z* 271, *m/z* 255, *m/z* 179 and *m/z* 151 were produced because of the fragmentation of quercetin. The fragments *m/z* 271 and *m/z* 255 were produced due to the loss of carbon monoxide (-28 amu) and carbon dioxide (-44 amu) respectively. The fragment ions at *m/z* 151 and *m/z* 179 were produced as a result of Retro Diel's alder fragmentation pattern. The fragmentation pathway followed by quercetin derivatives is shown in Figure 3.15.

Flavonoid glycosides are found in a wide variety of natural foods and have shown positive effects against dementia and Alzheimer's disease in various cell cultures and animal models [54]. In a transgenic mouse model, a flavonoid named nobiletin was found to improve the Aβ induced memory impairment. It also reduced the Aβ plaques formation in the hippocampus of APP-SL 7-5 Tg mice [55]. Luteolin another flavonoid, was found to inhibit the Aβ peptides with

an inhibition percentage >70% and >85% at the concentrations of 20 and 40 µM, respectively in both human “Swedish” mutant APP N2a and Tg2576 mouse-derived primary neuronal cells [56]. These previous studies indicated that the flavonoid glycosides identified from *S. brachypetala* could also play an important role in the anti-Alzheimer’s drug discovery.

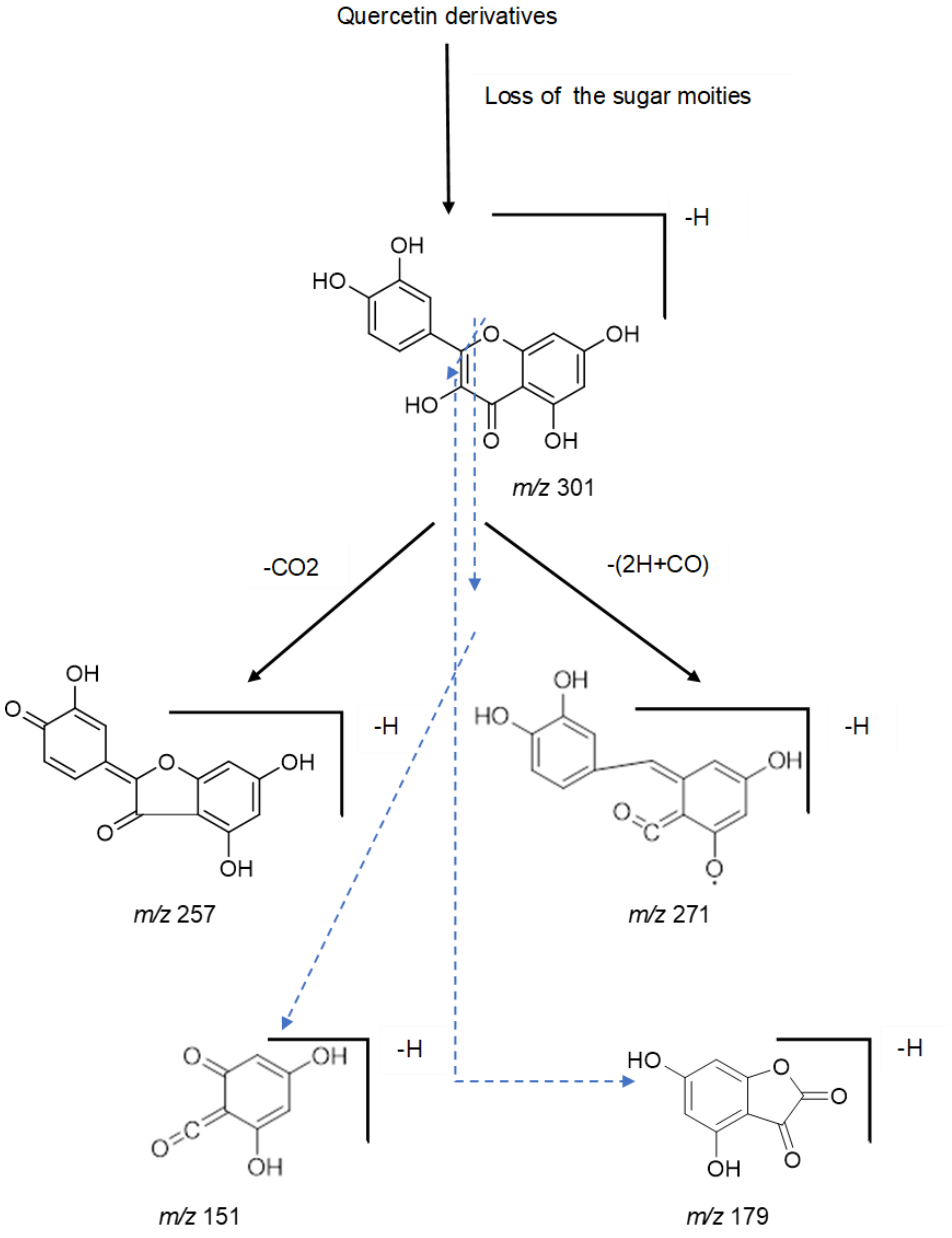


Figure 3.15: Fragmentation pattern of quercetin derivatives adapted from [43] and [39]

3.5.4. The effect of *C. paniculata* on APP processing and cytotoxicity

The extract of leaves of *C. paniculata* significantly reduced Aβ42 production in a dose-dependent manner. At concentrations 10, 25, 50 and 100 µg/ml, the leaf extract significantly reduced the Aβ42 levels by 29.03 ± 1.4%, 42.40 ± 1.7%, 47.50 ± 5.8% and 61.14 ± 9.3%, respectively (Figure 3.16).

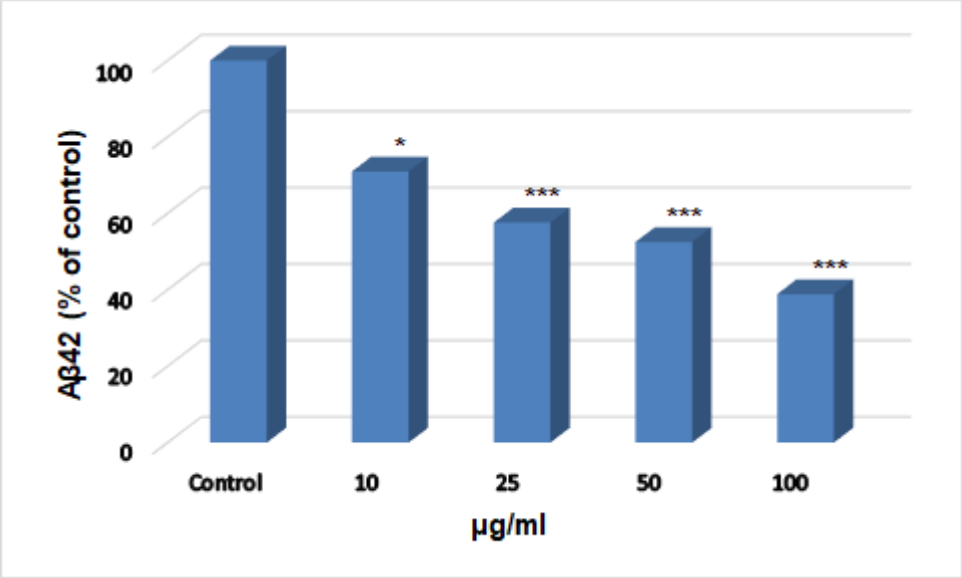


Figure 3.16: Change in the levels of Aβ42.Cells were incubated with indicated concentrations of leaf extract of *C. paniculata* for 8 h, the levels of Aβ42 were measured from the conditioned media by using specific ELISA methods. The levels of Aβ42 (n = 4) were decreased by the extract in a dose dependant manner. **, P<0.01; ***, P<0.001.

C. paniculata leaf extract (Figure 3.17) did not influence cell viability at low test concentrations but at high concentrations it induced some cytotoxicity. At the lower test concentrations of 10, 25 and 50 µg/ml *C. paniculata* leaf extract maintained cell viability at 105.4, 97.2 and 91.5%, respectively while at the high test concentration of 100 µg/ml, the leaf extract induced cytotoxicity with cell viability of 74.52%.

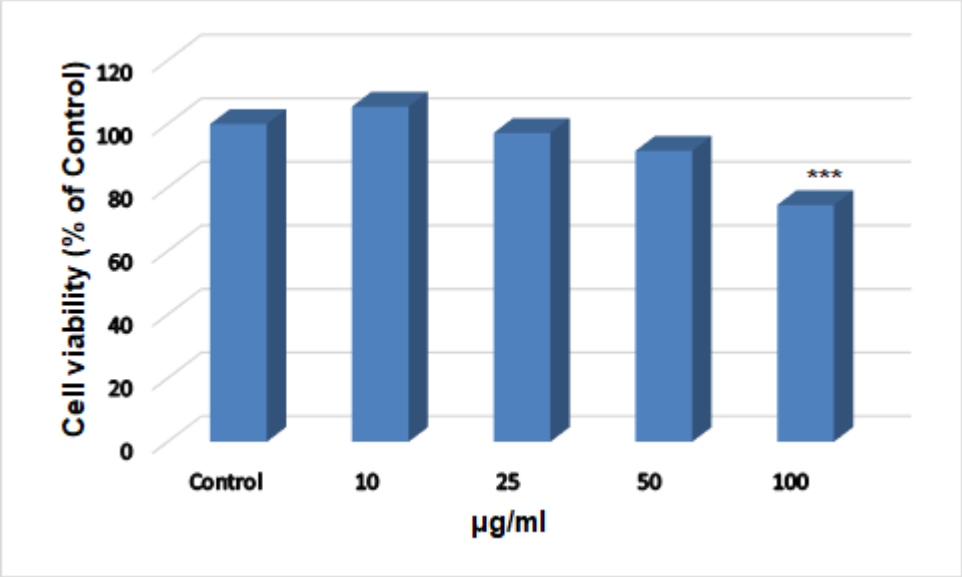


Figure 3.17: Cell viability measurement. Cells were incubated with indicated concentrations of extracts of leaves of *C. paniculata* for 8 h, and then incubated with EZ-Cytox solution for 1 h. The leaf extract of *C. paniculata* induced the decrease of cell viability at the highest test concentration of 100 µg/ml (n = 6).

The levels of APP proteolytic products from the conditioned media using specific ELISA kits for Aβ40, sAPPβ-sw and sAPPα were then measured. The level of Aβ40 was significantly decreased in a dose dependent manner by 47.7 ± 9.1%, 79.6 ± 2.2% and 90.19 ± 2.5% at 25, 50 and 100µg/ml of extract, respectively (Figure 3.18).

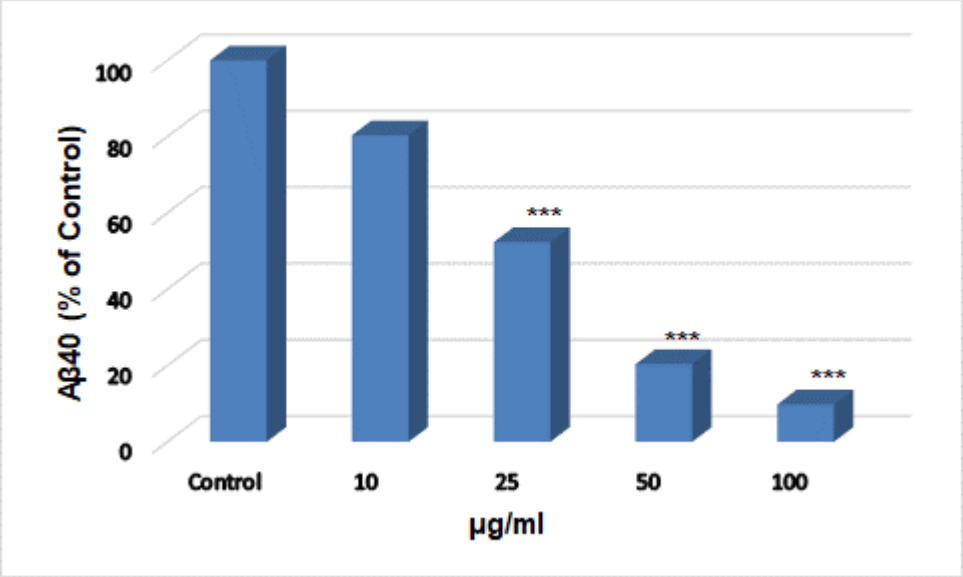


Figure 3.18: Change in the levels of Aβ40. Cells were incubated with indicated concentrations of leaf extract of *C. paniculata* for 8 h, the levels of Aβ40 were measured from the conditioned media by using specific ELISA methods. The levels of Aβ40 (n = 4) were decreased by the leaf extract in a dose dependant manner. **, P<0.01; ***, P<0.001.

The level of sAPPβ-sw was also significantly decreased in a dose dependent manner by 37.9 ± 4.4%, 56.6 ± 0.8% and 74.57 ± 1.5% at 25, 50 and 100 µg/ml of extract, respectively (Figure 3.19.). In addition, this extract also significantly reduced sAPPα production by 33.99 ± 3% and 49.47 ± 10% at 25 and 50 µg/ml, respectively as shown in Figure 3.20.

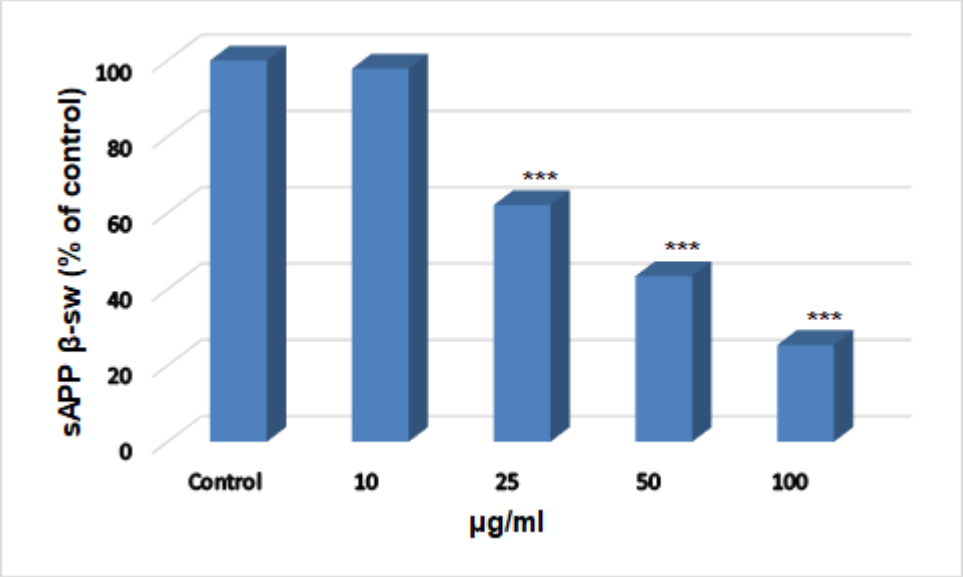


Figure 3.19: Change in the levels of sAPPβ. Cells were incubated with indicated concentrations of leaf extract of *C. paniculata* for 8 h, the levels of sAPPβ were measured from the conditioned media by using specific ELISA methods. The levels of sAPPβ (n = 4) were increased by the extract in a dose dependant manner **, P<0.01; ***, P<0.001.

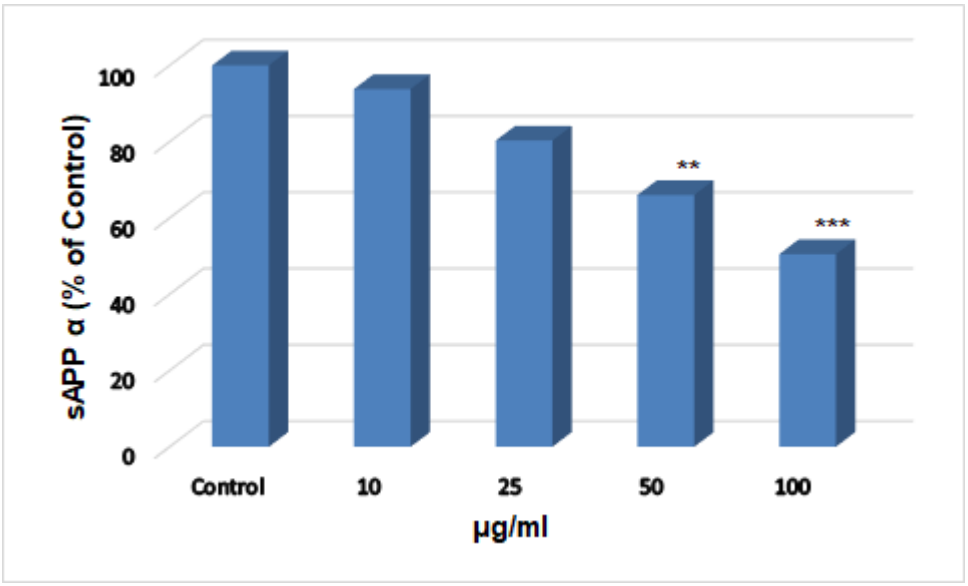


Figure 3.20: Change in the levels of sAPPα**.** Cells were incubated with indicated concentrations of leaf extract of *C. paniculata* for 8 h, the levels of sAPPα were measured from the conditioned media by using specific ELISA methods. The levels of sAPPα (n = 4) were increased by the extract in a dose dependant manner **, P<0.01; ***, P<0.001.

These results suggested that the DCM:MeOH extract of *C. paniculata* leaves decreased Aβ42 production at non-toxic concentrations through decreasing amyloidogenic processing of APP but there was no increase in the nonamyloidogenic processing of APP. The ability of *C. paniculata* leaf extract to reduce Aβ42 supported its efficacy in the development new drugs for the treatment of Alzheimer's disease. This study on the plant is the first report showing potent activity towards the reduction of Aβ42.

3.5.5. Chemical characterization of DCM:MeOH leaf extract of *C. paniculata*

The DCM:MeOH leaf extract of *C. paniculata* was analysed using the UPLC-QTOF-MS to identify the major peaks and the compounds which could be responsible for the reduction of Aβ42. The ionization was poor in the positive mode so it was not used for further analysis. In ESI negative mode, the peaks were mainly distributed within 6.16 to 11.38 minute range of the 18 minute chromatogram (Figure 3.21).

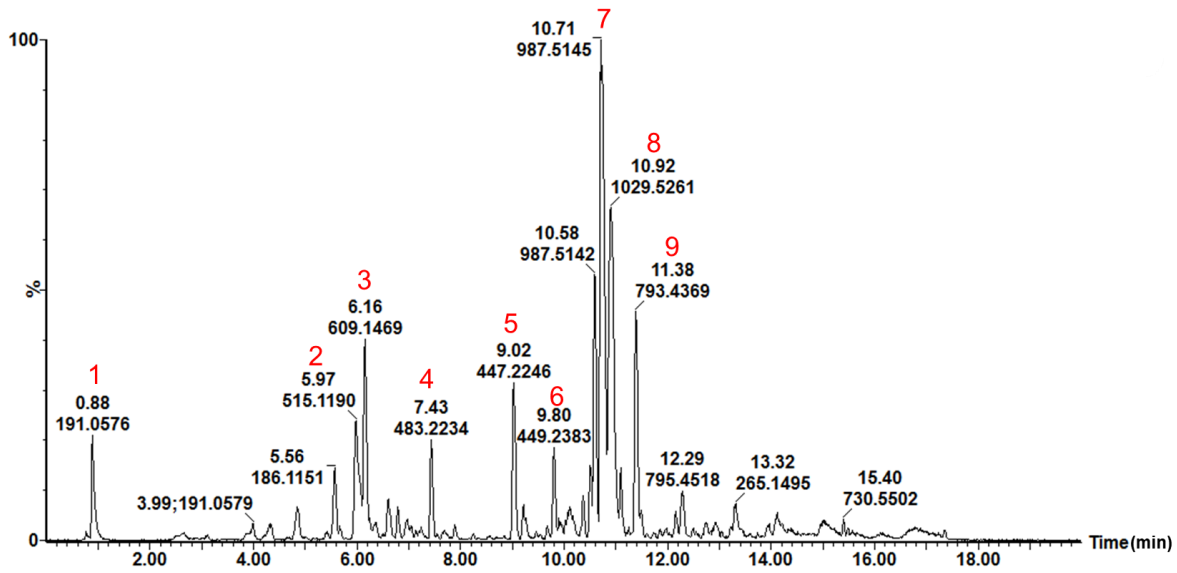


Figure 3.21: ESI negative mode BPI chromatogram of *C. paniculata* leaf extract**.**

This indicated the general elution region for polar to medium polar compounds. Using ESI negative mode, a total of nine compounds were tentatively identified shown in Table 3.3. Peak 1 was identified as quinic acid **(29)** with *m/z* 191.0576 $[M-H]^-$, peak 2 identified as 3,5-dicaffeoylquinic acid **(30)** with *m/z* 515.1190 $[M-H]^-$, peak 3 identified as rutin **(31)** with *m/z* 609.1469 $[M-H]^-$, peak 4 identified as valerianannoid E **(32)** with *m/z* 483.2234 $[M-H]^-$, peak 5 identified as acuminoside **(33)** with *m/z* 447.2246 $[M-H]^-$, peak 6 identified as dictamnoside D **(34)** with *m/z* 449.2383 $[M-H]^-$, peak 7 identified as 2''-O-β-D-glucopyranosylsaikosaponin B2 **(35)** with *m/z* 987.5145 $[M-H]^-$; peak 8 identified as clinoposaponin C **(36)** with *m/z* 1029.5261 $[M-H]^-$ and peak 9 identified as spinasaponin C **(37)** with *m/z* 793.4369 $[M-H]^-$.

Table 3.3: Tentative identification of compounds obtained from ESI-MS negative mode of DCM:MeOH extract of *C. paniculata* leaves

Peak	RT (min)	Acquired $[M-H]^-$ *m/z*	Formula of Possible structure	Theoretical $[M-H]^-$ m/z	Possible structure	Mass error (ppm)	MS/MS Data (Fragments)	Ref.
1	0.88	191.0576	$C_7H_{12}O_6$	191.0556	quinic acid	10.5	161.0456, 131.0485	-
2	5.97	515.1190	$C_{25}H_{24}O_{12}$	515.1190	3,5-dicaffeoylquinic acid	0.0	353.0883, 191.0572, 179.0363	[57]
3	6.16	609.1489	$C_{27}H_{30}O_{16}$	609.1514	rutin	-4.1	301.0353, 300.0292, 271.0259	[58]
4	7.43	483.2234	$C_{24}H_{36}O_{10}$	483.2230	valerianannoid E	0.8	277.1818, 173.0477, 135.0458	-
5	9.02	447.2246	$C_{21}H_{36}O_{10}$	447.2230	Acuminoside	3.6	315.1824, 161.0469	-
6	9.80	449.2383	$C_{21}H_{38}O_{10}$	449.2387	dictamnoside D	-0.9	161.0472, 101.0252	-
7	10.71	941.5082	$C_{48}H_{78}O_{18}$	941.5086	2''-O-ß-D-glucopyranosylsaikosaponin B2	-0.4	471.3481, 469.1565	[59]
8	10.92	983.5211	$C_{50}H_{80}O_{19}$	983.5216	clinoposaponin C	-0.5	471.3488, 469.1578	[60]
9	11.38	793.4372	$C_{42}H_{66}O_{14}$	793.4374	spinasaponin A	-0.3	631.3863	[61]

Peak 1 in the DCM:MeOH leaf extract of *C. paniculata* was observed at *m/z* 191.057 $[M-H]^-$ at retention time of 0.88 min. The molecular formula of the compound, $C_7H_{12}O_6$ was generated

through MassLynx with a normalized iFit value 0.025 **(supplementary data 9)**. The structure of the compound corresponding to this peak was tentatively identified as quinic acid **(29)** shown in Figure **3.26**. The MS/MS spectra **(supplementary data 10)** gave fragment ions at *m/z* 161.0456 with the loss of one hydroxyl ion and one oxygen atom. The other fragment ion was produced at *m/z* 131.0485 with the loss of formic acid and one hydroxyl ion.

In a previous study, it was reported that quinic acid did not prevent Aβ42 aggregation when tested on SH-SY_5Y cell line using thioflavin T (Th-T) bioassays with an IC_{50} value >100 µM [62]. Although in the previous studies quinic acid itself did not show any significant activity, its derivatives (discussed for peak 2) were active towards the prevention of Aβ42 aggregation [62].

Peak 2 had a *m/z* 515.1190 $[M-H]^-$ with retention time 5.97 min. The molecular formula, $C_{25}H_{24}O_{12}$ was generated through MassLynx with a normalized iFit value 0.444 **(supplementary data 11)**. The compound was tentatively identified as 3,5-dicaffeoylquinic acid **(30)** and the structure is shown in Figure 3.26. The MS/MS spectrum **(supplementary data 12)** produced the fragment ions at *m/z* 353.0883 $[M-H-162]^-$ with the loss of a caffeoyl moiety, a quinic acid fragment *m/z* 191.0572 $[M-H-162-162]^-$ with the loss of another caffeoyl moiety and a caffeic acid fragment ion at *m/z* 179,0363 $[M-H-336]^-$. The MS^3 base peak produced at *m/z* 191.0572 distinguished 3,5-dicaffeoylquinic acid from the isomers 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. The isomers 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid produced MS^3 base peak at *m/z* 173.0 [63], [57]. Therefore, the fragmentation pattern supported the identification of 3,5-dicaffeoylquinic acid.

The fragmentation pathway followed by 3,5-dicaffeoylquinic acid as an example is shown in Figure 3.23.

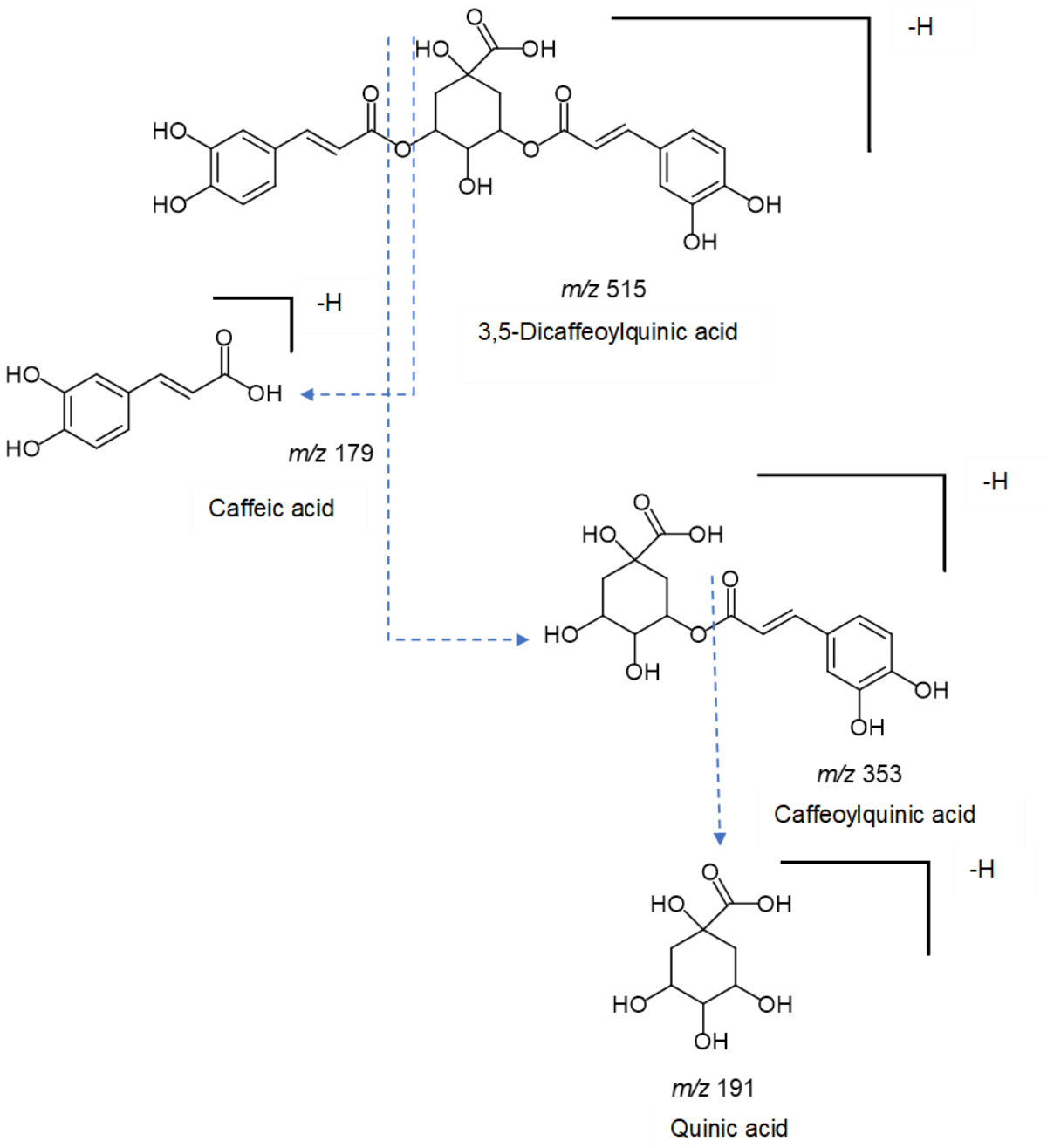


Figure 3.22: Fragmentation pattern of 3,5-Dicaffeoylquinic acid adapted from [57]

The 3,5-dicaffeoylquinic acid significantly improved the cognitive dysfunction caused because of the severe TMT induced neurotoxicity. It also inhibited AChE by recovering acetylcholine, a neurotransmitter in the cholinergic system [64]. The 3,5-dicaffeoylquinic acid isolated from

Aster scaber exhibited neuroprotective effects in Aβ-sensitive PC12 cells by significantly reducing the $A\beta_{25-35}$ cytotoxicity with a cell viability 90.0% or 75% when measured by MTT [65]. The 3,5-dicaffeoylquinic acid was also tested in behavioural tests (Y-maze, passive avoidance and Morris water maze test) in which it significantly improved the learning and memory deficits. It was found to prevent neuronal apoptosis by protecting mitochondrial activities [64].

The peak labelled as 3 produced a *m/z* 609.1469 $[M-H]^-$ with retention time 6.16 min. The molecular formula $C_{27}H_{30}O_{16}$ was generated through MassLynx with a normalised iFit value of 0.003 **(supplementary data 13)**. The compound was tentatively identified as rutin **(31)** and the structure of the compound is shown in Figure 3.26. It was further confirmed by comparing peak 6 in the DCM:MeOH extract of leaves of *C. paniculata* with a pure standard of rutin (Figure 3.24). A comparison of the MS/MS **(supplementary data 14)** fragmentation pattern of peak 6 (rutin) in the crude extract with that of the pure standard of rutin also confirmed the identity of peak 6 as rutin. Further, the retention time of the pure standard of rutin of 6.16 minutes (*m/z* 609.1454) with that of peak 6 in the crude extract retention time 6.16 min at *m/z* 609.1469 confirmed the identity of peak 6 as rutin.

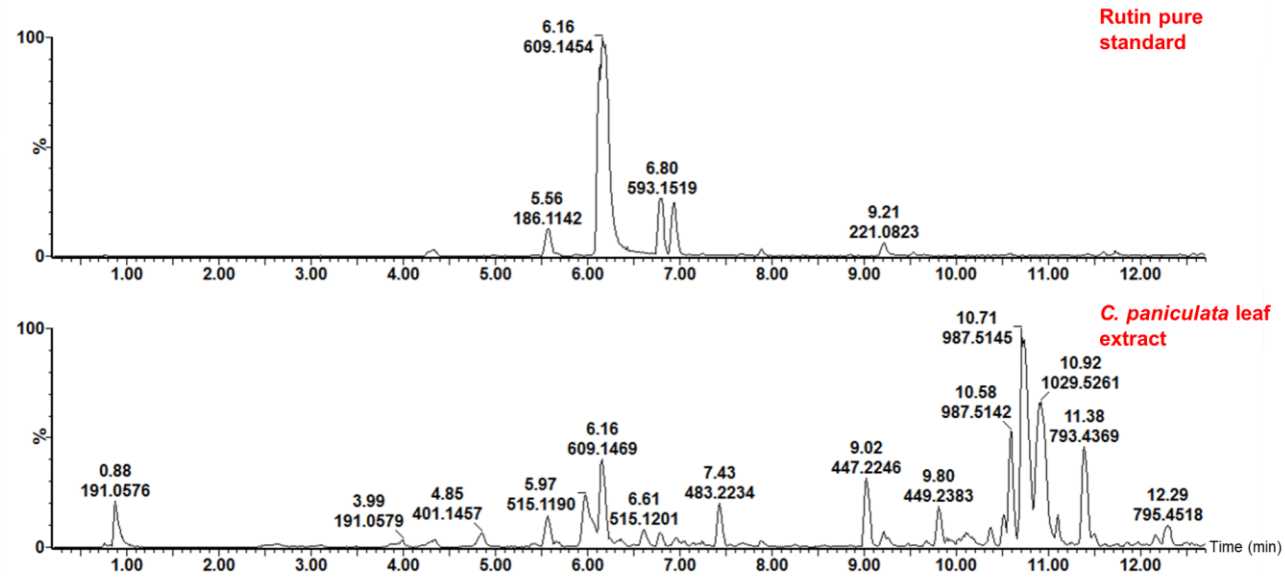


Figure 3.23: ESI negative mode BPI chromatogram of leaves of C. paniculata extracted with DCM:MeOH overlaid with standard rutin. The standard rutin had a retention time of 6.16 min and an m/z of 609.1454 which corresponded to peak 3 at retention time 6.16 min and m/z 609.1469 in the extract.

The MS^2 analysis gave a quercetin fragment at *m/z* 301.0344 $[M-308]^-$ with the successive loss of two hexose moieties, *m/z* 271.0257 $[M-308-CO-2H]^-$ and *m/z* 179.0221 due to the RDA fragmentation pattern. The fragmentation pattern was also supported with literature studies [40], [41]. Comparison of the accurate mass, retention time and the fragmentation pattern of peak 3 and the pure standard confirmed its structure (Figure 3.25).

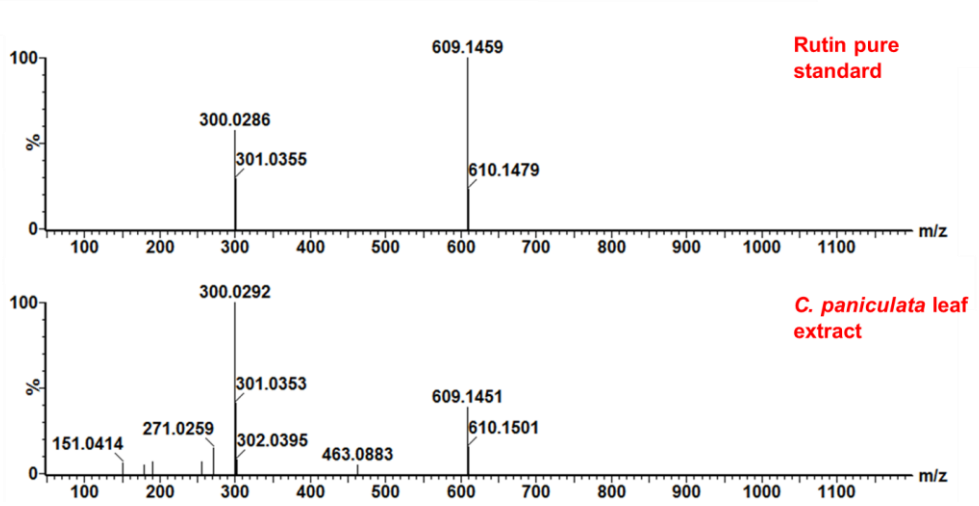


Figure 3.24: MS/MS peak 3 (rutin) in *C. paniculata* crude extract overlaid with the MS/MS of rutin pure standard

Rutin, is one of the major flavonoid glycosides isolated from *Gingko biloba*, which is one of the most investigated plant remedies for Alzheimer's disease, however its efficacy for the treatment of Alzheimer's is still controversial [66]. Rutin on oral administration significantly reduced the memory deficits in APPswe/PS1dE9 transgenic mice by decreasing Aβ oligomer levels. It also increased super oxide dismutase (SOD) activity and glutathione (GSH)/glutathione disulfide (GSSG) ratio and also decreased interleukin (IL)-1β and IL-6 levels in the brain. These results indicated that rutin as a promising agent for Alzheimer's disease [67]

The ESI negative mode for peak 4 gave a precursor ion peak at *m/z* 483.2234 and a molecular formula $C_{24}H_{36}O_{10}$ was generated through MassLynx with a normalised iFit value 0.075 **(supplementary data 15)**. A sesiquiterpenoid named valerianandoid E **(32)** was tentatively

identified and its structure is shown in Figure 3.26. MS/MS spectra gave the fragment ions at *m/z* 277.1818, *m/z* 173.0477 and *m/z* 135.0458 **(supplementary data 16)**.

The compound was previously isolated from *Valeriana jatamansi* which is used traditionally for the treatment of hypochondriasis, nervous disorders, migraine, hysteria, stress, depression, insomnia, neuralgia and neuroasthemia. Valeriananoids D–E isolated from *V. jatamansi* showed less than 10% AChE inhibition activity at a concentration of 50μM as compared to the positive control, tacrine with an inhibition percentage, 47.6% at 0.33 μM [68].

The ESI negative mode for peak 5 gave a precursor ion peak at *m/z* 447.2246 $[M-H]^-$ and a molecular formula $C_{21}H_{36}O_{10}$ was generated through MassLynx with a normalised iFit value of 0.050 **(supplementary data 17)**. The compound was identified as acuminoside **(33)** (6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside) and the structure of the compound is shown in Figure 3.26. The MS/MS analysis **(supplementary data 18)** produced one fragment ion at *m/z* 315.1824 by the loss of apiofuranosyl and another fragment at *m/z* 161.0469 with the loss of glucopyranosyl. This diglycoside is found in grapes [62] and does not have any previous scientific study related to Alzheimer's disease.

The ESI negative mode for peak 6 gave a precursor molecular ion peak at *m/z* 449.2383$[M-H]^-$ with retention time of 9.80 min. Molecular formula $C_{21}H_{38}O_{10}$ was generated through MassLynx with an normalised iFit value of 0.000 **(supplementary data 19)**. The compound was tentatively identified the eudesmane-type-sesquiterpene glycoside, dictamnoside D **(34)** and the structure is shown in Figure 3.26. The MS/MS spectrum **(supplementary data 20)** yielded a fragment ion at *m/z* 161.0472 with the loss of glycoside molecule and another fragment ion at *m/z* 101.0252.

The compound was previously isolated from the root bark of a Chinese medicinal plant *Dictamnus dasycarpus* Turcz [69]. The compound does not have any previous reports with any direct relation to Alzheimer's disease, but the *Dictamnus* species were found to exhibit neuroprotective activities [70].

The ESI negative mode for peak 7 gave a pseudomolecular ion peak at 987.5164 $[M+HCOO]^-$ and a molecular formula $C_{48}H_{78}O_{18}$ was generated through MassLynx with a normalised iFit value of 0.050 **(supplementary data 21)**. The compound was identified as a saikosaponin derivative 2''-O-ß-D-glucopyranosylsaikosaponin B2 **(35)** and its structure is shown in Figure 3.26. The fragment ions **(supplementary data 22)** were produced at *m/z* 471.3472 $[M-(2\times162)-146]^-$ with the succesive loss of sugar moities including two glucopyranosyl and one fucopyranosyl unit [59].

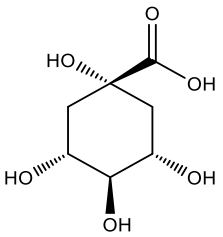
The compound was isolated from *Bupleurum longicaule* and from the roots of *Bupleurum wenchuanense* [59]. There is no previous study reported for this compound related to Alzheimer's disease. However there is some other saikosaponins of similar structure such as saikosaponin C which shown its effect on Alzheimer's disease by targeting amyloid β as well as tau protein [71]. Thus 2''-O-ß-D-glucopyranosylsaikosaponin B2 could be the active ingredient in the present study.

The ESI negative mode for peak 8 gave a pseudomolecular ion peak at 1029.5261$[M+HCOO]^-$ with retention time 10.92. The molecular formula $C_{50}H_{79}O_{19}$ was generated through MassLynx with a normalised iFit value of 0.001 **(supplementary data 23)**. The compound was identified as clinoposaponin C **(36)** and the structure of the compound is shown in Figure 3.26.The fragment ions **(supplementary data 24)** were produced at *m/z* 513.1736 $[M-(2\times162)-146]^-$ with the succesive loss of sugar moities including two glucopyranosyl and one fucopyranosyl. Another fragment ion was observed at *m/z* 471.3485 with the loss of the aglycone moiety.

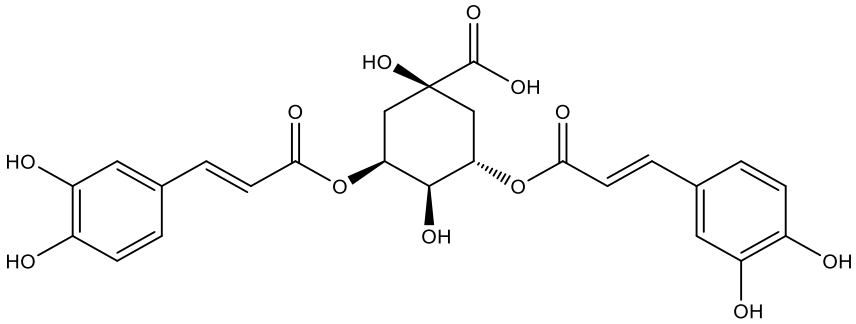
The compound showed moderate cytotoxic activities with IC_{50} values between 4.1 and 19.7 μM when it was tested against Hela, HCT-8, AGS, and MCF-7 human cancer cell lines [60]. The compound has not been reported for the treatment of Alzheimer's disease or other nervous disorders.

The ESI negative mode for peak 4 gave a pseudomolecular ion peak at 1029.5261[M+HCOO]$^-$ with retention time of 11.38 min. Molecular formula $C_{42}H_{66}O_{14}$ was generated through MassLynx with a normalised iFit value of 0.000 **(supplementary data 25)**. The compound was tentatively identified as oleanane type triterpenoid saponin, spinasaponin A **(37)** and its structure is shown in Figure 3.26. The MS/MS spectrum **(supplementary data 26)** yielded a fragment ion at m/z 631.385 [M-H-glucose]$^-$ corresponding to the loss of a glucose unit (162 amu).

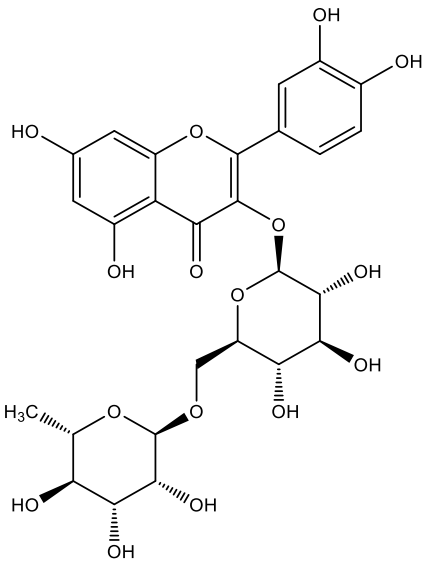
Oleanane type triterpenoid saponins were found to possess antitumour, antiulcer, antivirus, antihyperlipidemic, antiinflamatory and other biological activities due to the presence of sachharide moities [72].



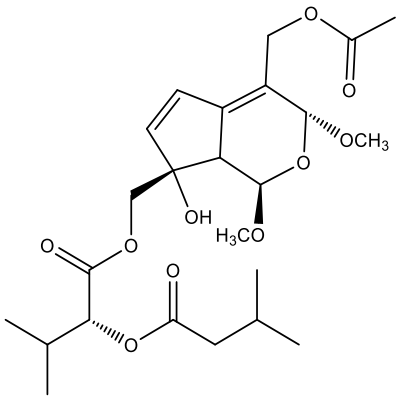
(29)



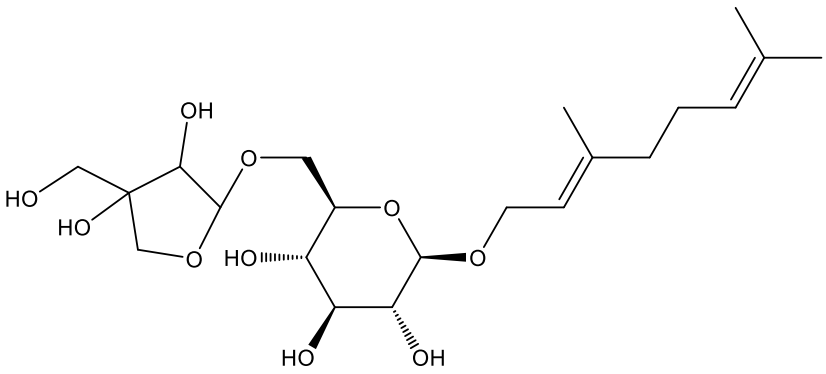
(30)



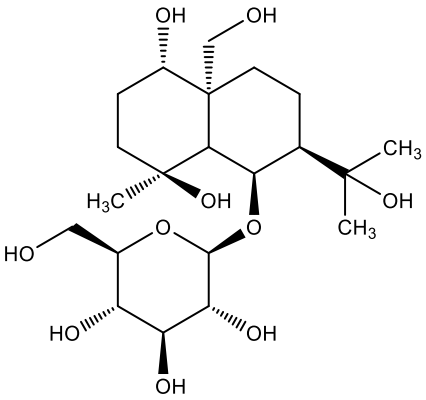
(31)



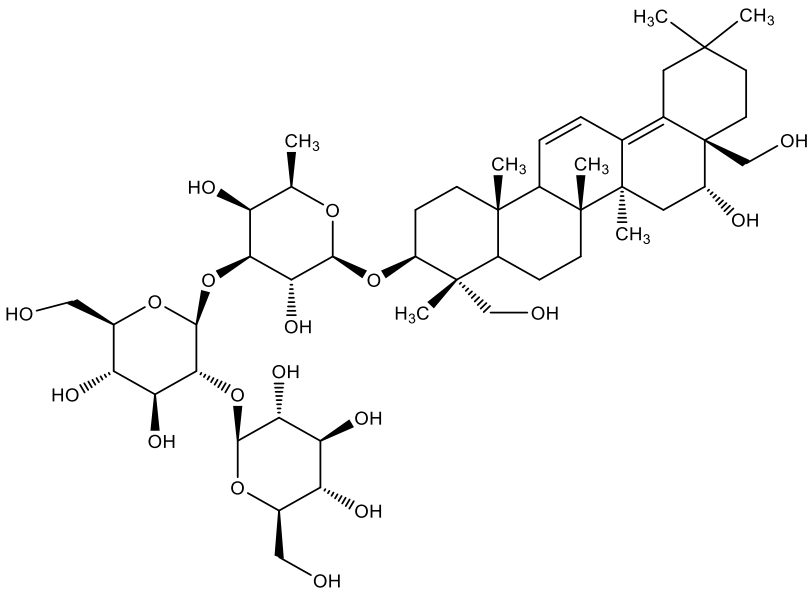
(32)


OH
HO
HO
O
O
O
HO
O
HO
OH

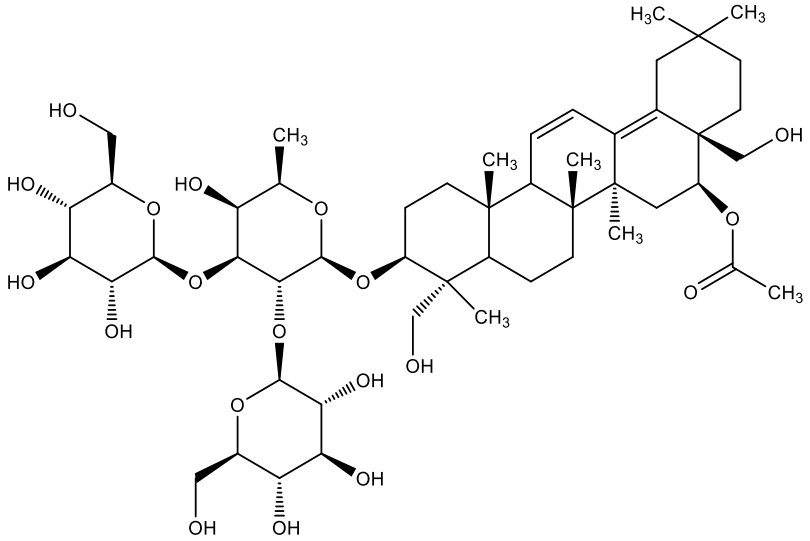
(33)


OH
OH
CH3
H3C
OH
OH
CH3
HO
O
O
HO
OH
OH

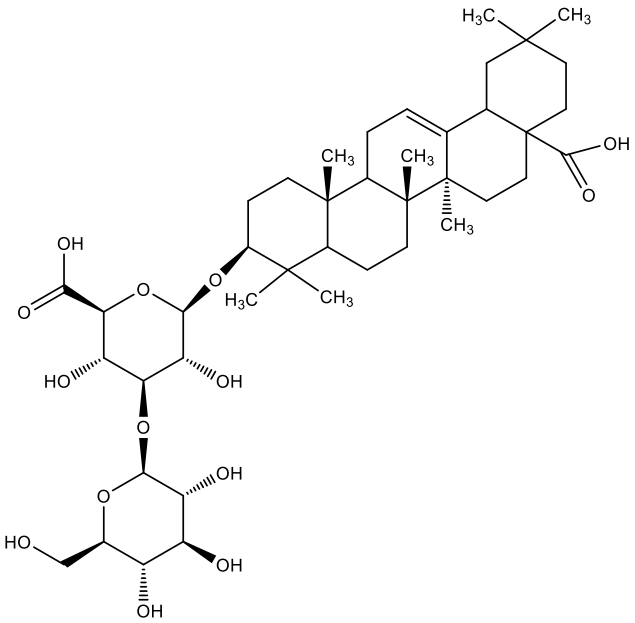
(34)


H3C
CH3
CH3
HO
O
CH3
CH3
OH
OH
CH3
O
O
H3C
OH
OH
HO
O
HO
OH
O
OH
O
OH
OH

(35)



(36)



(37)

Figure 3.25: Chemical structures of the compounds identified from *C. paniculata* leaf extract. Peak 1: quinic acid **(29)**, peak 2: 3,5- dicaffeoylquinic acid **(30)**, peak 3: rutin **(31)**, peak 4: valerianandoid E **(32)**, peak 5: acuminoside **(33)**, peak 6: dictamnoside D **(34)**, peak 7: 2''-O-ß-D glucopyranosylsaikosaponin B2 **(35)**, peak 8: clinosaponin C **(36)**, peak 9: spinasaponin A **(37)**

3.5.5. The effect of *H. arborescens* on Aβ42 production

The stem extract of *H. arborescens* significantly reduced Aβ42 production only at high test concentrations as shown in Figure 3.27. The Aβ42 levels were reduced by 75.78 ± 0.1% at 50 µg/ml and by 31.37 ± 0.2% at 25 µg/ml, while no reduction was seen at 5 and 10 µg/ml.

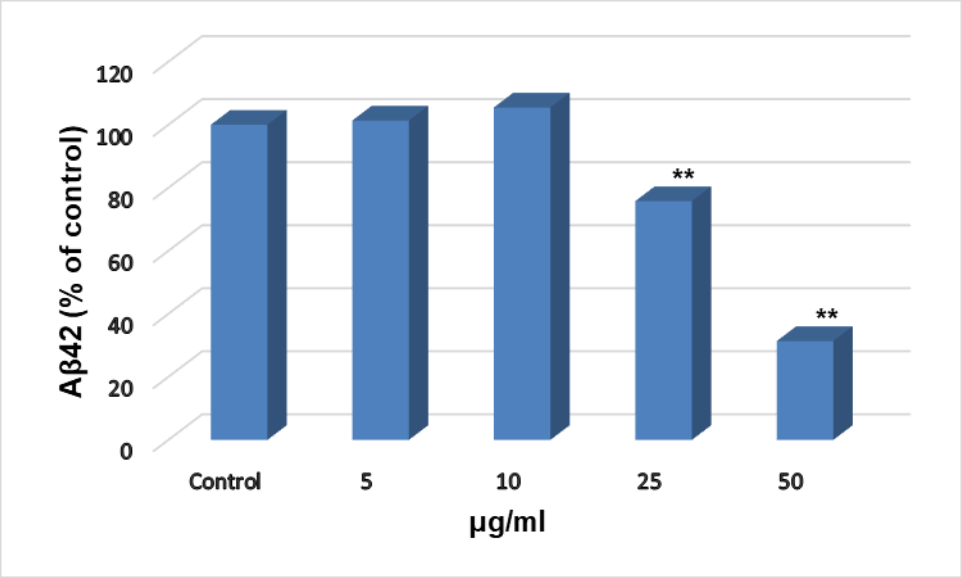


Figure 3.26: Change in the levels of Aβ42**.** Cells were incubated with indicated concentrations of stem extract of *H. arborescens* for 8 h, the levels of Aβ42 were measured from the conditioned media by using specific ELISA methods. The levels of Aβ42 (n = 4) were decreased by the extract on at 25 and 50 µg/ml. **, P<0.01; ***, P<0.001.

H. arborescens has no previous reported scientific studies related to any central nervous sytem disorder. This plant species is for the first time reported in the present study for the reduction of Aβ42 and supported the fact that plant is traditionally used for the nervous disorders. These results indicated that the compounds present in *H. arborescens* could be the active ingredients for the reduction of Aβ42 and can be developed into a potent drug for the treatment of Alzheimer's disease.

3.5.6. Chemical characterization of DCM:MeOH stem extract of *H. arborescens*

The DCM:MeOH stem extract of *H. arborescens* was analysed on UPLC-QTOF-MS to identify the major peaks and establish whether if these could be the active ingredients responsible for the reduction of Aβ42. The ES negative mode was not further analysed because of the poor ionisation. In ESI negative mode, the peaks were mainly distributed within 5.90 to 12.04 min range of the 18 min chromatogram (Figure 3.28).

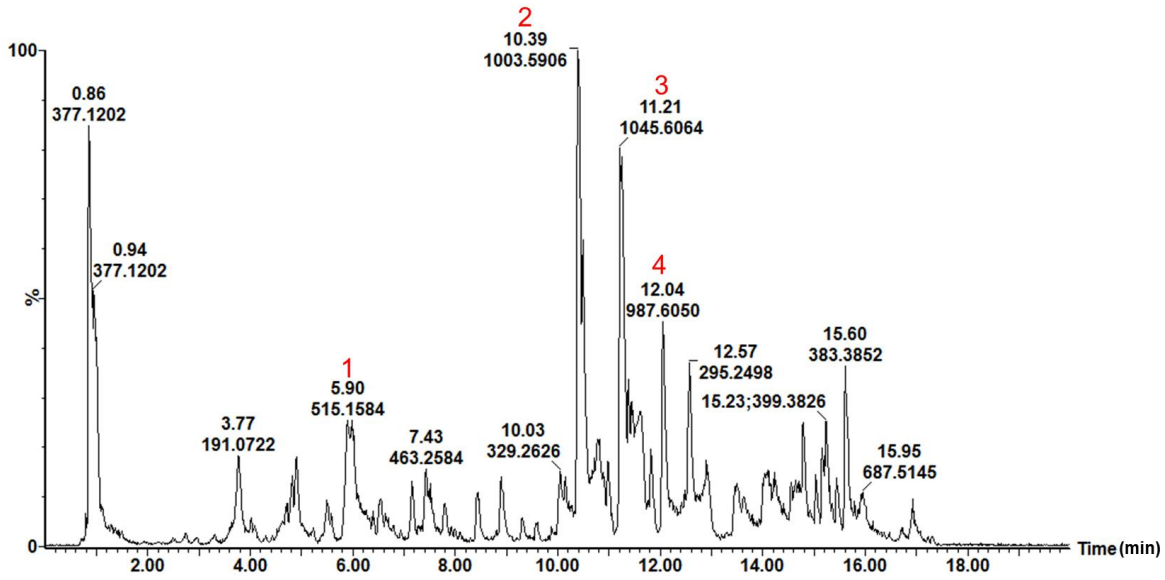


Figure 3.27: ESI negative mode BPI chromatogram of *H. arborescens* stem extract.

This indicated the general elution region for polar to medium polar compounds. Using ESI negative mode, mass fragmentation data and online search databases, a total of four compounds were tentatively identified which included one polyphenol and three triterpenoid saponins (Table 3.4), peak 1 identified as cynarin (1,5-dicaffeoylquinic acid) **(38)** with *m/z* 515.1584 $[M-H]^-$; peak 2 identified as alternoside IX **(39)** with *m/z* 1003.5906 $[M-HCOO]^-$; peak 3 identified as alternoside I **(40)** with *m/z* 1045.6064 $[M-HCOO]^-$ and peak 4 identified as saikogenin B4 **(41)** with *m/z* 987.6050 $[M-HCOO]^-$.

Table 3.4: Tentative identification of compounds obtained from ESI-MS negative mode of DCM:MeOH extract of H. arborescens (stem)

Peak	RT (min)	Acquired $[M-H]^-$ m/z	Formula of Possible structure	Theoretical $[M-H]^-$ m/z	Possible structure	Mass error (ppm)	MS/MS Data (Fragments)	Ref.
1	5.90	515.1192	$C_{25}H_{24}O_{12}$	515.1190	Cynarin	0.4	353.1182, 191.0722, 135.0551, 179.0321	[73]
2	10.39	957.5052	$C_{48}H_{78}O_{19}$	957.5059	alternoside IX	-0.2	795.5234, 633.4459	[74]
3	11.21	999.5168	$C_{50}H_{80}O_{20}$	999.5165	alternoside I	0.3	837.5388, 675.4644	[74]
4	12.04	941.5130	$C_{48}H_{78}O_{18}$	941.5110	saikogenin B_4	2.1	151.0066, 121.0306, 107.0152	[75]

ESI negative mode of peak 1 gave a molecular peak ion at *m/z* 515.1584 $[M-H]^-$ with a retention time 5.90 min. The molecular formula $C_{25}H_{24}O_{12}$ was generated through MassLynx with a normalised iFit value of 0.000 **(supplementary data 27)**. The compound was identified as polyphenol named cynarin (1,5- dicaffeoylquinic acid) **(38)** and the structure of the compound is shown in Figure 3.26.

This compound was also identified through integrated analytical workflow using UPLC-QTOF-MS and UNIFI informatics platform. In UNIFI, the iFit confidence is100% and exact mass error is -0.02 mDa **(supplementary data 28)**. The identified compound met the match criteria with the incorporated traditional medicinal library. The compound is provided with detailed information including compound name with retention time, molecular structure and fragment ion in the component plot **(supplementary data 29)**. The fragmentation patterns obtained

fom Masslynx and UNIFI were compared and found to be similar and both gave same molecular formula.

The fragment were produced at *m/z* 353.1182, *m/z* 191.0722, *m/z* 179.0321 and *m/z* 135.0551 **(supplementary data 30).** The same fragmentation pattern was obtained from a previous study which supported its identification as cynarin **(38)** [73]. 1,5-dicaffeoylquinic acid was found to partially reduce the Aβ cytotoxicity in peptide treated cultures (50 μM) in SH-SY5Y cell model system. It was also normalized Aβ peptide-induced tau gene expressions [76].

The ESI negative mode of peak 2 gave a psedomolecular peak ion at 1003.5906 $[M+HCOO]^-$ with a retention time 10.39 min. MassLynx was used to generate a molecular formula $C_{48}H_{78}O_{19}$ with a normalised iFit value of 0.050 **(supplementary data 31)**. The compound was tentatively identified as oleanene-type of compound called alternoside IX **(39)** and the structure of the compound is shown in Figure 3.29.

The fragment ions **(supplementary data 32)** were produced at *m/z* 795.5234 $[M-162]^-$ and *m/z* 633.4459 $[M-162-162]^-$ with the succesive loss of two sugar moities, glucopyranoside and rhamnopyranoside (-162 amu). There is no scientific study reported for this compound related to Alzheimer's disease and other mental disorders.

The ESI negative mode for peak 3 gave a pseudomolecular peak ion at 1045.6064 $[M+HCOO]^-$ with a retention time 11.21 minutes. The molecular formula $C_{50}H_{80}O_{20}$ was generated through MassLynx with a normalised iFit value of 0.000 **(supplementary data 33)**. The compound was identified as oleanene-type compound named alternoside I **(40)** and its structure is shown in Figure 3.29.

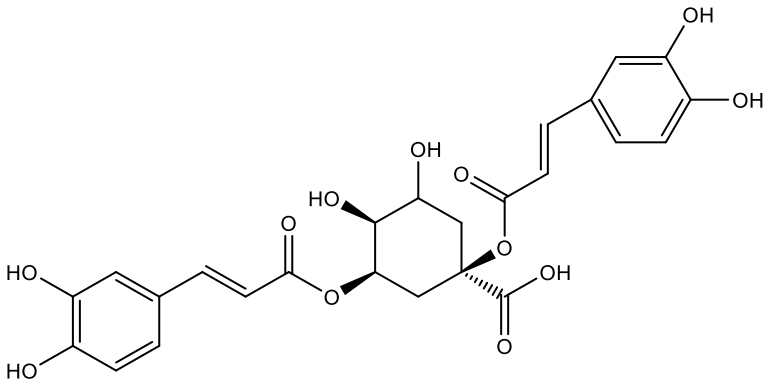
The fragment ions **(supplementary data 34)** were produced at *m/z* 837.5388 $[M-162]^-$ and *m/z* 675.4644 $[M-162-162]^-$ with the succesive loss of two sugar moities, glucopyranoside and rhamnopyranoside. The successive loss of the sugar moities showed the characteristic

fragmentation patterns of saponins. The compound was not reported previously for the reduction of Aβ42 or other bioassays related to Alzheimer's dsease.

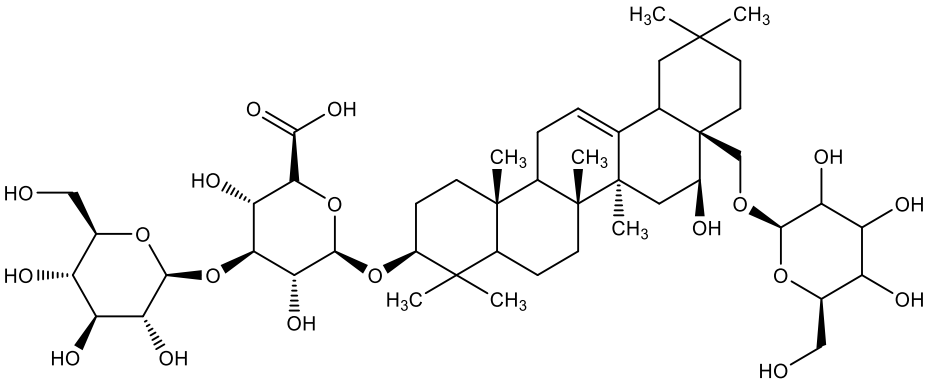
The molecular formula and fragmentation pattern found from the previous studies also supported the identification of both oleanene-type triterpenoids, alternoside IX **(39)** and alternoside I **(40)** [74].

The ESI negative mode for peak 4 gave a pseudomolecular ion peak at 987.6050 $[M+HCOO]^-$ with a retention time 12.04 min. The molecular formula $C_{48}H_{78}O_{18}$ was generated through MassLynx with a normalised iFit value of 0.000 **(supplementary data 35)**. The compound was identified as oleanene-type compound named saikogenin B_4 **(41)**, an oleonolic acid triterpenoid saponin with a structure shown in Figure 3.29.

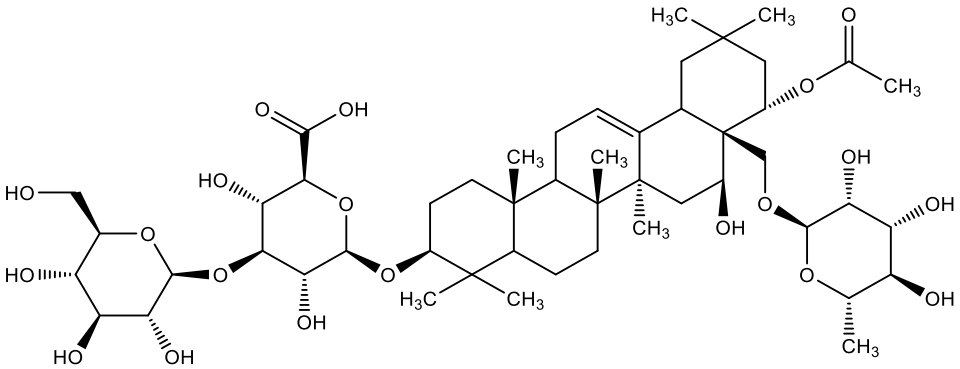
The fragment ions **(supplementary data 36)** were produced at *m/z* 779.4558 $[M-162]^-$ and *m/z* 617.4025 $[M-162-162]^-$ with the succesive loss of two hexose moities. The compound was previously isolated from the root extract of *Bupleurum chinese*, the plant which was reported for its potential as a mild sedative, antidepressant and anti-inflammatory. Furthermore, the oleanane triterpenoids including saikosapogenin B_4 isolated from *Bupleurum chinese* were found to exhibit neuroprotective properties and have the potential to be devloped for the treatment of Parkinson's disease, Alzheimer's disease and depression [77]. Based on this, the compound is highly probable to be an active ingredient for the reduction of Aβ42 in the stem of extract of the plant.



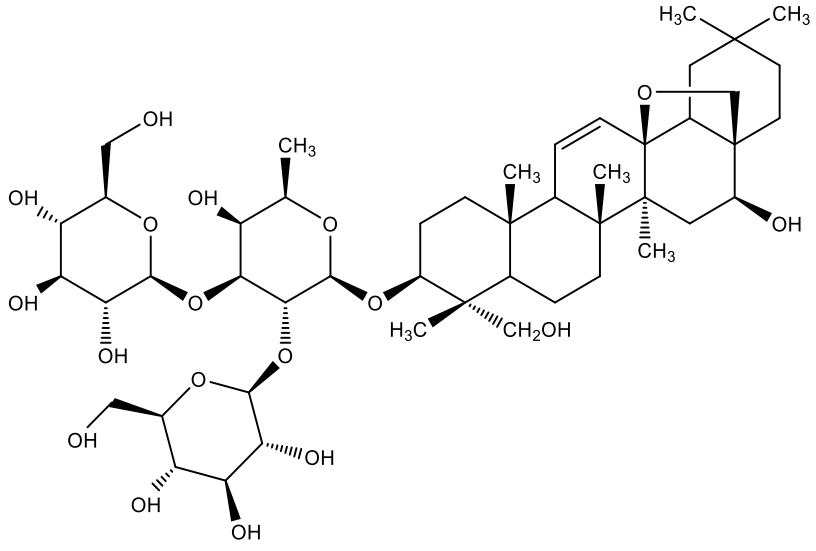
(38)



(39)



(40)



(41)

Figure 3.28: Chemical structures of the compounds identified from H. arborescens stem extract. Peak 1: cynarin **(38)** Peak 2: alternoside IX **(39)**, peak 3: alternoside I **(40)**, saikogenin B_4 **(41)**

The main class of compounds identified in *H. arborescens* stem extract using UPLC MS QTOF was saponins while a limited number of compounds have been identified in *C. paniculata* leaf extract. In previous studies it was found that most saponins contained an aglycone with molecuar mass 448, 456, 458, 472 or 474 Da with the presence of two to five sugar moieties [78]. The ESI MS/MS negative mode of these saponins showed similar of fragment pattern with the successive loss of several sugar moities such as hexose, pentose or deoxyhexose with m/z 162, 132 or 146 respectively [79]. This characteristic fragmentation pattern of triterpenoid saponins obtained from previous studies supported the tentative identification of saponins from leaf extract of *C. paniculata* and stem extract of *H. arborescens.*

Saponins were also found to exhibit neuroprotective activities in various bioassays. In an *in vitro* study using the Thioflavin T method, three acylated oleanane-type triterpene oligoglycosides, floraassamsaponins III, IV, and VII were found to significantly inhibit the Aβ42 aggregation with an inhibition percentage 73.4 ± 8.0 %, $p < 0.01$; 68.8 ± 8.4 %, $p < 0.01$; and 56.9 ± 13.2 %, $p < 0.01$ at 100 µM, respectively as compared to a positive control, morin which had an inhibition prcentage 54.0 ± 11.2 %, $p < 0.01$ at 100 µM [80].

Some other saponins including panax notoginseng saponins isolated from *Panax notoginseng*, ginsenosides from *Panax ginseng C. A. Mey.* and gypenosides isolated from *Gynostemma pentaphyllum* were found to exhibit potential anti-Alzheimer's activity [81].

These previously reported bioassays support the fact that the saponins identified from *C. paniculata* leaf extract and *H. arborescens* stem extract al in all likelihood the active compounds responsible for the reduction of Aβ42 and could lead to a the development of new anti-Alzheimer's therapeutics.

3.6. Conclusion

The three plants, *S. brachypetala* leaf extract, *C. paniculata* leaf extract and *H. arborescens* stem extract were found to significantly reduce the levels of Aβ42 either at higher

concentrations or in a dose dependant manner. This provided some evidence which substantiates the traditional use of the plant species for treating Alzheimer's disease and therefore warranted their further research. The plant extracts were further analysed on UPLC-QTOF-MS to idenfiy the major compounds which could be responsible for the decreased levels of Aβ42. ESI negative mode was used to identify the active ingredients as the ionization was poor for the positive mode for further analysis.

S. bracypetala leaf extract showed significant reduction in Aβ42 production only at higher concentrations i.e. 29.69 ± 4% at 50 µg/ml and by 62.17 ± 7% at 100 µg/ml.

Using UPLC-QTOF-MS, a total of five compounds were tentatively identified including four flavonoids, myricetin-3-*O*-alpha-L-rhamnopyranoside **(25)**, isoquercetin **(26)**, quercetin-3-*O*-rhamnoside **(27)**, quercetin **(28)**. Isoquercetin was confirmed by using a pure standard on UPLC-QTOF-MS. As these compounds were previously studied for their potential to treating Alzheimer's disease through Aβ reduction in different bioassays, in all likelihood the compounds would be be responsible for the Aβ42 reduction in *S. bracypetala*.

The extract of leaves of *C. paniculata* significantly reduced the Aβ42 levels in a dose dependent manner by 29.03 ± 1.4%, 42.40 ± 1.7%, 47.50 ± 5.8% and 61.14 ± 9.3% at concentrations 10, 25, 50 and 100 µg/ml, respectively. This is the first report for the reduction of Aβ42 and thus provides scientific evidence which substantiates its traditional use related to treatment of nervous disorder treatments.

Using ESI negative mode of UPLC-QTOF-MS, a total of nine compounds were tentatively identified as quinic acid **(29)**, 3,5-dicaffeoylquinic acid **(30)**, rutin **(31)**, valerianandoid E **(32)**, acuminoside **(33)**, dictamnoside D **(34)**, 2''-O-β-D-glucopyranosylsaikosaponin B2 **(35)**, clinoposaponin C **(36)**, spinasaponin C **(37)**. Rutin was confimed by using pure standard on UPLC-QTOF-MS. From these identified compounds, rutin was the most researched compound for Alzheimer's disease in previous studies. 3,5-dicaffeoylquinic acid was previously reported for AChE inhibition. The remaining identified compouds from this extract were not reported to be studied for treating Alzheimer's disease either through Aβ42 reduction

or by AChE inhibition. In the present study, rutin or 3,5-dicaffeoylquinic acid could be the active ingredients in *C. paniculata* leaf extract.

The stem extract of *H. arborescens* significantly reduced Aβ42 levels only at high test concentrations by 75.78 ± 0.1% at 50 µg/ml and by 31.37 ± 0.2% at 25 µg/ml.

Using ESI negative mode, a total of four compounds were tentatively identified which included one polyphenol, cynarin (1,5-dicaffeoylquinic acid) **(38)** and three triterpenoid saponin alternoside IX **(39)**, alternoside I **(40)** and saikogenin B_4 **(41)**. Cynarin was previously reported for the reduction of Aβ cytotoxicity while the other identified compounds, triterpenoid saponins were not examined for Alzheimer's disease. Thus in this study there is a possiblity that cynarin could be the active ingredient from *H. arborescens* leaf extract for the reduction of Aβ42.

From the present study it can be concluded that UPLC-QTOF-MS is an effective and quick technique to identify major compounds in complex plant extracts and based on published data of such compounds, the active ingredients can be established. The main class of compound identified from these plants were flavonoids, quinic acid derivatives and saponins. All these classes of compounds were previously scientifically tested for their neuroprotective effects in various bioassays and animal models and gave many positive results thereby no further development of the complex plant extracts was undertaken.

References

1. ***Schotia brachypetala* Sond.** [http://pza.sanbi.org/schotia-brachypetala]
2. Van Wyk B-E, Oudtshoorn Bv, Gericke N: **Medicinal Plants of South Africa**: Briza; 1997.
3. Symes C, Yoganand K: ***Schotia brachypetala*–a nectar cornucopia for birds**. *Bulletin of the American Bird Club* 2013, **20**:39-44.
4. ***Schotia brachypetala*** [http://pza.sanbi.org/schotia-brachypetala]
5. McGaw L, Jäger A, Van Staden J, Eloff J: **Variation in antibacterial activity of Schotia species**. *South African Journal of Botany* 2002, **68**(1):41-46.
6. Adewusi EA, Moodley N, Steenkamp V: **Antioxidant and acetylcholinesterase inhibitory activity of selected southern African medicinal plants**. *South African Journal of Botany* 2011, **77**(3):638-644.

7. Ndlovu G, Fouche G, Tselanyane M, Cordier W, Steenkamp V: **In vitro determination of the anti-aging potential of four southern African medicinal plants**. *BMC Complementary and Alternative Medicine* 2013, **13**(1):304.
8. Du K, Marston A, van Vuuren SF, van Zyl RL, Coleman C, Zietsman PC, Bonnet SL, Ferreira D, van der Westhuizen JH: **Flavonolacyl glucosides from the aril of *Schotia brachypetala* Sond. and their antioxidant, antibacterial and antimalarial activities**. *Phytochemistry Letters* 2014, **10**:cxxiii-cxxviii.
9. Drewes S: **Isolation of 3, 3′, 4′, 5, 5′-pentahydroxystilbene from Schotia species**. *Phytochemistry* 1971, **10**(11):2837-2838.
10. Morales M, Ros Barcelo A, Pedreno M: **Plant stilbenes: recent advances in their chemistry and biology**. *Advances in Plant Physiology* 2000, **3**:39-70.
11. Giacomini E, Rupiani S, Guidotti L, Recanatini M, Roberti M: **The use of stilbene scaffold in medicinal chemistry and multi-target drug design**. *Current Medicinal Chemistry* 2016, **23**(23):2439-2489.
12. Du K: **Isolation of bioactive constituents from seeds of *Schotia brachypetala* (Fabaceae) and *Colophospermum mopane* (Fabaceae)**. University of the Free State; 2011.
13. Sobeh M, ElHawary E, Peixoto H, Labib RM, Handoussa H, Swilam N, El-Khatib AH, Sharapov F, Mohamed T, Krstin S: **Identification of phenolic secondary metabolites from *Schotia brachypetala* Sond.(Fabaceae) and demonstration of their antioxidant activities in *Caenorhabditis elegans***. *PeerJ* 2016, **4**:e2404.
14. Hassaan Y, Handoussa H, El-Khatib AH, Linscheid MW, El Sayed N, Ayoub N: **Evaluation of plant phenolic metabolites as a source of Alzheimer's drug leads**. *BioMed Research International* 2014, **2014**.
15. Eldeen I, Elgorashi E, Van Staden J: **Antibacterial, anti-inflammatory, anti-cholinesterase and mutagenic effects of extracts obtained from some trees used in South African traditional medicine**. *Journal of Ethnopharmacology* 2005, **102**(3):457-464.
16. Stafford GI, Pedersen PD, Jäger AK, Van Staden J: **Monoamine oxidase inhibition by southern African traditional medicinal plants**. *South African Journal of Botany* 2007, **73**(3):384-390.
17. Tetyana P: **Micropropagation of *Cussonia paniculata*—a medicinal plant with horticultural potential**. *South African Journal of Botany* 2001, **67**(2):367-370.
18. Van Wyk B-E, Gericke N: **People's plants: A guide to useful plants of Southern Africa**: Briza Publications; 2000.
19. ***Cussonia paniculata* Eckl. & Zeyh** [PlantZAfrica.com]
20. De Villiers B, Van Vuuren S, Van Zyl R, Van Wyk B-E: **Antimicrobial and antimalarial activity of Cussonia species (Araliaceae)**. *Journal of Ethnopharmacology* 2010, **129**(2):189-196.
21. Dovgii I, Grishkovets V, Kachala V, Shashkov A: **Triterpene glycosides from *Cussonia paniculata*. I. Isolation and structure determination of glycosides A, B 1, B 2, C, D, G 2, H 1, and H 2 from Leaves of *Cussonia paniculata***. *Chemistry of Natural Compounds* 2005, **41**(2):200-204.
22. Adedapo AA, Sofidiya MO, Maphosa V, Moyo B, Masika PJ, Afolayan AJ: **Anti-inflammatory and analgesic activities of the aqueous extract of *Cussonia paniculata* stem Bark**. *Records of Natural Products* 2008, **2**(2):46.
23. Fouché G, Cragg G, Pillay P, Kolesnikova N, Maharaj V, Senabe J: **In vitro anticancer screening of South African plants**. *Journal of Ethnopharmacology* 2008, **119**(3):455-461.
24. Tetyana P, Prozesky E, Jäger A, Meyer J, Van Staden J, van Wyk B-E: **Some medicinal properties of *Cussonia* and *Schefflera* species used in traditional medicine**. *South African Journal of Botany* 2002, **68**(1):51-54.
25. Nkomo M, Nkeh-Chungag BN, Kambizi L, Ndebia EJ, Sewani-Rusike C, Iputo JE: **Investigation of the antinociceptive and anti-inflammatory properties of**

***Heteromorpha arborescens* (Apiaceae)**. *African Journal of Traditional, Complementary and Alternative Medicines* 2011, **8**(4).
26. Sunil C, Kumar V, Van Staden J: ***In vitro* alpha-glucosidase inhibitory, total phenolic composition, antiradical and antioxidant potential of *Heteromorpha arborescens* (Spreng.) Cham. & Schltdl. leaf and bark extracts**. *South African Journal of Botany* 2019, **124**:380-386.
27. Watt JM, Breyer-Brandwijk MG: **The Medicinal and Poisonous Plants of Southern and Eastern Africa being an account of their medicinal and other uses, chemical composition, Pharmacological effects and toxicology in man and animal**. *The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses, Chemical Composition, Pharmacological Effects and Toxicology in Man and Animal* 1962(Edn 2).
28. Van Wyk B-E: **The potential of South African plants in the development of new medicinal products**. *South African Journal of Botany* 2011, **77**(4):812-829.
29. Lundgaard NH, Prior RM, Light ME, Stafford GI, Van Staden J, Jäger AK: **COX-1 inhibition of Heteromorpha arborescens**. *South African Journal of Botany* 2008, **74**(2):335-337.
30. Recio MdC, Just MJ, Giner RM, Mañez S, Rios JL, Hostettmann K: **Anti-inflammatory activity of saikosaponins from *Heteromorpha trifoliata***. *Journal of Natural Products* 1995, **58**(1):140-144.
31. Adamu M, Bagla V, Eloff J: **Fractionation of *Heteromorpha arborescens* var abyssinica (Apiaceae) leaf extracts based on polarity leads to a marked change in cytotoxicity that may yield a commercially useful product**. *South African Journal of Botany* 2016, **103**:36-40.
32. Nkomo M, Kambizi L: **Antimicrobial activity of *Gunnera perpensa* and *Heteromorpha arborescens* var. abyssinica**. *Journal of Medicinal Plants Research* 2009, **3**(12):1051-1055.
33. Elisha IL, Dzoyem J-P, McGaw LJ, Botha FS, Eloff JN: **The anti-arthritic, anti-inflammatory, antioxidant activity and relationships with total phenolics and total flavonoids of nine South African plants used traditionally to treat arthritis**. *BMC Complementary and Alternative Medicine* 2016, **16**(1):307.
34. Recio M, Giner R, Manez S, Rios J, Marston A, Hostettmann K: **Screening of tropical medicinal plants for antiinflammatory activity**. *Phytotherapy Research* 1995, **9**(8):571-574.
35. Beuscher N, Bodinet C, Neumann-Haefelin D, Marston A, Hostettmann K: **Antiviral activity of African medicinal plants**. *Journal of Ethnopharmacology* 1994, **42**(2):101-109.
36. Louvel S, Moodley N, Seibert I, Steenkamp P, Nthambeleni R, Vidal V, Maharaj V, Klimkait T: **Identification of compounds from the plant species *Alepidea amatymbica* active against HIV**. *South African Journal of Botany* 2013, **86**:9-14.
37. Deng L, Shi AM, Liu HZ, Meruva N, Liu L, Hu H, Yang Y, Huang C, Li P, Wang Q: **Identification of chemical ingredients of peanut stems and leaves extracts using UPLC-QTOF-MS coupled with novel informatics UNIFI platform**. *Journal of Mass Spectrometry* 2016, **51**(12):1157-1167.
38. Wang, Qu D, Wang K: **Therapeutic approaches to Alzheimer's disease through stimulating of non-amyloidogenic processing of amyloid precursor protein**. *Eur Rev Med Pharmacol Sci* 2016, **20**(11):2389-2403.
39. Saldanha LL, Vilegas W, Dokkedal AL: **Characterization of flavonoids and phenolic acids in *Myrcia bella cambess*. Using FIA-ESI-IT-MSn and HPLC-PAD-ESI-IT-MS combined with NMR**. *Molecules* 2013, **18**(7):8402-8416.
40. Sánchez-Rabaneda F, Jáuregui O, Casals I, Andrés-Lacueva C, Izquierdo-Pulido M, Lamuela-Raventós RM: **Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (Theobroma cacao)**. *Journal of Mass Spectrometry* 2003, **38**(1):35-42.

41. Zhou C, Liu Y, Su D, Gao G, Zhou X, Sun L, Ba X, Chen X, Bi K: **A sensitive LC–MS–MS method for simultaneous quantification of two structural isomers, hyperoside and isoquercitrin: application to pharmacokinetic studies**. *Chromatographia* 2011, **73**(3-4):353-359.
42. Soong YY, Barlow PJ: **Isolation and structure elucidation of phenolic compounds from longan (*Dimocarpus longan* Lour.) seed by high-performance liquid chromatography–electrospray ionization mass spectrometry**. *Journal of Chromatography A* 2005, **1085**(2):270-277.
43. Chen M, Dai LH, Fei A, Pan SM, Wang HR: **Isoquercetin activates the ERK1/2-Nrf2 pathway and protects against cerebral ischemia-reperfusion injury in vivo and in vitro**. *Experimental and Therapeutic Medicine* 2017, **13**(4):1353-1359.
44. Lee KY, Hwang L, Jeong EJ, Kim SH, Kim YC, Sung SH: **Effect of neuroprotective flavonoids of *Agrimonia eupatoria* on glutamate-induced oxidative injury to HT22 hippocampal cells**. *Bioscience, Biotechnology, and Biochemistry* 2010, **74**(8):1704-1706.
45. Magalingam KB, Radhakrishnan A, Haleagrahara N: **Protective effects of flavonol isoquercitrin, against 6-hydroxy dopamine (6-OHDA)-induced toxicity in PC12 cells**. *BMC Research Notes* 2014, **7**(1):49.
46. Semwal DK, Semwal RB, Combrinck S, Viljoen A: **Myricetin: A dietary molecule with diverse biological activities**. *Nutrients* 2016, **8**(2):90.
47. Choi Y-J, Kim TD, Paik SR, Jeong K-J, Jung S-H: **Molecular simulations for anti-amyloidogenic effect of flavonoid myricetin exerted against Alzheimer's β-amyloid fibrils formation**. *Bulletin of the Korean Chemical Society* 2008, **29**(8):1505-1509.
48. Orhan IE, Kucukboyaci N, Calis I, Cerón-Carrasco JP, den-Haan H, Peña-García J, Pérez-Sánchez H: **Acetylcholinesterase inhibitory assessment of isolated constituents from Salsola grandis Freitag, Vural & Adıgüzel and molecular modeling studies on N-acetyltryptophan**. *Phytochemistry Letters* 2017, **20**:373-378.
49. Alrawaiq NS, Abdullah A: **A review of flavonoid quercetin: metabolism, bioactivity and antioxidant properties**. *International Journal of PharmTech Research* 2014, **6**(3):933-941.
50. Sabogal-Guáqueta AM, Munoz-Manco JI, Ramírez-Pineda JR, Lamprea-Rodriguez M, Osorio E, Cardona-Gómez GP: **The flavonoid quercetin ameliorates Alzheimer's disease pathology and protects cognitive and emotional function in aged triple transgenic Alzheimer's disease model mice**. *Neuropharmacology* 2015, **93**:134-145.
51. Ansari MA, Abdul HM, Joshi G, Opii WO, Butterfield DA: **Protective effect of quercetin in primary neurons against Aβ (1–42): relevance to Alzheimer's disease**. *The Journal of Nutritional Biochemistry* 2009, **20**(4):269-275.
52. Kanter M, Unsal C, Aktas C, Erboga M: **Neuroprotective effect of quercetin against oxidative damage and neuronal apoptosis caused by cadmium in hippocampus**. *Toxicology and Industrial Health* 2016, **32**(3):541-550.
53. Dajas F, Abin-Carriquiry JA, Arredondo F, Blasina F, Echeverry C, Martínez M, Rivera F, Vaamonde L: **Quercetin in brain diseases: Potential and limits**. *Neurochemistry International* 2015, **89**:140-148.
54. Guzzi C, Colombo L, Luigi AD, Salmona M, Nicotra F, Airoldi C: **Flavonoids and Their Glycosides as Anti-amyloidogenic Compounds: Aβ1–42 Interaction Studies to Gain New Insights into their Potential for Alzheimer's Disease Prevention and Therapy**. *Chemistry–An Asian Journal* 2017, **12**(1):67-75.
55. Onozuka H, Nakajima A, Matsuzaki K, Shin R-W, Ogino K, Saigusa D, Tetsu N, Yokosuka A, Sashida Y, Mimaki Y: **Nobiletin, a citrus flavonoid, improves memory impairment and Aβ pathology in a transgenic mouse model of Alzheimer's disease**. *Journal of Pharmacology and Experimental Therapeutics* 2008, **326**(3):739-744.

56. Rezai-Zadeh K, Douglas Shytle R, Bai Y, Tian J, Hou H, Mori T, Zeng J, Obregon D, Town T, Tan J: **Flavonoid-mediated presenilin-1 phosphorylation reduces Alzheimer's disease β-amyloid production**. *Journal of Cellular and Molecular Medicine* 2009, **13**(3):574-588.
57. Clifford MN, Knight S, Kuhnert N: **Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS n**. *Journal of Agricultural and Food Chemistry* 2005, **53**(10):3821-3832.
58. Sousa EAd, Da Silva AA, Cavalheiro AJ, Lago JHG, Chaves MH: **A new flavonoid derivative from leaves of *Oxandra Sessiliflora* RE Fries**. *Journal of the Brazilian Chemical Society* 2014, **25**(4):704-708.
59. Si-Qi L, Long-Ze L, Cordell GA: **Saikosaponin derivatives from *Bupleurum wenchuanense***. *Phytochemistry* 1993, **33**(5):1197-1205.
60. Wang S, Ma G, Zhong M, Yu S, Xu X, Hu Y, Zhang Y, Wei H, Yang J: **Triterpene saponins from *Tabellae Clinopodii***. *Fitoterapia* 2013, **90**:14-19.
61. Yoshikawa M, Harada E, Matsuda H, Murakami T, Yamahara J, Murakami N: **Elatosides A and B, potent inhibitors of ethanol absorption in rats from the bark of *Aralia elata* Seem: the structure-activity relationships of oleanolic acid oligoglycosides**. *Chemical and Pharmaceutical Bulletin* 1993, **41**(11):2069-2071.
62. Minig M, Mazzaferro LS, Erra-Balsells R, Petroselli G, Breccia JD: **α-Rhamnosyl-β-glucosidase-catalyzed reactions for analysis and biotransformations of plant-based foods**. *Journal of Agricultural and Food Chemistry* 2011, **59**(20):11238-11243.
63. Heyman HM, Senejoux F, Seibert I, Klimkait T, Maharaj VJ, Meyer JJM: **Identification of anti-HIV active dicaffeoylquinic-and tricaffeoylquinic acids in *Helichrysum populifolium* by NMR-based metabolomic guided fractionation**. *Fitoterapia* 2015, **103**:155-164.
64. Kang JY, Park SK, Guo TJ, Ha JS, Lee DS, Kim JM, Lee U, Kim DO, Heo HJ: **Reversal of trimethyltin-induced learning and memory deficits by 3, 5-dicaffeoylquinic acid**. *Oxidative Medicine and Cellular Longevity* 2016, **2016**.
65. Hur JY, Soh Y, Kim B-H, Suk K, Sohn NW, Kim HC, Kwon HC, Lee KR, Kim SY: **Neuroprotective and neurotrophic effects of quinic acids from *Aster scaber* in PC12 cells**. *Biological and Pharmaceutical Bulletin* 2001, **24**(8):921-924.
66. Canevelli M, Adali N, Kelaiditi E, Cantet C, Ousset P-J, Cesari M: **Effects of Gingko biloba supplementation in Alzheimer's disease patients receiving cholinesterase inhibitors: data from the ICTUS study**. *Phytomedicine* 2014, **21**(6):888-892.
67. Xu P-x, Wang S-w, Yu X-l, Su Y-j, Wang T, Zhou W-w, Zhang H, Wang Y-j, Liu R-t: **Rutin improves spatial memory in Alzheimer's disease transgenic mice by reducing Aβ oligomer level and attenuating oxidative stress and neuroinflammation**. *Behavioural Brain Research* 2014, **264**:173-180.
68. Jugran AK, Rawat S, Bhatt ID, Rawal RS: ***Valeriana jatamansi*: An herbaceous plant with multiple medicinal uses**. *Phytotherapy Research* 2019, **33**(3):482-503.
69. Yang S, Li Z, Wang J, Ruan J, Zheng C, Huang P, Han L, Zhang Y, Wang T: **Eudesmane-Type Sesquiterpene Glycosides from *Dictamnus dasycarpus* Turcz**. *Molecules* 2018, **23**(3):642.
70. Lv M, Xu P, Tian Y, Liang J, Gao Y, Xu F, Zhang Z, Sun J: **Medicinal uses, phytochemistry and pharmacology of the genus Dictamnus (Rutaceae)**. *Journal of Ethnopharmacology* 2015, **171**:247-263.
71. Kim BM: **The role of saikosaponins in therapeutic strategies for age-related diseases**. *Oxidative Medicine and Cellular Longevity* 2018, **2018**.
72. Sha Y, Yan M-C, Liu J, Liu Y, Cheng M-S: **Facile synthesis of oleanolic acid monoglycosides and diglycosides**. *Molecules* 2008, **13**(7):1472-1486.
73. Ferracane R, Graziani G, Gallo M, Fogliano V, Ritieni A: **Metabolic profile of the bioactive compounds of burdock (*Arctium lappa*) seeds, roots and leaves**. *Journal of Pharmaceutical and Biomedical Analysis* 2010, **51**(2):399-404.

74. Yoshikawa K, Ogata H, Arihara S, CHANG H-C, Wang J-D: **Antisweet natural products. XIII. Structures of alternosides IX from *Gymnema alternifolium***. *Chemical and Pharmaceutical Bulletin* 1998, **46**(7):1102-1107.
75. Chemli R, Babadjamian A, Faure R, Boukef K, Balansard G, Vidal E: **Arvensoside A and B, triterpenoid saponins from *Calendula arvensis***. *Phytochemistry* 1987, **26**(6):1785-1788.
76. Gray NE, Morré J, Kelley J, Maier CS, Stevens JF, Quinn JF, Soumyanath A: **Caffeoylquinic acids in *Centella asiatica* protect against amyloid-β toxicity**. *Journal of Alzheimer's Disease* 2014, **40**(2):359-373.
77. Li D-Q, Zhou L, Wang D, Wu J, Li L-Z, Huang X-X, Liu Q-B, Wu Y-Y, Lin S, Yang J-Y: **Neuroprotective oleanane triterpenes from the roots of *Bupleurum chinense***. *Bioorganic & Medicinal Chemistry Letters* 2016, **26**(6):1594-1598.
78. Khakimov B, Tseng L, Godejohann M, Bak S, Engelsen S: **Screening for triterpenoid saponins in plants using hyphenated analytical platforms**. *Molecules* 2016, **21**(12):1614.
79. Chen Q, He L, Mo C, Zhang Z, Long H, Gu X, Wei Y: **Rapid evaluation of chemical consistency of artificially induced and natural Resina Draconis using Ultra-Performance Liquid Chromatography Quadrupole-Time-of-Flight Mass Spectrometry-based chemical profiling**. *Molecules* 2018, **23**(8):1850.
80. Matsuda H, Nakamura S, Morikawa T, Muraoka O, Yoshikawa M: **New biofunctional effects of the flower buds of *Camellia sinensis* and its bioactive acylated oleanane-type triterpene oligoglycosides**. *Journal of Natural Medicines* 2016, **70**(4):689-701.
81. Gao J, Inagaki Y, Li X, Kokudo N, Tang W: **Research progress on natural products from traditional Chinese medicine in treatment of Alzheimer's disease**. *Drug Discoveries & Therapeutics* 2013, **7**(2):46-57.

Chapter 4
Isolation and characterization of compounds from *Xysmalobium undulatum* targeting Aβ42 reduction for the treatment of Alzheimer's disease

4.1. Introduction

4.1.1. Botany and geographical distribution of *Xysmalobium undulatum*

Xysmalobium undulatum (Apocynaceae) is a robust annual herb, 0.5 - 2.0 m tall. It sprouts annually in spring and dies in winter. The leaves are large (80 - 270 × 10 - 75 cm), hairy, heart shaped and almost stalkless; the branches are thick, erect and hairy and the roots are fleshy, it has a white inside and latex present in all parts. Many flowered, stalked inflorescences develop in the axils of the leaves. The flowers are creamish green to yellow and produced mainly in December and the fruits are usually large, inflated ovoid follicle, pale green, short hairy and many are seeded [1], [2].

The common names of *X. undulatum* are milkbush, milkwort, Uzara, wild cotton and wave-leaved *Xysmalobium* while in Afrikaans it is known as bitterhout [2]. These plants grow mainly in grasslands and moist places. The genus *Xysmalobium* comprises of about 40 species in Africa and of the 40 species, about 24 species are found in southern Africa. The plant is extensively harvested for its medicinal use in South Africa.

The plant is also widely distributed in Kenya, Tanzania, Malawi, Zambia, Angola, Namibia, Botswana, Zimbabwe, Mozambique, Lesotho, Swaziland and found in all the provinces of South Africa [3].

Figure 4.1: Picture showing *X. undulatum* plant (image taken by Anuradha Thakur at the time of collection).

4.1.2. Traditional medicinal uses

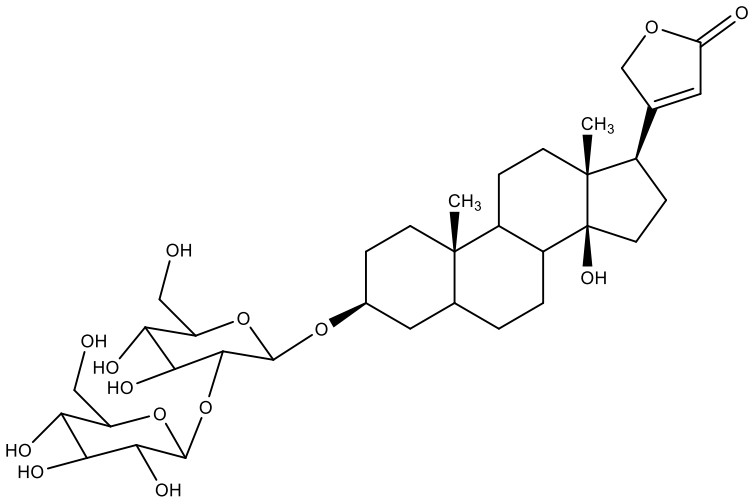
Traditionally the plant is used in Botswana for headache and abdominal pains [4] while it has also been recorded for the treatment of diarrhoea, dysentery, dysmenorrhoea, stomach cramps, wounds and indigestion [5]. The root extracts are used to treat acute diarrhoea and menstrual cramps since early 1900s in Europe and have been marketed as ‘uzarae radix’ [2]. The Xhosa people sometimes mixed *X. undulatum* with *Pachycarpus shinzianus* (Schltr.) N.E.Br. to treat intestinal problems as they have the same medicinal properties. The Tswana people chew a piece of the root as an antidote to food poisoning, the Mpondo people use powdered root or root decoction to treat dysentry and the Nama people ingest root as stomach carminative and for the treatment of diarrhoea and lumpy skin disease of cattle [2]. In Kenya a root decoction is taken to treat headache, in Zambia it is taken for the treatment of malaria,

typhoid and other fevers and in Zimbabwe the pulverized root is eaten with porridge as an aphrodisiac. The latex and plant decoction is used as a remedy for wounds, sores and snakebites. The Sotho people in South Africa eat the young leaves mixed in porridge [3]. The tuber infusions of this plant are also used for the treatment of diarrhoea and headache [6]. It is also used for the treatment of skin diseases, infected sores, wounds and urinary tract infections [7].

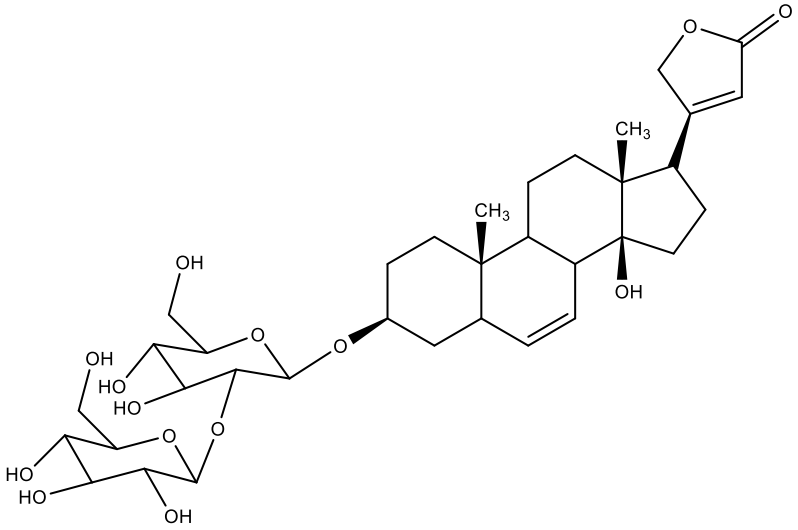
4.1.3. Phytochemistry

The phytochemical investigations showed that the main class of compounds in *X. undulatum* is cardenolide glycosides. The compounds isolated from *X. undulatum* root are uzarin, xysmalorin, uzarigenin, xysmalogenin, allouzarin, alloxysmalorin, allouzarigenin, alloxysmalogenin, ascleposide, corogluaucigenin, corogluaucigenin-3-*O*-glucoside, desglucouzarin, smalogenin, desglucoxysmalorin, uzaroside, pregnenolone and β-sitosterol [2].

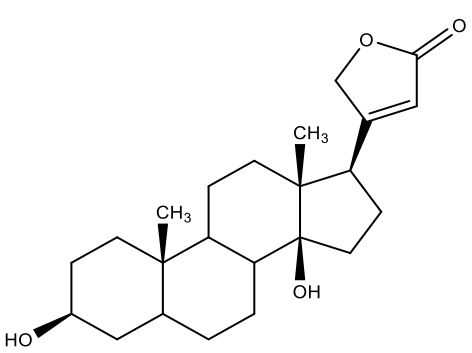
Four major cardenolide gycosides were isolated named uzarin, allouzarin, xysmalorin and alloxysmalorin from the leaf extract [8] and the structures of these compounds are shown in Figure 4.2. A biglucosyl moiety was also identified through the presence of anomeric protons in the ^{1}H NMR while their β configuration was confirmed by the large coupling constants of the two anomeric protons (7.7Hz).The COSY spectrum in DMSO-d_6 established 1→2, glycosidic linkage, i.e. glucopyranosyl-(1→2)-glucopyranose.


O
O
CH3
CH3
OH
OH
O
O
OH
HO
HO
O
O
HO
HO
OH

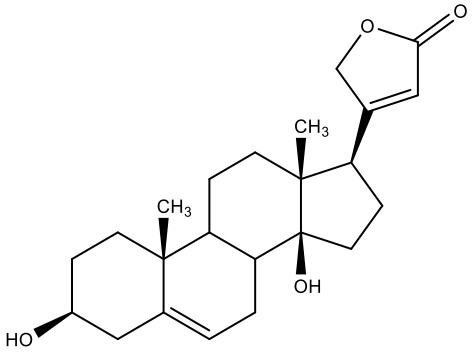
(42)


O
O
CH3
CH3
OH
OH
O
O
OH
HO
HO
O
O
HO
HO
OH

(43)



(44)



(45)

Figure 4.2: Structure of main compounds isolated from *X.undulatum,* uzarin **(42)**, xysmalorin **(43)**, uzarigenin **(44)** and xysmalogenin **(45)**

4.1.4. Previously reported biological assaying

Previous studies reported that *X. undulatum* possess a strong affinity towards serotonin uptake transport protein which plays an important role in the symptoms of depression and Alzheimer's diseases [9]. Another study has suggested that the ethyl acetate root extract of *X. undulatum* exhibited some acetylcholinesterase inhibitory activity by displaying an IC_{50} value of 0.0005 ± 0.000 mg/ml [10]. Previous findings showed that *X. undulatum* extracts exhibited antidepressant-like *in vitro* and *in vivo* effects [11]. The ethanol root extract of the plant exhibited antiplasmodial activity with an IC_{50} value 6µg/ml. Uzara whole plant extract exhibited antimicrobial activity with a recorded MIC value of 3.125 mg/ml against four bacteria *Bacillus subtilis* (ATCC6051), *Escherichia coli* (ATCC11775), *Klebsiella pneumoniae* (ATCC13883) and *Staphylcoccus aureus* (ATCC12600) using neomycin as a reference standard as well as *Candida albicans* (ATCC10231) with amphotericin B as a reference standard [2]. In an *in vitro* study for acetylcholinesterase (AChE) inhibition performed using Ellman's colourimetric method, the ethyl acetate root extract of *X. undulatum* exhibited the AChE inhibition with an IC_{50} value of 0.5 ng/ml as compared to control galanthamine with an IC_{50} value of 0.053 ng/ml [2]. These values are significantly lower for galanthamine as compared to the other Aβ42 inhibition tests using galanthamine as control. As an example in one of the previous studies,

galanthamine was reported to inhibit amyloid aggregations at all the concentrations tested from 25-1000 µM [12]. In another study it was found that release of Aβ42 was decreased by 37% with 10 µM galanthamine in SH-SY5Y cells [13].

4.1.5. History of commercialisation of *X. undulatum* (Uzara)

Carl Peter Thunberg, described Uzara under the synonymous name *Asclepias undulata* in European scientific literature [14]. The plant was a part of Thunberg herbarium and was said to be used as remedy for the colic [15]. Further Smith described the plant under a synonym name *Xysmalobium lapathifolium* and mentioned the uses of root as tonic and as a remedy for healing the wounds [16].

H.W.A Hopf from Melsungen, was the first person who brought Uzara roots to Europe. While travelling through South Africa, he suffered from severe dysentery and obtained Uzara roots from a traditional healer to treat dysentery. In this way he became familiar with Uzara roots and brought the roots with him when he returned to Melsungen, Germany, where he left the root specimen to the pharmacological institute for further investigations. At the institute, August Gurber (1864-1937) isolated three unidentified compounds from the roots during his investigation. Following further research Uzara was commercialized and introduced into German pharmaceutical market in 1909. It is marketed as a drug under the name “Uzara” for the treatment of acute diarrhoea [17].

4.1.6. Pharmacological effects of active ingredients from *X. undulatum*

The cardenolide glycosides, major compounds from *X. undulatum* are also known as cardiotonic steroids, a class of naturally occuring drugs which has both beneficial as well as toxic effects on the heart. Although the toxicity remains a serious problem, these compounds in lower doses are widely used in the treatment of congestive heart failure and cardiac arrhythmia. They have positive inotropic effect on myocardial contraction and atrioventricular conduction. The compounds increase the tone, excitability and contractility of the cardiac

muscle which helps the proper functioning of the weakened heart [18]. The cardenolide glycosides act by inhibiting the plasma membrane Na^+, K^+ ATPase thereby making them useful in the drugs for the treatment of heart problems [19]. The varying structures of cardenolides does not affect their binding at Na^+, K^+ ATPase [20]. They also exhibit diuretic activity due to increased renal circulation, which relieves edema often associated with heart failure. Howerver in large doses they cause cardiac arrest in systole, and can also lead to death [18]. The major and best known cardenolide glycoside isolated from *X. undulatum* is uzarin. It inhibits intensitinal motility through affecting the visceral smooth muscles and consequently proven to have antidiarrhoel activity [18].

4.2. Materials and Methods

4.2.1. Collection and extraction of plant material

Fresh leaf material from *X. undulatum* were collected from the experimental farm of University of Pretoria, South Africa with the help of the curator, Jason Samuels. Plant identification was done at the H.G.W.J. Schweickerdt Plant Herbarium at Universiy of Pretoria where a voucher specimen was kept for further reference. The collected leaves were oven dried at 60˚C and ground to a fine coarse powder. The powdered leaves (300 g) were extracted repeatedly (3 times) for 8 hours with 2 l of DCM:MeOH (1:1) at room temperature using a magnetic stirrer. After filtration, the solvent was concentrated using a rotary vaccum evaporater at 50-60˚C and then dried under high vaccum for 24 h and the resulting extract stored in the cold room (Figure 4.3).

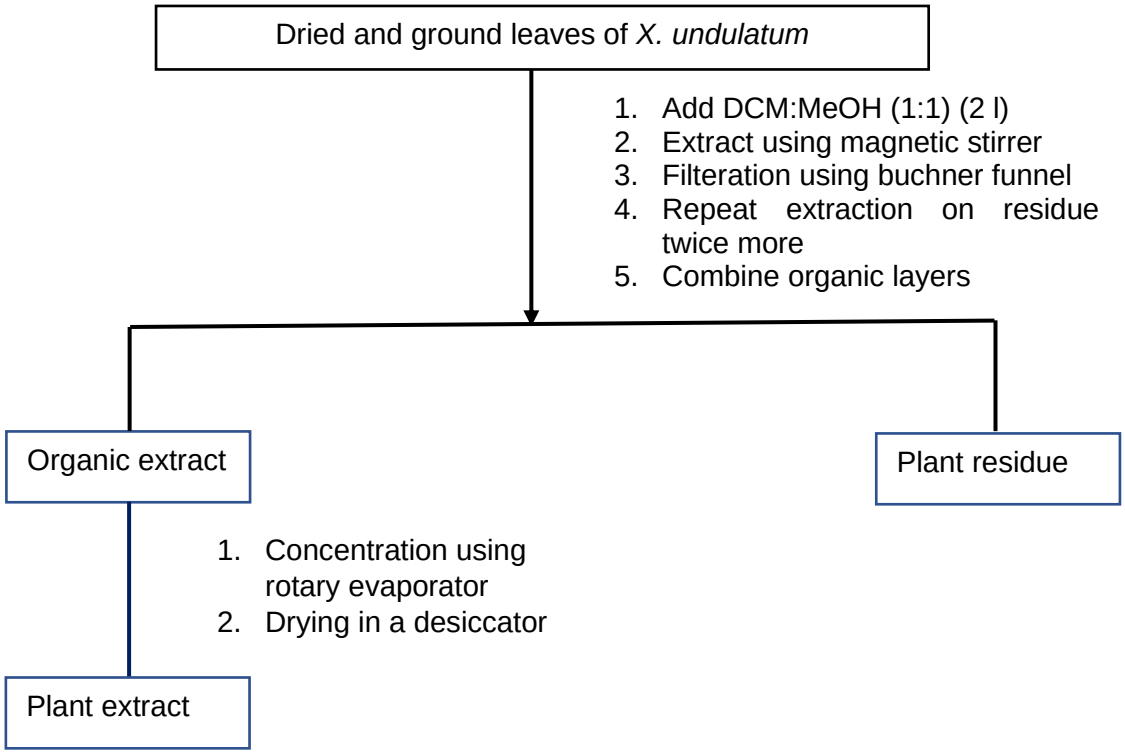


Figure 4.3: Flow diagram for the extraction of dried leaves of *X. undulatum*

4.2.2. Fractionation using silica gel column chromatography

The crude extract was subjected to silica gel (0.063-0.2 mm/70-230 mesh) gravity column chromatography using a glass silica column of 107 cm length and 7cm internal diameter. A stepwise gradient of DCM:Hexane (50:50 to 80:20), DCM (100%) and DCM:MeOH (95:5 to 70:30) was used for elution with the solvent gradient flow rate of 2 ml/min. The fractions were monitored using TLC silica gel plates and visualised under a UV lamp at 254 nm followed by staining using the vanilin stain. After visualisation, the similar fractions were combined based on Rf values. These fractions were then screened for Aβ42 reduction activity. The active fractions were further purified using semi-preparative HPLC-MS.

4.2.3. Isolation and purification of compounds using preparative HPLC-MS

The active fractions were purified using preparative HPLC-MS on a Waters chromatographic system with Waters PDA (2998) and MS detector (Waters, Milford, MA, USA). Each of the active fractions fractions (70mg) was diluted to 1 ml with methanol. A X-Bridge preparative C18 column (19×250 mm, i.d., 5µm particle size, Waters) was used for the separation while the mobile phase consisted of 0.1% ammonia in water (solvent A) and acetonitrile (solvent B) at a flow rate of 20 ml/min. Gradient elution was applied as follows: 95% A : 5% B for 1 min, 50% A : 50% B (1:00-8:00 min), 15% A : 85% B (8:00-13:00 min), 95% A : 5% B (13:00-13:20 min). Injection volume was 150 µl. Data were collected using MassLynx4.1^{TM} (Waters, USA) software. The preparative HPLC system was interfaced with QDa mass spectrometer (Waters, USA) and the negative ion mode selected. The probe temperature and source temperature were set at 600ºC and 120ºC, respectively. The capillary and cone voltage were set to 800 and 10 V, respectively. Data was collected between 100 and 650 *m/z.* The pure compounds were collected using a fraction collector and subsequently combined and concentrated using the speed vaccum. The compounds were later analyzed by UPLC-MS and their structures are confirmed by NMR.

4.2.4. Chemical profiling using UPLC-QTOF-MS

The crude extracts and fractions were analyzed using a Waters Acquity UPLC system (Waters corp.,MAUSA), equipped with a binary solvent delivery system and an autosampler. The instrument was centrally operated by MassLynx 4.1 software (Waters Inc., Milford, Massachusetts, USA) for data acquisition. The separation was achieved on Waters BEH C18 1.7 µm particle size (2.1 mm × 100 mm). The mobile phase consisted of solvent A: water with 0.1% formic acid and solvent B: methanol with 0.1% formic acid. The gradient elution was optimized as follows: 3% B (0-0.1 min), 100% B (0.3-14.00 min), 100% B (14.00-16.00 min), 3% B (16.00-16.50 min), 3% B (16.50-20.00 min). The flow rate was 0.3 ml/min for the entire run, giving a total run time of 20 min and the injection volume was 5µl. The instrument was calibrated by direct infusion of 5 nM sodium formate solution at a flow rate of 10 µl/min over a

mass range of 50-1200 Da. The following MS source parameters were set for both positive and negative mode: source temperature 100 ºC, sampling cone 15 V, extraction cone 4.0 V, desolvation temperature 400 ºC, cone gas flow 10.0 l/h, desolvation gas flow 700 l/h, capillary 2.0 kV. Both positive and negative modes were obtained, however the results were analyzed using the negative mode as the higher intensity peaks were obtained from this mode. Compounds were tentatively identified based on their accurate mass generated from MassLynx V 4.1, iFit value, MS/MS fragmentations (product ions) and by the use of Metlin, Metfusion, Pubchem, Reaxys, Scifinder, Scopus, Chemspider, Chemical Entities of Biological Interest (ChEBI) and Massbank libraries.

4.2.5. NMR analysis

Structure elucidation of isolated compounds was carried out using NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were acquired on a Bruker Fourier 400 spectrometer (^{1}H at 400.21 MHz and ^{13}C at 100.63 MHz) and 500 spectrometer (^{1}H at 500.10 MHz and ^{13}C at 125.75 MHz). Chemical shifts were reported in ppm, referenced to residual solvent resonances (methanol-d4 δH 3.31, δC 49.0 ppm).

4.2.6. Cell culture

For cell culture methodology, see section 2.2.3 of chapter 2.

4.2.7. sAPPα, sAPPβ, Aβ peptide assay

For sAPPα, sAPPβ, Aβ peptide assay, see section 3.4.4 of chapter 3.

4.2.8. Cell viability measurement

For cell viability measurement, see section 3.4.5 of chapter 3.

4.2.9. Statistical analysis

For statistical analysis, see section 2.2.5 of chapter 2.

4.3. Results and discussion

4.3.1. Collection and extraction of plant material

Fresh leaves (900 g) of *X. undulatum* was collected from the experimental farm of University of Pretoria. A voucher specimen (PRU 124301) was deposited at H.G.W.J. Schweickerdt Herbarium of University of Pretoria. After oven drying of the leaves, 200 g of dried material was obtained. The three times repeated extraction using DCM:MeOH of the dried leaves gave 17 grams of the extract in a 8.5% yield. The weight and percentage yield of the plant material (fresh and dried leaves) and plant extract are shown in the Table 4.1.

Table 4.1: Weight and percentage yield of crude extracts from the collected of *X. undulatum*

Sample	Extract	Weight (g)	% yield (w/w)
X. undulatum leaf material	Fresh plant material	900	
	Dried and ground plant material	200	22.2
	Dry DCM:MeOH extract	17	8.5

4.3.2. The effect of the leaf extract of *X. undulatum* on Aβ42 production

The bioassaying was made possible with support of Korean Institute of Science and Technology (KIST). Special thanks to the strong collaboration of Dr Yoon Sun Chun, Ansun Park in Dr Hyun OK Yang's laboratory at KIST. I also acknowledge the work done by KIST in the training on running the assays and interpreting of data during my research visit to their laboratories. The effect of *X. undulatum* leaf extract on Aβ42 production at 0.5, 1, 5, and 10 μg/ml extract for 8 h, showed significant decrease in the level of Aβ42 production in a dose-

dependent manner (Figure 4.4). The leaf extract significantly reduced the Aβ42 production by 49.8 ± 4.0%, 71.4 ± 1.6% and 72.9 ± 1.1% at concentrations 1, 5 and 10 µg/ml, respectively.

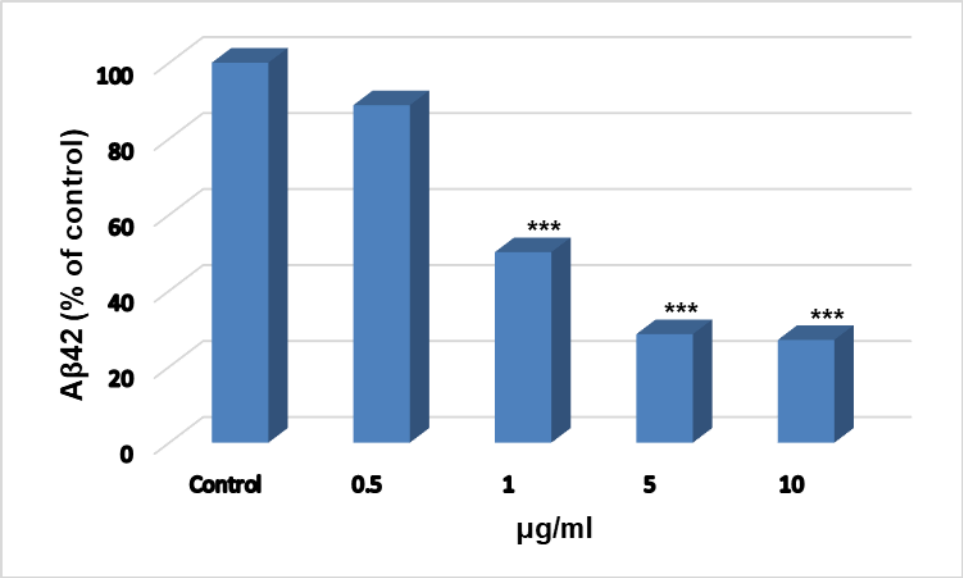


Figure 4.4: Change in the levels of Aβ42. Cells were incubated with indicated concentrations of leaf extract of *X. undulatum* for 8 h, the levels of Aβ42 were measured from the conditioned media by using specific ELISA methods. The levels of Aβ42 (n = 4) were significantly decreased by the extract at concentrations 1, 5 and 10 µg/ml. **, P<0.01; ***, P<0.001.

The leaf extract of *X. undulatum* induced the decrease in cell viability to a small extent (Figure 4.5). The cell viability was decreased by 9.5 ± 6%, 13.6 ± 7.3%, 15.5 ± 4.2% and 22.0 ± 2.8% at the concentrations 0.5, 1, 5 and 10 µg/ml, respectively.

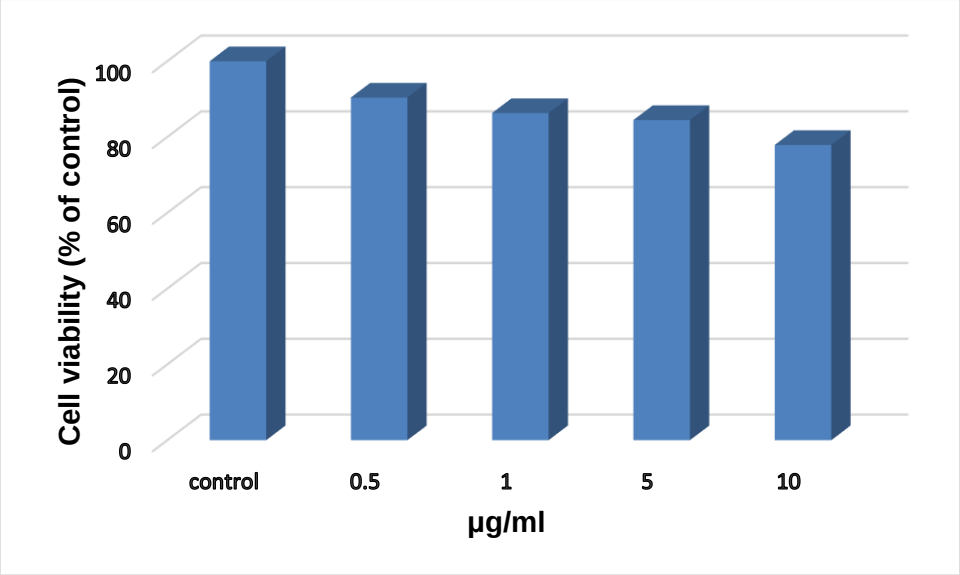


Figure 4.5: Cell viability measurements. Cells were incubated with indicated concentrations of extracts of leaves of *X. undulatum* for 8 h, and then incubated with EZ-Cytox solution for 1 h. The extract of leaves of *X. undulatum* induced the decrease of cell viability to small extent at all concentrations (0.5, 1, 5 and 10 µg/ml) (n = 6).

The secreted level of APP proteolytic products from the conditioned media using specific ELISA kits for Aβ40, sAPPβ-sw and sAPPα were then measured. Secreted level of Aβ40 were decreased by 26.2 ± 3.2%, 66.4 ± 1.4% and 73.3 ± 1% at 1, 5 and 10 µg/ml of extract, respectively (Figure 4.6).

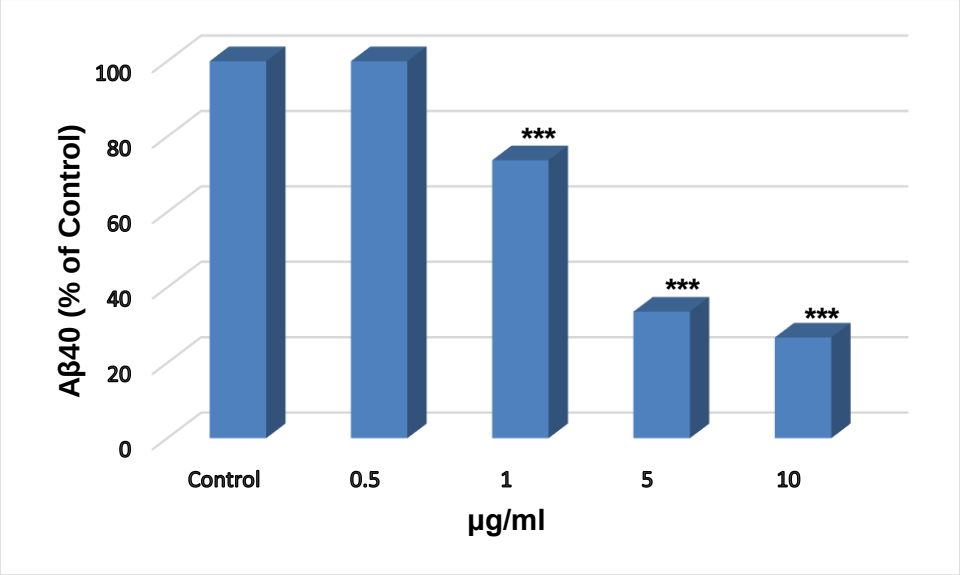


Figure 4.6: Change in the levels of Aβ40. Cells were incubated with indicated concentrations of leaf extract of *X. undulatum* for 8 h, the levels of Aβ40 were measured from the conditioned media by using specific ELISA methods. The levels of Aβ40 (n = 4) were decreased by the extract only at the higher concentrations. **, P<0.01; ***, P<0.001.

Secreted level of sAPPβ-sw were also reduced by 34.5 ± 1%, 49.1 ± 4.8% and 52.7 ± 2% at 1, 5 and 10 µg/ml of extract, respectively (Figure 4.7). In contrast, this extract significantly increased the secreted level of sAPPα in a dose-dependent manner. The sAPPα level was increased by two-fold at 10 µg/ml of the *X. undulatum* leaf extract. Secreted level of sAPPα level was increased by 23.2 ± 2.0 and 42.1 ± 2.0 at 5 and 10 µg/ml of extract, respectively (Figure 4.8). This data suggests that the leaf extract increased the non-amyloidogenic processing of APP, while it decreased amyloidogenic processing of APP, leading to the reduction of Aβ level.

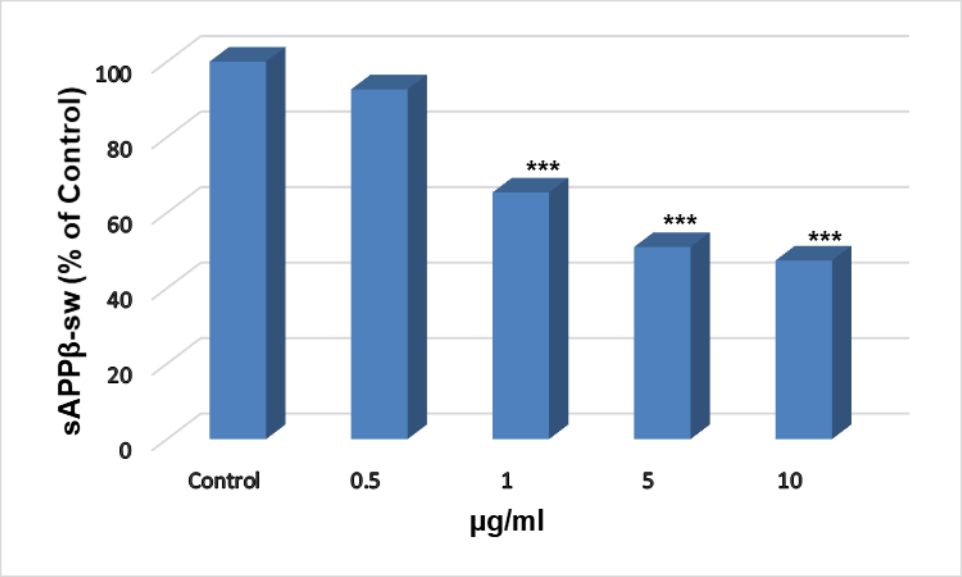


Figure 4.7: Change in the levels of sAPPβ-sw. Cells were incubated with indicated concentrations of leaf extract of *X. undulatum* for 8 h, the levels of sAPPβ-sw were measured from the conditioned media by using specific ELISA methods. The levels of sAPPβ-sw (n = 4) were decreased by the extract at higher test concentrations. **, P<0.01; ***, P<0.001.

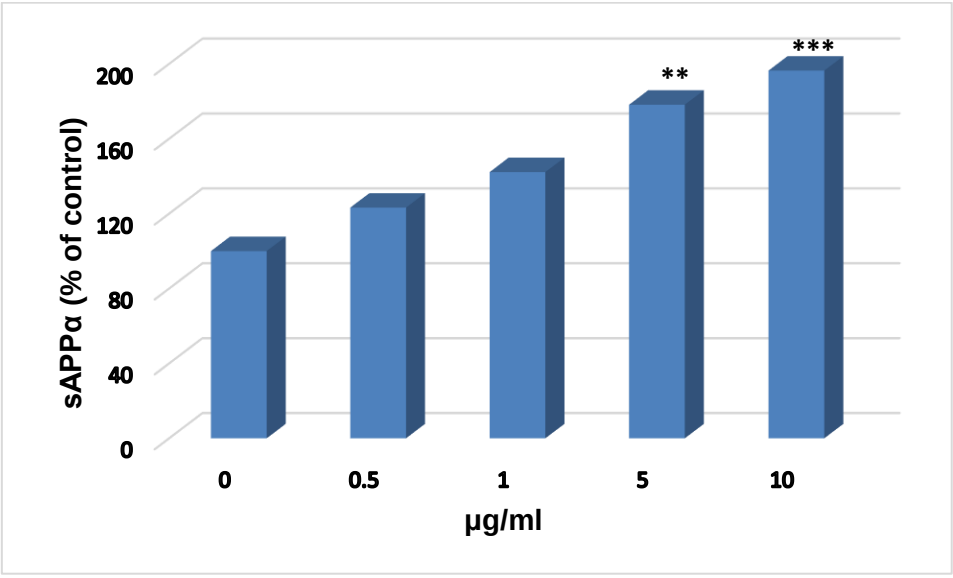


Figure 4.8: Change in the levels of sAPPα. Cells were incubated with indicated concentrations of leaf extract of *X. undulatum* for 8 h, the levels of sAPPα were measured from the conditioned media by using specific ELISA methods. The levels of sAPPα (n = 4) were increased by the extract. **, P<0.01; ***, P<0.001.

These results suggest that *X. undulatum* leaf extract potently decreased Aβ42 production at non-toxic concentrations through decreasing amyloidogenic processing of APP and increasing the nonamyloidogenic processing of APP.

Although the leaf extract *X. undulatum* has some cytotoxicity, it potently induced the decrease in Aβ42 production. The plant was chosen for further studies to determine the active ingredients responsible for its activity of Aβ42 reduction. In addition, the leaf extract showed its efficacy to exhibit neuroprotective properties through inhibition of Aβ42 and simultaneously increasing sAPPα secreted levels. Both these features are responsible for memory enhancement [21].

4.3.3. UPLC-MS-QTOF analysis of *X. undulatum* leaf extract

UPLC-MS-QTOF analysis was performed in ESI negative mode for the DCM: MeOH leaf extract of *X. undulatum* to identify the major peaks and the compounds which could be responsible for the reduction of Aβ42. In ESI negative mode, the peaks were mainly distributed within the 3.20 to 6.40 min range of the 18 min chromatogram (Figure 4.9)

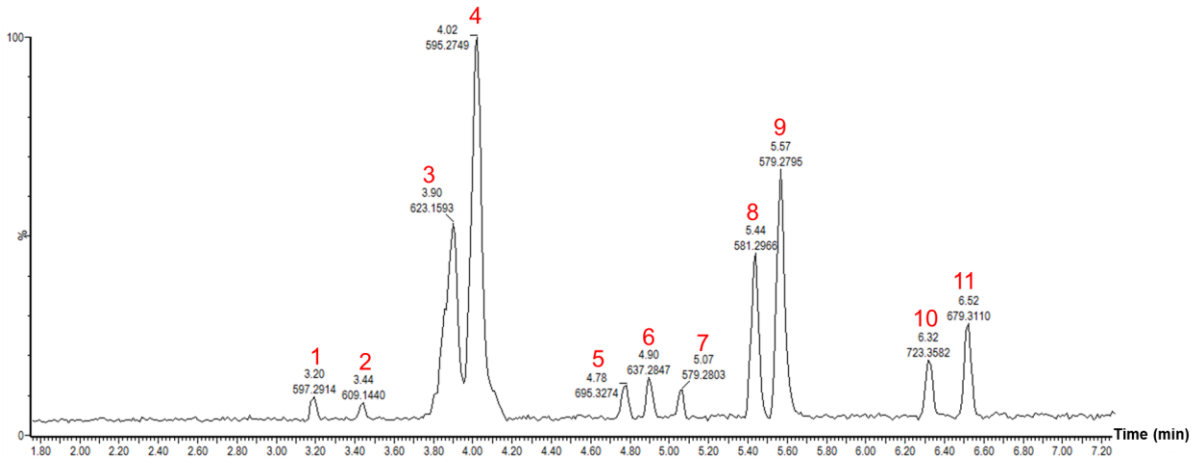


Figure 4.9: ESI negative mode BPI chromatogram of *X. undulatum* leaf extract.

In total, nine compounds were successfully detected and tentatively identified which included seven cardenolides, two flavone C-glycosides (rutin and isorhamnetin-3-*O*-rutinoside), based on the iFit values, mass error, mass fragmentation patterns and published data (Table 4.2.). The different electronic database, Chemspider, Pubchem, Mass Bank, Scifinder, Scopus, Reaxys, Metalin Scripps and ChEBI (Chemical Entities of Biological Interest) were used for the tentative identification of compounds.

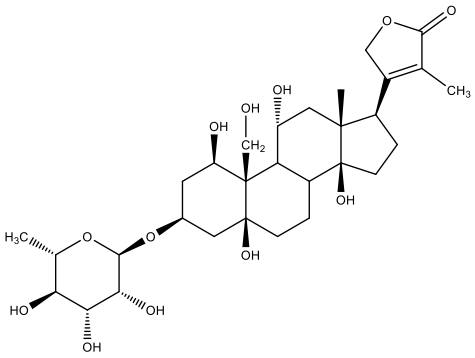
Table 4.2: Tentative identification of compounds obtained from ESI-MS negative mode of DCM: MeOH extract of *X. undulatum* (leaf)

Peak	RT (min)	Acquired $[M-H]^-$ *m/z*	Formula of Possible structure	Theoretical $[M-H]^-$ *m/z*	Tentative name	Mass Error (ppm)	MS/MS Data (Fragments)	Ref.
1	3.20	597.2914	$C_{30}H_{46}O_{12}$	597.2911	22-methylouabain	0.5	587.2650, 433.2082, 304.9143, 174.9560	Reaxys
2	3.44	609.1440	$C_{27}H_{30}O_{16}$	609.1455	rutin	-2.4	301.0256, 300.0309,	[22]
3	3.90	623.1593	$C_{28}H_{32}O_{16}$	623.1612	isorhamnetin-3-O-rutinoside (ramnazin-3-O-rutinoside)	-3.0	315.0490, 299.0174, 271.0222	[23]
4	4.02	549.2687	$C_{29}H_{42}O_{10}$	549.2699	glucocorotoxi-genin	-2.1	387.2144	http://www.chemspider.com
5	4.78	695.3274	$C_{35}H_{52}O_{14}$	695.3278	xysmalorin	-0.5	533.2762, 387.2171	[24]
6	4.90	637.2847	$C_{32}H_{46}O_{13}$	637.2860	unidentified	-2.0	533.2756, 256.8793, 174.9565	-
7	5.07	579.2740	$C_{30}H_{44}O_{11}$	579.2805	unidentified	-11.2	460.7869 414.8052 338.8590	-

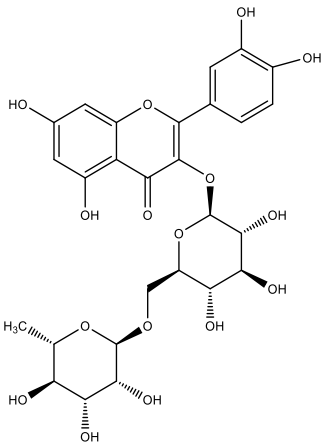
							274.8797	
8	5.44	535.2877	$C_{29}H_{44}O_9$	535.2907	desglucouzarin	-5.6	373.2382	[25]
9	5.57	533.2766	$C_{29}H_{42}O_9$	533.2750	gofruside	3.0	533.2797, 387.2174	Reaxys
10	6.32	677.3536	$C_{36}H_{54}O_{12}$	677.3537	corotoxigenin-3-O-β-digitalopyranosyl-(1→4)-O-β-digitoxopyanoside	-0.1	517.2767	[26]
11	6.52	679.3110	$C_{38}H_{48}O_{11}$	679.3118	12β-(acetyloxy)-(3β,5β)-3-[4-carboxy-3-[4-(acetyloxy)-phenyl]-1-oxobutoxy]-14-hydroxycard-20(22)-enolide	-1.1	163.0398, 145.0296	https://pubchem.ncbi.nlm.nih.gov

Peak 1 exhibited a precursor ion $[M-H]^-$ at *m/z* 597.2194 which corresponded with the molecular formula $C_{30}H_{46}O_{12}$. MS/MS spectra of deprotonated precursor ion showed different fragment ions at *m/z* 433.2082 $[M-164.0112]^-$ with the loss of a glucoside moiety. The use of the electronic database, Reaxys, and the proposed molecular formula led to the tentative identification of a cardenolide, 22-methylouabain **(46)**. The mass spectrum of peak 2 showed a molecular ion at *m/z* 609.1455 $[M-H]^-$, producing a quercetin daughter ion at *m/z* 301.0256 $[M-308.1199]^-$ with the loss of rhamnosyl-glucoside moiety. The elemental composition proposed the molecular formula $C_{27}H_{30}O_{16}$. From all the observed data, the compound was tentatively identified as rutin **(47)**. The MS analysis of peak 3 showed a molecular ion at *m/z* 623.1593 with molecular formula $C_{28}H_{32}O_{16}$, releasing one MS^2 aglycone fragment ion of isorhamnetin at *m/z* 315.0490 $[M-308.1103]^-$. This fragment ion showed the loss of rhamnosyl-glucoside moiety. The previous studies and the mass spectrum analysis led to the identification of isorhamnetin-3-*O*-rutinoside (ramnazin-3-O-rutinoside) **(48)** [27], [23]. Loss of rhamnosyl-glucoside fragment in rutin and isorhamnetin-3-O-rutinoside is a characteristic of flavone C-glycosides [27]. The MS analysis of peak 4 presented a pseudo molecular ion at *m/z* 595.2749 $[M-HCOO]^-$ and was assigned the elemental composition $C_{30}H_{44}O_{12}$ (calculated

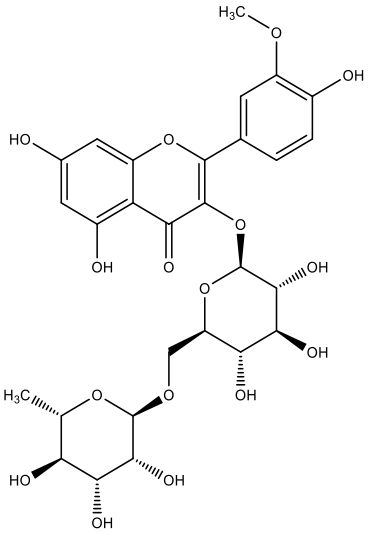
mass 550.2778). The MS/MS spectra gave the fragment ion at *m/z* 387.2144 [M-162.0543]$^-$ with the loss of a glucoside moiety. This typical fragmentation pattern led to the tentative identification of the cardenolide, glucocorotoxigenin **(49)**. The mass spectrum of peak 5 presented a molecular ion at *m/z* 695.3274 [M-H]$^-$, corresponding to the formula $C_{35}H_{52}O_{14}$. Two MS^2 fragment ions, one at *m/z* 533.2756 [M-162.0518]$^-$ and the other at *m/z* 387.2171 [M-162.0518-146.0585]$^-$ were detected and corresponded to the loss of pentosylglucoside and rhamnosylglucoside, respectively. In accordance with literature and the mass fragmentation pattern, the compound was tentatively identified as xysmalorin **(43)** [24], for which the structure of the compound is shown in Figure 4.1. Peak 8 presented a pseudomolecular ion at *m/z* 581.2966 [M-HCOO]$^-$ with a molecular formula $C_{30}H_{46}O_{11}$ (calculated mass 536.2985). The MS/MS spectrum produced the fragment ion at *m/z* 373.2382 [M-162.0495]$^-$ with the loss of glucoside moiety. The typical fragmentation pattern of cardenolides and the literature study led to the tentative identification of compound as desglucouzarin **(50)** [25]. Peak 9 presented a pseudomolecular ion at *m/z* 579.2795 [M-HCOO]$^-$ with a molecular formula $C_{30}H_{44}O_{11}$ (calculated mass 533.2750). The characteristic fragment signals at *m/z* 387.2174 [M-146.0582]$^-$ indicated the loss of a deoxyhexose moiety. Based on the literature and fragmentation pattern, the compound was identified as the cardenolide, gofruside **(51)**. The MS analysis of peak 10 showed a pseudo molecular ion peak at *m/z* 723.3582 with a predicted formula $C_{36}H_{54}O_{12}$ (calculated mass 678.3615). The fragment ions were detected at *m/z* 517.2767 [M-160.0926]$^-$ with the loss of digitalose unit. Based on the previous study and the fragmentation pattern, this peak was tentatively identified as corotoxigenin-3-O-β-digitalopyranosyl-(1→4)-O-β-digitoxopyanoside **(52)** [26]. Finally, peak 11 presented molecular ion at *m/z* 679.3110 [M-H]$^-$. Through the elemental composition, the molecular formula was predicted as $C_{38}H_{48}O_{11}$ with the release of fragment ion at *m/z* 163.0398 [M-516.2712]$^-$. All this data including that in Pubchem led to the tentative identification of this peak as 12β-(acetyloxy)-(3β,5β)-3-[4-carboxy-3-[4-(acetyloxy)phenyl]-1-oxobutoxy]-14-hydroxycard-20(22)-enolide **(53)**. The structures of the identified compounds are shown in Figure 4.10.



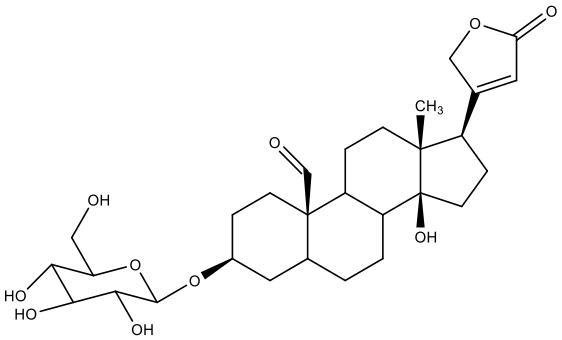
(46)



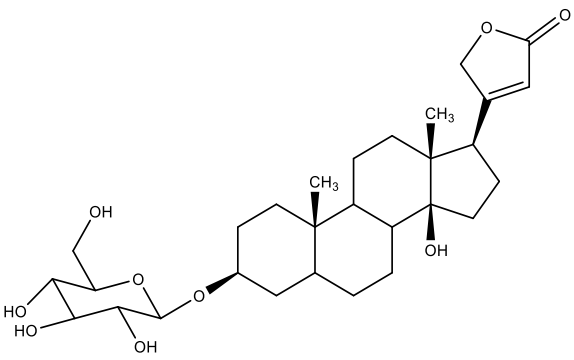
(47)



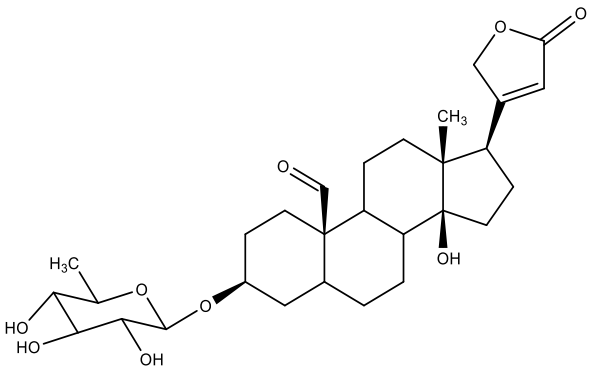
(48)



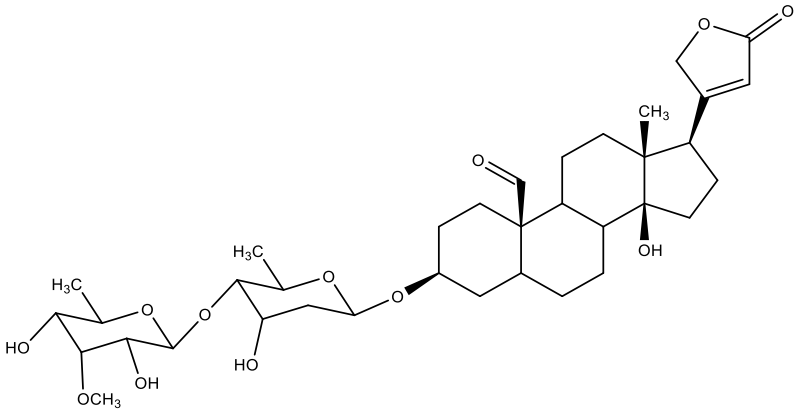
(49)



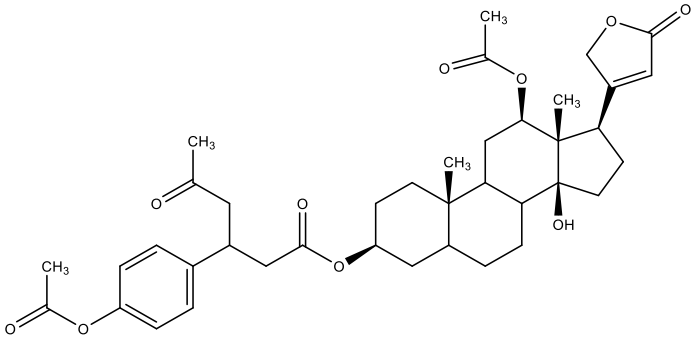
(50)



(51)



(52)



(53)

Figure 4.10: Structures of 22-methylouabain **(46)**, rutin **(47)**, isorhamnetin-3-O-rutinoside (Ramnazin-3-O-rutinoside) **(48)**, glucocorotoxigenin **(49)**, desglucouzarin **(50)**, gofruside **(51)**, corotoxigenin-3-O-β-digitalopyranosyl-(1→4)-O-β-digitoxopyanoside **(52)**, 12β-(Acetyloxy)-(3β,5β)-3-[4-carboxy-3-[4-(acetyloxy)-phenyl]-1-oxobutoxy]-14-hydroxycard-20(22)-enolide **(53)** identified in DCM:MeOH leaf extract of *X. undulatum*

4.3.4. Fractionation using silica gel column chromatography

Silica gel column chromatography of the leaf extract (17g) using the gradient solvent system resulted in the collection of 46 fractions. Fractions were analysed by TLC and those fractions which showed similar profiles based on compounds with similar Rf values were combined to give 15 fractions as shown in Figure 4.11 which were evaluated for their Aβ42 production.

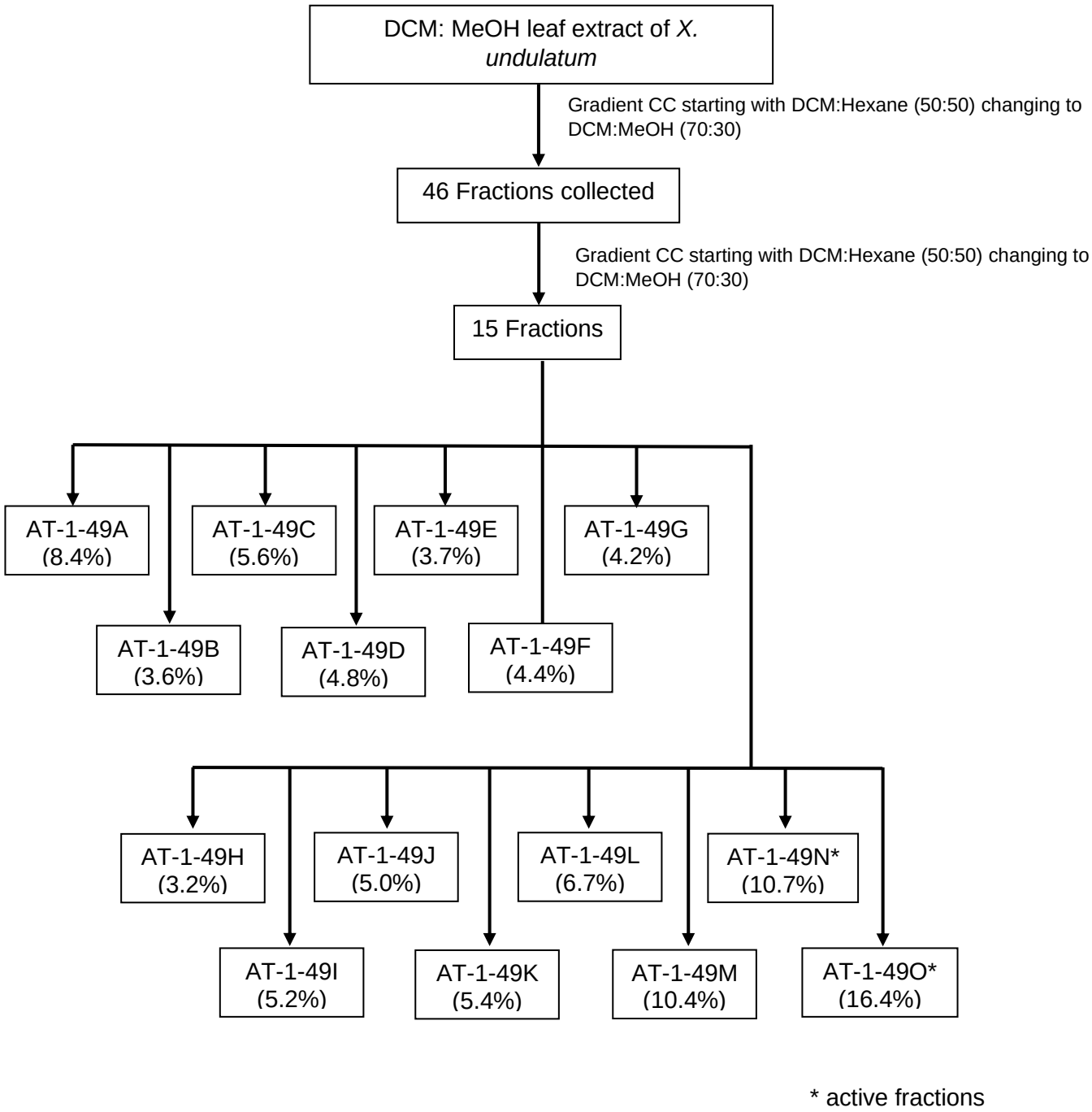


Figure 4.11:Flow diagram for the fractionation of the leaf extract of *X.undulatum* using silica gel column chromatography. Value in the brackets indicated the percentage yield (*w/w*) of the fractions obtained from silica gel column.

4.3.5. Bioassaying of fractions for Aβ42 production

The 15 fractions (AT-1-49A to AT-1-49O) were evaluated for their activity on Aβ42 production by incubating the HeLa cells with 10 µg/ml of each fraction for 8 h and the levels of Aβ42

measured from the conditioned media by specific ELISA. Two fractions (AT-1-49N and AT-1-49O) potently decreased the Aβ42 secretion by 73.6 ± 0.8% and 65.3 ± 0.01%, respectively, while all other fractions showed no or minimal reduction of Aβ42 as shown in Figure 4.12. The results indicated that the silica gel column chromatography successfully separated the active from the non-active fractions. The two active fractions were further purified to isolate the active compounds.

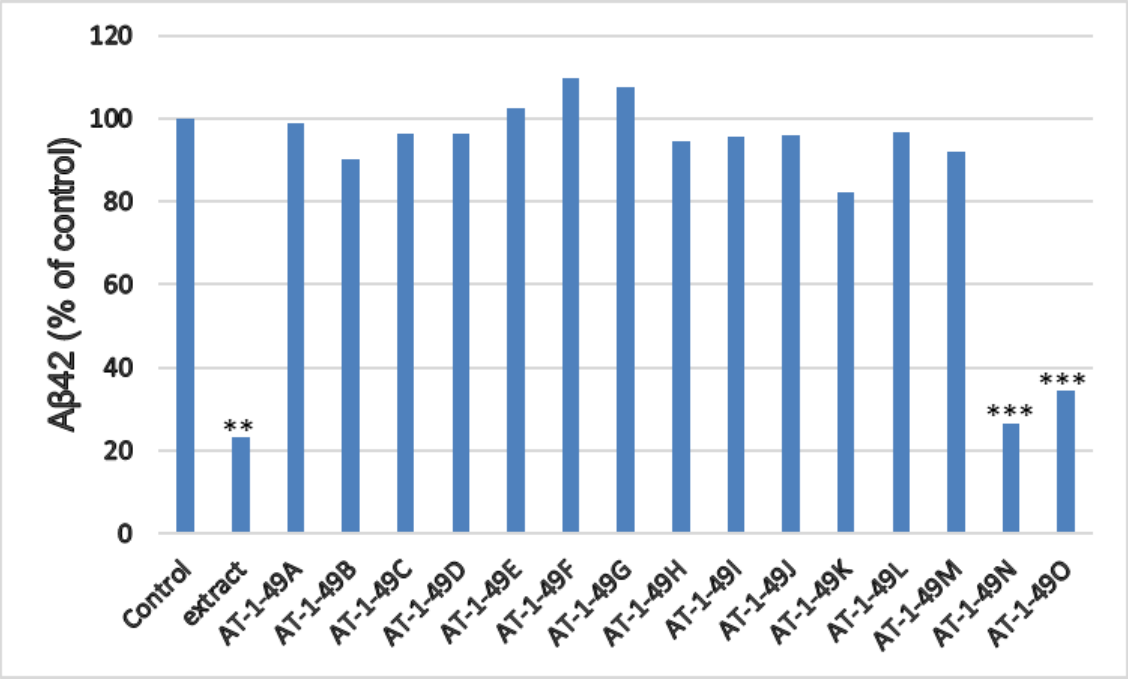


Figure 4.12: Effect of fractions on the production of Aβ42 in APPsw-transfected HeLa cells.15 Fractions were obtained from leaf extract of *X. undulatum* by silica gel column chromatography. Cells were incubated with 10 µg/ml extract and 10 µg/ml fractions for 8 h, and the level of Aβ42 was measured from the conditioned media by specific ELISA methods. The two fractions, AT-1-49N and AT-1-49O, potently decreased the secreted level of Aβ42 (n = 2). *, P<0.05; **, P<0.01; ***, P<0.001.

4.3.6. UPLC- MS analysis the active fractions

UPLC-MS analysis of the active fractions tentatively identified a mixture of cardenolides. Based on literature reports, it was found that the cardenolides in ESI MS negative mode formed abundant adduct ions $[M+HCOO]^-$ while pseudo molecular ions $[M-H]^-$ were obtained in ESI-MS/MS experiments [28]. The similar pattern was observed in the active fractions AT-

1-49N and AT-1-49O which supported the presence of cardenolides. In ESI-MS negative mode for fraction AT-1-49N (Figure 4.13), peak 1 with *m/z* 637.2856, peak 2 with *m/z* 433.2226, peak 3 with *m/z* 579.2813, peak 4 with *m/z* 723.3585 and peak 6 with *m/z* 709.3793 were obtained as formate adduct ions $[M+HCOO]^-$ while their pseudo molecular ions $[M-H]^-$ observed at *m/z* 591.2797, *m/z* 387.2164, *m/z* 533.2763, *m/z* 677.3526 and *m/z* 663.3740, respectively in the ESI MS/MS spectra **(supplementary data 1 to 5)**. All the peaks except peak 2 and peak 6 were analysed earlier as descibed in section 4.1.2.

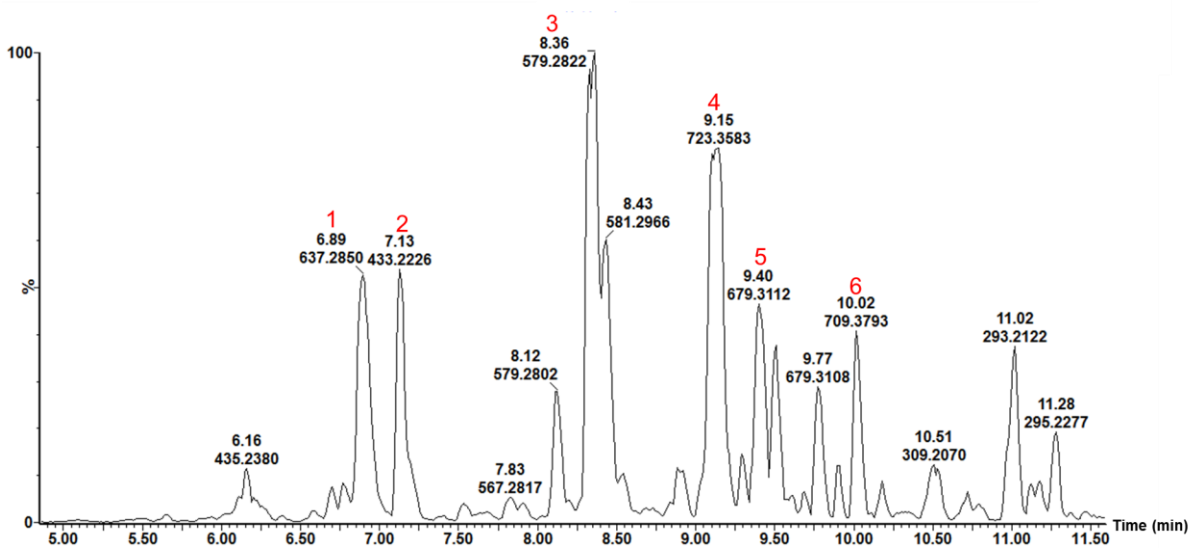
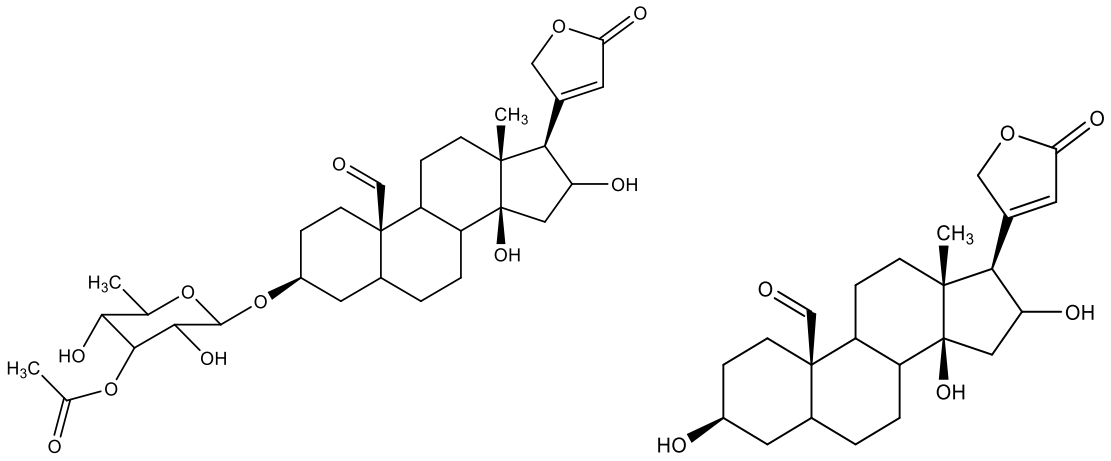


Figure 4.13: ESI negative BPI chromatogram of active fraction AT-1-49N

Peak 2 and peak 6 had not been dectected in the crude parent DCM:MeOH leaf extract. This indicated either their poor ionization or low concentrations in the extract. Peak 1 (637.2850) showed a formic acid adduct ion in fraction AT-1-49N while this adduct did not appear in MS/MS fragmentation pattern of the DCM:MeOH extract due to its poor isonisation. The molecular formula for these compounds were generated through MassLynx and the compounds were identified using online database searches (Table 4.3). The structures of the compounds are shown in Figure 4.14.

Table 4.3: Tentative identification of compounds obtained from ESI-MS negative mode of fraction AT-1-49N obtained from DCM: MeOH extract of *X. undulatum* (leaf)

Peak	RT (min)	Acquired [M-H]- *m/z*	Formula of Possible structure	Theoretical[M-H]$^-$ *m/z*	Tentative name	Mass Error (ppm)	Ref.
1	6.89	591.2797	$C_{31}H_{44}O_{11}$	591.2805	adonitoxigenin-(2-acetyl-rhamnosid)	-1.3	Reaxys
2	7.13	387.2164	$C_{23}H_{32}O_5$	387.2171	corotoxigenin	-1.8	Reaxys
3	10.02	699.3505	$C_{49}H_{48}O_4$	699.3474	unidentified	-	-



(54) **(55)**

Figure 4.14: Structures of adonitoxigenin-(2-acetyl-rhamnosid) **(54)**, crotoxigenin **(55)**

Similarly formate adduct ions $[M+HCOO]^-$ were observed in fraction AT-1-49O (Figure 4.15) with *m/z* 581.2970 and *m/z* 595.2756 while their pseudo molecular ions obtained in MS/MS spectra were *m/z* 535.2970 and *m/z* 549.2701, respectively **(supplementary data 6 and 7).** The analysis of these compounds were examined earlier as descibed in section 4.1.2.

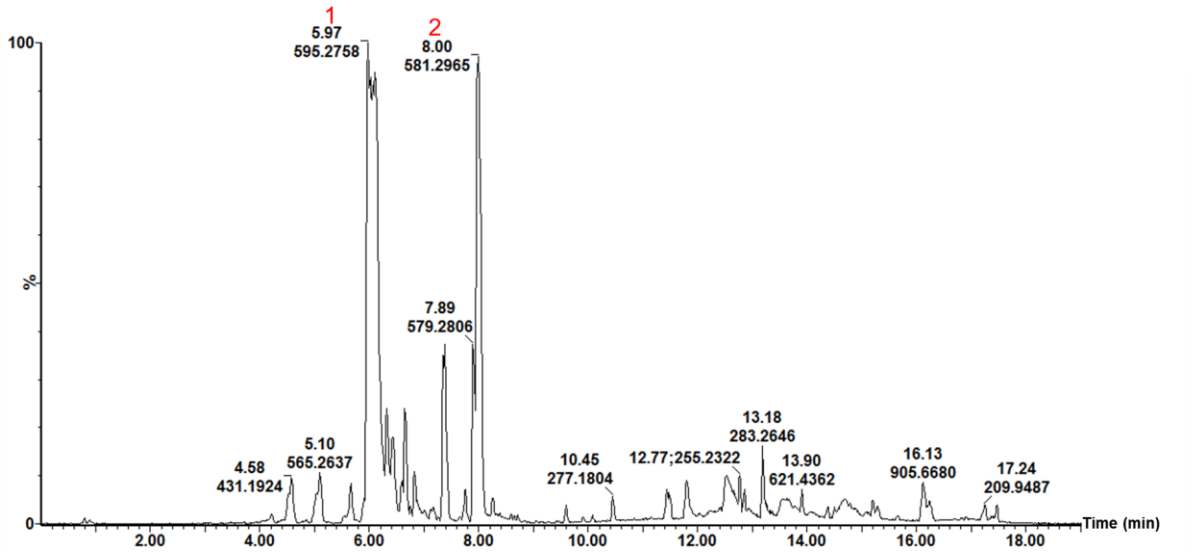


Figure 4.15: ESI negative BPI chromatogram of fraction AT-1-49O.

The common class of compounds, cardenolide glycosides was identified from both the active fractions AT-1-49N and AT-1-49O. This indicated that the cardenolide glycosides could be the active ingredients in the present study. These fractions were further purified using preparative HPLC.

4.3.7. Isolation and purification of compounds from active fractions using preparative HPLC

AT-1-49N was refined by using mass-directed purification using preparative HPLC. The fractions were collected in test tubes by using mass triggered fractionation. The fractions with the similar masses were combined and evaporated to dryness using rotary vacuum evaporator. The purity of compounds was further confirmed with UPLC-MS and NMR analysis. All *m/z* values in the ESI negative mode (Figure 4.16) corresponding to major peaks labelled as XU01 (637.2850), XU02 (433.2226), XU03 (579.2822), XU04 (723.3583), XU05 (679.3112), XU06 (709.3793) were targeted and collected. Of these major peaks, three XU01 (4 mg), XU03 (3.5 mg) and XU04 (4.9 mg) were collected in pure form with sufficient quantities to allow further

analysis. ESI negative-mode BPI chromatograms of pure compounds XU01, XU03 and XU04 is shown in figure 4.16.

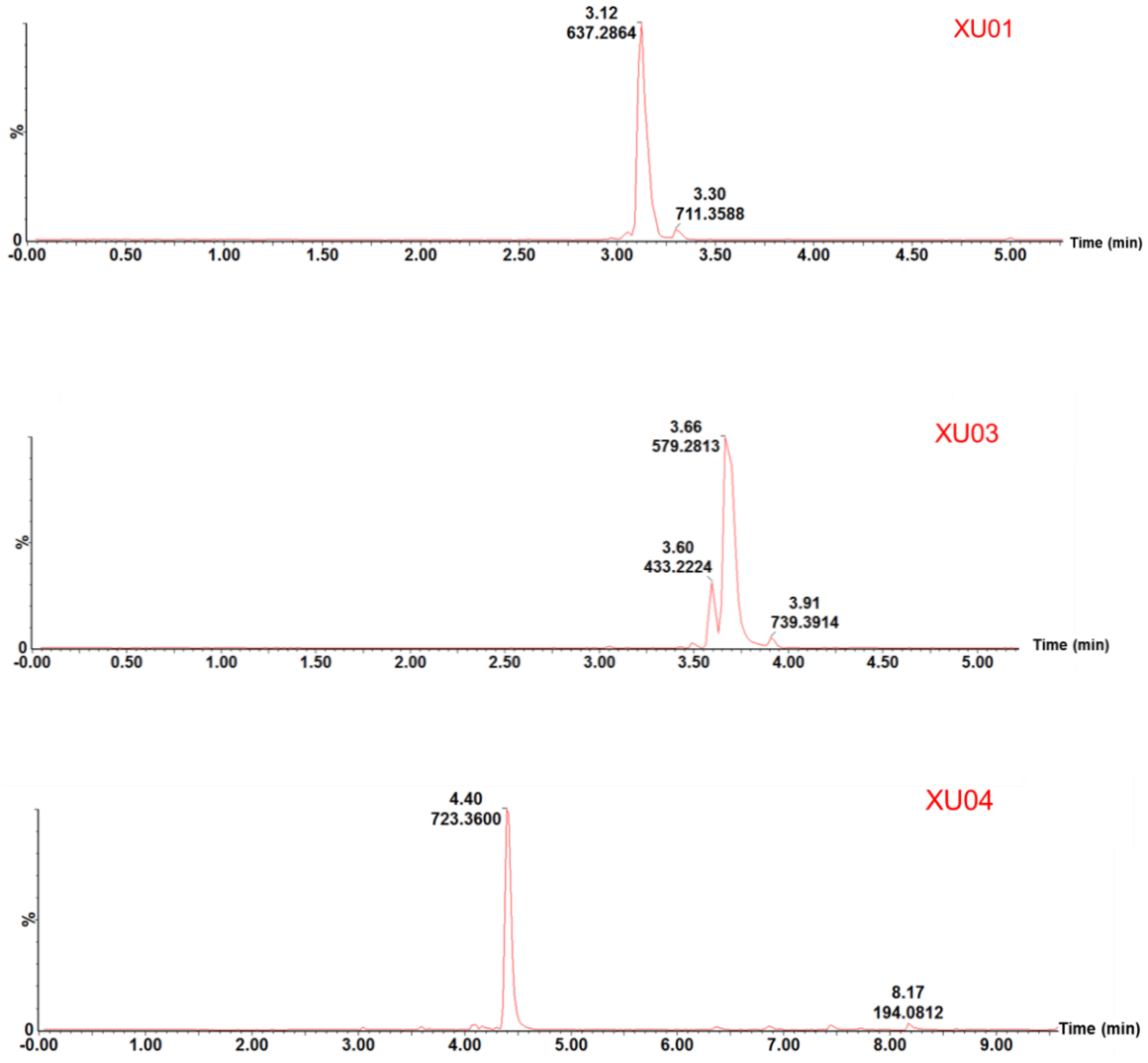


Figure 4.16: ESI negative-mode BPI chromatograms of pure compounds XU01, XU03 and XU04 from active fraction AT-1-49N.

Similarly, AT-1-49O was also resolved using mass-directed purification preparative HPLC. The *m/z* values in the ESI negative mode (Figure 4.13) corresponding to major peaks 595.2758 and 581.2965 were targeted and collected in pure form. These were labelled as XU07 (5 mg) and XU08 (4 mg). The ESI negative-mode BPI chromatograms of pure compounds XU07 and XU08 is shown in Figure 4.17.

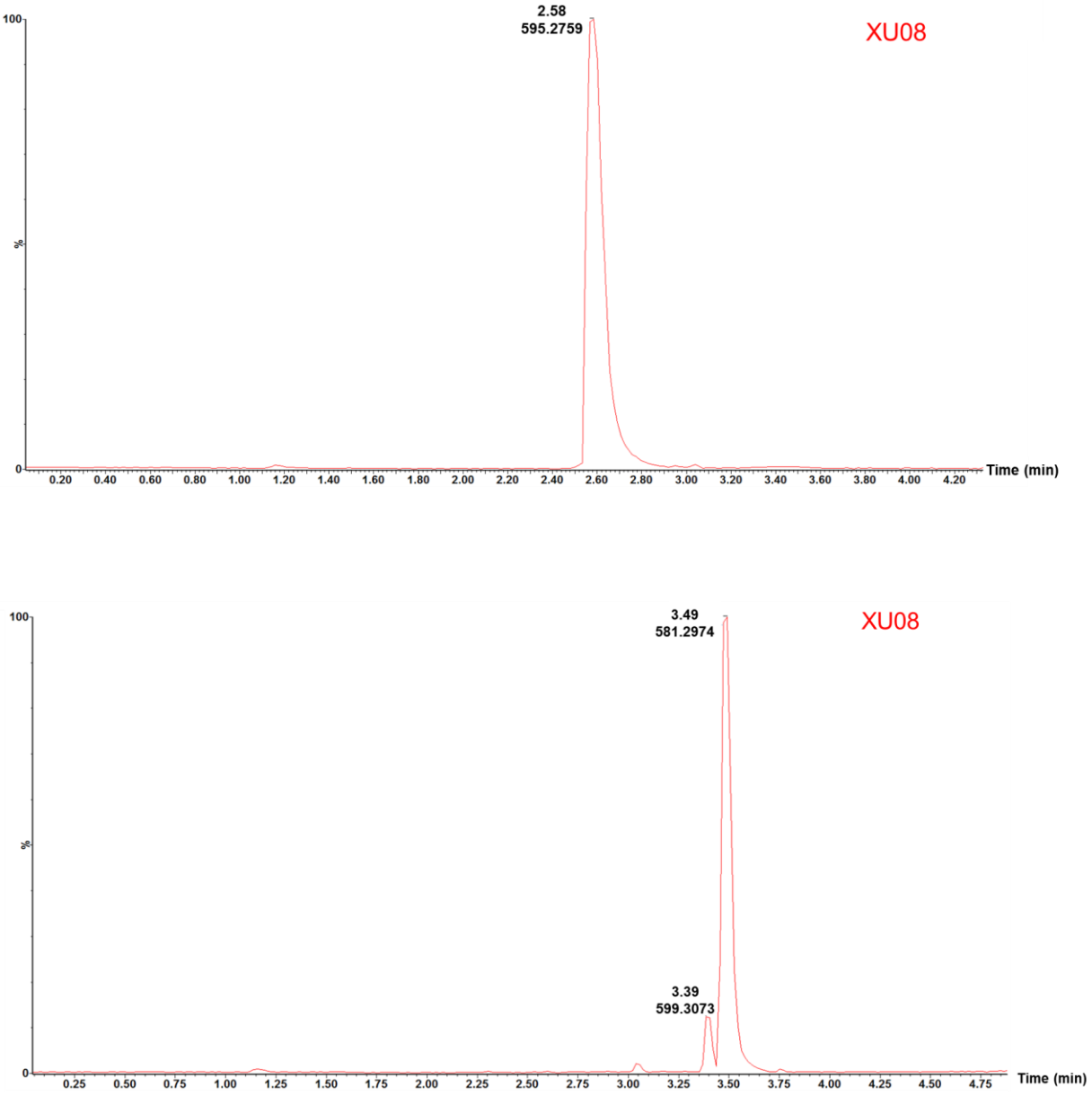


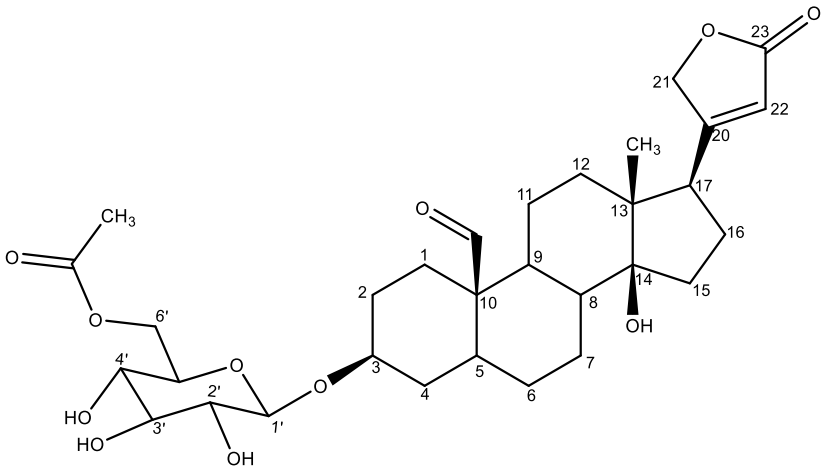
Figure 4.17: ESI negative-mode BPI chromatograms of pure compounds XU07 and XU08 from active fraction AT-1-49O.

4.3.8. Structural elucidation using NMR

4.3.8.1. Acetylated glycosydated crotoxogenin (56)

The pure compound (XU01) was identified as the novel acetylated glycosydated crotoxigenin **(56)** based on the NMR data and mass spectral analysis. It was isolated as a white amorphous powder with a mass of 4 mg. It had a molecular formula of $C_{31}H_{44}O_{11}$ (calculated mass

592.2883), as deduced from its pseudomolecular precursor ion at *m/z* 637.2851[M-HCOO]$^-$ based on the QTOF mass spectrum. These data confirmed the presence of cardenolide glycoside. The formula was further confirmed by the number of protons in ^{1}H NMR and of carbon atoms in the ^{13}C NMR spectrum **(Supplementary data 6, 7)**. The structure of the compound **(56)** is shown in Figure 4.18.



(56)

Figure 4.18: Structure of acetylated glycosydated crotoxogenin **(56)**

The ^{1}H NMR spectrum (MeOD-*d4*, 400 MHz) of the compound showed the H-21 protons at δ 5.03 (1H, dd) and δ 4.96 (1H, dd) and an olefinic proton at δ 5.92 (1H, s, H-22) as part of the lactone ring which indicated the characteristic feature of the cardenolide system. The other prominent signal indicated the presence of one upfield methyl proton at δ 0.98 (3H, s, H-18). The spectrum also indicated one downfield aldehydic proton at δ 9.57 (1H, s, H-19) and overall pattern of the ^{1}H NMR indicated that the compound was a cardenolide with one sugar unit as an anomeric proton signal was observed at δ 4.40 (1H, d, H-1′). The chemical shift at δ 2.08 (3H, s, 5′-OCOCH$_3$) confirmed the presence of an acetate group, in all likelihood attached to the sugar moiety.

The ^{13}C NMR spectrum had a total of 31 signals and the assignments were done together with the DEPT 135 spectra **(supplementary data 8)**. The methylene signal at δ 73.9 was assigned

to C-21, δ 116.4 assigned to C-22 olefinic carbon, δ 176.9 assigned to the C-23 carbonyl group and δ 177.8 assigned to the quaternary C-20, all of which confirmed the presence of the butenolide ring. Two prominent signals at δ 14.8 assigned to C-18 and at δ 206.5 assigned to C-19 indicated the position of methyl carbon and carbonyl carbon, respectively. The β hydroxyl group at C-14 was confirmed by the downfield chemical shift at δ 84.5. One anomeric carbon was revealed at δ 101.3 which was assigned to C-1′. The carbon at δ 171.2 (5′-$OCOCH_3$) revealed the presence of acetate group and carbon at δ 19.3 represent the position of methyl group.

The HSQC and COSY correlations **(supplementary data 9, 10)** led to the assignment of the proton and carbon signals. The chemical shifts for ^{1}H and ^{13}C were shown in Table 4.4.

Figure 4.19: ^{1}H NMR and ^{13}C NMR data for glycosydated crotoxigenin in MeOD-*d4*

Position	**^{13}C (ppm)**	**^{1}H (ppm), *J* (Hz)**	**COSY**	**HMBC**
1a	28.1	1.62, m,		C-9
1b		1.51, m		
2a	33.0	1.96, m,		C-3, C-10
2b		1.78, m		
3	77.8	3.74, m		
4a	25.9	1.87, m,		
4b		1.37, m		
5	41.6	1.89, m		C-9
6a	25.1	1.92, m,		C-5
6b		1.35, m		
7a	21.2	1.80, m,		C-6, C-8
7b		1.34, m		
8	35.0	1.98, m		C-5
9	35.1	1.83, m		
10	50.5	-		
11a	20.3	1.79, m,		C-9, C-10
11b		1.51 m		
12	39.3	1.88, m		C-9
12b		1.57, m		
13	49.6	-		
14	84.5	-		
15a	31.3	2.21 m,		
15b		1.72 m		
16a	26.5	2.17, m		C-14
16b		1.61, m		
17	50.4	2.80 (q, 14.6)		C-13, C-14, C-21, C-22
18	14.8	0.97, s		C-12, C-13, C-14
19	206.5	9.57, s		C-9
20	175.8	-		

21a	73.9	5.03 (dd, 18.5, 1.57),		C-22
21b		4.96 (dd, 18.1, 1.73)		
22	116.4	5.92 s		C-17, C-21, C-23
23	176.9	-		
1′	101.3	4.40 (d, 7.57)	H-2′	C-3
2′	73.6	3.17, m	H-1′, H-3′	C-1′
3′	70.2	3.36, m	H-2′	
4′	73.7	3.30, m	H-5′	
5′	76.4	3.47, m	H-4′	
6′a	63.4	4.20 (dd, 11.6, 5.14)	H-5′	C-2′, C-5′ -OCOCH$_3$
6′b		4.30 (dd, 11.6, 1.84)		
5′- OCO	171.2	-		
5′- CH$_3$	19.3	2.08 s		C-5′ -OCO

The strong HMBC correlation **(supplementary data 11)** between the proton at δ 2.80 (H-17) with C-21 (δ 73.9) and C-22 (δ 116.4) confirmed that the *α,β*-unsaturated *γ*-lactone was connected at C-17 (δ 50.4). The proton at δ 9.57 (H-19) is correlated to C-9 (δ 35.1) confirmed the position of aldehydic proton. HMBC correlations between C-5′- CH$_3$ with C-5′- OCO confirmed the presence of acetate group. HMBC correlations also established the linkages of the glycosidic bonds and the point of attachment of the saccharide chain to the aglycone based on the correlation between δ 4.40 (H-1′) to δ 77.8 (C-3) . The proton at δ 3.10 (H-2′) correlating with δ 101.3 (C-1′) and δ 73.7 (C-4′) ; proton δ 3.30 (H-4′) correlating with δ 76.48 (C-5′) and δ 63.40 (C-6′) confirmed a single sugar moiety.

The large coupling constant of the anomeric proton at (δ 4.40, 7.57 Hz) indicated the β-orientation of the glycoside moiety. There was a COSY correlation between the anomeric proton at δ 4.4 (H-1') and the proton at δ 3.17 (H-2') while another correlation was observed between the proton at δ 3.17 (H-2') and the proton at δ 3.36 (H-3'). The proton at δ 3.30 (H-4') showed a COSY correlation with proton at δ 3.47 (H-5'). The proton at δ 3.47 (H5') coupled with the two methylene protons at δ 4.20 (C6'a) and δ 4.30 (C6'b) to give a multiplet. These methylene protons also coupled which each other, hence the two sets of doublets of doublets at δ 4.20 (*dd,* 11.6 Hz, 5.14 Hz, H-6'a) and δ 4.30 (*dd,* 11.6 Hz, 1.84 Hz, H-6').

The HMBC and COSY relations are shown in the Figure 4.19.

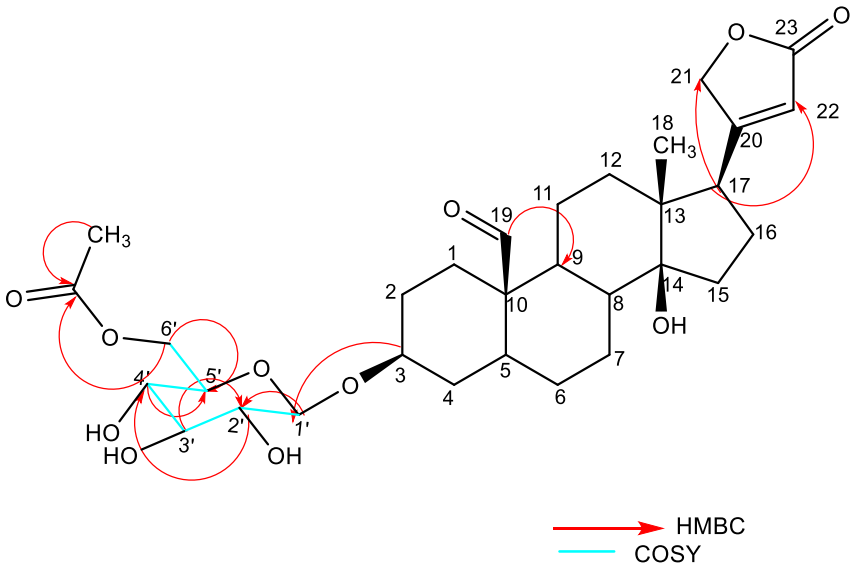


Figure 4.20: Key HMBC and COSY correlations of acetylated glycosydated crotoxogenin **(56)**

4.3.8.2. Xysmalogenin-3, β-D-glucopyranoside (57)

The pure compound (XU03) obtained from fraction AT-1-49N was identified as a known compound xysmalogenin-3, β-D-glucopyranoside **(57)** based on the NMR data, mass spectral analysis and previous published studies [2]. However, the exact and complete NMR data for this compound was not previously published. It was isolated as a white solid weighing 3.5 mg and had a molecular formula of $C_{29}H_{42}O_9$ (calculated mass 534.2828), as deduced from its pseudomolecular precursor ion at *m/z* 579.2822 $[M\text{-}HCOO]^-$ based on the QTOF mass spectrum. These data confirmed the presence of cardenolide glycoside. The formula was further confirmed by the number of protons in ^1H NMR and of carbon atoms in the ^{13}C NMR spectrum **(supplementary data 12, 13)**. The structure of the compound **(57)** is shown in Figure 4.20.

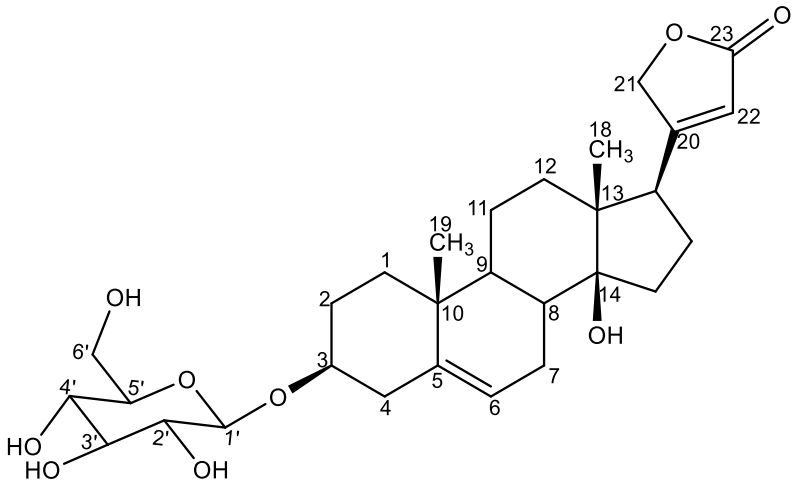


Figure 4.21: Structure of xysmalogenin-3, β-D-glucopyranoside **(57)**

The ^{1}H NMR spectrum (MeOD-*d4*, 400 MHz) of the compound showed the H-21 protons at δ 5.02 (1H, dd) and δ 4.92 (1H, dd) and an olefinic proton at δ 5.91 (1H, s, H-22) as a part of the lactone ring which indicated the characteristic feature of the cardenolide system. The spectrum showed the presence of one more double bonds at δ 5.44 (1H, s, H-6). The other prominent signals indicated one high field methyl proton at δ 0.92 (1H, s, H-18), and another high field proton at δ 1.03 (1H, s, H-19). The spectrum indicated the cardenolide with one sugar unit, with a signal for one anomeric proton at δ 4.38 (1H, dd, H-1′), where the large coupling constant (7.68 Hz) confirmed the β- coupling of the sugar unit to the aglycone.

The ^{13}C NMR spectrum had a total of 31 signals and the assignments were done together with the DEPT 135 spectra **(supplementary data 14)**. The methylene signal at δ 75.2 was assigned to C-21, δ 117.8 assigned to C-22 olefinic carbon, δ 178.2 assigned to the C-23 carbonyl group and δ 177.1 assigned to the quaternary C-20, all of which confirmed the presence of butenolide ring. Two prominent signals at δ 16.1 assigned to C-18 and at δ 19.8 assigned to C-19 indicated the position of two methyl carbons. The β hydroxyl group at C-14 was confirmed by downfield chemical shift at δ 86.3. One anomeric carbon revealed at δ 102.3 was assigned to C-1′.

The proton and carbon signals are assigned based on the HSQC **(supplementary data 15)** and COSY correlations **(supplementary data 16)**. The chemical shifts for ^{1}H and ^{13}C were shown in Table 4.5.

Figure 4.22: ^{1}H NMR and ^{13}C NMR data for xysmalogenin-3, β-D-glucopyranoside in MeOD-*d4*

Position	**^{13}C (ppm)**	**^{1}H (ppm), *J* (Hz)**	**COSY**	**HMBC**
1	38.9	2.27 m		C-3, C-5, C-6
2a	30.5	1.92 m,		
2b		1.62 m		
3	79.6	3.60 m		
4	40.1	1.52 m		
5	140.8	-		
6	122.7	5.44 m	H-7	C-8
7a	27.4	2.26 m,	H-6	C-5, C-6
7b		2.22 m		
8	38.3	1.72 m		
9	47.7	1.24 m		
10	38.3			
11	22.0	1.53 m		
12	39.4	2.44 m		
13	50.7	-		
14	86.3	-		
15	33.8	1.74m		
16	28.1	1.73 m		C-14, C-15
17	52.1	2.86 (q, 14.62)		C-12, C-21, C-22, C-23
18	16.1	0.92 s		C-12, C-13, C-14
19	19.8	1.03 s		C-5, C-9, C-10
20	177.1	-		
21a	75.2	5.02, (dd, 18.44, 1.65)		C-22, C-23
21b		4.92, (dd, 18.44, 1.65)		
22	117.8	5.91 s		C-13, C-20, C-21
23	178.2	-		
1′	102.3	4.38 (dd, 7.68)	H-2′	C-3
2′	75.1	3.14 m		C-1′, C-3′
3′	78.0	3.25 m		
4′	71.5	3.25 m		
5′	77.7	3.35 m		
6′a	63.6	3.84 m,	H-5′	C-4′, C-5,
6′b		3.65 m		

The α,β- unsaturated γ- lactone was determined to be connected at C-17 through the strong HMBC **(supplementary data 17)** correlations between proton at δ 2.86 (H-17) to δ 177.1 (C-21), δ 117.8 (C-22) and δ 178.8 (C-23). The glycosidic linkage and the point of attachment of to the genin moiety was established by HMBC correlations from δ 4.38 (H-1′) to δ 79.6 (C-3). HMBC correlations between δ 3.14 (H-2′) with δ 102.3 (C-1′) and δ 78.0 (C-3′); (δ 3.84) H-6′a with δ 77.7 (C-5′) and δ 71.5 (C-4′) confirmed the sugar unit.

A large coupling constant H1′/H2′(δ 4.38, 7.68 Hz) indicated a diaxial relationship for anomeric proton H1′ and another proton H2′. This large coupling constant also indicated the β-pyranose form for glycoside by verifying the β- orientation of glycoside. COSY correlations were also observed between δ 5.44 (H-6) and δ 2.26 (H-7). The HMBC and COSY relations are shown in Figure 4.21.

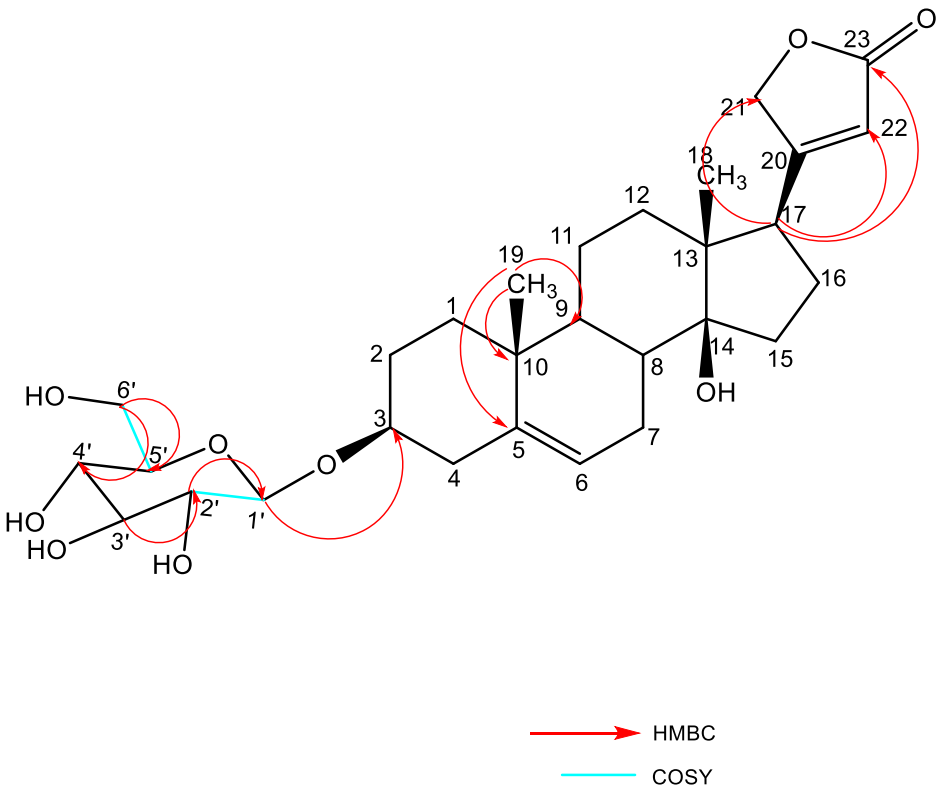


Figure 4.23: Key HMBC and COSY correlations of xysmalogenin-3,β-D-glucopyranoside **(57)**

4.3.8.3. Crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside (58)

The pure compound (XU04) obtained from fraction AT-1-49N was identified as a known compound, crotoxigenin-3-*O*-β-digitalopyranosyl-(1-4)-*O*-β-digitoxopyanoside **(58),** based on the NMR data, mass spectral analysis and previous published studies [29], [26]. It was isolated as a white amorphous powder (4.9 mg). It had a molecular formula $C_{36}H_{54}O_{12}$ (calculated mass 677.3537), as deduced from its pseudo molecular precursor ion at *m/z* 723.3582 $[M\text{-}HCOO]^-$ based on the QTOF mass spectrum. These data confirmed the presence of cardenolide glycoside. This is the first report of this cardenolide isolated from *X. undulatum*. The formula was further confirmed by the number of protons in ^1H NMR and of carbon atoms in the ^{13}C NMR spectrum **(supplementary data 18, 19)**. The structure of the compound **(58)** is shown in Figure 4.22.

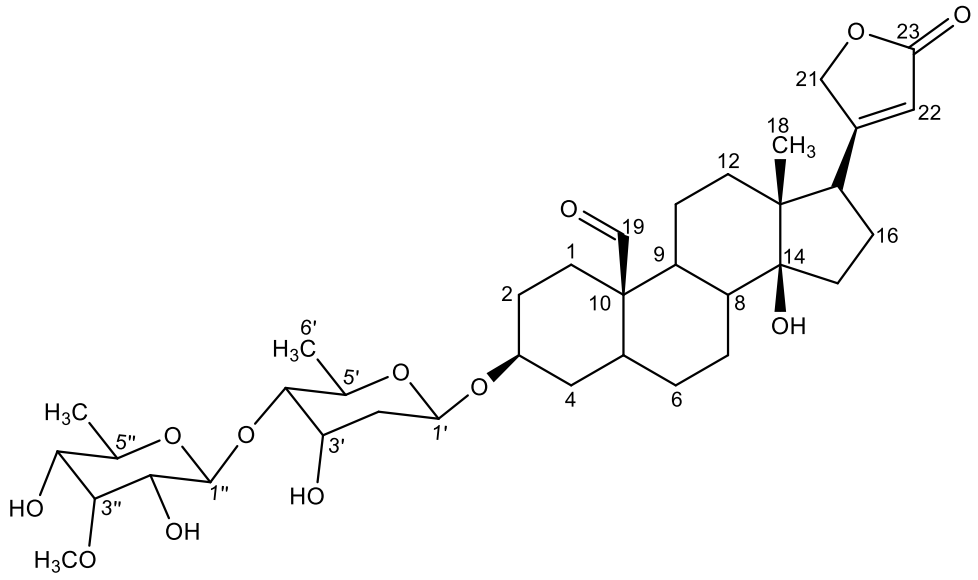


Figure 4.24: Structure of compound crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**

The ^{1}H NMR spectrum (MeOD-*d4*, 400 MHz) of the compound showed the H-21 protons at δ 5.06 (1H, dd) and δ 4.90 (1H, dd) and an olefinic proton at δ 5.94 (1H, s, H-22) as part of the lactone ring which indicated the characteristic feature of the cardenolide system. The other prominent signal indicated the presence of one upfield methyl proton at δ 0.98 (3H, s, H-18). The spectrum also indicated one downfield aldehydic proton at δ 9.44 (1H, s, H-19). The ^{1}H NMR indicated the presence of two sugar units as one anomeric proton signal at δ 4.95 (1H, d, H-1′) and other at δ 4.37 (1H, d, H-1″). The chemical shift at δ 3.48 (3H, s, 3″-OCH3) confirmed the presence of methoxy group attached to one sugar moiety. The overall ^{1}H NMR confirmed the presence of cardenolide.

The ^{13}C NMR spectrum had a total of 36 signals and the assignments were done together with the DEPT 135 spectra **(supplementary data 20)**. The methylene signal at δ 73.9 was assigned to C-21, δ 116.4 assigned to C-22 olefinic carbon, δ 176.1 assigned to the C-23 carbonyl group and δ 177.1 assigned to the quaternary C-20, all of which confirmed the presence of butenolide ring. Two prominent signals at δ 14.8 assigned to C-18 and at δ 206.7 assigned to C-19 indicated the position of methyl carbon and aldehydic carbon, respectively. The β hydroxyl group at C-14 was confirmed by downfield chemical shift δ 84.5. Two anomeric carbons were revealed at δ 95.7 assigned to C-1′ and δ 104.7 assigned to C-1″. The carbon at δ 55.9 assigned to position 3″-OCH3 revealed the presence of methoxy group.

The HSQC and COSY correlations **(supplementary data 21 and 22)** led to the assignment of the proton and carbon signals and the ^{1}H and ^{13}C NMR data of compound **(58)** corresponds to the NMR data published by [29] as shown in Table 4.6.

Table 4.4: ^{1}H NMR and ^{13}C NMR data for crotoxogenin 3-*O*-glucopyranoside in MeOD-*d4* compared to literature reports for the compound in [29] in DMSO-*d6*.

Isolated crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside (17)			**^{1}H NMR and ^{13}C NMR literature data of compound (58) in DMSO-d6**	
Position	**^{13}C (ppm)**	**^{1}H (ppm), *J* (Hz)**	**^{13}C (ppm)**	**^{1}H (ppm), *J* (Hz)**
1	31.3	2.2, m	31.4	2.42 (dt,13.5,3.5,3.5)
2	28.7	1.8, 1.6 m	32.5	2.1, 1.6 m
3	72.0	4.08 s	76.0	4.02 br,s

4	20.6	1.44 m	35.6	1.44
5	34.5	1.86 m	42.8	1.66
6	27.9	1.84, 1.53 m	30.3	1.88, 1.25 m
7	24.6	1.83, 1.59 m	28.2	1.71, 1.22 m
8	41.6	1.88 m	48.5	1.65 m
9	29.5	2.20 m	42.7	1.74 m
10	50.8	-	51.3	-
11	21.4	1.61 m	21.7	1.46,1.19 m
12	39.4	1.54, 1.63 m	39.4	1.49, 1.35 m
13	49.6	-	49.4	-
14	84.7	-	85.0	-
15	21.7	1.77 m	26.8	2.41, 1.62 m
16	26.5	2.2, 1.9 m	27.7	2.11, 1.87 m
17	50.8	2.8 (dd, 8.7, 5.9)	50.9	2.76 (dd, 9.4,5.4)
18	14.8	0.98 s	15.6	0.87 s
19	206.7	9.44 s	208.3	9.97 s
20	176.0	-	174.4	-
21	73.9	5.06 (dd,18.4, 1.68), 4.9 (dd, 18.2, 1.79)	73.5	5.01 (dd, 18.2,1.8) 4.80 (dd, 18.2,1.8)
22	116.4	5.94 s	117.8	5.89 br.s
23	177.2	-	174.6	-
1′	95.7	4.95 (dd, 18.4, 1.4)	95.3	4.94 (dd, 1.9,1.2)
2′	37.5	1.99, 1.77 m	37.3	1.97, 1.73 m
3′	67.4	4.30 (q, 6.17, 3.0)	66.8	4.24 (q, 6.4,3.2)
4′	82.7	3.26 (dd, 9.4, 2.8)	82.8	3.22 (dd, 9.3,2.9)
5′	67.1	3.87 m	67.9	3.85 m
6′	15.5	1.30 (d, 6.2)	16.5	1.36 (d, 6.5)
1′ ′	104.7	4.37 (d, 7.8)	103.8	4.32 (d, 7.8)
2′ ′	69.9	3.66 (q, 12.5, 6.7)	70.3	3.7 (dd, 9.5, 7.8)
3′ ′	82.9	3.15 (dd, 9.6, 3.1)	83.4	3.27 (dd, 9.5,3.3)
4′ ′	68.3	3.89 m	67.8	3.22 m
5′ ′	70.0	3.58 (dd, 7.5, 1.9)	70.6	3.63 (dd, 6.1, 1.4)
6′ ′	17.1	1.32 (d, 6.2)	18.2	1.32 (d, 6.2)
3′ ′ -OCH3	55.9l	3.48 s	57.7	3.52 s

The strong HMBC correlation **(supplementary data 23)** from proton at δ 2.8 (H-17) with δ 73.9 (C-21), δ 116.4 (C-22) and δ 177.0 (C-23) indicated that the *α,β*-unsaturated *γ*-lactone to be connected at C-17 (δ 50.5); protons at δ 5.02 and δ 4.92 (C-21) correlated with δ 175.8 (C-20). The proton at δ 9.44 (H-19) correlated to δ 29.5 (C-9) and δ 34.5 (C-5) confirmed the position of aldehydic proton.

HMBC correlations also established the linkages of the glycosidic bonds and the point of attachment of the saccharide chain to the aglycone by a δ 4.95 (H-1′) correlation to δ 4.02 (H-

3) and a proton at δ 4.37 (H-1″) correlation to δ 3.26 (H-4′) which indicated a 1-4 linkage between the sugar units.

The two anomeric protons δ 4.95 (br dd) and δ 4.37 (d, 7.8Hz) indicated the presence of two sugar moieties (based on the large coupling you can indicate the apha or beta linkage). The COSY correlations δ 4.95 (H-1′) – δ 1.77 (H_2-2'); δ 4.30 (H-3′) – δ 3.26 (H-4′); δ 3.89 (H-5′) – δ1.33 (H-6′) confirmed the sugar to be digitoxose and the COSY correlations of the second sugar moiety, δ 4.37 (H-1″) – δ 3.58 (H-2″); δ 3.14 (H-3″) – δ 3.87 (H-4″); δ 3.66 (H-5″) – δ 1.30 (H-6″) confirmed the sugar to be digitalose. The chemical shifts for the digitoxose and digitalose were confirmed based the comparison to literatutre data [30],[31]. The HMBC and COSY correlations are shown in Figure 4.23.

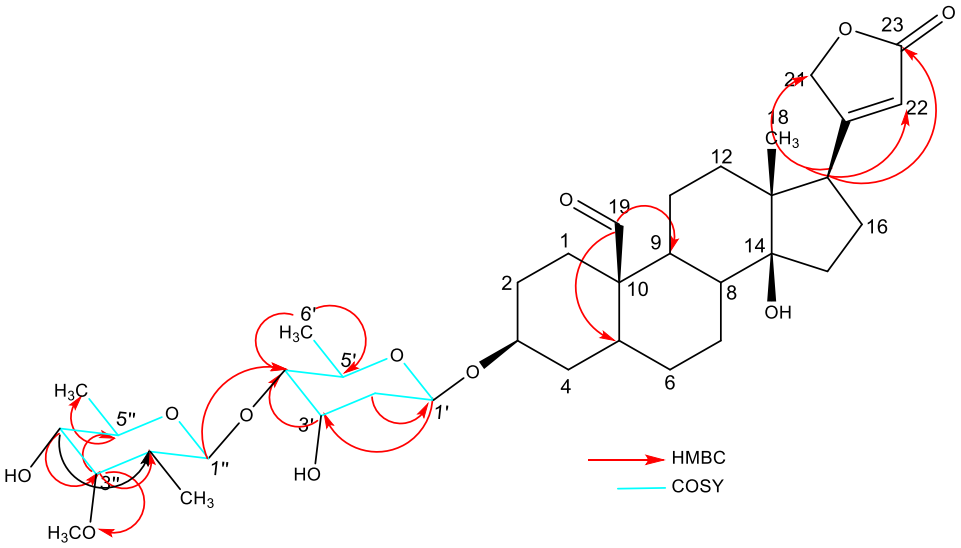
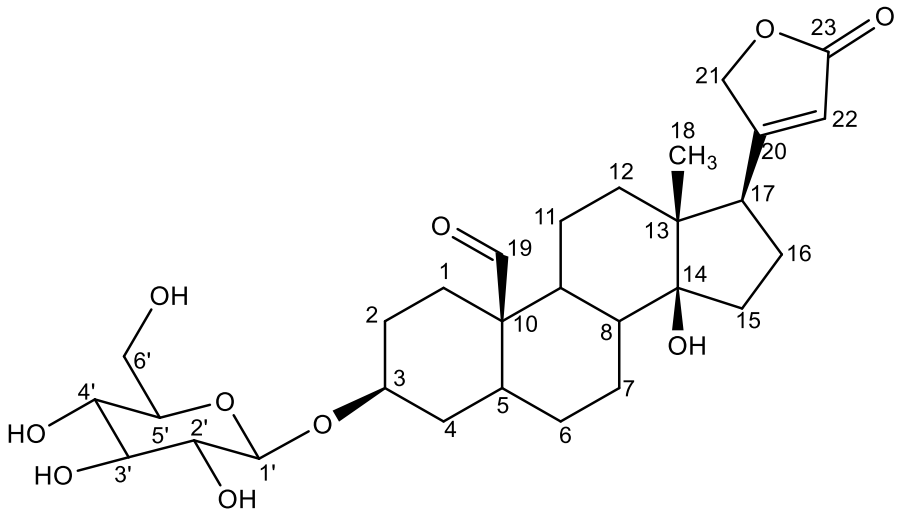


Figure 4.25: Key HMBC and COSY correlations of crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**

Crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside, a cardenolide glycoside was originally isolated from *Alafia* sp. belongs to family Apocynaceae, a traditional medicinal plant from South of Madagascar for which the leaves of the plant are used traditionally for the treatment of malaria. This study reports the first isolation of the compound from the leaves of *X.undulatum.*

4.3.8.4. Crotoxigenin 3-O-glucopyranoside (59)

The pure compound (XU07) obtained from fraction AT-1-49N was identified as a known compound crotoxigenin 3-*O*-glucopyranoside **(59)** based on the NMR data, mass spectral analysis and previous published studies. It was isolated as a white crystalline solid (5 mg). It had a molecular formula of $C_{29}H_{42}O_{10}$ (calculated mass 552.2934), as deduced from its pseudomolecular precursor ion at *m/z* 595.2758 $[M\text{-}HCOO]^-$ based on the QTOF mass spectrum. These data confirmed the presence of cardenolide glycoside. The formula was further confirmed by the number of protons in ^1H NMR and of carbon atoms in the ^{13}C NMR spectrum **(supplementary data 24, 25)**. The structure of the compound **(59)** is shown in Figure 4.24.



(59)

Figure 4.26: Structure of compound crotoxigenin 3-O-glucopyranoside **(59)**

The ^1H NMR spectrum (MeOD-*d4*, 400 MHz) of the compound showed the H-21 protons at δ 5.04 (1H, dd) and δ 4.92 (1H, dd) and an olefinic proton at δ 5.92 (1H, s, H-22) as part of the lactone ring which indicated the characteristic feature of the cardenolide system. The other prominent signal indicated the presence of one high field methyl proton at δ 0.95 (3H, s, H-18). The spectrum also indicated one downfield aldehydic proton at δ 9.54 (1H, s, H-19) and

overall pattern of the ^{1}H NMR indicated that the compound was a cardenolide with one sugar unit as an anomeric proton signal was observed at δ 4.42 (1H, d, H-1′).

The ^{13}C NMR spectrum had a total of 29 signals and the assignments were done together with the DEPT 135 spectra **(supplementary data 26)**. The methylene signal at δ 73.6 was assigned to C-21, δ 116.4 assigned to C-22 olefinic carbon, δ 176.9 assigned to the C-23 carbonyl group and δ 175.8 assigned to the quaternary C-20, all of which confirmed the presence of butenolide ring. Two prominent signals at δ 16.1 assigned to C-18 indicated a methyl carbon and at δ 206.8 assigned to C-19 indicated the position of aldehydic carbon. The β hydroxyl group at C-14 was confirmed by downfield chemical shift δ 84.5. One anomeric carbon at δ 100.8 was assigned to C-1′.

The HSQC and COSY **(supplementary data 27, 28)** correlations led to the assignment of the proton and carbon signals. The ^{1}H and ^{13}C NMR data of compound **(59)** ompared favorably to the NMR data based on the literature data for the compound [32] shown in Table 4.7.

Table 4.5: ^{1}H NMR and ^{13}C NMR data for desglucouzarin in MeOD-*d4* compared to those reported by [32] in DMSO-*d6*.

Isolated Crotoxogenin 3-O-glucopyranoside (18)			**^{1}H NMR and ^{13}C NMR literature data for compound (59) in DMSO-d6**	
Position	**^{13}C (ppm)**	**^{1}H (ppm), *J* (Hz)**	**^{13}C (ppm)**	**^{1}H (ppm), *J* (Hz)**
1	28.0	1.83 m, 1.54 m	28.03	1.85 m,1.50 m
2	31.3	1.85 m	30.36	1.80 m
3	77.0	3.82 m	76.78	3.05 m
4	25.9	1.85 m, 1.33 m	30.20	1.82 m, 1.14 m
5	41.6	1.89 m	42.03	1.45 m
6	24.9	1.76 m	26.27	1.74 m
7	20.3	1.48 m	21.41	1.60 m
8	35.2	1.96 m	47.57	1.43 m
9	35.0	1.79 m	35.49	1.81 m
10	50.4	-	49.99	-
11	21.2	1.82 m, 1.32 m	21.41	1.60 m
12	39.4	1.51 m	42.18	1.44 m
13	49.6	-	49.19	-
14	84.5	-	83.37	-
15	32.9	1.71 m	31.59	1.94 m, 1.55 m
16	26.5	2.16 m	27.17	2.13 m
17	50.5	2.8 (q, 14.6)	51.23	2.72 m
18	14.8	0.95 s	15.54	0.69 s

19	206.8	9.54 s	209.66	9.94 s
20	175.8	-	173.96	-
21	73.7	5.04 (d, 1.65), 4.92 (dd, 18.5, 1.74)	73.48	4.90 dd
22	116.4	5.91 s	116.38	5.89 s
23	176.9	-	176.31	-
1′	100.8	4.42 d (7.80)	100.71	4.20 d
2′	73.9	3.14 (dd, 9.03, 1.07)	75.75	3.59 m
3′	76.5	3.26	73.23	2.85 m
4′	70.3	3.27 m	70.11	3.02 m
5′	76.7	3.35m	76.78	3.06 m
6′	61.4	3.87 m, 3.65 m	61.14	3.64m, 3.40m

The strong HMBC correlation **(supplementary data 29)** of proton at H-17 (δ 2.8) with δ 73.7 (C-21) , δ 116.4 (C-22) and δ 176.9 (C-23) established that the *α,β*-unsaturated *γ*-lactone to be connected at C-17 (δ 50.53) ; the methyl protons at δ 0.95 (H-18) correlated to δ 39.4 (C-12), δ 49.6 (C-13) and δ 84.5 (C-14) which confirmed the position of the methyl group at C-18; the proton δ 9.54 (H-19) correlated with δ 35.0 (C-9) which indicated the position of the aldehydic group at C-19.

Correlations between proton δ 4.42 (H-1′) with δ 77.0 (C-3) established the glycosidic linkage to be on psition C-3 of the aglycone; proton δ 3.14 (H-2′) with δ 76.5 (C-4′) and δ 100.8 (C-1′) ; proton δ 3.35 (H-3′) with δ 70.3 (C-4′) and δ 3.27 (H-5′); proton δ 3.26 (H-4′) with δ 70.3 (C-5′) ; proton δ 3.27 (H-5′) with δ 76.5 (C-4′) and δ 61.4 (C-6′); protons at δ 3.68 and δ 3.48 (H-6′) with δ70.3 (C-5′) confirmed the presence of the sugar moiety.

A large coupling constant H1′/H2′ (δ 4.42, 7.8 Hz) indicated a diaxial relationship for anomeric proton H1′ and proton H2′. This large coupling constant also indicated the β- glycosidic linkage. The second large coupling constant H2′/H3′ (δ 3.14, 9.07) confirmed that H2′ was also axial. The small coupling constant H3′/H4′ indicated the proton at H′4 to be equatorial assignment. COSY correlations were also observed between H5′ (δ 3.29) with two protons at H6′a (δ 3.87) and H6′b (δ 3.67). These two methylene protons are also coupled to each other and gave a set of doublet of doublet at δ 3.67 (12.0Hz, 5.2Hz) while at δ 3.87, the spin multiplicity was not clear due to overlapping of signals. The HMBC and COSY relations are shown in Figure 4.25.

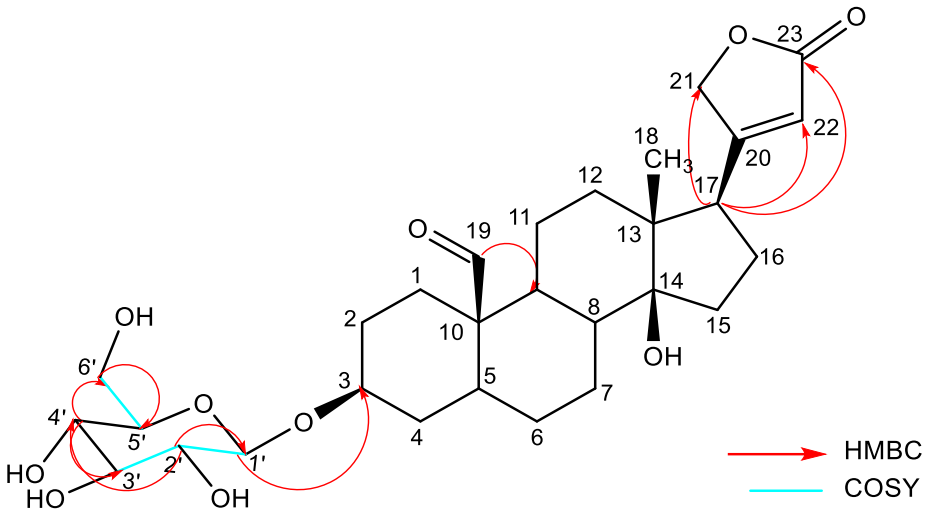
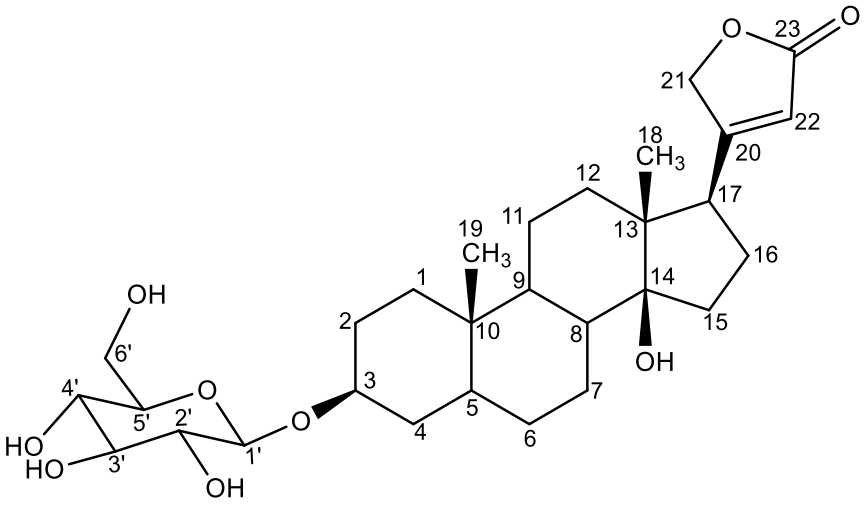


Figure 4.27: Key HMBC and COSY correlations of crotoxigenin 3-O-glucopyranoside **(59)**

4.3.8.5. Desglucouzarin (60)

The pure compound (XU08) obtained from fraction AT-1-49N was identified as a known compound desglucouzarin **(60)** based on the NMR data, mass spectral analysis and previous studies [25]. It was isolated as a white crystalline solid (4 mg). It had a molecular formula of $C_{29}H_{44}O_9$ (calculated mass 536.2985), as deduced from its pseudomolecular precursor ion at *m/z* 581.2965 $[M\text{-}HCOO]^-$ based on the QTOF mass spectrum. This data confirmed the presence of cardenolide glycoside. The formula was further confirmed by the number of protons in ^1H NMR and of carbon atoms in the ^{13}C NMR spectrum **(supplementary data 30, 31)**. The structure of the compound **(60)** is shown in Figure 4.26.



(60)

Figure 4.28: Structure of desglucouzarin **(60)**

The ^{1}H NMR spectrum (MeOD-*d4*, 400 MHz) of this compound showed the protons δ 5.01 and δ 4.95 (2H, dd, H-21) and an olefinic proton δ 5.92 (1H, s, H-22) attached to the lactone ring indicated the characteristic feature of cardenolide system. The other prominent signal of one high field methyl proton at δ 0.96 (3H, s, H-18), was suggestive of cardenolide nucleus together with the other high field proton at δ 0.92 (3H, s, H-19). The spectrum indicated a cardenolide with one sugar unit in which anomeric proton signal was observed at δ 4.40 (1H, dd, H-1′), where the large coupling constant (7.80 Hz) confirmed the β-coupling of the sugar unit to the aglycone.

The ^{13}C NMR spectrum had a total of 29 signals and the assignments were done together with the DEPT 135 spectra **(supplementary data 32)**. The methylene signal at δ 73.7 was assigned to C-21, δ 116.8 assigned to C-22 olefinic carbon, δ 176.1 assigned to the C-23 carbonyl group and δ 177.1 assigned to the quaternary C-20, all of which confirmed the presence of butenolide ring. Two prominent signals at δ 22.3 assigned to C-18 and at δ 15.1 assigned to C-19 indicated the position of two methyl carbons. The β hydroxyl group at C-14

was confirmed by downfield chemical shift at δ 85.3. One anomeric carbon at δ 102.3 was assigned to C-1′.

The HSQC and COSY correlations **(supplementary data 33, 34)** led to the assignment of the proton and carbon signals. The ^{1}H and ^{13}C NMR data of compound. The ^{1}H and ^{13}C NMR data of compound **(60)** and the corresponding published data for the compound [25] is shown in Table 4.8.

Table 4.6: ^{1}H NMR and ^{13}C NMR data for desglucouzarin **(60)** in MeOD-*d4* compared to that which is reported in DMSO-*d6* [25]

Isolated desglucouzarin (19)			**^{1}H NMR and ^{13}C NMR literature data of compound (60) in DMSO-*d6***	
Position	**^{13}C (ppm)**	**^{1}H (ppm), *J* (Hz)**	**^{13}C (ppm)**	**^{1}H (ppm), *J* (Hz)**
1	34.6	1.07, 1.82 m	36.6	0.92,1.75 m
2	33.9	1.65, 1.89 m	29.0	1.75 m
3	78.2	3.80 m	76.4	3.05 m
4	32.1	1.77 m	33.9	1.14, 1.63 m
5	41.6	1.45 m	43.7	0.95 m
6	26.9	1.41 m	28.5	1.13, 1.23 m
7	26.6	1.91 m	27.2	0.92, 1.94 m
8	41.8	1.67 m	40.8	1.42 (dt, 2.93)
9	36.2	1.83 m	49.4	0.87 (dt, 2.93)
10	34.7	-	35.4	-
11	20.8	1.49, 1.83 m	20.8	1.18, 1.4 m
12	39.6	1.54 m	38.8	1.3, 1.42 m
13	49.8	-	48.6	-
14	85.3	-	83.6	-
15	31.9	1.79, 2.2 m	32.1	1.55t, 1.92t
16	26.4	2.22,1.86 m	26.3	1.75, 1.96 m
17	50.8	2.88 (dd, 9.1, 5.9)	50.1	2.72 (dd, 9.6, 5.4)
18	22.3	0.96 s	15.7	0.72 s
19	15.1	0.92 s	12.0	0.75 s
20	177.1	-	176.4	-
21	73.7	4.95, 5.01 (dd, 1.49, 18.4)	73.2	4.88 m, 4.95 (dd, 1.47, 18.32)
22	116.8	5.92 s	116.2	5.89 s
23	176.1	-	173.9	-
1′	100.8	4.4 (d, 7.80)	100.6	4.2 (d, 8.06)
2′	73.9	3.18 (dd, 9.07, 1.12)	73.5	2.85 m
3′	76.7	3.36 m	76.8	3.10 m
4′	70.2	3.28 m	70.1	3.01 m
5′	76.5	3.28 m	76.4	3.50 m
6′	61.3	3.87 m, 3.67 (ddd, 1.9, 5.2, 12.0)	61.1	3.35 m, 3.65 (ddd, 2, 5.4, 12.0)

The strong HMBC correlation **(supplementary data 35)** of proton at δ 2.88 (H-17) with δ 73.7 (C-21), δ 116.8 (C-22) and δ 176.1 (C-23) indicated that the *α,β*-unsaturated *γ*-lactone was connected at δ 50.8 (C-17). The methyl proton at δ 0.96 (H-19) correlated to δ 41.6 (C-5) and δ 34.7 (C-10) which confirmed the position of the methyl protons at C-19. Correlations between the anomeric proton H1′ (δ 4.4) with δ 78.2 (C-3) established the glycosidic linkage and confirmed that the sugar unit is attached to C-3.

A large coupling constant H1′/H2′ (δ 4.4, 7.8 Hz) indicated a diaxial relationship for anomeric proton H1′ and other proton H2′. This large coupling constant also indicated the β-pyranose form for glycoside by verifying the β-orientation of glycoside. The second large coupling constant H2′/ H3′ (9.07 Hz) confirmed that H2′ was also axial. The small coupling constant H3′/H4′ indicated the proton at H4′ to be equatorial assignment. COSY correlations were also observed between δ 3.29 (H5′) with two protons at δ 3.87and δ 3.67 (H6′). These two methylene protons also coupled to each other and gave a set of doublet of doublet at δ 3.67 (12.0 Hz, 5.2 Hz) while at δ 3.87, the spin multiplicity was not clear due to overlap of signals. The HMBC and COSY relations are shown in Figure 4.27.

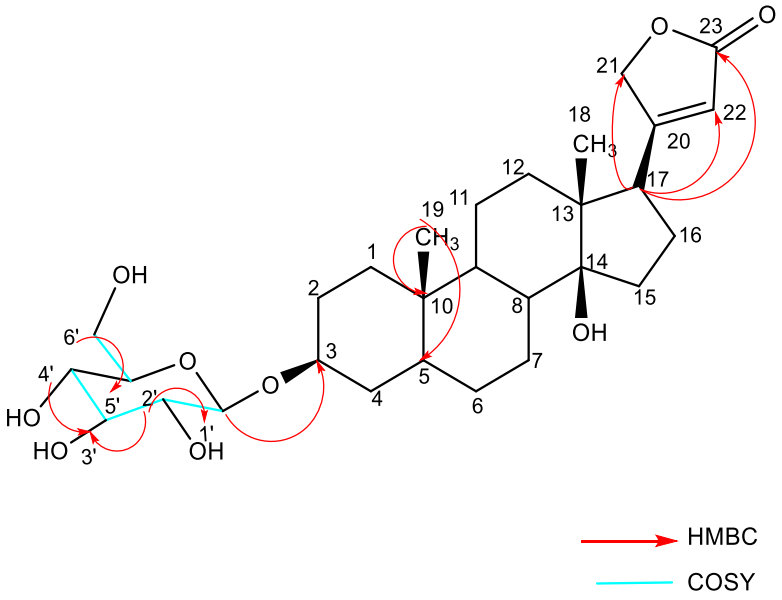


Figure 4.29: Key HMBC and COSY correlations of desglucouzarin **(60)**

The compound was previously obtained from *Asclepias subulata*, *Asclepias syriaca* L. and *Asclepias linaria* Cav., and showed significant antiproliferative activity on MCF-7, T47D, SK-

BR-3 and Hs578T cells. It showed a strong antiproliferative activity with an IC_{50} value 0.90 ± 0.02 [32]. This compound was also isolated from aerial parts of *Pergularia tomentosa* L. [25]. The compound was also previously reported in *X. undulatum* [2].

4.9. The effect of the isolated compounds on Aβ42 production

The four compounds acetylated glycosydated crotoxigenin **(56)**, xysmalogenin-3, β-D-glucopyranoside **(57)**, crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**, desglucouzarin **(60)** isolated from *X. undulatum* leaf extract significantly decreased the Aβ42 levels as compared to the negative control by 75.04 ± 0.85%, 74.66 ± 0.1%, 65.55 ± 0.19% and 69.15 ± 0.25%, respectively as shown in Figure 4.28. The compound crotoxigenin 3-*O*-glucopyranoside **(59)** did not show any significant reduction in Aβ42 levels. The results clearly indicated that the isolated from compounds from the active fractions were responsible for the effect of Aβ42 production in *X.undulatum* extract.

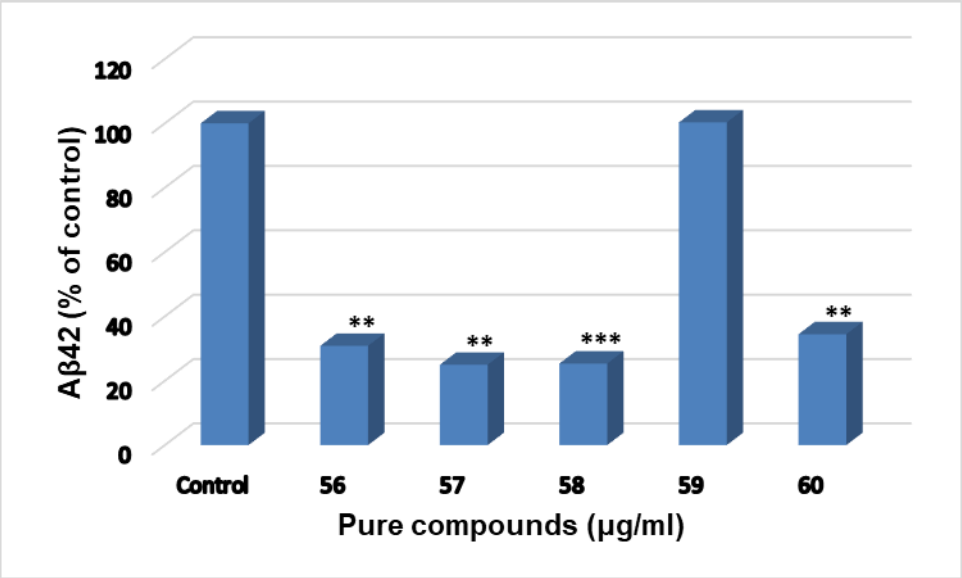


Figure 4.30: Change in the level Aβ42 by compounds of *X. undulatum* extract. Cells were incubated with 10µM concentrations of compounds acetylated glycosydated crotoxogenin **(56),** xysmalogenin-3, β-D-glucopyranoside **(57)**, crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**, crotoxigenin 3-O-glucopyranoside **(59)** and desglucouzarin **(60)** for 8 h. The level of Aβ42 was measured from the conditioned media by using specific ELISA. The level of Aβ42 was decreased by compounds **56**, **57**, **58** and **60** (n=4), **, $P < 0.01$; *** , $P < 0.001$.

The active compounds were then treated at different concentrations in APPsw-transfected HeLa cells for 8 h. Compound **(56)** significantly decreased the levels of Aβ42 by 35.5 ± 2.1, 62.83 ± 1.6 %, 71.8 ± 2.4% and 76.9 ± 4.4% at concentrations 1, 2.5, 5 and 10 μM, respectively (Figure 4.29). Compound **(57)** significantly decrease the Aβ42 levels by 24.7 ± 0.0, 37.6 ± 0.1, 49.5 ± 0.0 and 52.3 ± 1.9 at 1, 2.5, 5 and 10 μM respectively (Figure 4.30). Compound **(58)** significantly decreased the levels of Aβ42 by 19.1± 1.2 %, 48.6 ± 7.2%, 68.1 ± 3.1% and 74.5 ± 0.9% at concentrations 0.025, 0.05, 0.1 and 0.25 μM, respectively (Figure 4.31) while the levels of Aβ42 was significantly decreased by 31.2 ± 6.2%, 63.8 ± 1.1%, 73.2 ± 3.2% and 74.9 ± 5.4% at 1, 2.5, 5 and 10 μM for compound **(60)**, respectively (Figure 4.32).

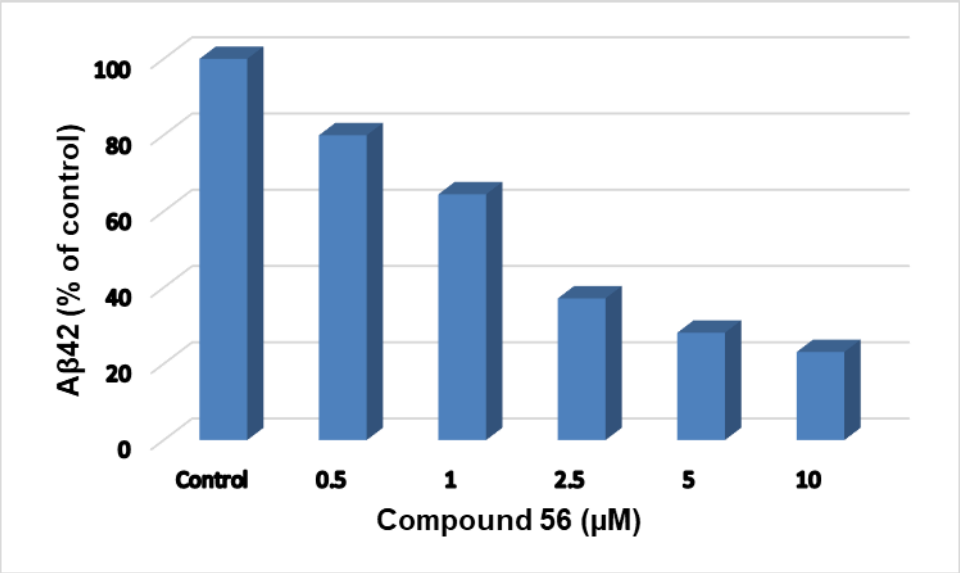


Figure 4.31: Change in the level Aβ42 by acetylated glycosydated crotoxogenin **(56)**. Cells were incubated with indicated concentrations of compound **56** for 8 h. The level of Aβ42 was measured from the conditioned media by using specific ELISA. The level of Aβ42 was decreased by compound **56** in a dose dependant manner (n=4), **, P < 0.01; *** , P < 0.001.

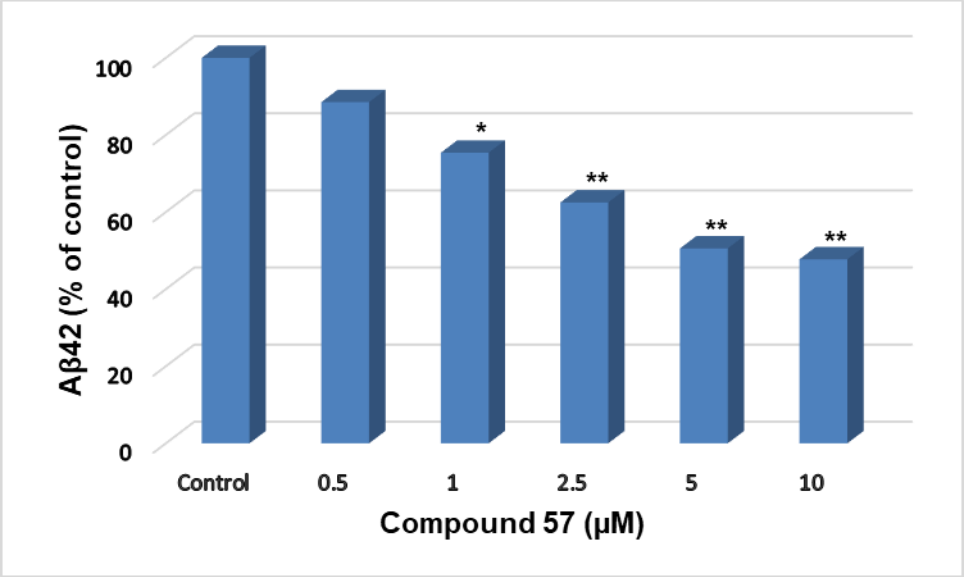


Figure 4.32: Change in the level Aβ42 by acetylated glycosydated crotoxogenin **(57)**. Cells were incubated with indicated concentrations of compound **57** for 8 h. The level of Aβ42 was measured from the conditioned media by using specific ELISA. The level of Aβ42 was decreased by compound **57** in a dose dependant manner (n=4), **, P < 0.01; *** , P < 0.001.

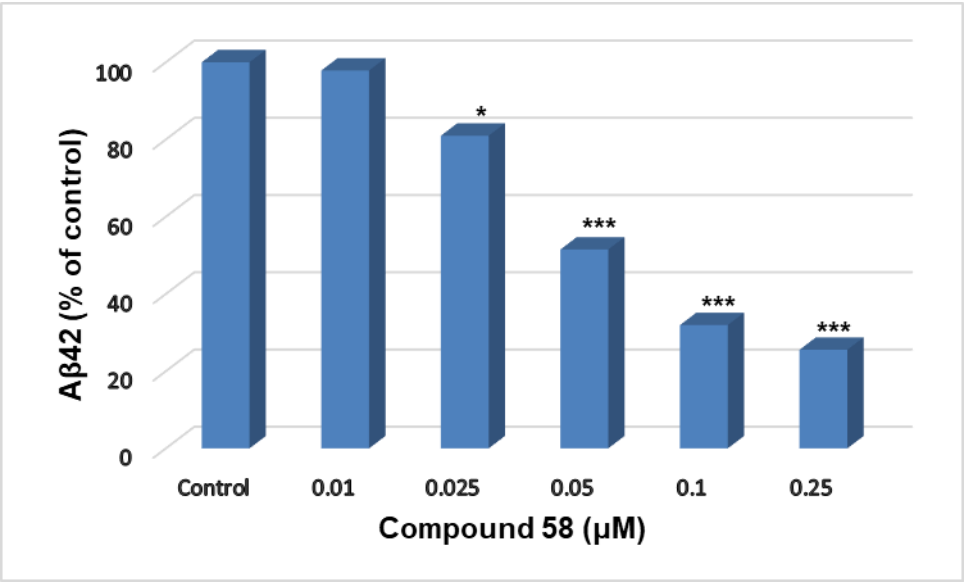


Figure 4.33: Change in the level Aβ42 by crotoxigenin-3-O-β-digitalopyranosyl-(1-4)-O-β-digitoxopyanoside **(58)**. Cells were incubated with indicated concentrations of compound **58** for 8 h. The level of Aβ42 was measured from the conditioned media by using specific ELISA. The level of Aβ42 was decreased by compounds **58** in a dose dependant manner (n=4), **, P < 0.01; *** , P < 0.001.

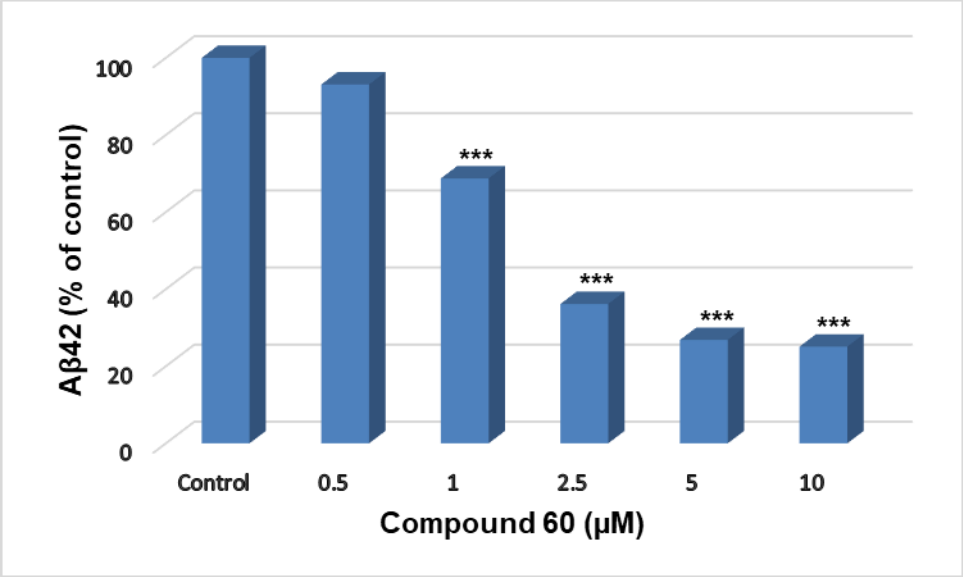
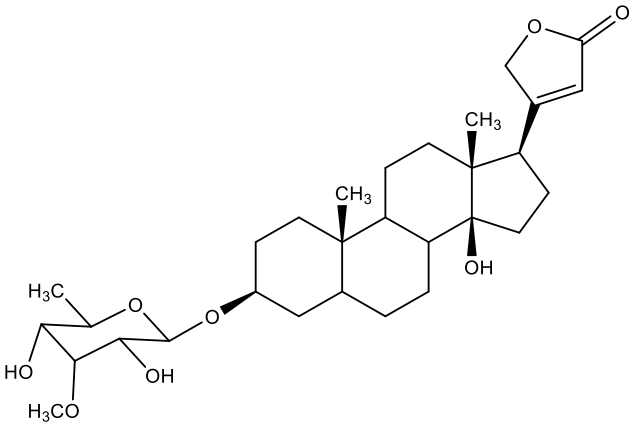


Figure 4.34: Change in the level Aβ42 by compound desglucouzarin **(60)**. Cells were incubated with indicated concentrations of compound **60** for 8 h. The level of Aβ42 was measured from the conditioned media by using specific ELISA. The level of Aβ42 was decreased by compounds **60** (n=4), **, P < 0.01; *** , P < 0.001.

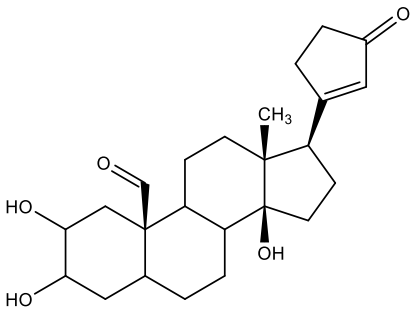
All these pure compounds acetylated glycosydated crotoxigenin **(56)**, xysmalogenin-3, β-D-glucopyranoside **(57)**, crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**, crotoxigenin 3-*O*-glucopyranoside **(59)** and desglucouzarin **(60)** isolated from the leaves of *X. undulatum* have examined for the first time for Aβ42 reduction in the present study. Four of the compounds, except crotoxigenin 3-*O*-glucopyranoside **(59),** showed significant activity. Since all the compounds have the butenolide ring attached to C-17 of the aglycone as part of their structures, it appears that this moiety may not be the pharmacophore responsible for the activity especially the fact that compound **(59)** had no activity at the highest test concentration of 10 μM (Figure 4.28). This is suggestive that the remaining units of the molecules i.e. the aglycone unit and the carbohydrate units a play a more significant the role in the reduction of Aβ42. This is promising as any future work should involve the removal of the butenolide ring and testing the glycosidated aglycones for their potential to reduce Aβ42. The added advantage of this is that the cardiotoxicity could be removed as it is known that the butenolide ring is mainly responsible for toxicity [33], making the compounds more pharmaceutically attractive. The compound **(58)**, crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-

digitoxopyanoside was for the first time isolated from this plant while compound **(56)**, acetylated glycosydated crotoxogenin has been reported as the novel cardenolide. The compound **(58)** was the most promising of the four active compounds since as it was tested at a very low concentrations (test concentration of 0.025 μM) compared to the other compounds (lowest test concentration 1 μM) and showed significant reduction of Aβ42 at 0.025 μM, at least 40 time more potent than any of the other cardenolides.

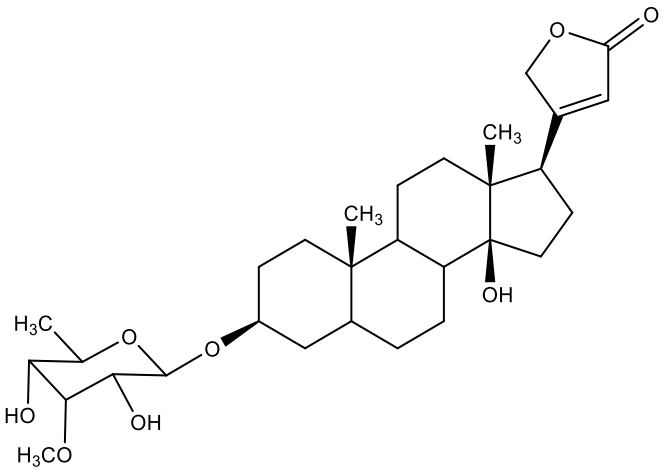
In a brain slice assay for ischemic stroke, neriifolin **(60)** showed a significant neuroprotective effect included good delayed therapeutic potential exceeding 6 h as compared to no delay [34]. In a docking model generated for identifying the AChE inhibition, a cardenolide, calotropagenin **(61)** isolated from *Calotropis procera*, was found to exhibit significant binding affinity towards the AChE with a significant Grid based Ligand Docking with Energetics (GLIDE) score of -13.07 as compared to the GLIDE score of -11.23 for Aricept AChE complex [35]. In another study, a structurally similar cardenolide, odoroside H **(62)**, isolated from the leaves of *Nerium oleander* showed CNS depressant activity in *in vivo* studies. These compounds showed decrease in motor activity in mice at a dosage of 25 mg/kg [36].



(60)



(61)



(62)

Figure 4.35: Structure of neriifolin **(60)**, calotropagenin **(61)** and odoroside H **(62)**

Neriifolin **(60)** and odoroside **(62)** are structurally similar to the cardiac glycosides such as digitoxin, digoxin and ouabain which are medically recognised because of their Na^+/K^+-ATPase inhibition action [34]. The isolated compounds in the present study also have similar skeletal structures. Therefore, it indicates that the isolated compounds in the present study can also be able to inhibit the AChE and can be examined for the *in vivo* animal models to check its ability to be develop into an Alzheimer's disease.

There is limited research showing the efficacy of cardenolides for the treatment of Alzheimer's disease with no reported research showing activity of cardenolides towards the reduction of Aβ42 which is an important factor for the treatment of Alzheimer's disease. The current study has shown for the first time that cardenolides from *X. undulatum* can significantly reduce the levels of Aβ42.

4.4. Conclusion

X. undulaum has many traditional uses such as treatment of diarrhoea, dysentery, dysmenorrhoea, stomach cramps, wounds and indigestion. *X.undulatum* is a commercialised plant in Europe for the treatment of diahrroea and traded as Uzara. Although the plant has

been extensively researched and well-documented for its antidepressant effect, acetylcholinesterase inhibitory effects and selective serotonin reuptake inhibitor (SSRI) activity, there is only limited literature available showing its inhibitory effects for the reduction of Aβ42, an important factor in the tratment of Alzheimer's disease. In the present study, the DCM:MeOH leaf extract significantly reduced the Aβ42 production by 49.8 ± 4.0%, 71.4 ± 1.6% and 72.9 ± 1.1% at concentrations 1, 5 and 10 µg/ml, respectively. To the best of our knowledge, this study is the first report for the reduction of Aβ42 as a treatment for Alzheimer's disease. The active leaf extract was then analysed on UPLC-QTOF-MS to identify the main classes of compounds. MS analysis revealed the presence of cardenolides and flavonoids. The DCM:MeOH leaf extract was subjected to bioassay guided fractionation using silica gel column chromatography. A total of 15 fractions were collected after the TLC analysis and combining fractions with common compounds based on their Rf values. Of these, two fractions AT-1-49N and AT-1-49O were found to be active towards the redution of Aβ42. These fractions were analysed by UPLC-QTOF-MS to identify the active ingredients. Cardenolide glycosides identified in both the active fractions through the MS analysis. These fractions were further purified using mass directed purification using preparative HPLC. Three peaks labelled as XU01 (637.2850), XU03 (579.2822) and XU04 (723.3583) and two peaks XU07 (595.2758) and XU09 (581.2965) were collected from the two active fractions. The structures of these compounds were elucidated using mass spectral analysis and ^{1}H, ^{13}C NMR and 2D NMR NMR. The compounds were identified as acetylated glycosydated crotoxogenin **(56),** xysmalogenin-3, β-D-glucopyranoside **(57),** crotoxigenin-3-O-β-digitalopyranosyl-(1-4)-O-β-digitoxopyanoside **(58),** crotoxigenin-3-O-glucopyranoside **(59)** and desglucouzarin **(60)** and were tested for Aβ42 reduction. The four cardenolide glycosides, acetylated glycosydated crotoxogenin **(56)**, xysmalogenin-3, β-D-glucopyranoside **(57)**, crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**, desglucouzarin **(60)** significantly decreased the Aβ42 levels at 10µM as compared to the negative control by 75.04 ± 0.85%, 74.66 ± 0.1%, 65.55 ± 0.19% and 69.15 ± 0.25%, respectively. The compound crotoxigenin-3-O-glucopyranoside **(59)** did not show any reduction in Aβ42 levels. Dose response studies

showed that the Aβ42 reduction for compound **(58)** potently reduced Aβ42 levels at a low concentration of 0.025 μM clearly indicating that it was the most potent compoound of all the isolated cardenolide glycosides. All compounds were structurally similar and contained the characacteristic lactone ring. As compound **(59)** was inactive, this indicated that the lactone ring may not be repsonsible for Aβ42 reduction. Further research could focus on the reduction of the lactone ring in the compound **(58)** and further bioassaying of the analogues to measure their Aβ42 reduction potential. The overall bioassay-guided fractionation was succesul for the the leaf extract of *X.undulatum* as the pure active ingredients were isolated and identified, often a major challenge to the natural product chemist.

Refrences

1. Van Wyk B-E, Gericke N: **People's plants: A guide to useful plants of Southern Africa**: Briza Publications; 2000.
2. Vermaak I, Enslin GM, Idowu TO, Viljoen AM: **Xysmalobium undulatum (uzara)–review of an antidiarrhoeal traditional medicine**. *Journal of ethnopharmacology* 2014, **156**:135-146.
3. Schmelzer GH, Gurib-Fakim A: **Plant resources of tropical Africa 11 (2): medicinal plants 2**: PROTA Foundation; 2013.
4. Hutchings A, van Staden J: **Plants used for stress-related ailments in traditional Zulu, Xhosa and Sotho medicine. Part 1: Plants used for headaches**. *Journal of Ethnopharmacology* 1994, **43**(2):89-124.
5. Van Wyk B-E, Van Oudtshoorn B, Gericke N: **Medicinal plants of South Africa**, 2nd ed. edn. Pretoria :: Briza Publications; 2009.
6. Amusan OO, Sukati NA, Dlamini PS, Sibandze FG: **Some Swazi phytomedicines and their constituents**. *African Journal of Biotechnology* 2007, **6**(3).
7. Pujol J: **Nature Africa: The Herbalist Handbook: African Flora, Medicinal Plants**. *Natural Healers Foundation, Durban* 2000.
8. Ghorbani M, Kaloga M, Frey H-H, Mayer G, Eich E: **Phytochemical Reinvestigation of Xysmalobium undulatum Roots (Uzara) 1**. *Planta medica* 1997, **63**(04):343-346.
9. Nielsen ND, Sandager M, Stafford GI, van Staden J, Jäger AK: **Screening of indigenous plants from South Africa for affinity to the serotonin reuptake transport protein**. *Journal of Ethnopharmacology* 2004, **94**(1):159-163.
10. Adewusi EA, Moodley N, Steenkamp V: **Antioxidant and acetylcholinesterase inhibitory activity of selected southern African medicinal plants**. *South African Journal of Botany* 2011, **77**(3):638-644.
11. Pedersen ME, Szewczyk B, Stachowicz K, Wieronska J, Andersen J, Stafford GI, van Staden J, Pilc A, Jäger AK: **Effects of South African traditional medicine in animal models for depression**. *Journal of ethnopharmacology* 2008, **119**(3):542-548.

12. Matharu B, Gibson G, Parsons R, Huckerby TN, Moore SA, Cooper LJ, Millichamp R, Allsop D, Austen B: **Galantamine inhibits β-amyloid aggregation and cytotoxicity**. *Journal of the neurological sciences* 2009, **280**(1-2):49-58.
13. Li Q, Wu D, Zhang L, Zhang Y: **Effects of galantamine on β-amyloid release and beta-site cleaving enzyme 1 expression in differentiated human neuroblastoma SH-SY5Y cells**. *Experimental gerontology* 2010, **45**(11):842-847.
14. Meve U, Liede S: **Asclepiadoideae and Periplocoideae (Apocynaceae sl) of the Thunberg herbarium**. *Nordic Journal of Botany* 1999, **19**(2):129-138.
15. Thunberg CP: **Travels in Europe, Africa, and Asia, Made Between the Years 1770 and 1779; in Four Volumes: Containing travels in the empire of Japan, and in the islands of Java and Ceylon, together with the voyage home**, vol. 4: F. and C. Rivington, and sold; 1795.
16. Smith A: **A contribution to South African materia medica: Chiefly from plants in use among the natives**: JC Juta; 1895.
17. Helmstädter A: **Xysmalobium undulatum (Uzara) research–how everything began**. *Journal of ethnopharmacology* 2015, **164**:385-387.
18. Heinrich M, Barnes J, Prieto-Garcia J, Gibbons S, Williamson EM: **Fundamentals of Pharmacognosy and Phytotherapy E-Book**: Elsevier Health Sciences; 2017.
19. El-Mallakh RS, Brar KS, Yeruva RR: **Cardiac glycosides in human physiology and disease: update for entomologists**. *Insects* 2019, **10**(4):102.
20. Van Wyk B-E, Wink M: **Medicinal plants of the world**: CABI; 2017.
21. Wang, Qu D, Wang K: **Therapeutic approaches to Alzheimer's disease through stimulating of non-amyloidogenic processing of amyloid precursor protein**. *Eur Rev Med Pharmacol Sci* 2016, **20**(11):2389-2403.
22. Sousa EAd, Da Silva AA, Cavalheiro AJ, Lago JHG, Chaves MH: **A new flavonoid derivative from leaves of Oxandra Sessiliflora RE Fries**. *Journal of the Brazilian Chemical Society* 2014, **25**(4):704-708.
23. Hefny Gad M, Tuenter E, El‐Sawi N, Younes S, El‐Ghadban EM, Demeyer K, Pieters L, Vander Heyden Y, Mangelings D: **Identification of some Bioactive Metabolites in a Fractionated Methanol Extract from Ipomoea aquatica (Aerial Parts) through TLC, HPLC, UPLC‐ESI‐QTOF‐MS and LC‐SPE‐NMR Fingerprints Analyses**. *Phytochemical analysis* 2018, **29**(1):5-15.
24. Ghorbani, Kaloga M, Frey H-H, Eich E: **Phytochemical Reinvestigation of Xysmalobium undulatum Roots (Uzara)**. *Planta Medica* 1990, **56**(06):550-550.
25. Gohar AA, El-Olemy M, Abdel-Sattar E, El-Said M, Niwa M: **Cardenolides and ß-sitosterol glucoside from Pergularia tomentosa L**. *Nat Prod Sci* 2000, **6**:142-146.
26. Razakarivelo JF, Rafanomezantsoa RM, Krebs HC, Ravelonjato BA, Randrianasolo R, Rakotondramanga MF: **Secondary Metabolites from Brachylaena Merana, an Endemic Plant from Madagascar Rain Forest**. *Journal of Pharmacognosy and Phytochemistry* 2015, **4**(1).
27. Barros L, Dueñas M, Ferreira I, Baptista P, Santos-Buelga C: **Phenolic compounds profile in wild edible greens from Portugal obtained by HPLC-DADESI/MS**. *Food Chem* 2011, **127**:169-173.
28. Grosa G, Allegrone G, Del Grosso E: **LC–ESI-MS/MS characterization of strophanthin-K**. *Journal of pharmaceutical and biomedical analysis* 2005, **38**(1):79-86.
29. Rakotondramanga M, Raharisololalao A, Rakotoarimanga J, Krebs HC, Rasoanaivo L, Razakarivony A, Randrianasolo R: **Cardenolide and Steroid Glycosides from Alafia sp., an Antimalarial Plant from Madagascar**. *Chemistry of natural compounds* 2016, **52**(5):865-869.
30. Tal DM, Karlish SJ, Gottlieb HE: **An NMR study of new cardiac glycoside derivatives**. *Magnetic Resonance in Chemistry* 2016, **54**(3):260-262.

31. Qiu S-X, Cordell GA, Kumar BR, Rao YN, Ramesh M, Kokate C, Rao AVNA: **Bisdesmosidic pregnane glycosides from Caralluma lasiantha1**. *Phytochemistry* 1999, **50**(3):485-491.
32. Rascón-Valenzuela L, Velázquez C, Garibay-Escobar A, Medina-Juárez L, Vilegas W, Robles-Zepeda R: **Antiproliferative activity of cardenolide glycosides from Asclepias subulata**. *Journal of ethnopharmacology* 2015, **171**:280-286.
33. Schneider G, Wolfling J: **Synthetic cardenolides and related compounds**. *Current Organic Chemistry* 2004, **8**(14):1381-1403.
34. Wang JK, Portbury S, Thomas MB, Barney S, Ricca DJ, Morris DL, Warner DS, Lo DC: **Cardiac glycosides provide neuroprotection against ischemic stroke: discovery by a brain slice-based compound screening platform**. *Proceedings of the National Academy of Sciences* 2006, **103**(27):10461-10466.
35. Rohit M, Ashok T, Vijaykumar R, Kashniyal K: **Molecular docking study of cassia tora, brassica campestris and calotropis procera as acetylcholinesterase inhibitor**. *Indian J Pharm Educ* 2016, **50**:116-122.
36. Siddiqui BS, Sultana R, Begum S, Zia A, Suria A: **Cardenolides from the methanolic extract of Nerium oleander leaves possessing central nervous system depressant activity in mice**. *Journal of natural products* 1997, **60**(6):540-544.

Chapter 5
Experimental

5.1. Materials

The plants under investigation were identified by the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria. The 18 selected plant species *Centella asiatica*, *Heteromorpha arborescens*, *Mondia whitei*, *Stapelia gigantea*, *Tabernaemontana elegans*, *Cussonia spicata*, *Cussonia paniculata*, *Bulbine natalensis*, *Catha edulis*, *Cotyledon orbiculata*, *Schotia brachypetala*, *Tetradenia riparia*, *Trichilia dregeana*, *Plumbago auriculata*, *Ruta graveolens*, *Zanthoxylum capense*, *Buddleja salvifolia*, *Cissus quadrangularis* were obtained from the Pretoria University gardens (University of Pretoria, cnr Lynnwood Road and Roper Street, Hatfield, Pretoria, South Africa); one plant, *Xysmalobium undulatum* was collected from the experimental farm of University of Pretoria; two plants, *Commelina africana* and *Ziziphus mucronata* were collected from Dinokeng Nature Reserve Pretoria (N1 North, Hammanskraal, 0400). Voucher specimens were preserved at H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria.

For extraction and silica gel column chromatography, analytical grade dichloromethane (≥99 %) (Merck), methanol (≥99.5 %) (Merck), hexane (≥99 %) (Sigma-Aldrich), and ethyl acetate (99.5 %) (Sigma-Aldrich) were used. For LC-MS and preparative HPLC-MS, the solvents used were ultra-pure HPLC grade methanol/ water/ acetonitrile (Romil-UpS^{TM}, Microsep, South Africa), formic acid (99% purity) (Thermo Scientific, South Africa), ammonium hydroxide ≥25% in H_2O which was the eluent additive (Fluka® Analytical, Sigma-Aldrich, South Africa). Vanillin (Analytical grade, Radchem) was used for staining TLC plates. Standards isoquercetin, quercetin and rutin were purchased from Sigma Aldrich, South Africa.

Silica gel 60 (0.063-0.20 mm) was used for column chromatography and Silica gel plates 60 F254 (Merck, South Africa) was used for analysis and visualized using a UV Detector at 254

and 360 nm (Spectroline®, Model ENF-240/FE, Spectronics Corporation Westbury, New York, USA).

5.2. Extraction process

Plant parts were cut into small pieces and then dried in oven (PROLAB) at 60˚C. The dried material was ground to fine coarse powder using a laboratory mill (POLYMIX ® PX-MFC 90 D/ Kinematika). The dried ground material (50 g) was placed separately in an Erlenmeyer flask (1 L). A mixture of dichloromethane and methanol (200 ml) in a 1:1 ratio was added to the flask. The plant material was extracted continuously by shaking using a magnetic stirrer for 8 h. The extraction process was repeated three times. The dried leaf material of X. undulatum (200 g) was separately placed in an Erlenmeyer flask (2 L) and extracted with the dichloromethane and methanol (1000 ml) in a 1:1 ratio. The plant material was extracted 3 times under similar conditions.

All the plant extracts were then filtered separately through a Whattman No.1 (GE Healthcare, Life Sciences) using a Buchner funnel connected to a vacuum pump. All the filtrates were combined separately and placed in a round bottom flask. The plant extract was then concentrated using a rotary evaporator to dryness and the extracts weighed to calculate the extraction yield. The plant residue was discarded.

5.3. Chromatographic techniques

5.3.1. Thin layer chromatography

TLC was performed on precoated Kieselgel 60 with florescent indicator UV_{254} (MACHEREY-NAGEL), 0.20 mm thick, with glass support. The TLC plates were examined under a short wavelength, 254 nm UV light and subsequently by using a specific spray reagent. The following mixture of solvents were used as eluting solvents for the TLC analysis and column chromatography for the plant extracts of *X. undulatum* in order to attain the improved separation: Hexane:EtOAc (95:5), Hexane:EtOAc (90:10), Hexane:EtOAc (80:20),

DCM:MeOH (95:5), DCM:MeOH (90:10), DCM:MeOH (80:20), DCM:EtOAc (95:5), DCM:EtOAc (90:10), DCM:EtOAc (80:20), EtOAc:MeOH:HCOOH:H2O (8:1:1:2). Vanillin staining was used to reveal the presence of compounds. The staining reagent was prepared by adding 6 g vanillin in a 100ml container. Ethanol (95 ml, 96%) was added to the vanillin to give a clear solution followed by careful addition of 1.5 ml of concentrated sulfuric acid. The solution was stored in the dark. TLC plates were dipped into the vanillin stain and dried with a hair dryer for visualization.

5.3.2. UPLC-QTOF-MS analysis

The crude extracts and fractions were analyzed using a Waters Acquity UPLC system (Waters corp., MA USA), equipped with a binary solvent delivery system and an autosampler. The instrument was centrally operated by MassLynx 4.19 software (Waters Inc., Milford, Massachusetts, USA) for data acquisition. The sample (1mg/ml) was prepared by dissolving dried extract, fraction or pure compounds in 100% methanol. The solution was centrifuged at 10 000 g for 10 minutes to remove the particulates. The separation of compounds was achieved on Waters BEH C18 1.7 µm particle size (2.1 mm × 100 mm). The column temperature was maintained at 40ºC. The mobile phase consisted of solvent A: water with 0.1% formic acid and solvent B: methanol with 0.1% formic acid. The gradient elution was optimized as follows: 3% B (0-0.1 min), 100% B (0.3-14.00 min), 100% B (14.00-16.00 min), 3% B (16.00-16.50 min), 3% B (16.50-20.00 min). The flow rate was 0.3 ml/min for the entire run, giving a total run time of 20 minutes and the injection volume was 5µl. A Waters Synapt G2 high definition QTOF mass spectrometer equipped with an ESI source was used to acquire negative ion data. The instrument was calibrated by direct infusion of 5 nM sodium formate solution at a flow rate of 10 µl/min over a mass range of 50-1200 Da.

The following MS source parameters were set for both positive and negative mode: source temperature 100 ºC, sampling cone 15 V, extraction cone 4.0 V, desolvation temperature 400 ºC, cone gas flow 10.0 L/h, desolvation gas flow 700 L/h, capillary 2.0 kV. Both positive and

negative modes were obtained, but the results were analyzed using the negative mode as the higher intensity peaks were obtained in the negative mode. A 2 ng/µl leucine encephalin solution was used as lockspray solution throughout all the acquisitions. It was constantly infused at a rate of 3 µl/min through a separate orthogonal ESI probe to compensate for experimental drift in mass accuracy. The trap collision energy was 28 V.

Compounds were tentatively identified based on their accurate mass generated from MassLynx V 4.1, iFit value, MS/MS fragmentations (product ions). The electronic databases Metlin, Metfusion, Pubchem, Chemspider, Chemical Entities of Biological Interest (ChEBI) Massbank, Reaxys, Scifinder and Scopus were used for the tentative identification of compounds. The compounds were identified by comparing the mass fragmentation pattern generated by the instrument with that in electronic data bases and literature.

A novel software, integrated MassLynx with UNIFI (Waters UNIFI Scientific Library Component Listing version: 01) informatics was also used for identifying the compounds present in crude extract. This software analyses the chromatographic and mass spectral components of the extract or samples by using the different libraries including UNIFI Toxiology Library, UNIFI Pesticide Library, UNIFI Traditional Medicine Library (Library type, Green Tea and Chinese Traditional Medicine) incorporated in UNIFI software. The Chinese Traditional Medicine library contains more than 6000 compounds isolated from 600 herbs [1]. In order to identify the compounds, the raw data from the MassLynx was imported to UNIFI software where the software integrates the data acquisition, data mining and generates a report through library searching. The UNIFI data analysis automatically matches the compound information collected from MassLynx with the Chinese Traditional Medicinal library and gives possible chemical identifications. The software provides the elemental composition to generate the possible formula for accurate mass peak based on fragmentation pattern matching. It also provides the i-Fit confidence score and a formula with a score greater than 0.8 given as a priority in the list of possible identifications. Further, the identified compounds with a mass difference of less than 5 mDa between the theoretical and instrument exact mass reading were selected for further identification and are provided with the detailed information including

compound name, molecular formula, average molecular mass, monoisotopic retention time, exact mass error in mDa and ionization mode.

5.3.3. Silica gel column chromatography

For the column chromatography, a glass silica column of 107 cm length and 7 cm internal diameter with a reservoir of 2 L capacity was used. The column was packed with a slurry suspension formed by mixing 1500 g of dried silica gel (0.063-0.2 mm/70-230 mesh) with hexane. The dried DCM:MeOH (1:1) *X. undulatum* leaf extract (17 g) was re-dissolved in the same solvent system and mixed with 12g of silica gel 60. The mixture was stirred gently with the help of glass rod to prepare a suspension. Then the solvent was gently evaporated by using a rotary evaporator until the sample was dry. The resulting powdered sample was poured slowly onto the bed of the silica gel layer and allowed to settle. Initially DCM:Hexane (50:50 to 80:20) solvent system was used as the eluent. Later, the polarity was increased to DCM (100%) and DCM:MeOH (95:5 to 70:30) as the eluants with the solvent flow rate of 2 ml/min. The separation provided a total of 46 fractions. These fractions were further analysed by TLC using silica gel plates (60 F_{254}) and visualised under UV light at 254nm. Pasteur pipettes were used for spotting the samples. The TLC plates were also dipped in the vanillin stain to detect the compounds. The TLC plates were dried by using a hair dryer after staining. After visualisation, the similar fractions were combined based on their Rf values. After combining the similar fractions, 15 fractions (AT-1-49A to AT-1-49O) were obtained and two fractions (AT-1-49N and AT-1-49O) were selected for further purification using prep HPLC-MS based on their promising biological activity.

5.3.4. Isolation of compounds using preparative HPLC-MS

Isolation of compounds from the active fractions (AT-1-49N and AT-1-49O) of *X. undulatum* DCM:MeOH leaf extract was performed on Waters chromatographic system with Waters PDA (2998) and MS detector (Waters, Milford, MA, USA). For the sample preparation, two active fractions (70 mg each) were separately dissolved in HPLC grade methanol (100 ml). The

solution was centrifuged for 10 min and then filtered through a 0.22 µm (Millipore) membrane syringe filter unit. Six major peaks labelled as XU01 to XU06 from sample AT-1-49N and two major peaks labelled as XU07 and XU08 from AT-1-49O were targeted for isolation. The injection volume was 150 µl per injection.

The chromatographic conditions were optimized on a preparative column to achieve well resolved chromatograms. The separation was achieved on an X-Bridge preparative C18 column (19×250 mm, i.d., 5µm particle size, Waters). The mobile phase consisted of 0.1% ammonia in water (solvent A) and acetonitrile (solvent B) at a flow rate of 20ml/min. Gradient elution was applied as follows: 95% A : 5% B for 1 min, 50% A : 50% B (1:00-8:00 min), 15% A : 85% B (8:00-13:00 min), 95% A : 5% B (13:00-13:20 min). Injection volume was 150 µl. The total run time was 20 minutes. Data were collected using MassLynx4.1^{TM} (Waters, USA) software. The preparative HPLC system was interfaced with QDa mass spectrometer (Waters, USA) and the negative ion mode selected. The probe temperature and source temperature were set at 600ºC and 120ºC, respectively. The capillary and cone voltage were set to 800 and 10 V, respectively. Data were collected between 100 and 650 *m/z.* The pure compounds were collected using a fraction collector and subsequently combined and concentrated speed vaccum. The compounds were later analyzed by UPLC-MS and their structures are confirmed by NMR.

5.4. Bioassay screenings

5.4.1. APPsw-transfected HeLa cell culture

HeLa cells stably transfected with Swedish mutant form of APP (APPsw) were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin, 100 µg/ml streptomycin, 260 µg/ml Zeocin and 400 µg/ml G418 at 37˚C in a humified atmosphere of 5% CO2 and 95% air.

Fetal bovine serum is vitally important as a source of growth and adhesion factors, hormones, lipids and minerals for the cell culture in basal media. It also regulates the cell membrane

permeability and serve as a carrier. Penicillin/ Streptomycin is used to avoid contamination. Antibiotics Zeocin and G418 are used for the selection of APPsw-transfected HeLa cell.

5.4.2. Cell culture protocol

Spent cell culture media was removed and discarded from culture vessel. Cells were washed using PBS (5 ml). Wash solution was gently added to the side of vessel opposite to the attached cell layer to avoid disturbing the cell layer. The wash step was used to remove any traces of serum, calcium and magnesium that would inhibit the action of the dissociation reagent. The wash solution was removed from the cell culture. The pre-warmed dissociation reagent such as trypsin-EDTA (1 ml) was added to the side of flask. Culture vessel was then incubated at room temperature for approximately 2-3 min. The actual incubation time varies with the cell line used. The cells were observed under the microscope for detachment. An equivalent of 5 ml of pre-warmed complete growth medium was added to inactivate trypsin-EDTA. The growth medium was dispersed by pipetting over the cell layer surface several times. The cells were transferred to a 15 ml conical tube and centrifuged at 1000 g for 3 min. The centrifuge speed and time also vary with the cell type used. The cell culture media was removed. The cell pellet was resuspended in 5 ml of pre-warmed complete growth medium and a sample was removed for counting. To subculture after 3 days, the cells (0.5 ml) were transferred to a T75 flask with cell culture media 10-12 ml. To subculture after 4 days, the cells (0.3 ml) were transferred to a T75 flask with 10-12 ml culture media. The total number of cells were determined by using a hemocytometer.

5.4.3. Sample preparation

The samples were prepared by dissolving the stock solution of the compound in DMSO, kept at -20ºC. Before each experiment, the solution was diluted to the final concentration in fresh media. In order not to effect cell growth, the final DMSO concentration did not exceed 0.5 % in all experiments.

5.4.4. Cell Viability Measurement

Cell viability was measured by MTT assay using an EZ-Cytox kit (Daeil Lab Co, Ltd, Republic of Korea) according to the manufacturer's instructions. APPsw-transfected HeLa cells in a 96 well plate were cultured with sample solubilized in DMSO for 8 h, and then incubated with EZ-Cytox solution for 1 h at 37°C. The absorbance was measured on a microplate reader (BIO-TEK® DowerWave XS, Winooski, VT, USA) with a software capable of measurement at or near 450 nm. The results were expressed as the percentage of MTT reduction relative to the absorbance of control cells.

5.4.5. sAPPα, sAPPβ, Aβ peptide assay

APPsw-transfected HeLa cells at 80% confluence in a 35 mm dish were cultured with the samples solubilized in dimethyl sulphoxide (DMSO) for 8 h in serum free medium. The conditioned medium was analyzed by a sandwich ELISA (Invitrogen) for detection of Aβ42 and Aβ40 according to the supplier's instruction. The levels of sAPPα and sAPPβ-sw were measured from the conditioned medium using the specific ELISA (IBL) according to the supplier's instruction. The APPsw-transfected HeLa cells with DMSO (vehicle) was used as a negative control.

5.4.6. Statistical analysis

Data was expressed as mean ± SEM. Statistical comparisons between controls and treated experimental groups were performed using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

5.5. Structure elucidation using NMR

^{1}H and ^{13}C NMR spectra were acquired at room temperature on a Bruker Avarice III 400 spectrometer (^{1}H at 400.21 MHz and ^{13}C at 100.63 MHz) and 500 spectrometer (^{1}H at 500.10 MHz and ^{13}C at 125.75 MHz). Deuterated CD_3OD (Merck, Switzerland) was used to dissolve

the compounds. Chemical shifts were reported in ppm, referenced to residual solvent resonances (methanol-*d4* δH 3.31, δC 49.0 ppm). Bruker TopSpin 4.0.6 software was used to process the spectra. The coupling constant *"J"* was given in Hertz (Hz) while the signal multiplicity was reported as: *s* = singlet, *d* = doublet, *dd* = doublet of doublet, *q* = quartet, *m* = multiplet.

References

1. Rohit M, Ashok T, Vijaykumar R, Kashniyal K: **Molecular docking study of *Cassia tora*, *Brassica campestris* and *Calotropis procera* as acetylcholinesterase inhibitor**. *Indian J Pharm Educ* 2016, **50**:116-122.

Chapter 6
Conclusion

Alzheimer's disease is an irreversible, slow and progressive brain disorder that destroys memory and other thinking abilities. It is the most common type of dementia. The abnormal cleavage of APP (Amyloid precursor protein), an integral membrane produces the accumulated forms of beta amyloid (Aβ42), to form the plaques in brain which are the main causes of Alzheimer's disease.

The traditional plants play an important role in the treatment of many diseases like cancer, HIV-AIDS, malaria and Alzheimer's disease. One of the FDA approved drug for the treatment of Alzheimer's disease is galanthamine, an alkaloid (natural product). South Africa has a wide plant diversity. 15% of these plant species are used for medicinal purposes [1] and about 80% of South African people use traditional medicines for their health care [2]. The goal of this study was to identify, characterize and develop new natural ingredients from the South African medicinal plants for the treatment of Alzheimer's disease by targeting the reduction of Aβ42 resulting in decreased levels of plaques.

For the selection of the plants in this study, different database searches Google Scholar, Scifinder, Reaxys, Chemspider, Pubchem, Scopus were used for the selection of plant species by using the keywords; African medicinal plants and traditional medicinal plants in combination with the memory loss, mental illness, depression, Alzheimer's disease, dementia, anxiety, epilepsy, forgetfulness, convulsion, hysteria, sedative effects, acetylcholinesterase inhibition which resulted in the selection of 51 plant species. Due to the broad search and wide search terms, a scoring system was developed and used to prioritize, which resulted in 21 plant species being selected for further studies. The different plant parts of the plant species were collected and extracted to give a total of 34 extracts which were screened for Aβ42 reduction. Among them, four plant extracts, stems of *H. arborescens*, leaves of *X. undulatum,* leaves of *C. paniculata and* leaves of *S. brachypetala,* potently decreased the levels of Aβ42 by 68.6 ±

0.2, 76.9 ± 1%, 57.5 ± 1.3% and 44.8 ± 0.1%, respectively at 50 µg/ml. The stem extract of *H. arborescens*, leaf extracts of *X. undulatum* and *C. paniculata* were for the first times screened for Aβ42 reduction. In the case for *H. arborescens* and *C. paniculata* which are traditionally used for the treatment of mental disorders, the reduction in Aβ42 levels provided some scientific evidence authenticating their traditional uses and further exploration of their potential to be further developed into anti-Alzheimer's ingredients. The ability for the reduction of Aβ42 for *S. brachypetala was* demonstrated in other assays in published literature while *X. undulatum* has been previously reported for acetylcholinesterase inhibition [3], [4]. These four extracts were selected for the further study to identify and or isolate the active ingredients responsible for Aβ42 reduction.

The three plants, *S. brachypetala* leaf extract, *C. paniculata* leaf extract and *H. arborescens* stem extract were assayed for their Aβ42 reduction in dose dependent studies. The plant extracts were further analyzed on UPLC-QTOF-MS to identify the active ingredients which could be responsible for the decreased levels of Aβ42. ESI negative mode was used to identify the active ingredients as the ionization was poor for the positive mode for further analysis

S. bracypetala leaf extract showed significant reduction in Aβ42 production only at higher concentrations i.e. 29.69 ± 4% at 50 µg/ml and by 62.17 ± 7% at 100 µg/ml. A total of four compounds belong to the class flavonoids, myricetin-3-*O*-alpha-L-rhamnopyranoside **(25)**, isoquercetin **(26)**, quercetin-3-*O*-rhamnoside **(27)**, quercetin **(28)** were identified through UPLC-MS analysis. These compounds were previously identified in the same plant and their potential to treat Alzheimer's disease through Aβ reduction was published in different bioassays [4]. Based on these findings, the compounds are in all likelihood responsible for the Aβ42 reduction in *S. bracypetala*.

The extract of leaves of *C. paniculata* significantly reduced the Aβ42 levels in a dose dependent manner by 29.03 ± 1.4%, 42.40 ± 1.7%, 47.50 ± 5.8% and 61.14 ± 9.3% at concentrations 10, 25, 50 and 100 µg/ml, respectively. This is the first report for the reduction of Aβ42 and thus provides scientific evidence which substantiates its traditional use related to

treatment of nervous disorder treatments. The UPLC-QTOF-MS analysis led to the identification of nine compounds; quinic acid **(29)**, 3,5-dicaffeoylquinic acid **(30)**, rutin **(31)**, valerianannoid E **(32)**, acuminoside **(33)**, dictamnoside D **(34)**, 2''-O-β-D-glucopyranosylsaikosaponin B2 **(35)**, clinoposaponin C **(36)**, spinasaponin C **(37)**. The presence of rutin was confirmed by using pure standard for the UPLC-QTOF-MS analysis. Rutin is a well-researched compound for the Alzheimer's disease in previous studies [5], [6] . 3,5-dicaffeoylquinic acid was previously examined for AChE inhibition [7]. Based on these studies rutin and/or 3,5-dicaffeoylquinic acid could be responsible for the activity in *C. paniculata*.

The stem extract of *H. arborescens* significantly reduced Aβ42 levels only at high test concentrations by 75.78 ± 0.1% at 50 µg/ml and by 31.37 ± 0.2% at 25 µg/ml. A total of four compounds were tentatively identified as cynarin (1,5-dicaffeoylquinic acid) **(38)** and three triterpenoid saponin alternoside IX **(39)**, alternoside I **(40)** and saikogenin B4 **(41)** using the UPLC-QTOF-MS. Cynarin was previously reported for the reduction of Aβ cytotoxicity while the other identified compounds, triterpenoid saponins were not examined for Alzheimer's disease. In this study cynarin could be the active ingredient from *H. arborescens* leaf extract for the reduction of Aβ42.

X. undulatum leaf extract was highly potent against Aβ42 reduction in the present study based on the data, it was further explored for its potential to treat Alzheimer's disease through the reduction of Aβ42. The plant is commercialised in Europe for the treatment of diarrhoea and traded as the commercial brand Uzara. The plant is also used traditionally for treatment of many diseases such as dysentery, diarrhoea, headache, malaria, typhoid, skin diseases, infected sores and wounds. Although the plant has been widely researched and well-known for its antidepressant effect, acetylcholinesterase inhibitory effects and selective serotonin reuptake inhibitor (SSRI) activity, there is limited published data available showing its inhibitory effects for the reduction of Aβ42, an important target in the treatment of Alzheimer's disease. In the present study, the DCM:MeOH leaf extract significantly reduced the Aβ42 production

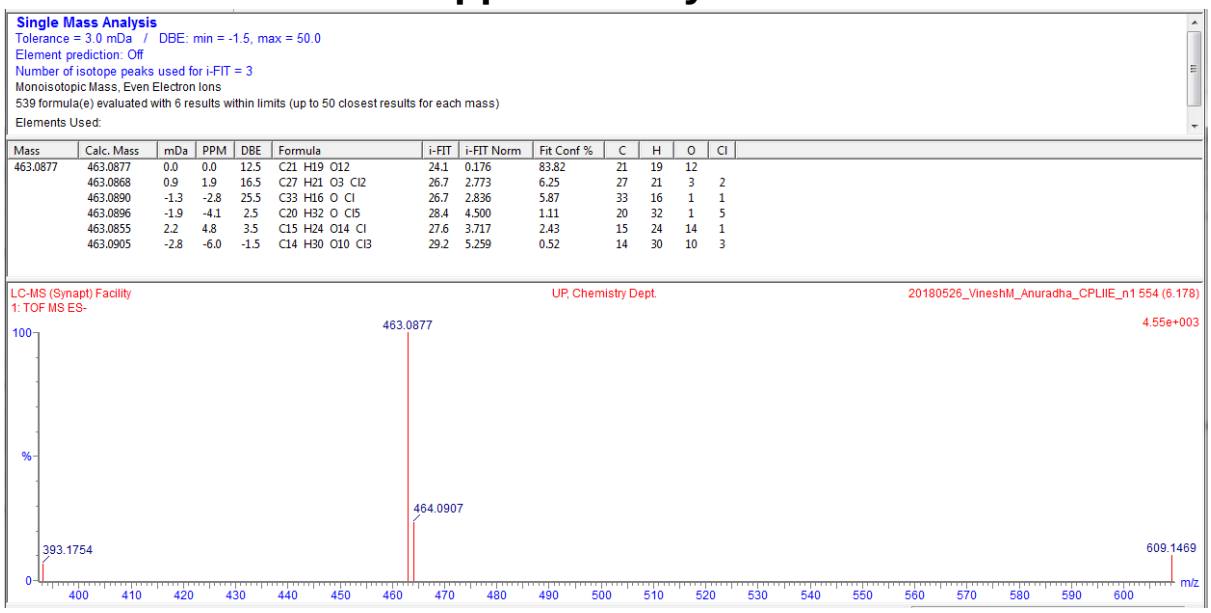
by 49.8 ± 4.0%, 71.4 ± 1.6% and 72.9 ± 1.1% at concentrations of 1, 5 and 10 µg/ml, respectively. To the best of our knowledge, this is the first study examining the plant extracts for its Aβ42 production as a potential for treating Alzheimer's diseases. The main class of compounds in the extract was identified as cardenolide glycosides through the analysis of the samples UPLC-QTOF-MS confirming other previous findings [8]. In order to identify the active ingredients between the several cardenolides present in the crude extract, bioassay-guided fraction was undertaken. The crude DCM:MeOH extract was fractionated using silica gel column to produce a total of 46 fractions which were combined based on their similar TLC profiles to give a final 15 combined fractions. These fractions were then tested for Aβ42 levels and only two fractions (AT-1-49N and AT-1-49O) were found to significantly exhibit Aβ42 reduction. These fractions were once again analysed on UPLC-QTOF-MS to identify the compounds and the results revealed the presence of cardenolide glycosides in the two active fractions. These fractions were further purified using mass directed purification using preparative HPLC which resulted in the isolation of five pure compounds. The structure elucidation of the compounds was undertaken using mass spectral and NMR analysis which resulted in the identification of acetylated glycosydated crotoxogenin **(56)**, xysmalogenin-3, β-D-glucopyranoside **(57)**, crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**, crotoxigenin-3-O-glucopyranoside **(59),** desglucouzarin **(60)**. These compounds were tested for Aβ42 reduction and four cardenolide glycosides, acetylated glycosydated crotoxogenin **(56)**, xysmalogenin-3, β-D-glucopyranoside **(57)**, crotoxigenin-3-*O*-*β*-digitalopyranosyl-(1-4)-*O*-*β*-digitoxopyanoside **(58)**, desglucouzarin **(60)** were found to significantly decreased the Aβ42 levels at 10µM by 75.04 ± 0.85%, 74.66 ± 0.1%, 65.55 ± 0.19% and 69.15 ± 0.25%, respectively as compared to the negative control. Although, the compound crotoxigenin-3-O-glucopyranoside **(59)** was found to be inactive, structurally the characteristic lactone ring was present. This pointed to the probability that the lactone is not responsible for the activity towards the Aβ42 reduction. As it is known that the lactone ring is the main moiety responsible for the cardiotoxic effects [9], any analogues synthetically prepared lacking the lactone ring may still retain Aβ42 reduction while also decreasing

cardiotoxicity. Further dose response studies of the 4 cardenolides identified crotoxigenin-3-O-β-digitalopyranosyl-(1-4)-O-β-digitoxopyanoside **(58)** as the most potent compound as it significantly reduced Aβ42 levels at the low concentration of 0.025 μM, indicating that this is the most active compound responsible for Aβ42 reduction in the plant and exhibits the potency to develop into anti-Alzheimer's drug. The bioassay-guided approach of identifying the active ingredient in *X. undulatum* responsible for the reduction of Aβ42 level was highly successful and resulted in identification of 4 cardenolides active active ingredient, with one being the most active at nanomolar levels, which compares favourably to conventional pharmaceutical drugs. Often the approach of bioassay-guided fraction is a challenge to natural product chemists as bioactivity is lost during fractionation for various reasons, however in this case potency of the fractions and the final compounds increased during the purification process. The future work will involve the prediction of binding sites in Aβ42 protein by docking studies and the removal of the butenolide ring and testing the glycosidated aglycones for their potential to reduce Aβ42.

References

1. Abdalla MA, McGaw LJ: **Bioprospecting of South African plants as a unique resource for bioactive endophytic microbes**. *Frontiers in Pharmacology* 2018, **9**.
2. Street R, Prinsloo G: **Commercially important medicinal plants of South Africa: a review**. *Journal of Chemistry* 2012, **2013**.
3. Vermaak I, Enslin GM, Idowu TO, Viljoen AM: ***Xysmalobium undulatum* (Uzara)–review of an antidiarrhoeal traditional medicine**. *Journal of Ethnopharmacology* 2014, **156**:135-146.
4. Hassaan Y, Handoussa H, El-Khatib AH, Linscheid MW, El Sayed N, Ayoub N: **Evaluation of plant phenolic metabolites as a source of Alzheimer's drug leads**. *BioMed Research International* 2014, **2014**.
5. Canevelli M, Adali N, Kelaiditi E, Cantet C, Ousset PJ, Cesari M: **Effects of Gingko biloba supplementation in Alzheimer's disease patients receiving cholinesterase inhibitors: data from the ICTUS study**. *Phytomedicine* 2014, **21**(6):888-892.
6. Xu PX, Wang SW, Yu XI, Su YJ, Wang T, Zhou WW, Zhang H, Wang YJ, Liu RT: **Rutin improves spatial memory in Alzheimer's disease transgenic mice by reducing Aβ oligomer level and attenuating oxidative stress and neuroinflammation**. *Behavioural Brain Research* 2014, **264**:173-180.
7. Kang JY, Park SK, Guo TJ, Ha JS, Lee DS, Kim JM, Lee U, Kim DO, Heo HJ: **Reversal of trimethyltin-induced learning and memory deficits by 3, 5-dicaffeoylquinic acid**. *Oxidative Medicine and Cellular Longevity* 2016, **2016**.
8. Ghorbani M, Kaloga M, Frey H-H, Mayer G, Eich E: **Phytochemical reinvestigation of *Xysmalobium undulatum* roots (Uzara)**. *Planta Medica* 1997, **63**(04):343-346.
9. Erlij D, Elizalde A: **Rapidly reversible inhibition of frog muscle sodium pump caused by cardiotonic steroids with modified lactone rings**. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1974, **345**(1):49-54.

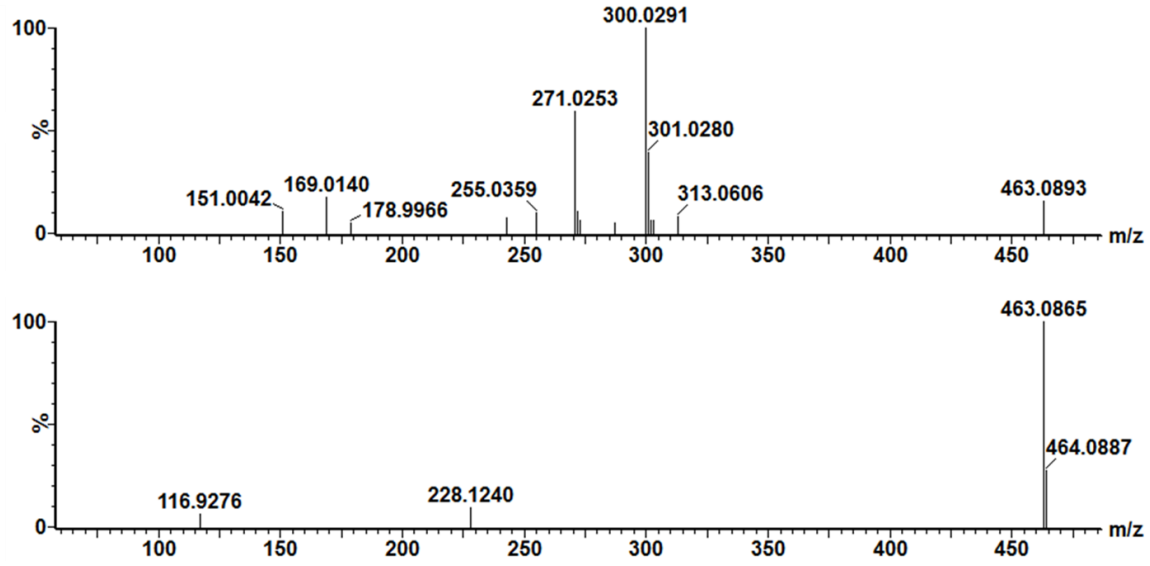
Supplementary Data



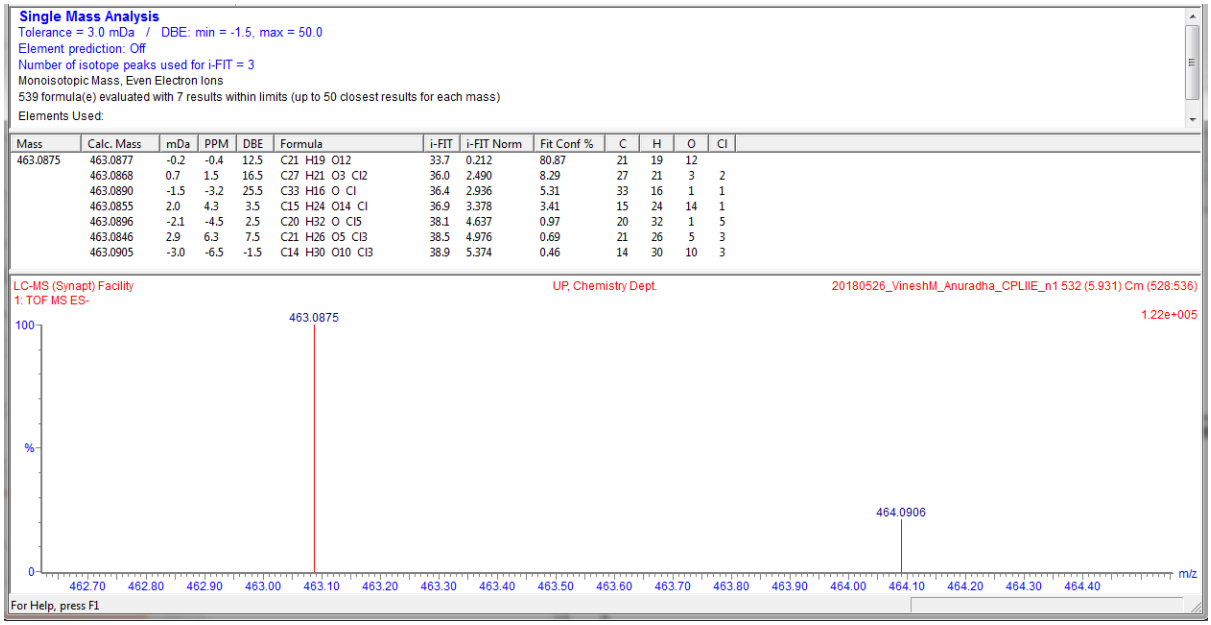
Single Mass Analysis
Tolerance = 3.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
539 formula(e) evaluated with 6 results within limits (up to 50 closest results for each mass)
Elements Used:

Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O	Cl
463.0877	463.0877	0.0	0.0	12.5	C21 H19 O12	24.1	0.176	83.82	21	19	12	
	463.0868	0.9	1.9	16.5	C27 H21 O3 Cl2	26.7	2.773	6.25	27	21	3	2
	463.0890	-1.3	-2.8	25.5	C33 H16 O Cl	26.7	2.836	5.87	33	16	1	1
	463.0896	-1.9	-4.1	2.5	C20 H32 O Cl5	28.4	4.500	1.11	20	32	1	5
	463.0855	2.2	4.8	3.5	C15 H24 O14 Cl	27.6	3.717	2.43	15	24	14	1
	463.0905	-2.8	-6.0	-1.5	C14 H30 O10 Cl3	29.2	5.259	0.52	14	30	10	3

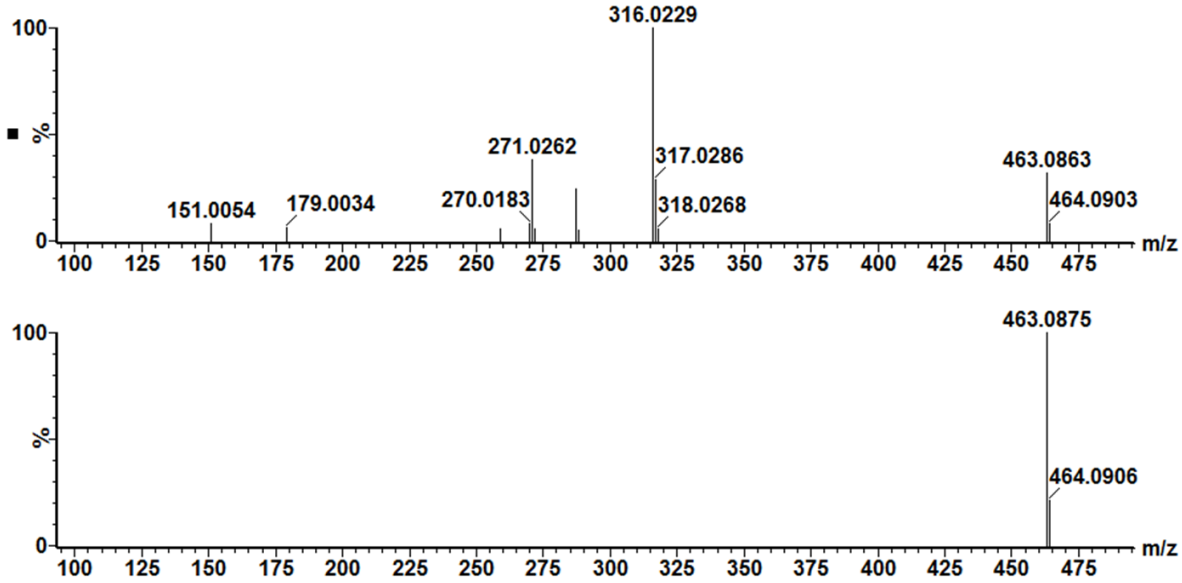
Supplementary data 1: iFit value of isoquercetin **(25)** in *S.brachypetala* leaves extracted with DCM:MeOH



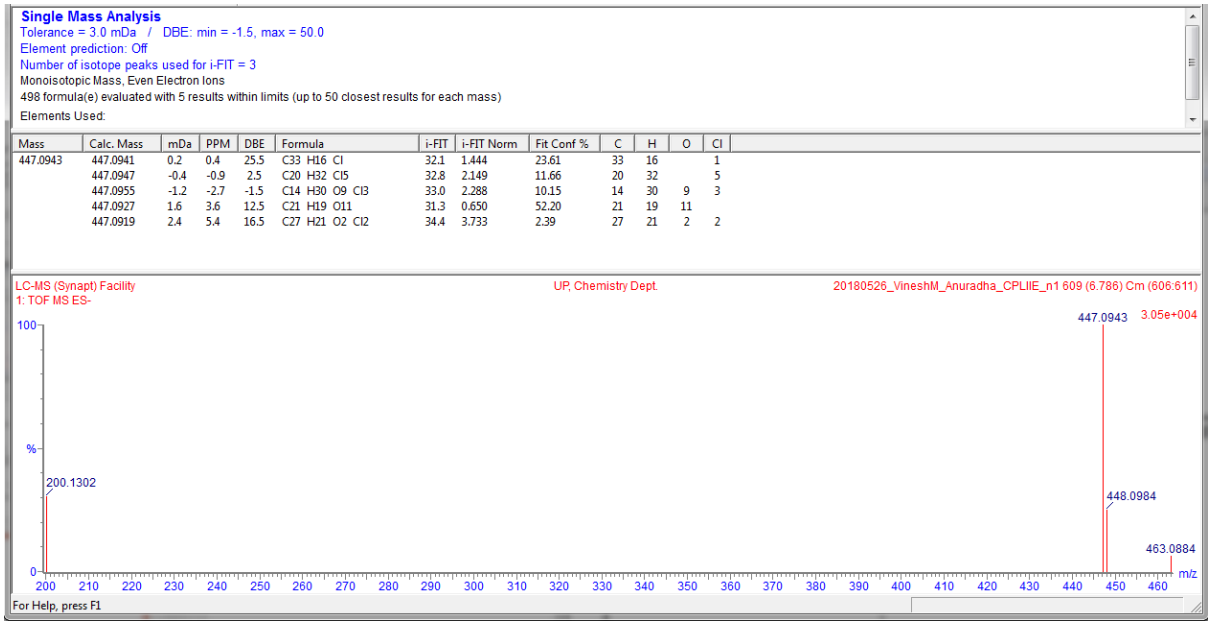
Supplementary data 2: MS and MS/MS data of isoquercetin **(25)** in the DCM:MeOH extract of leaves of *S. brachypetala*



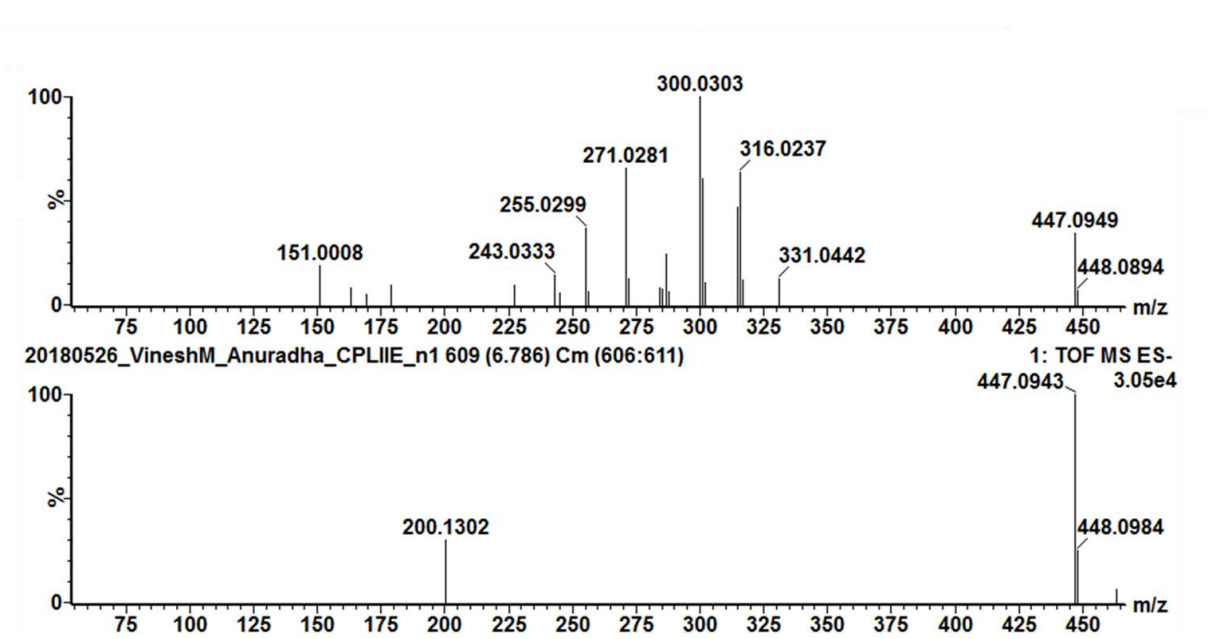
Supplementary data 3: iFit value of myricetin-3-O-alpha-L-rhamnopyranoside **(26)** in *S. brachypetala* leaves extracted with DCM:MeOH



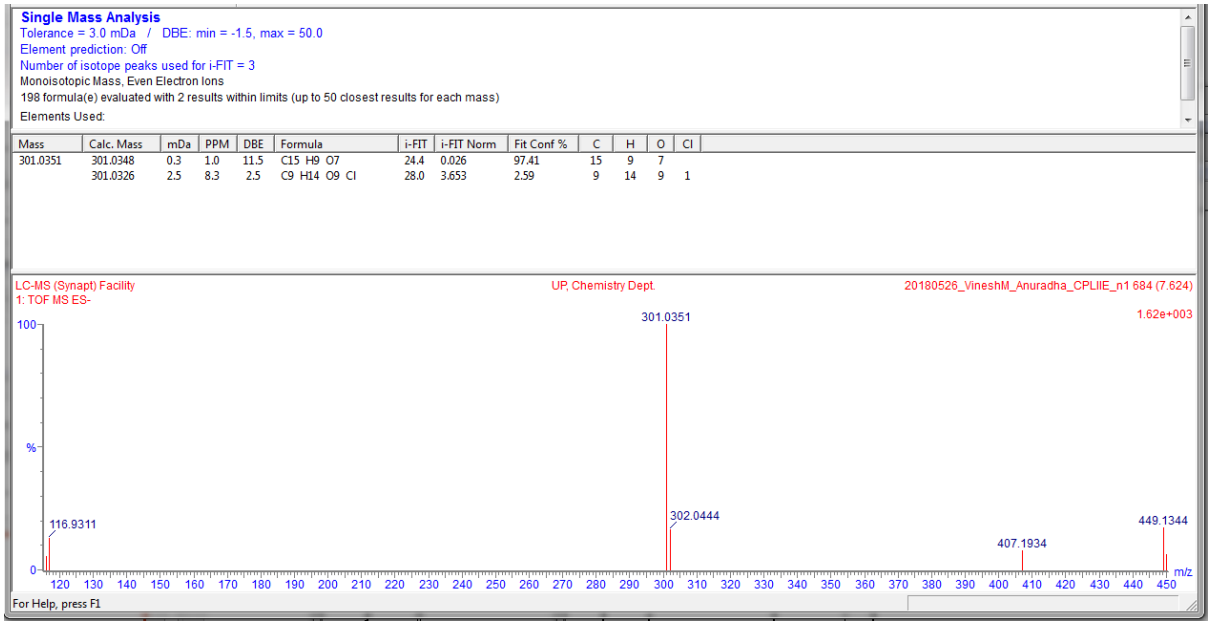
Supplementary data 4: MS and MS/MS data of myricetin-3-O-alpha-L-rhamnopyranoside **(26)** in the DCM:MeOH extract of leaves of *S. brachypetala*



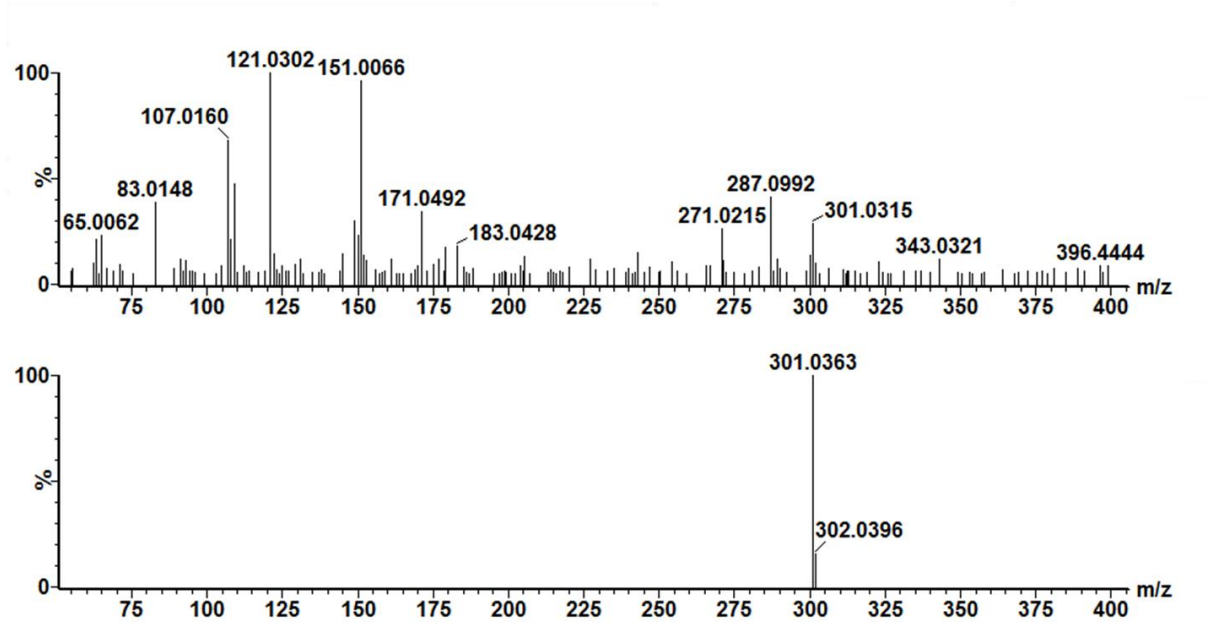
Supplementary data 5: iFit value of quercetin-3-O-rhamnoside **(27)** in *S.brachypetala* leaves extracted with DCM:MeOH



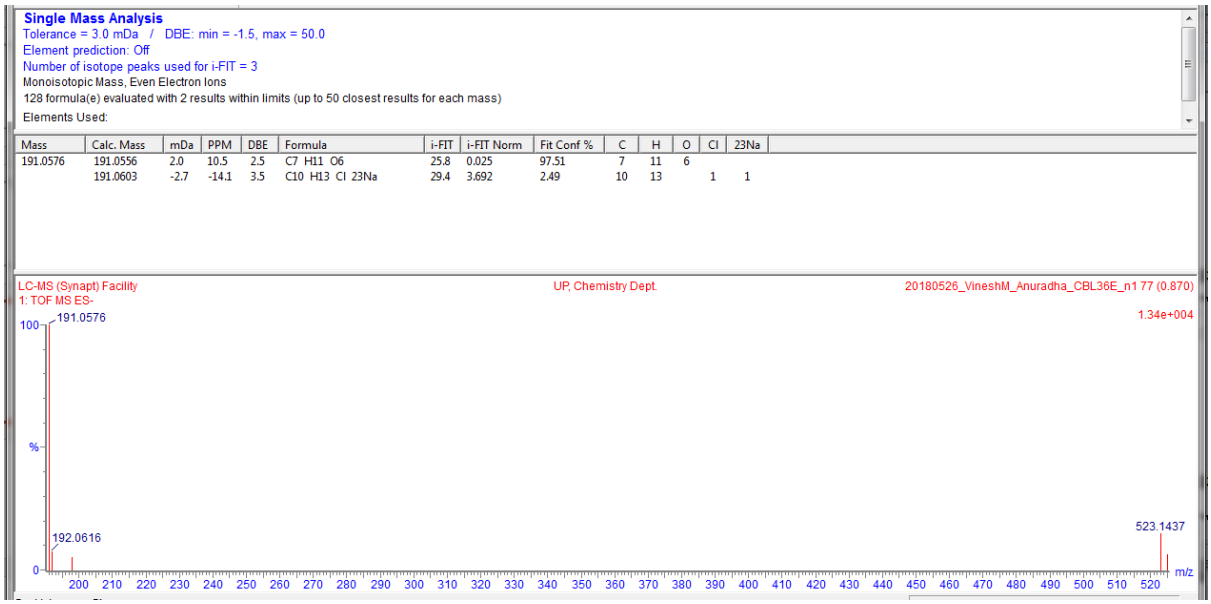
Supplementary data 6: MS and MS/MS data of quercetin-3-O-rhamnoside **(27)** in the DCM:MeOH extract of leaves of *S. brachypetala*



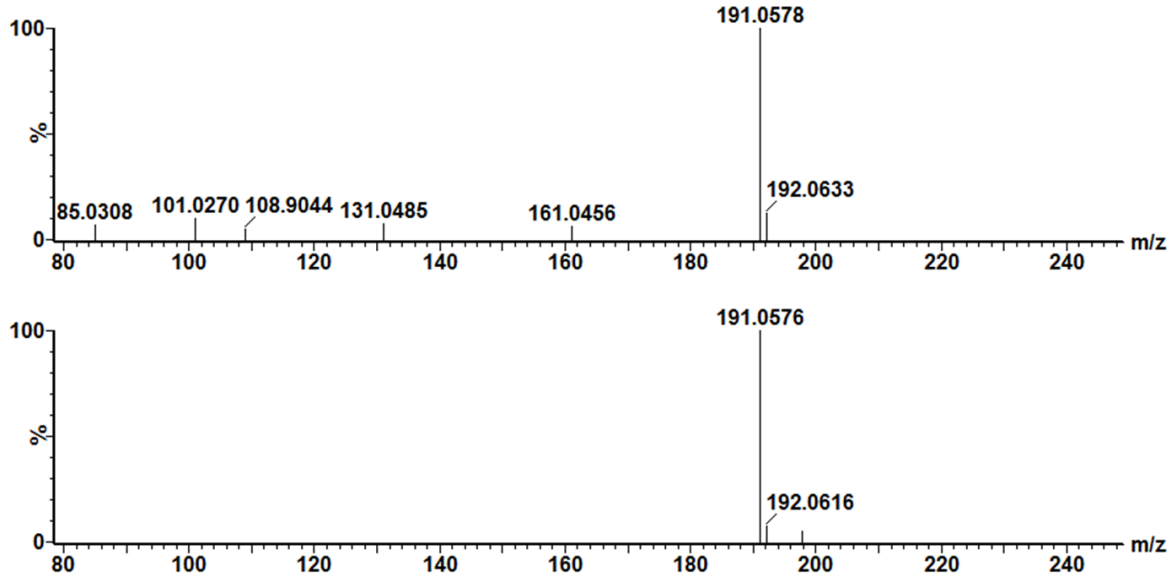
Supplementary data 7: iFit value of quercetin **(28)** in *S.brachypetala* leaves extracted with DCM:MeOH



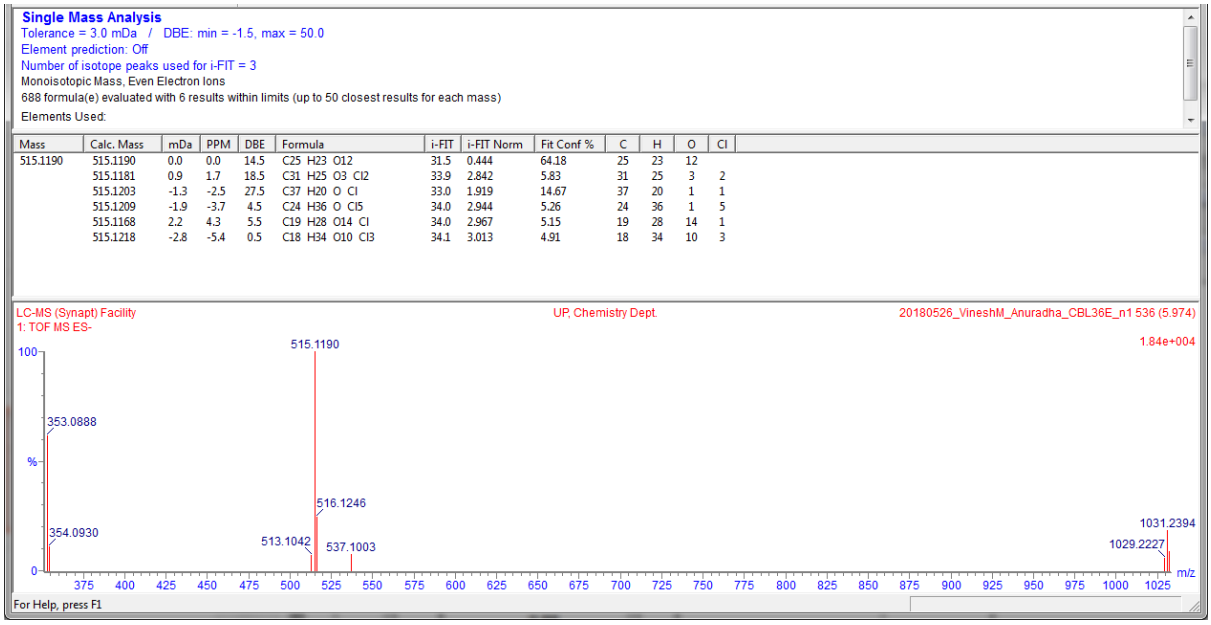
Supplementary data 8: MS and MS/MS data of quercetin **(28)** in the DCM:MeOH extract of leaves of *S. brachypetala*



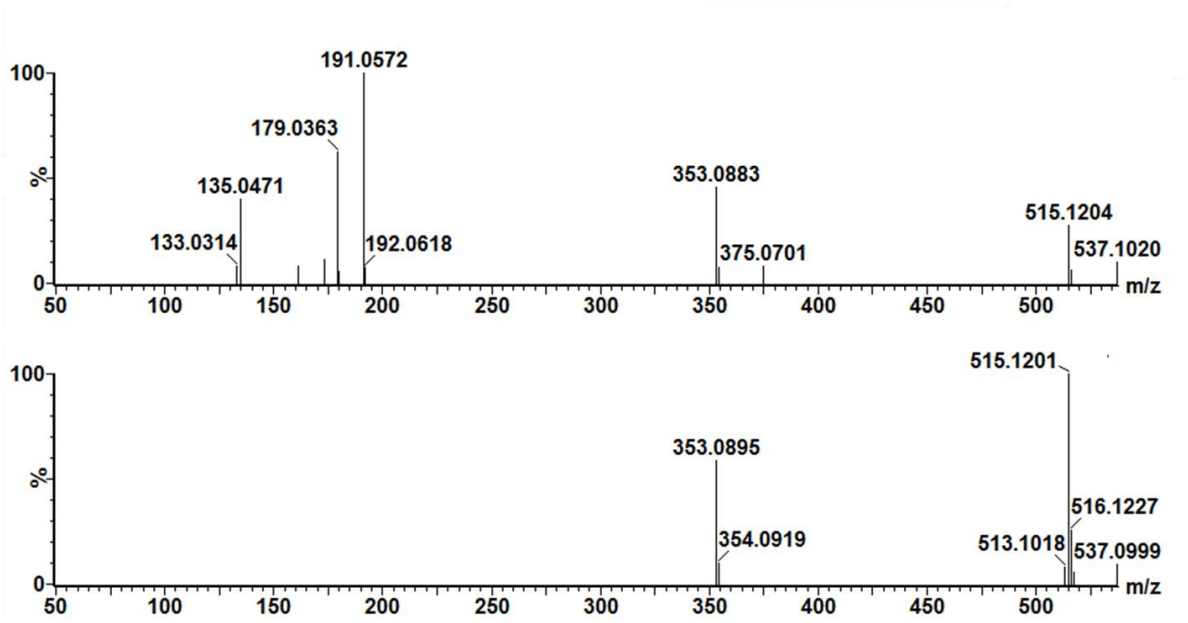
Supplementary data 9: iFit value of quinic acid **(29)** in *C. paniculata* leaves extracted with DCM:MeOH



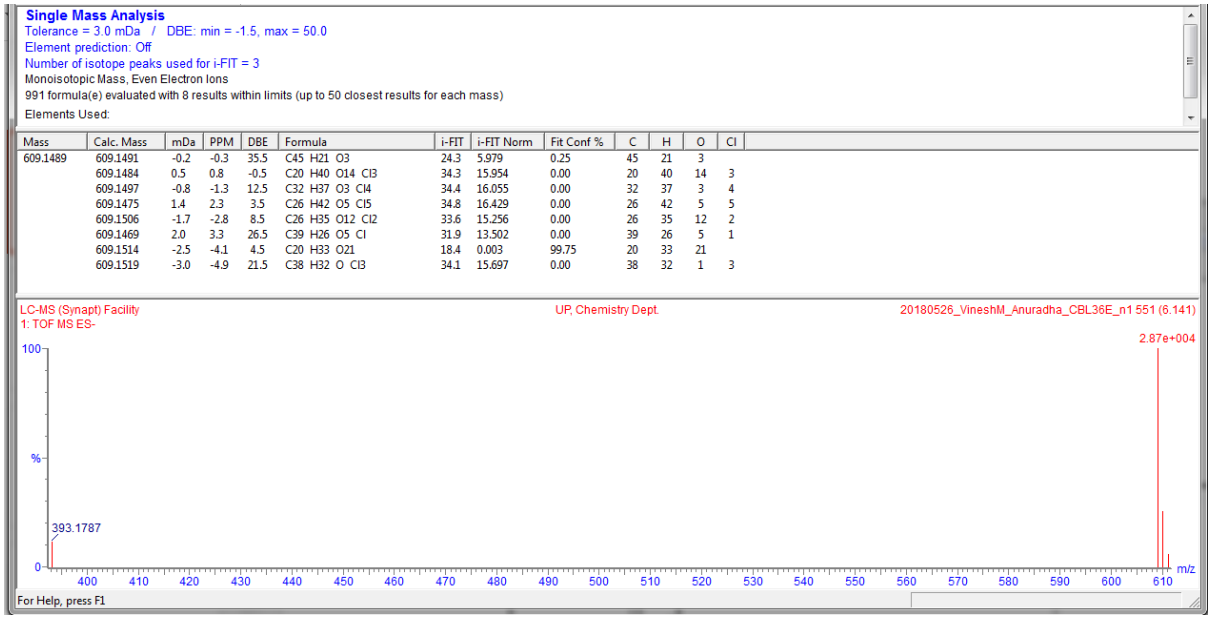
Supplementary data 10: MS and MS/MS data of quinic acid **(29)** in the DCM:MeOH extract of leaves of *C. paniculata*



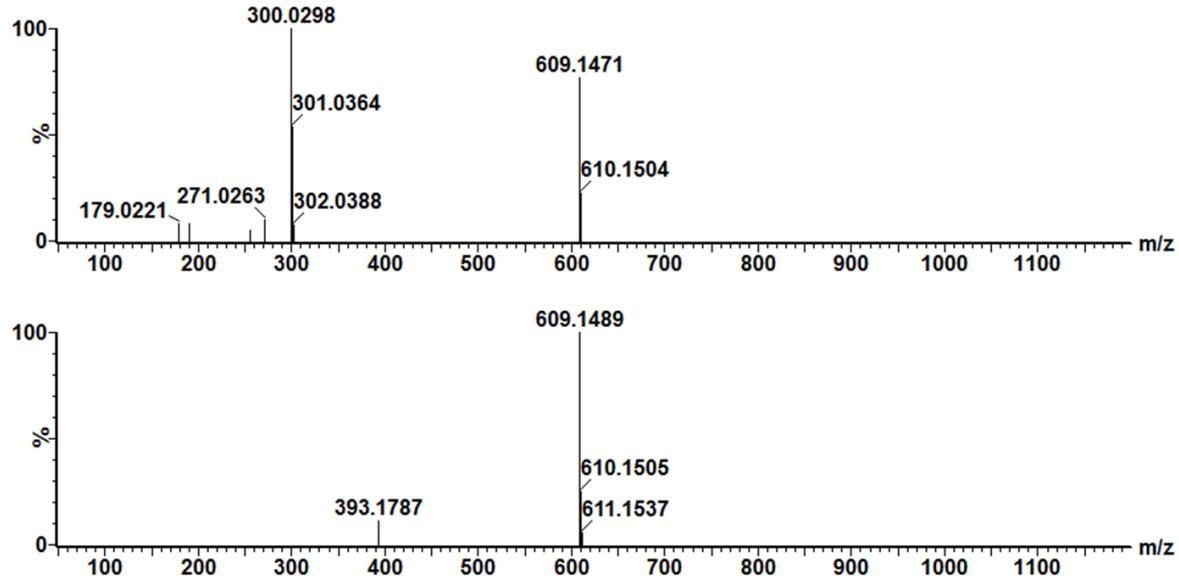
Supplementary data 11: iFit value of 3,5- dicaffeoylquinic acids **(30)** in *C. paniculata* leaves extracted with DCM:MeOH



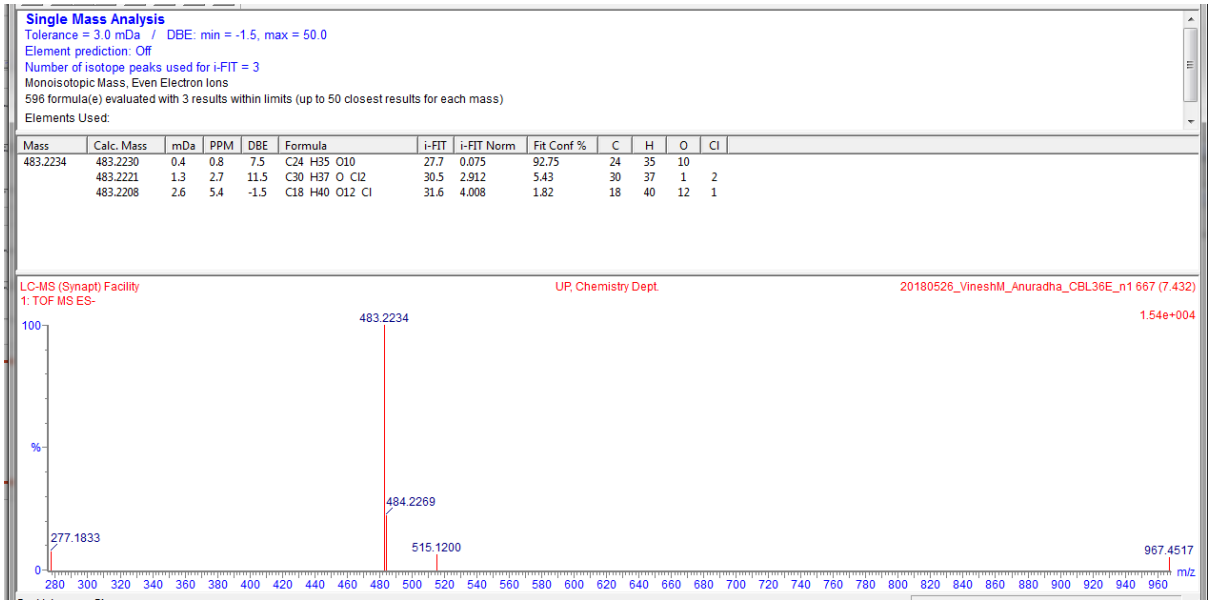
Supplementary data 12: MS and MS/MS data of 3,5- dicaffeoylquinic acids **(30)** in the DCM:MeOH extract of leaves of *C. paniculata*



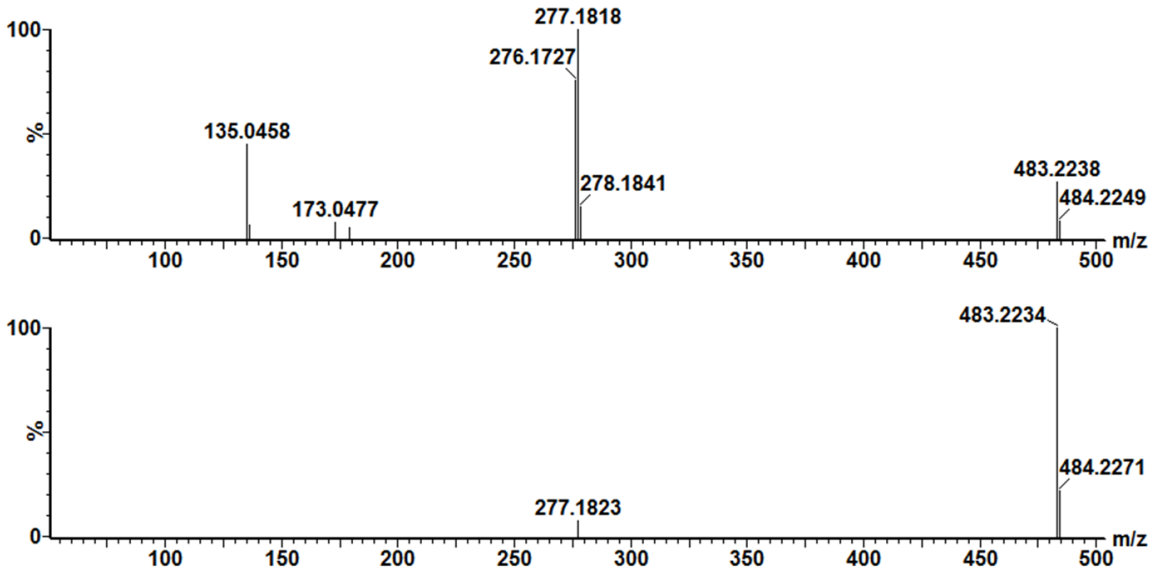
Supplementary data 13: iFit value of rutin **(31)** in *C. paniculata* leaves extracted with DCM:MeOH



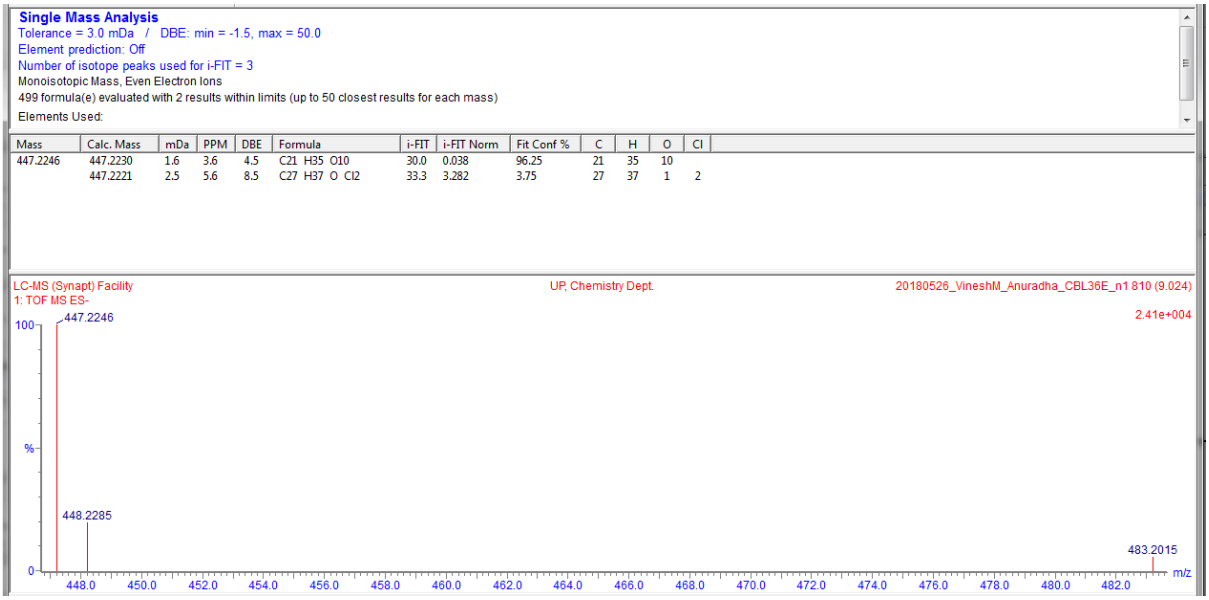
Supplementary data 14: MS and MS/MS data of rutin **(31)** in the DCM:MeOH extract of leaves of *C. paniculata*



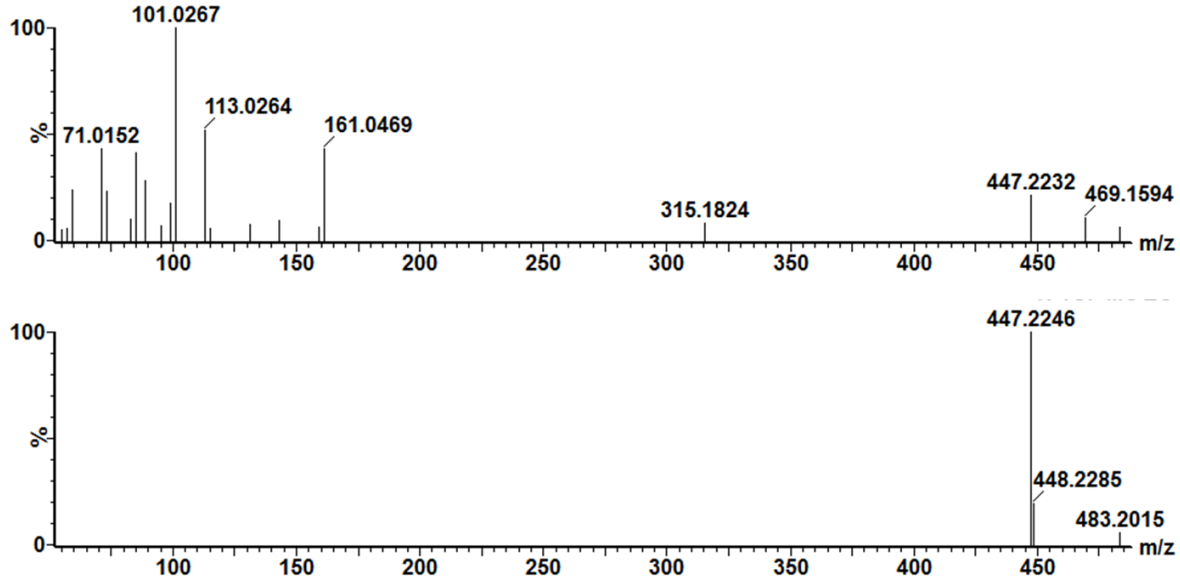
Supplementary data 15: iFit value of valerianannoid E **(32)** in *C. paniculata* leaves extracted with DCM:MeOH



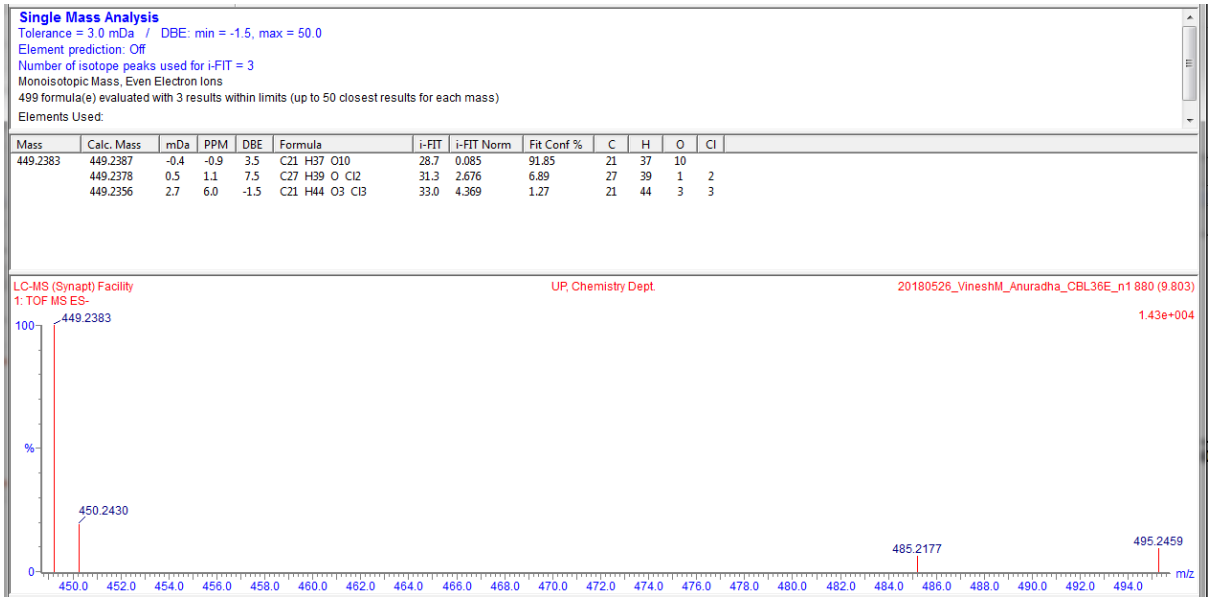
Supplementary data 16: MS and MS/MS data of valerianannoid E **(32)** in the DCM:MeOH extract of leaves of *C. paniculata*



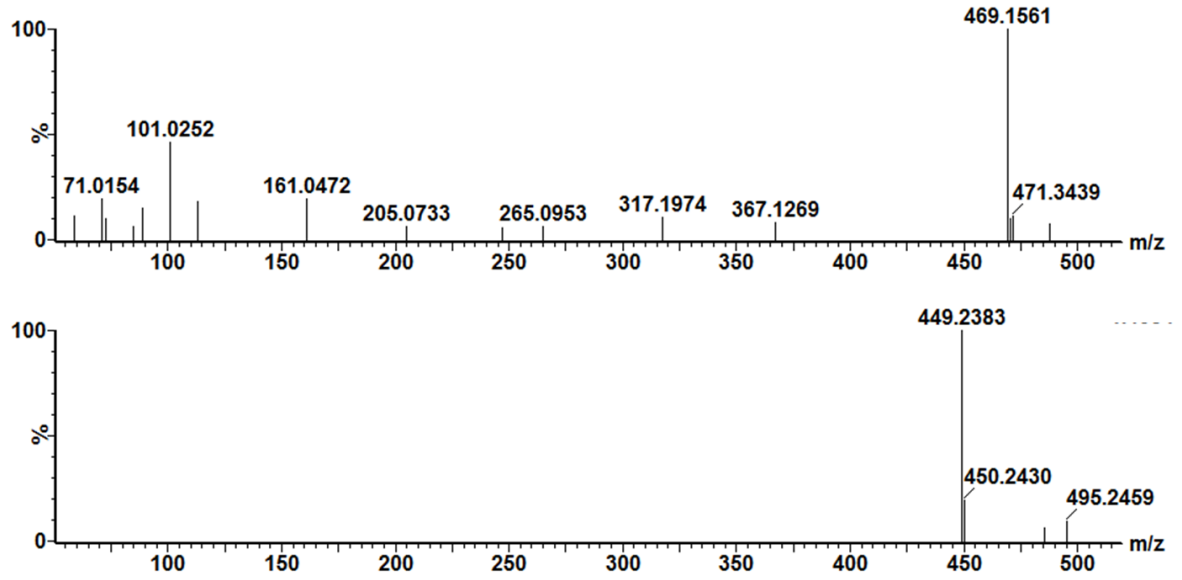
Supplementary data 17: iFit value of acuminoside **(33)** in *C. paniculata* leaves extracted with DCM:MeOH



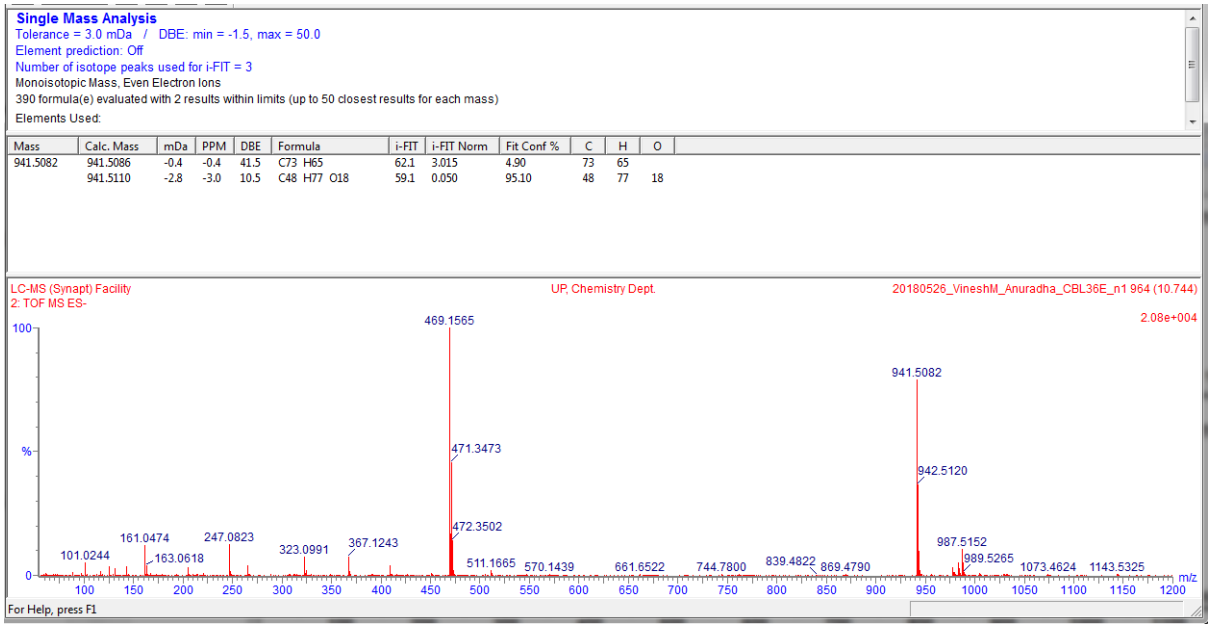
Supplementary data 18: MS and MS/MS data of acuminoside **(33)** in the DCM:MeOH extract of leaves of *C. paniculata*



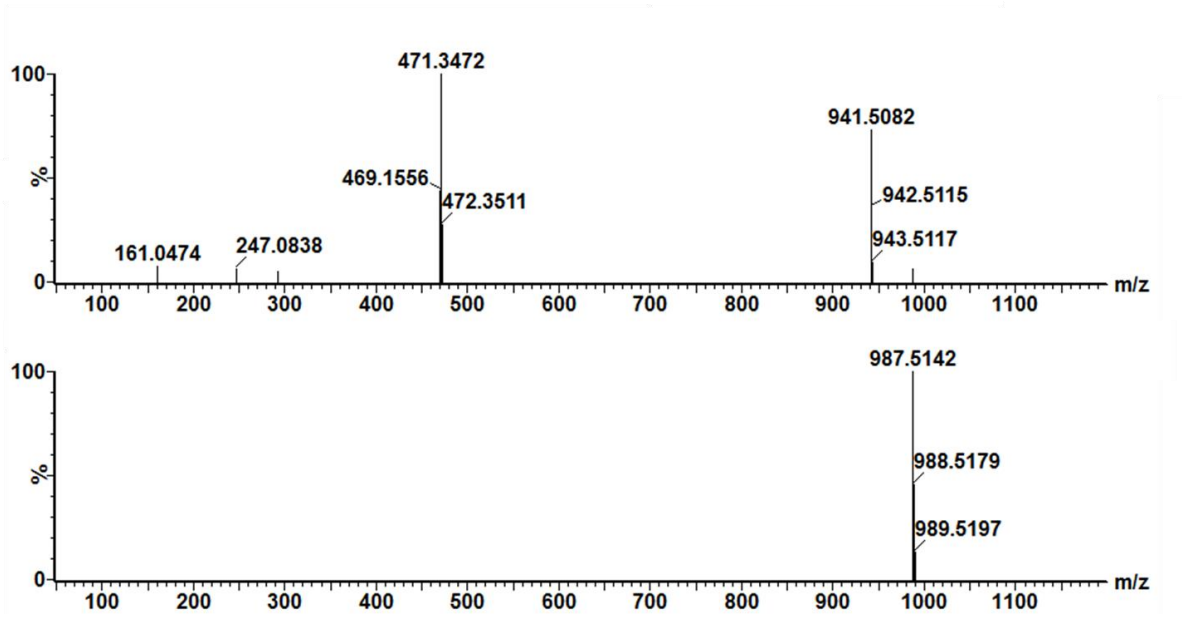
Supplementary data 19: iFit value of dictamnoside D **(34)** in *C. paniculata* leaves extracted with DCM:MeOH



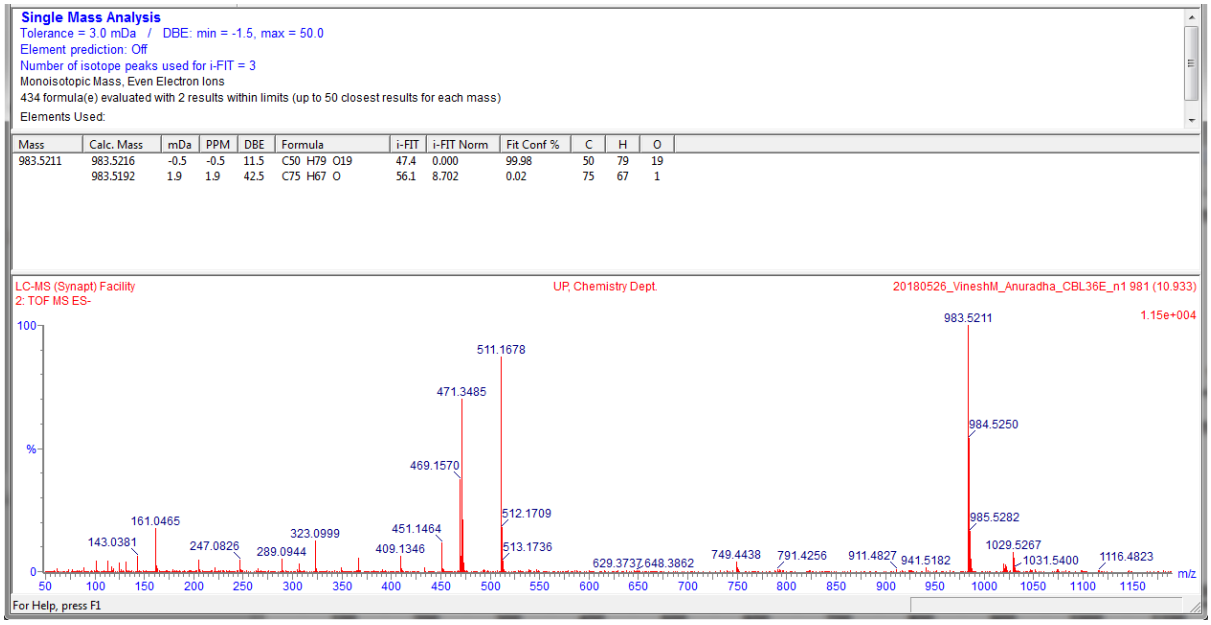
Supplementary data 20: MS and MS/MS data of dictamnoside D **(34)** in the DCM:MeOH extract of leaves of *C. paniculata*



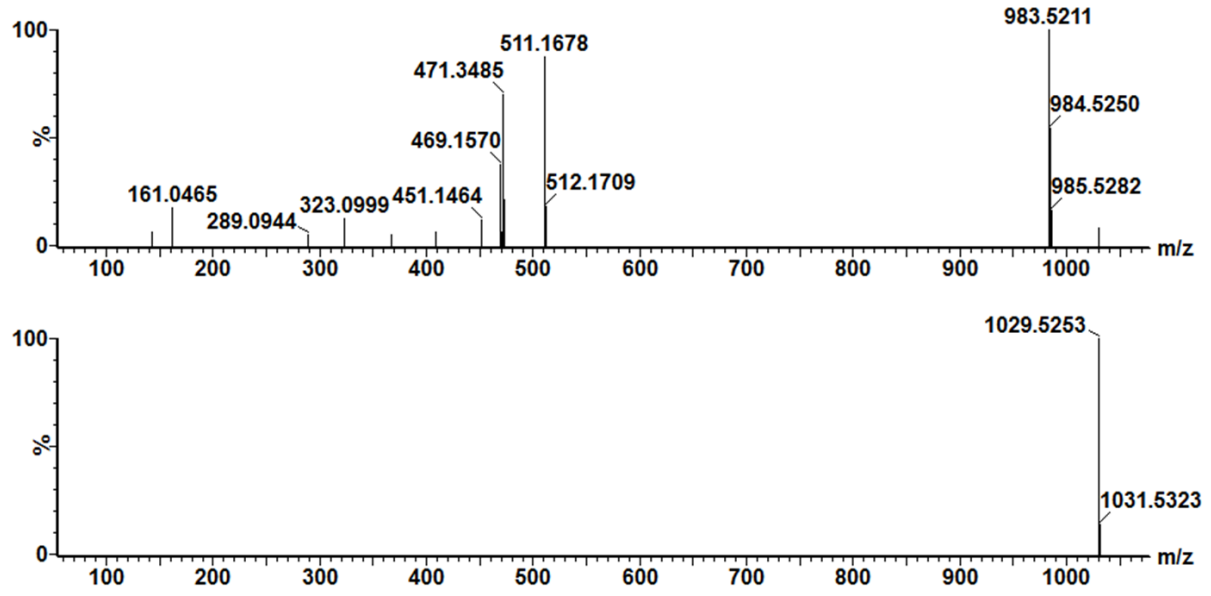
Supplementary data 21: iFit value of 2"-O-ß-D-glucopyranosylsaikosaponin B2 **(35)** in *C. paniculata* leaves extracted with DCM:MeOH



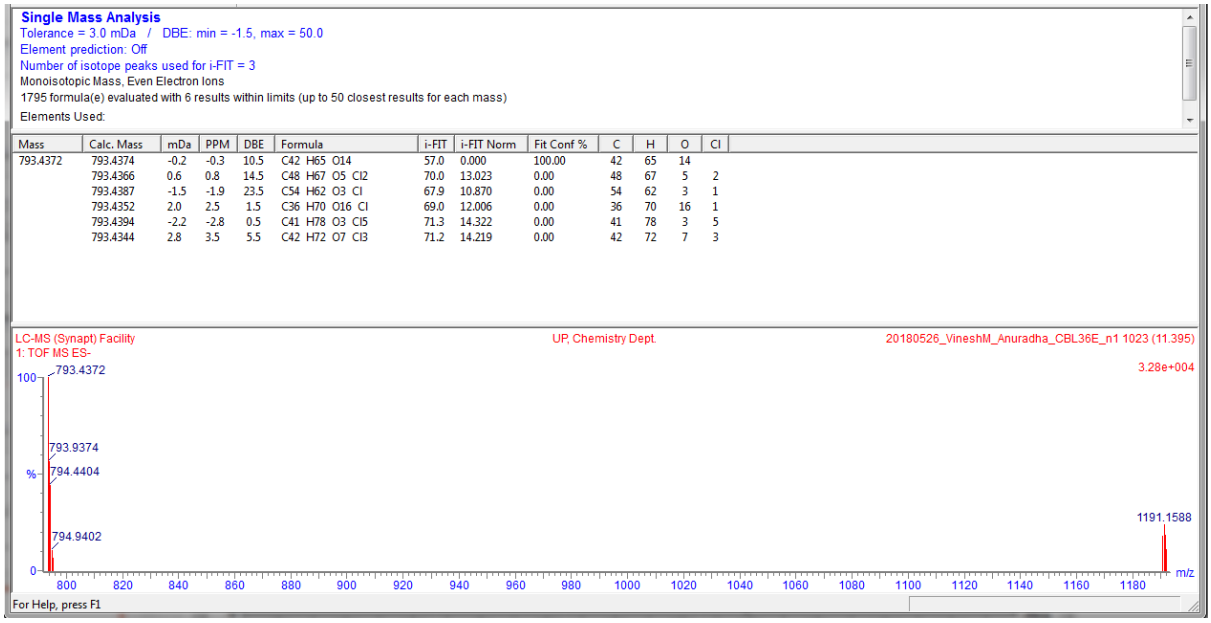
Supplementary data 22: MS and MS/MS data of 2"-O-ß-D-glucopyranosylsaikosaponin B2 **(35)** in the DCM:MeOH extract of leaves of *C. paniculata*



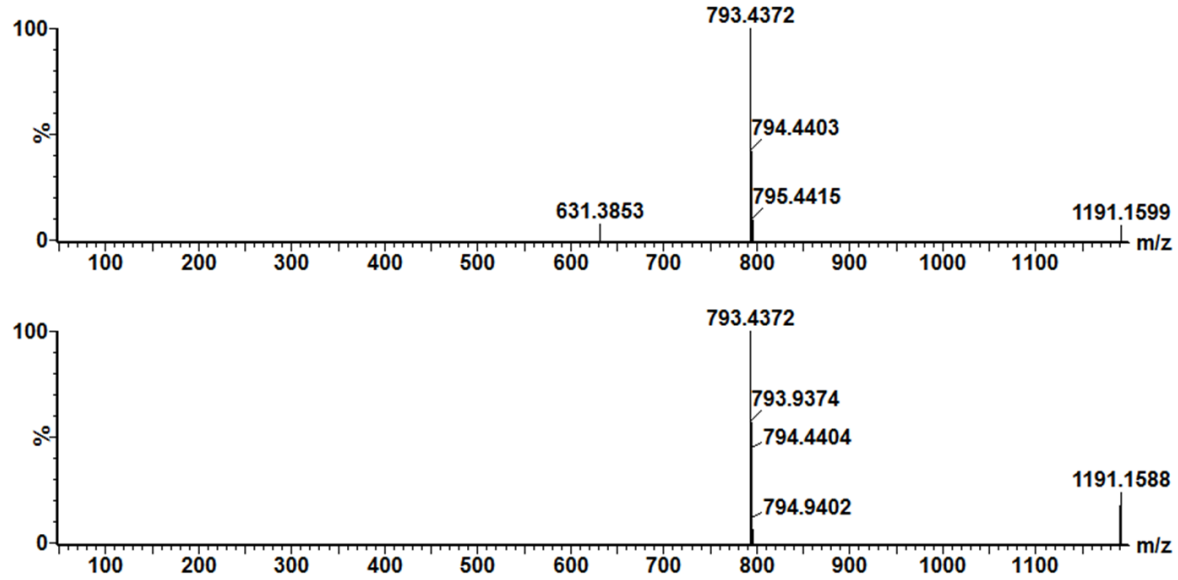
Supplementary data 23: iFit value of clinosaponin C **(36)** in *C. paniculata* leaves extracted with DCM:MeOH



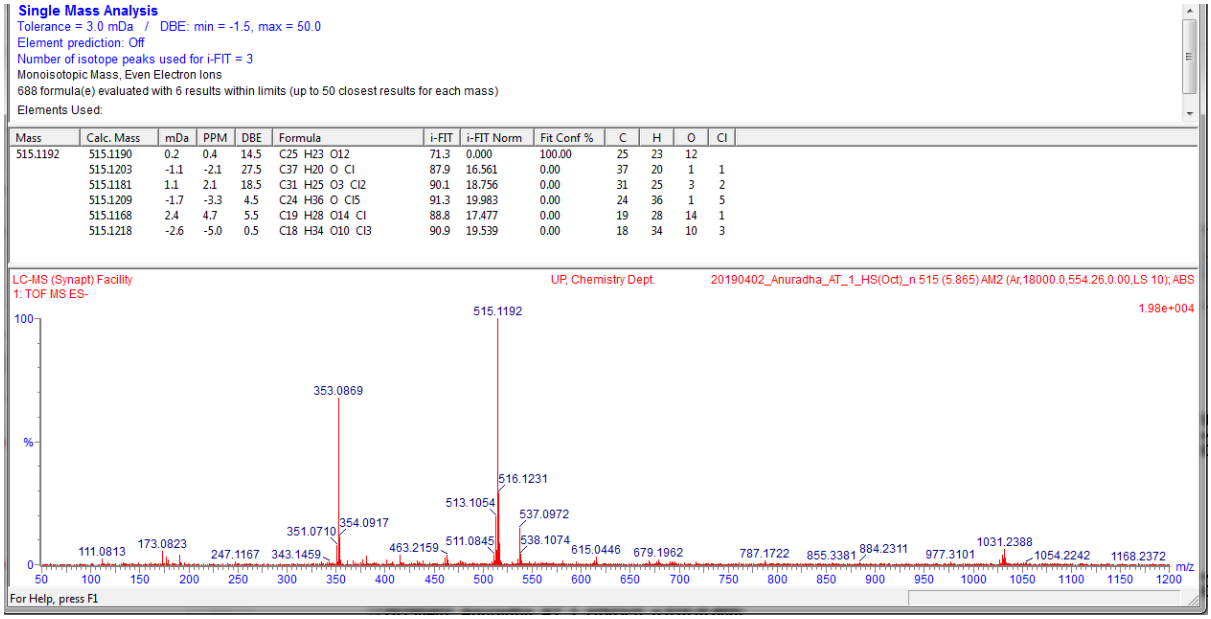
Supplementary data 24: MS and MS/MS data of clinosaponin C **(36)** in the DCM:MeOH extract of leaves of *C. paniculata*



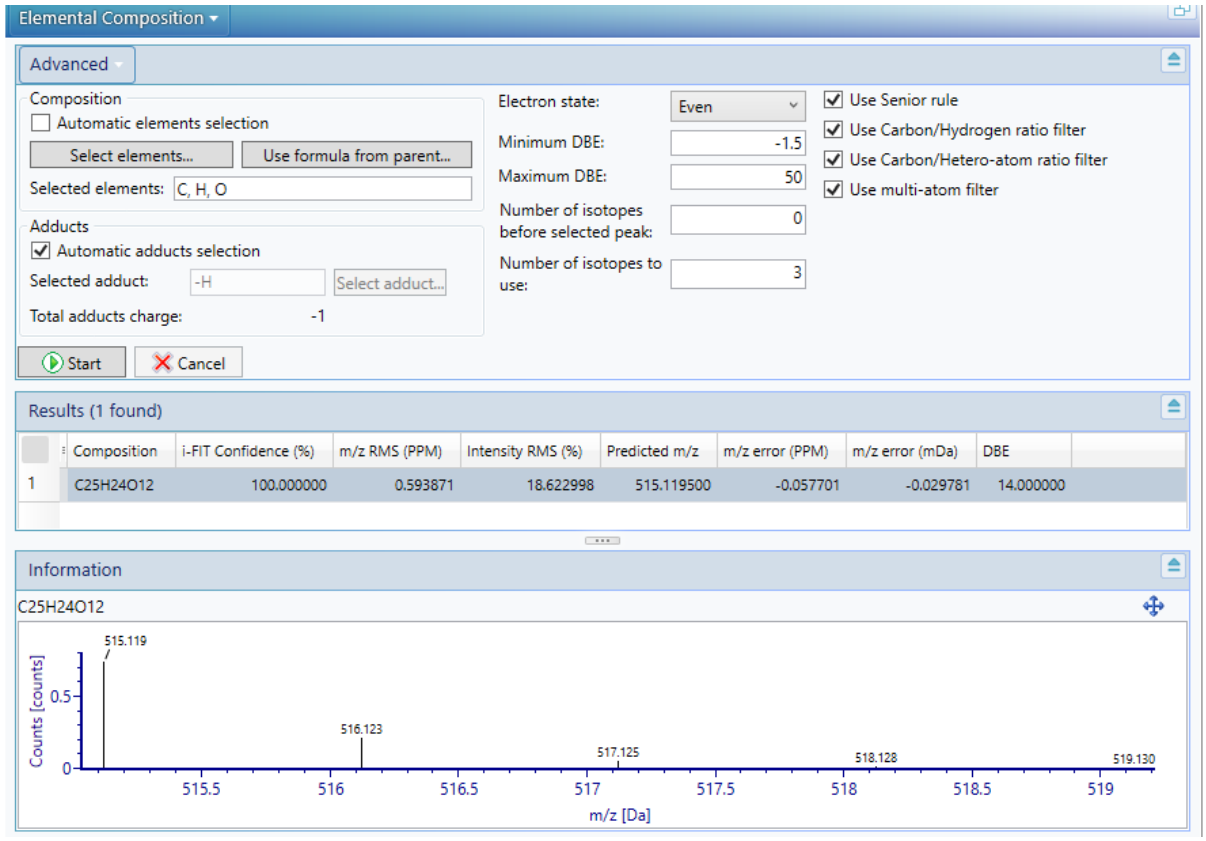
Supplementary data 25: iFit value of spinasaponin A **(37)** in *C. paniculata* leaves extracted with DCM:MeOH



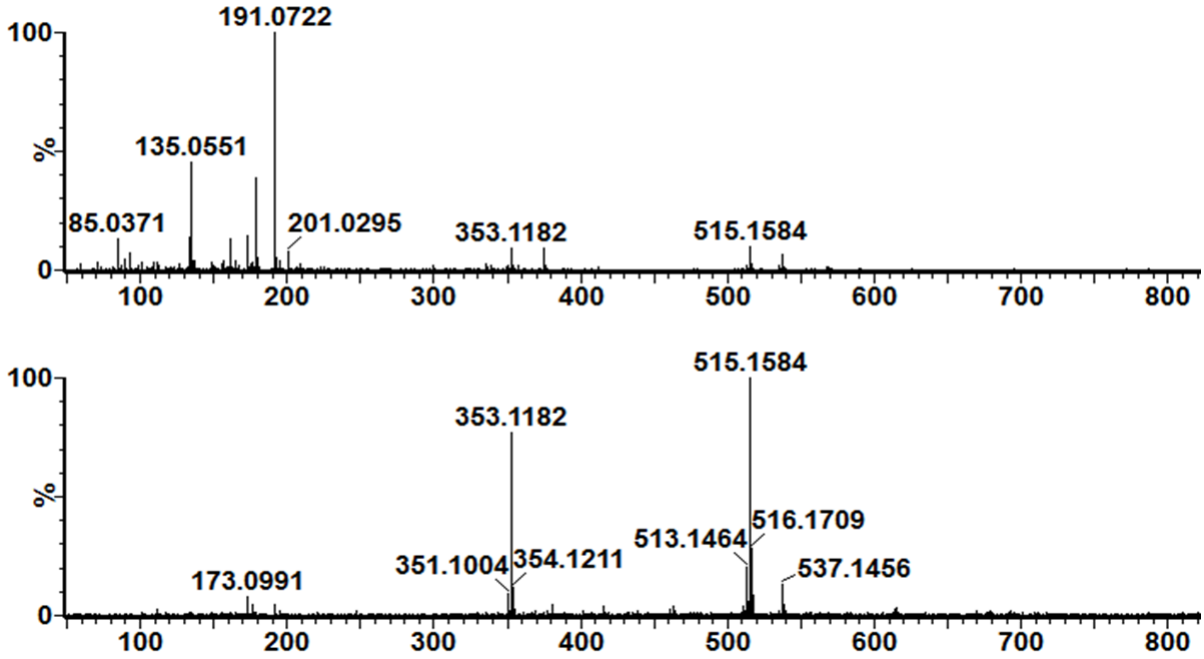
Supplementary data 26: MS and MS/MS data of spinasaponin A **(37)** in the DCM:MeOH extract of leaves of *C. paniculata*



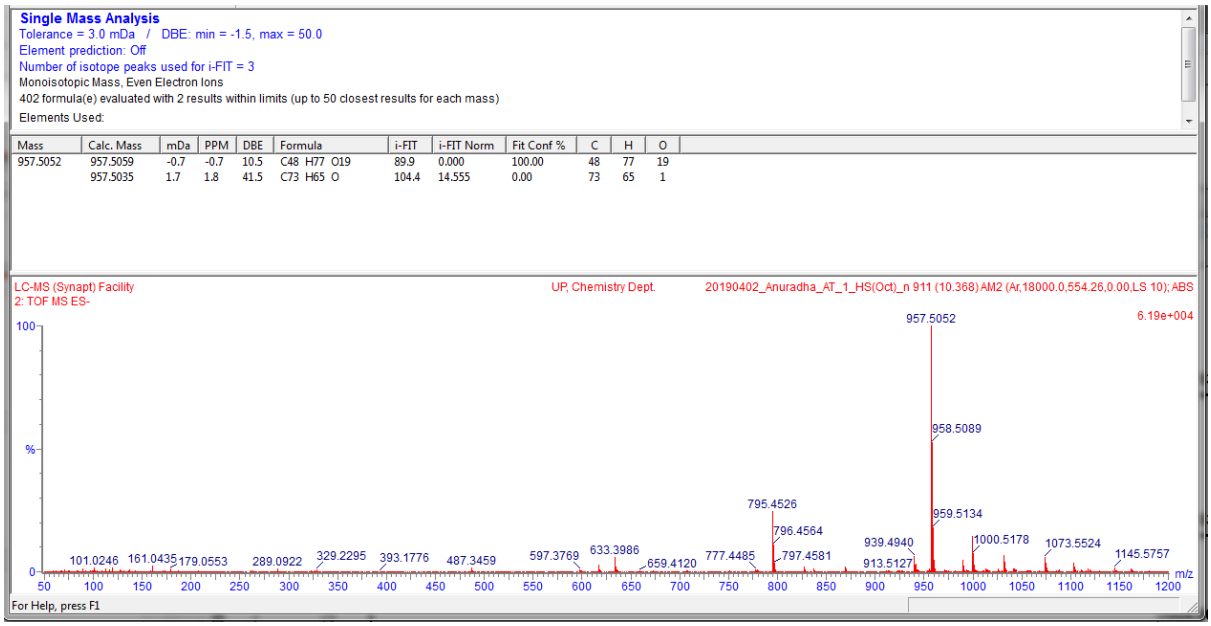
Supplementary data 27: iFit value of 1,5-dicaffeoylquinic acid **(38)** in *H. arborescens* stems extracted with DCM:MeOH



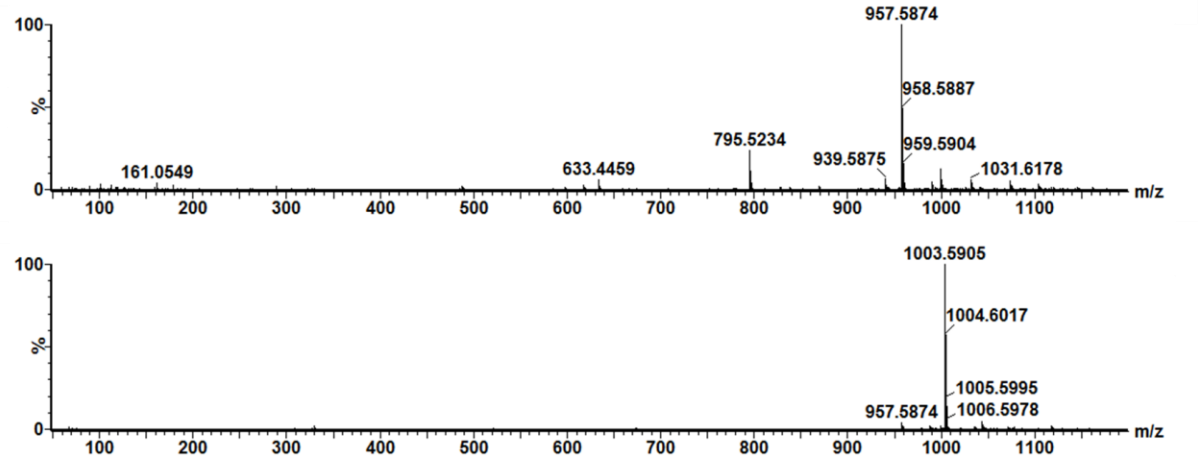
Supplementary data 28: Elemental composition by using UNIFI for 1,5-dicaffeoylquinic acid **(38)** in *H. arborescens* stems extracted with DCM:MeOH



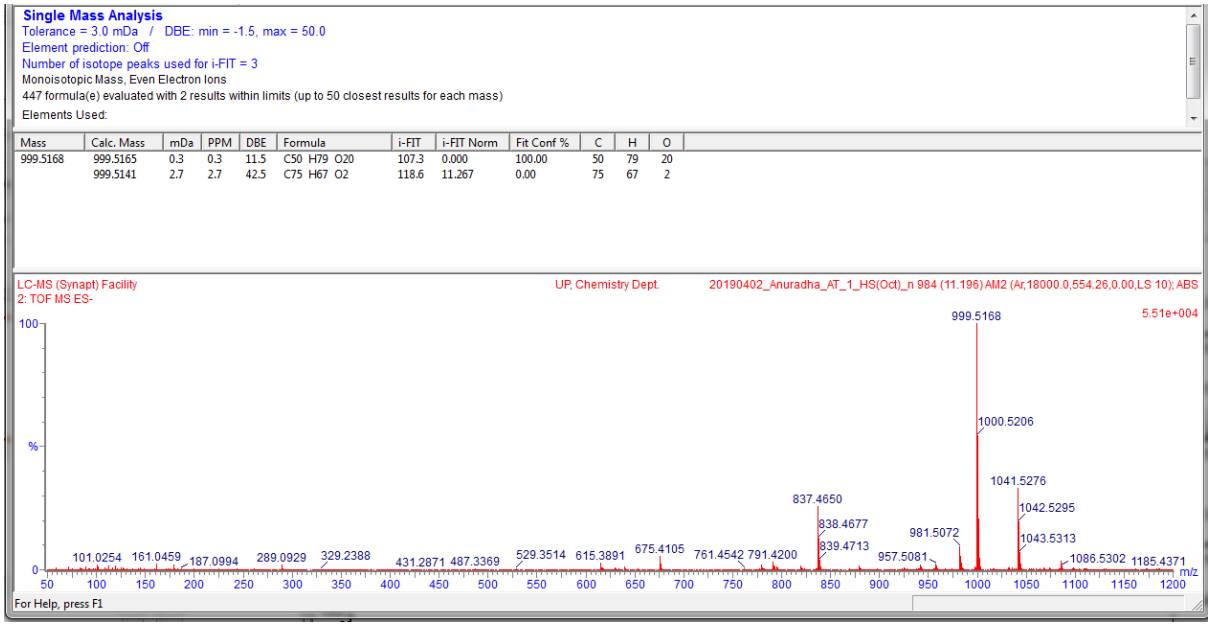
Supplementary data 29: MS and MS/MS data of 1,5-dicaffeoylquinic acid **(38)** in the DCM:MeOH extract of leaves of *H. arborescens*



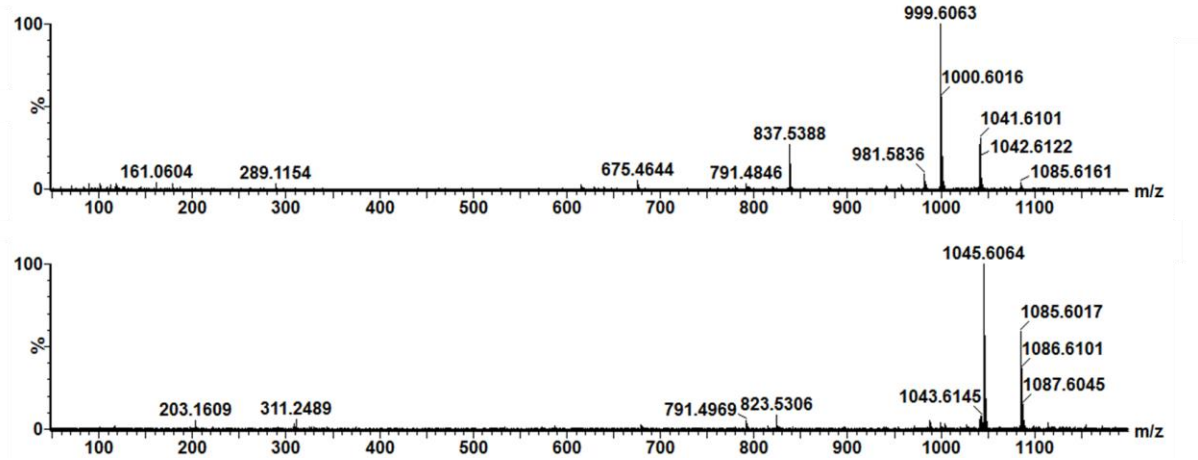
Supplementary data 30: iFit value of alternoside IX **(39)** in *H. arborescens* stems extracted with DCM:MeOH



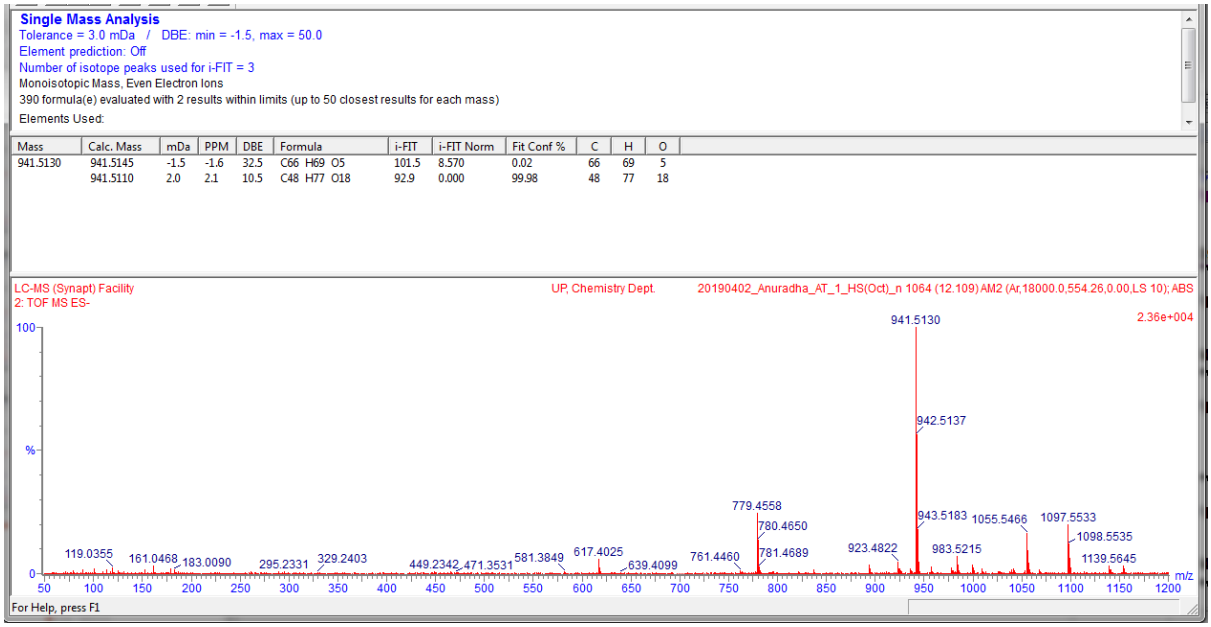
Supplementary data 31: MS and MS/MS data of alternoside IX **(39)** in the DCM:MeOH extract of leaves of *H. arborescens*



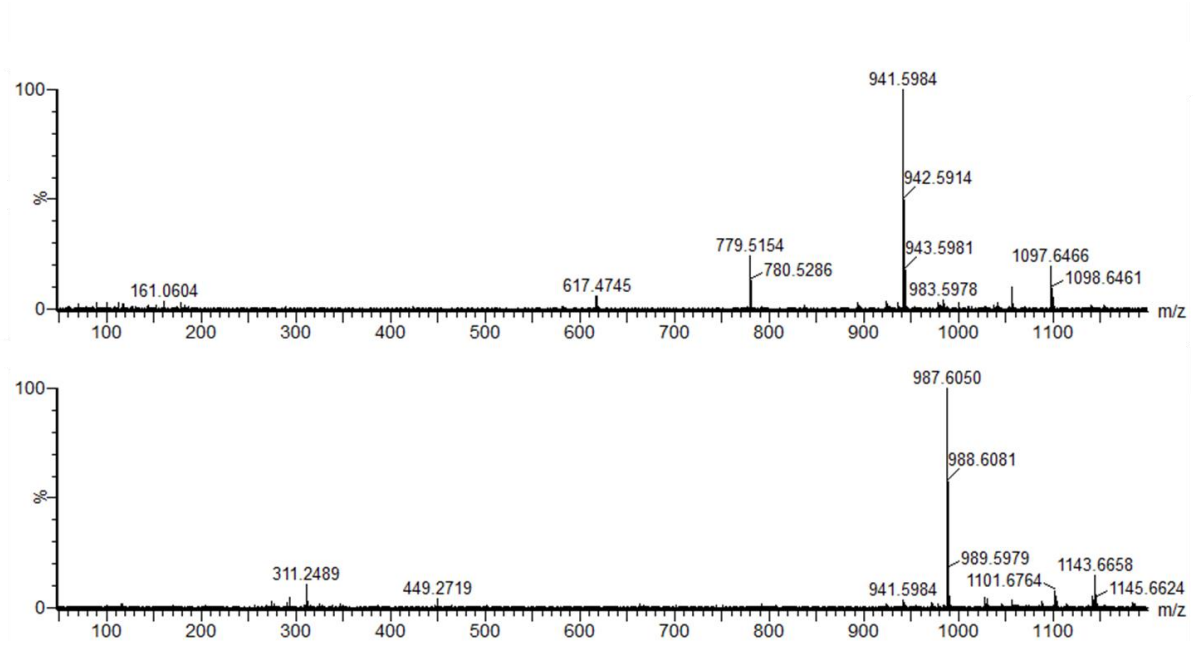
Supplementary data 32: iFit value of alternoside I **(40)** in *H. arborescens* stems extracted with DCM:MeOH



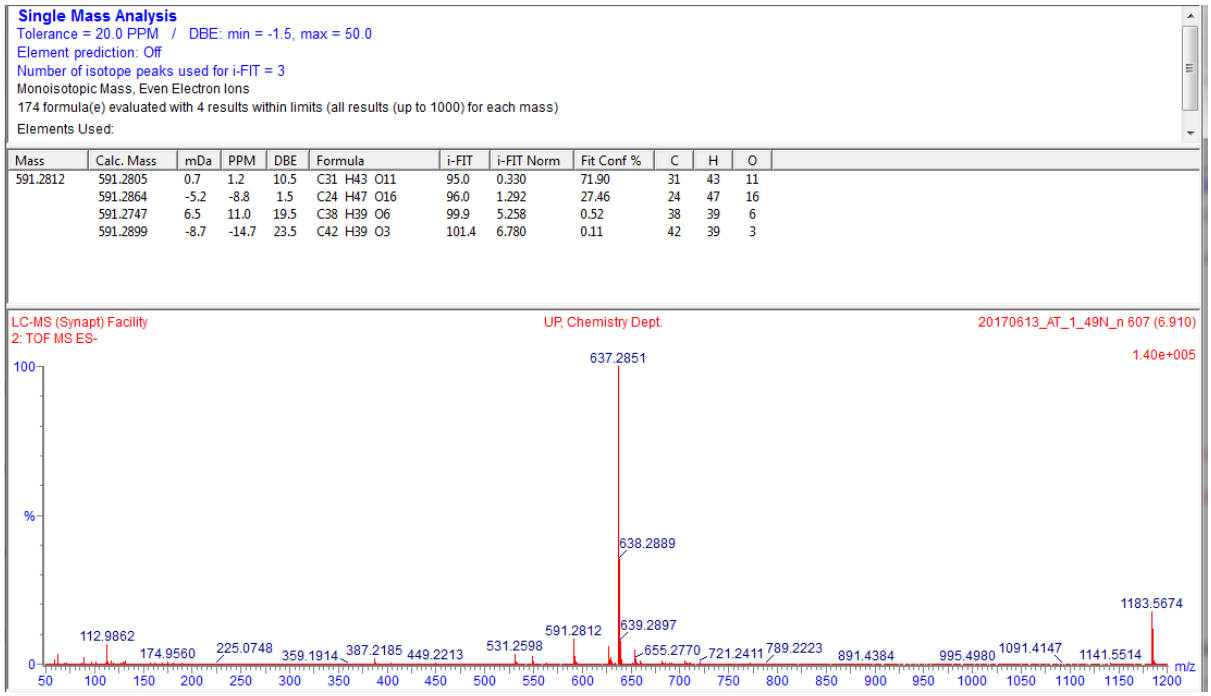
Supplementary data 33: MS and MS/MS data of alternoside I **(40)** in the DCM:MeOH extract of leaves of *H. arborescens*



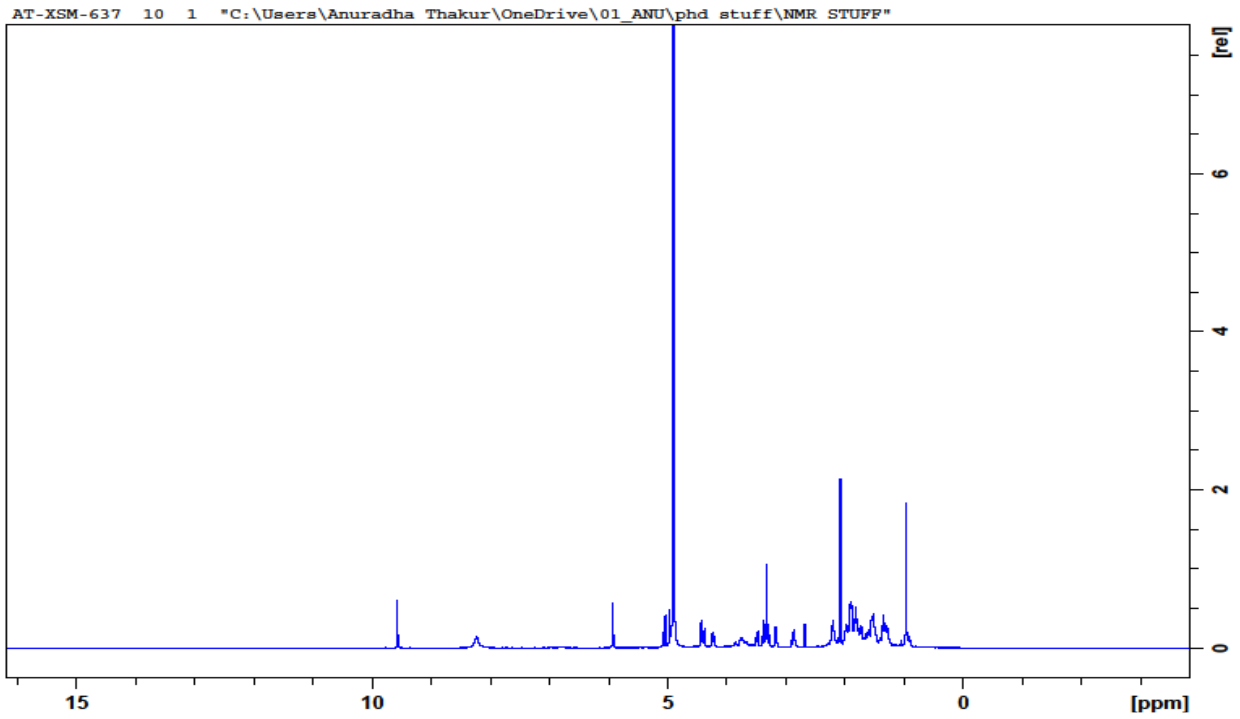
Supplementary data 34: iFit value of saikogenin B_4 **(41)** in *H. arborescens* stems extracted with DCM:MeOH



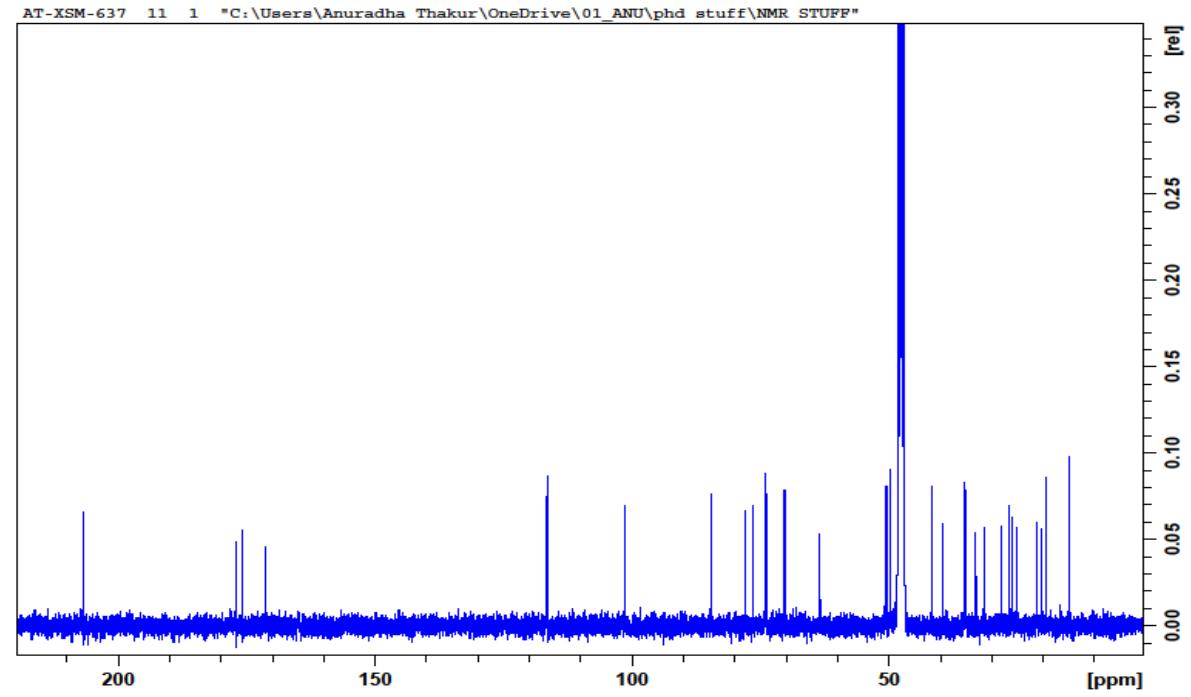
Supplementary data 35: MS and MS/MS data of saikogenin B_4 **(41)** in the DCM:MeOH extract of leaves of *H. arborescens*



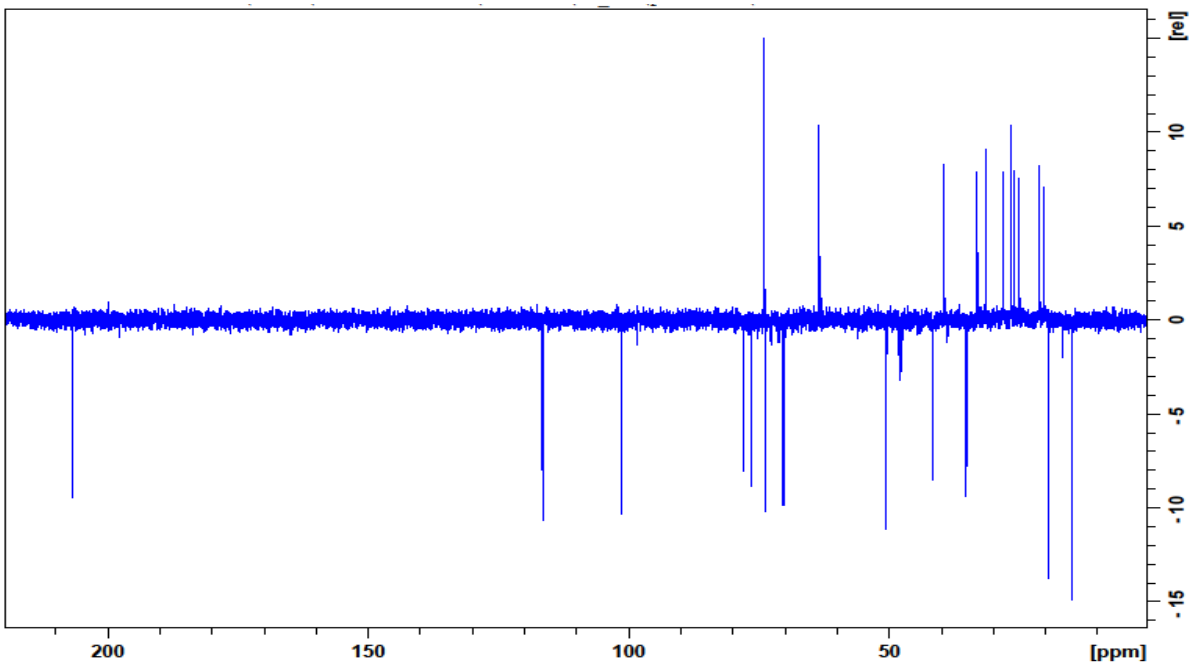
Supplementary data 36: iFit value of acetylated glycosydated crotoxigenin **(56)** in *X. undulatum* leaves extracted with DCM:MeOH



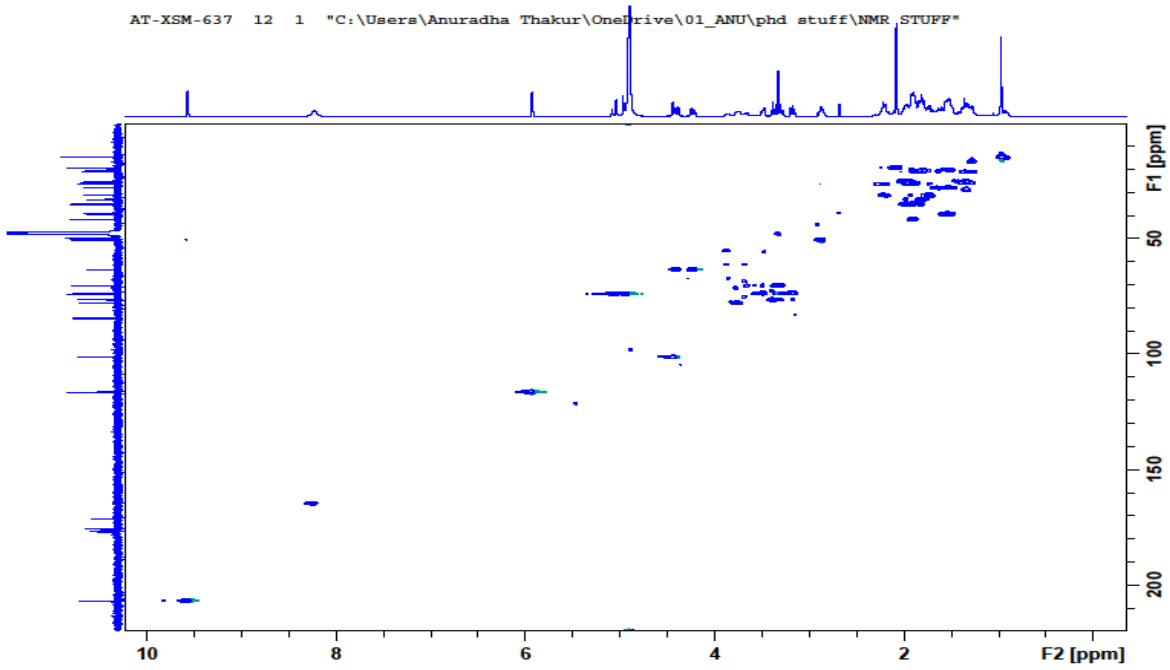
Supplementary data 37:[1]H NMR for compound 3 acetylated glycosydated crotoxigenin **(56)** in MeOD-*d4*



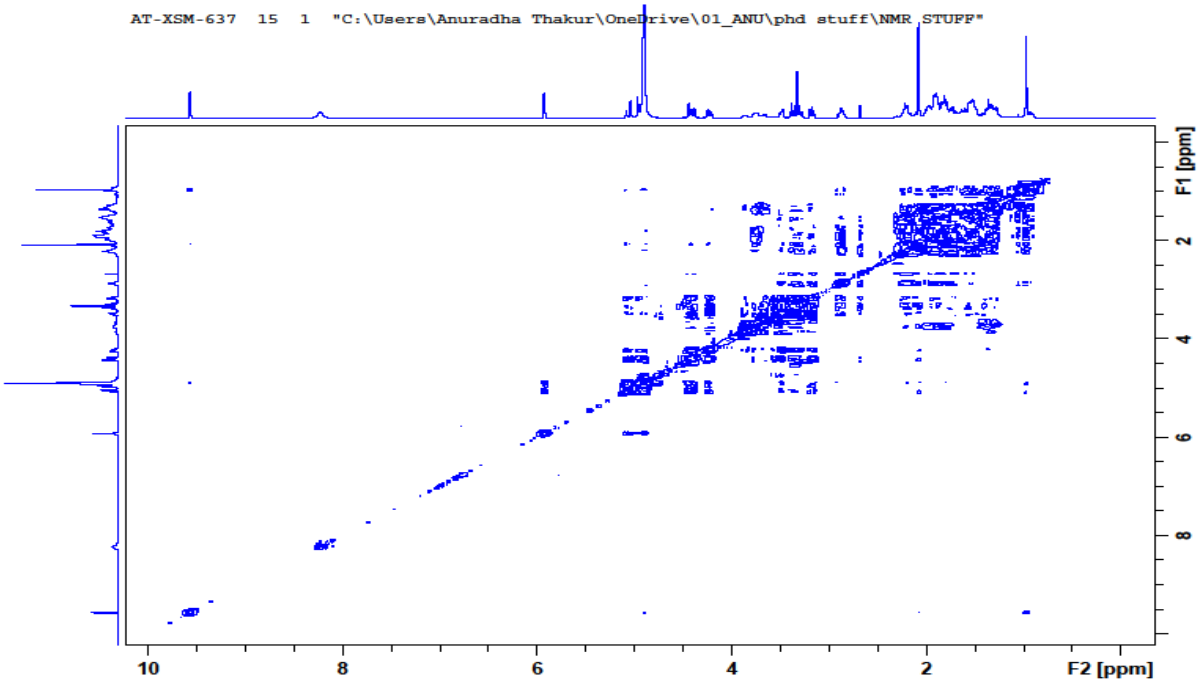
Supplementary data 38: [13]C NMR for compound 3 acetylated glycosydated crotoxigenin **(56)** in MeOD-*d4*



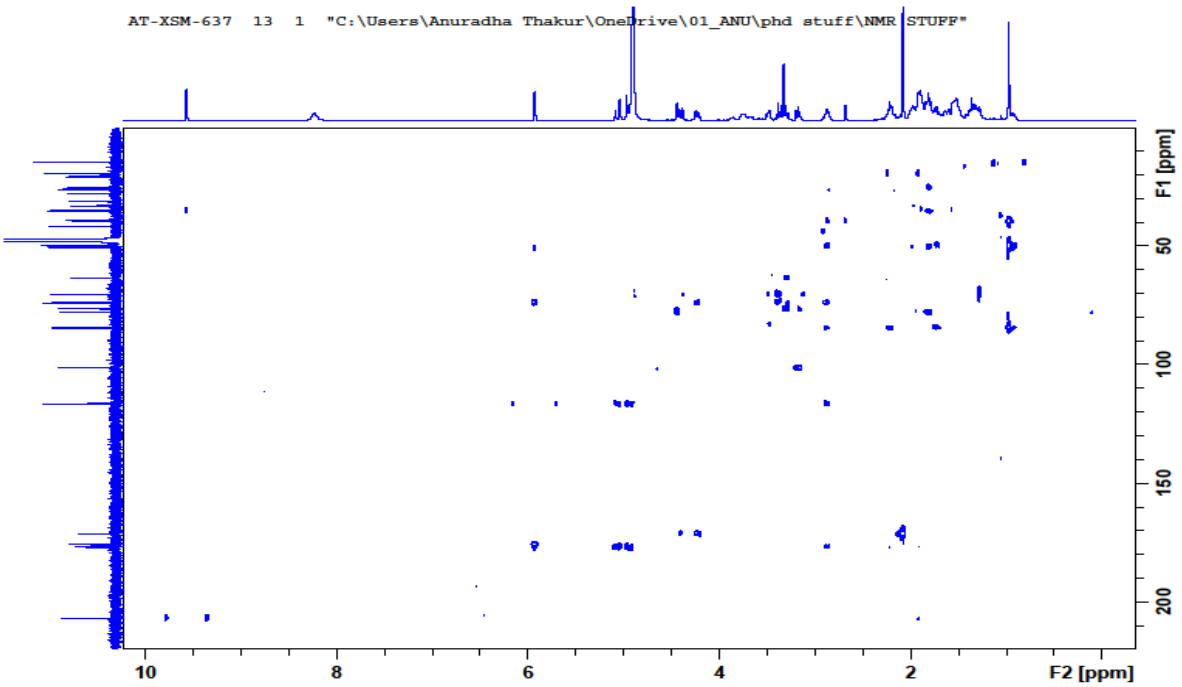
Supplementary data 39:DEPT 135 for acetylated glycosydated crotoxigenin **(56)** in MeOD-*d4*



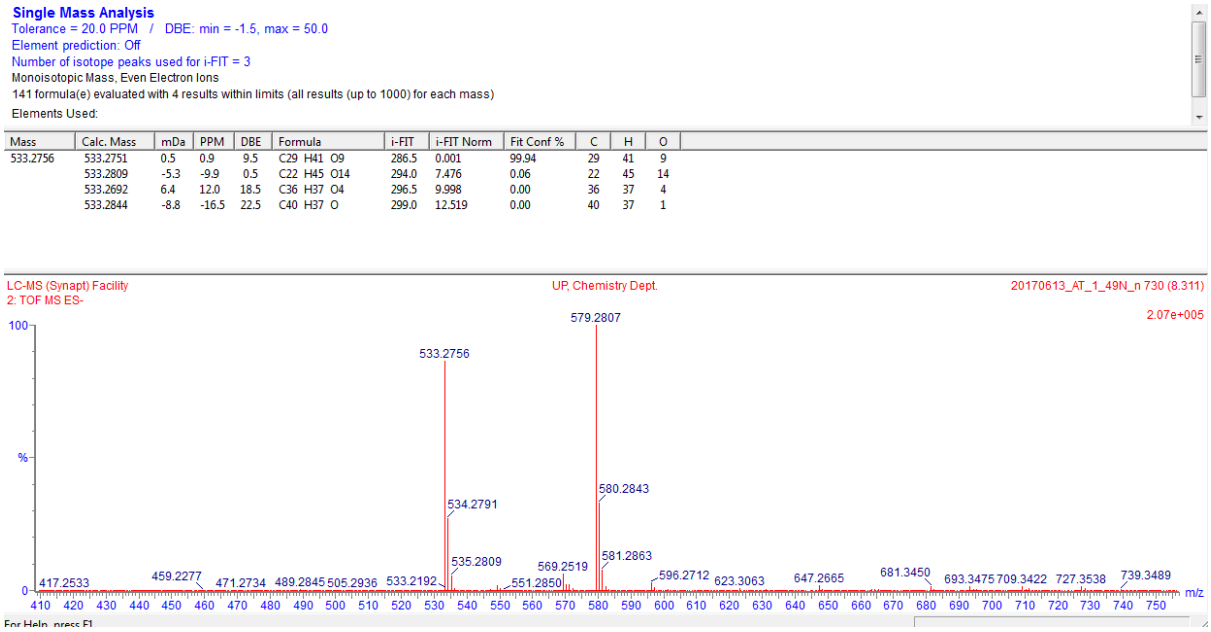
Supplementary data 40:HSQC spectrum for acetylated glycosydated crotoxogenin **(56)** in MeOD-*d4*



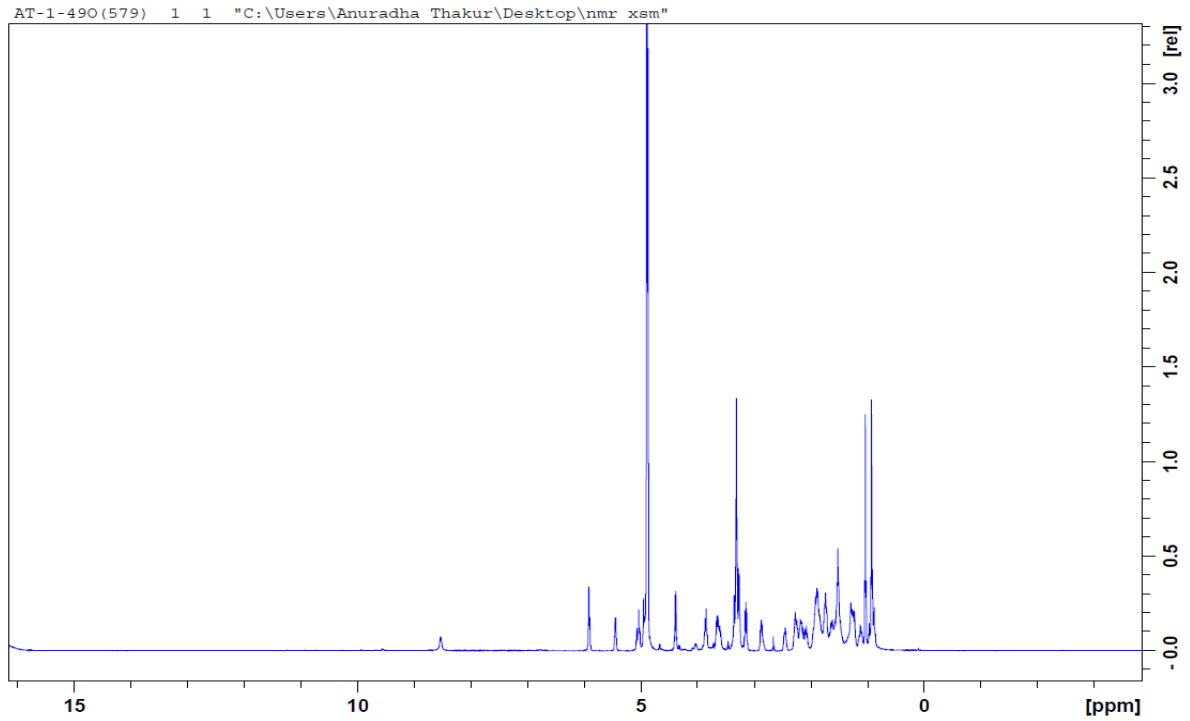
Supplementary data 41: COSY spectrum for acetylated glycosydated crotoxogenin **(56)** in MeOD-*d4*



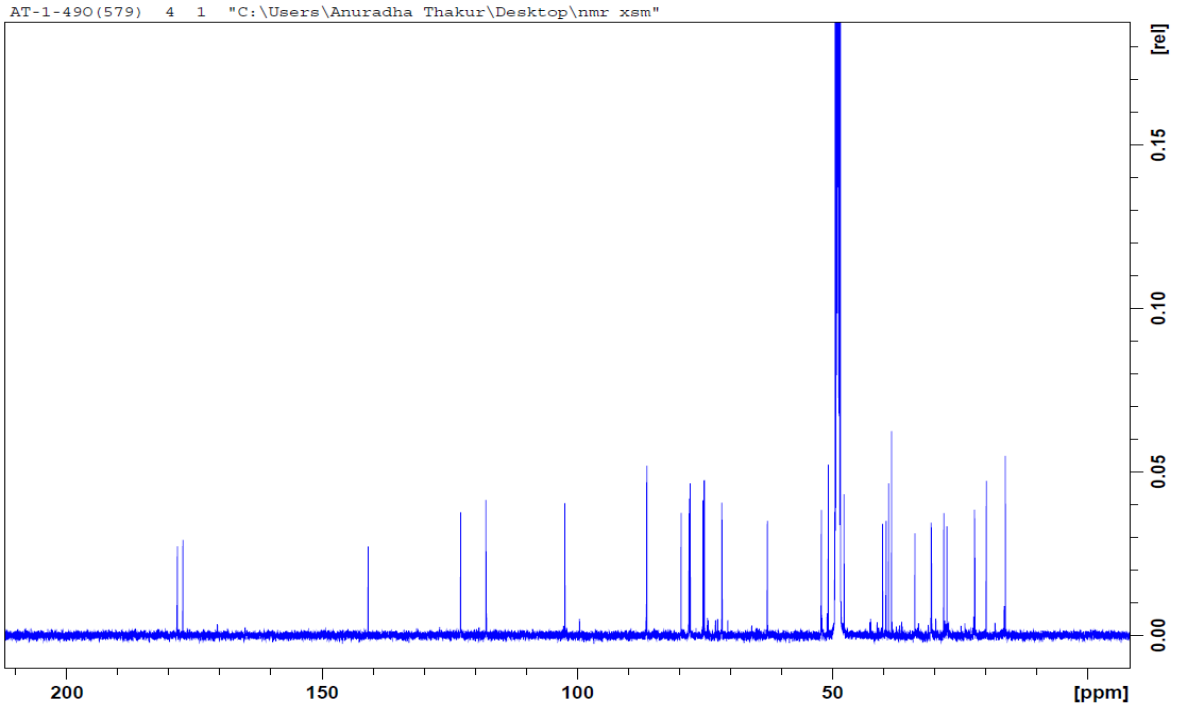
Supplementary data 42: HMBC for acetylated glycosydated crotoxigenin **(56)** in MeOD-*d4*



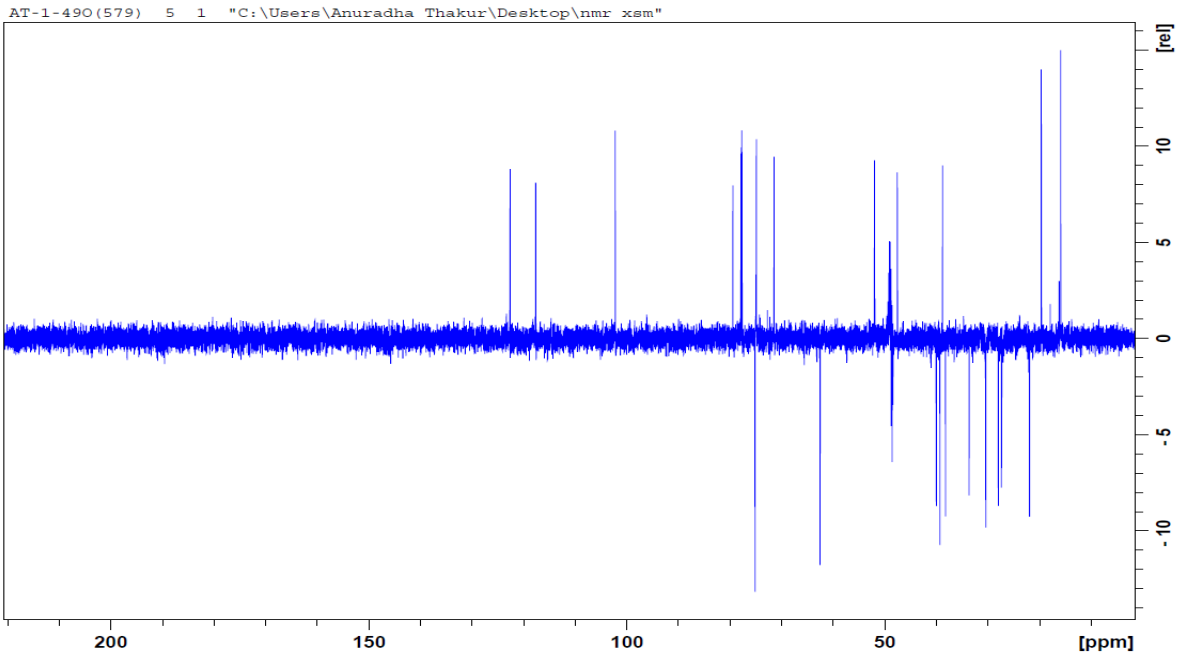
Supplementary data 43: iFit value of Xysmalogenin-3, β-D-glucopyranoside **(57)** in *X. undulatum* leaves extracted with DCM:MeOH



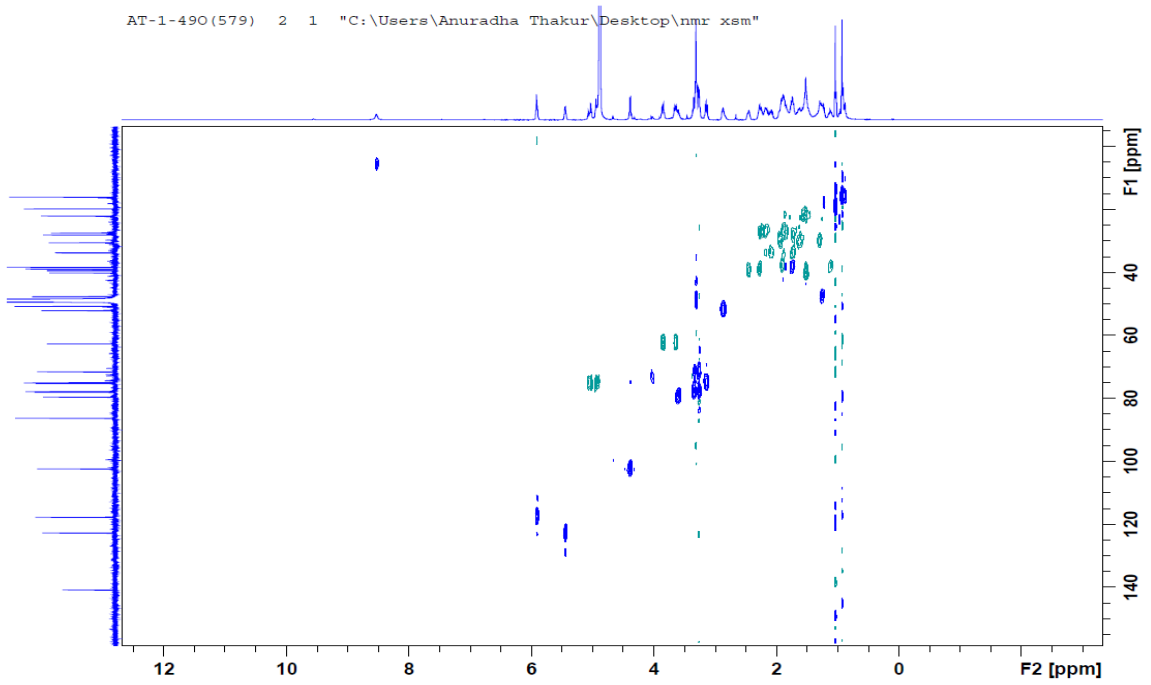
Supplementary data 44:^{1}H NMR for Xysmalogenin-3, β-D-glucopyranoside **(57)** in MeOD-*d4*



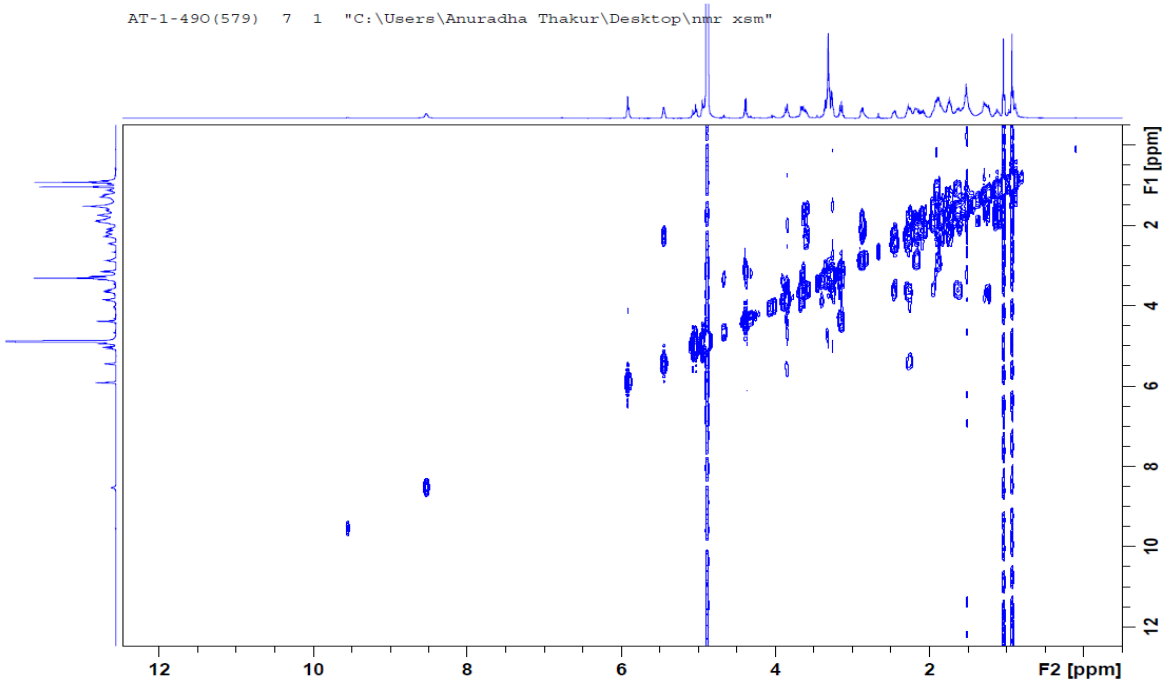
Supplementary data 45: ^{13}C NMR for compound Xysmalogenin-3, β-D-glucopyranoside **(57)** in MeOD-*d4*



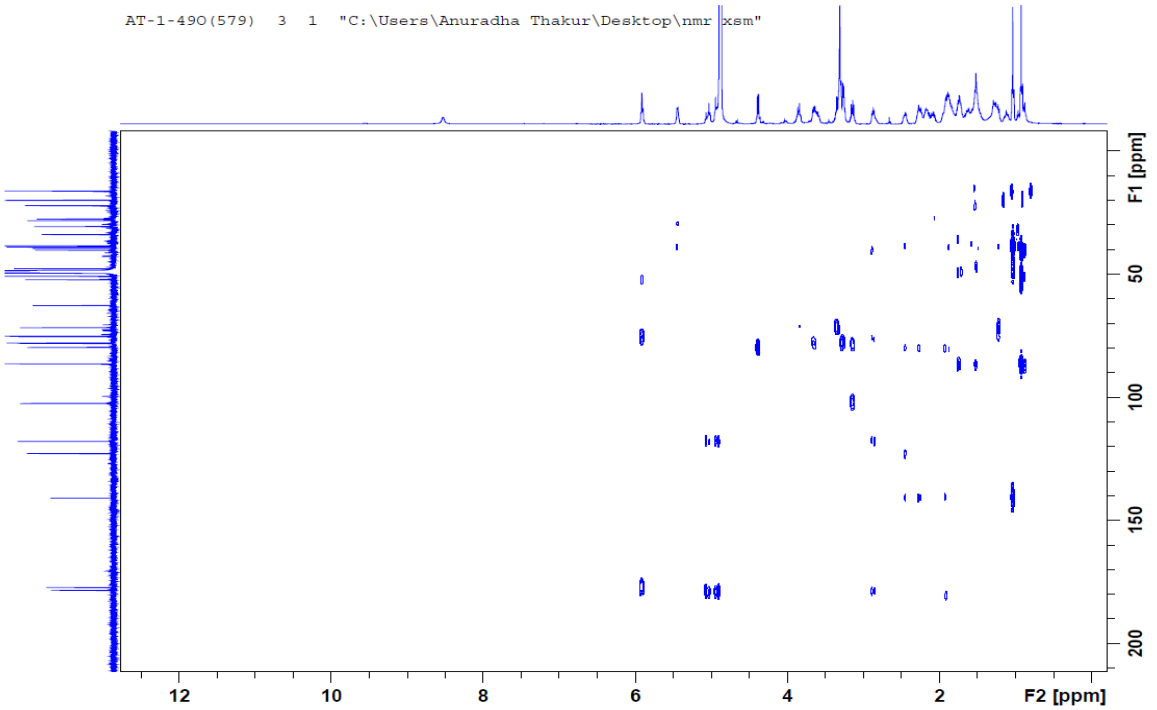
Supplementary data 46: DEPT 135 for Xysmalogenin-3, β-D-glucopyranoside **(57)** in MeOD-*d4*



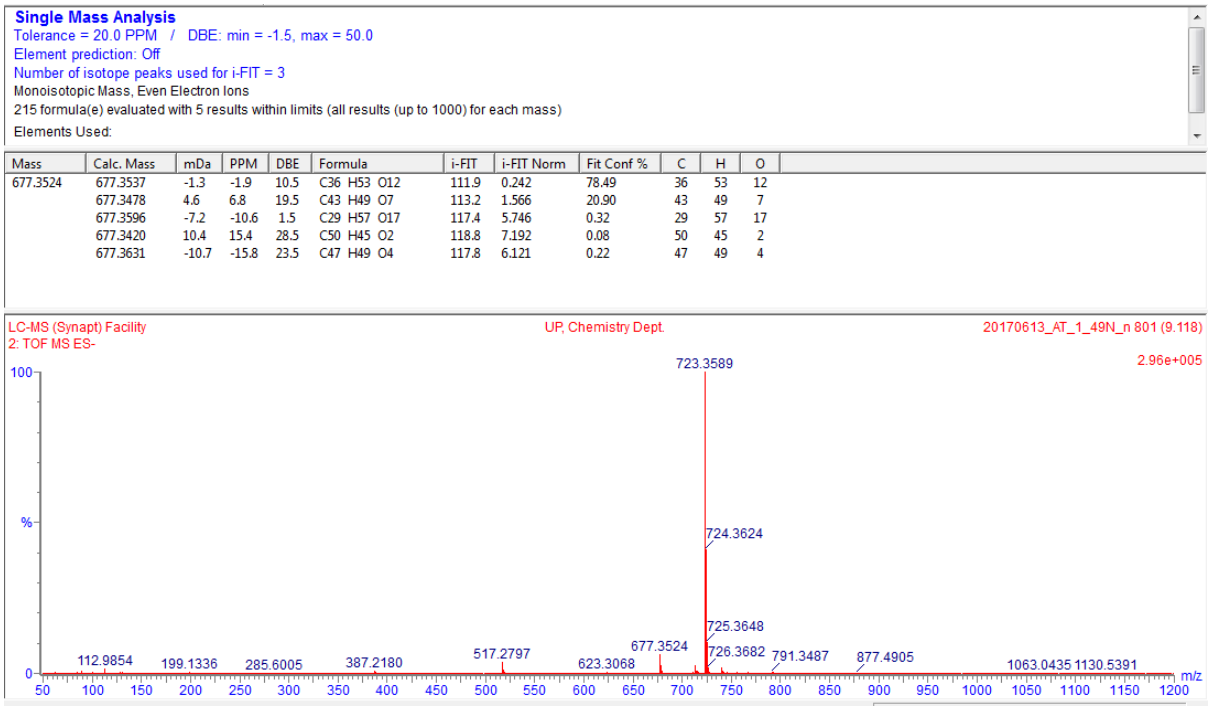
Supplementary data 47: HSQC for Xysmalogenin-3, β-D-glucopyranoside **(57)** in MeOD-*d4*



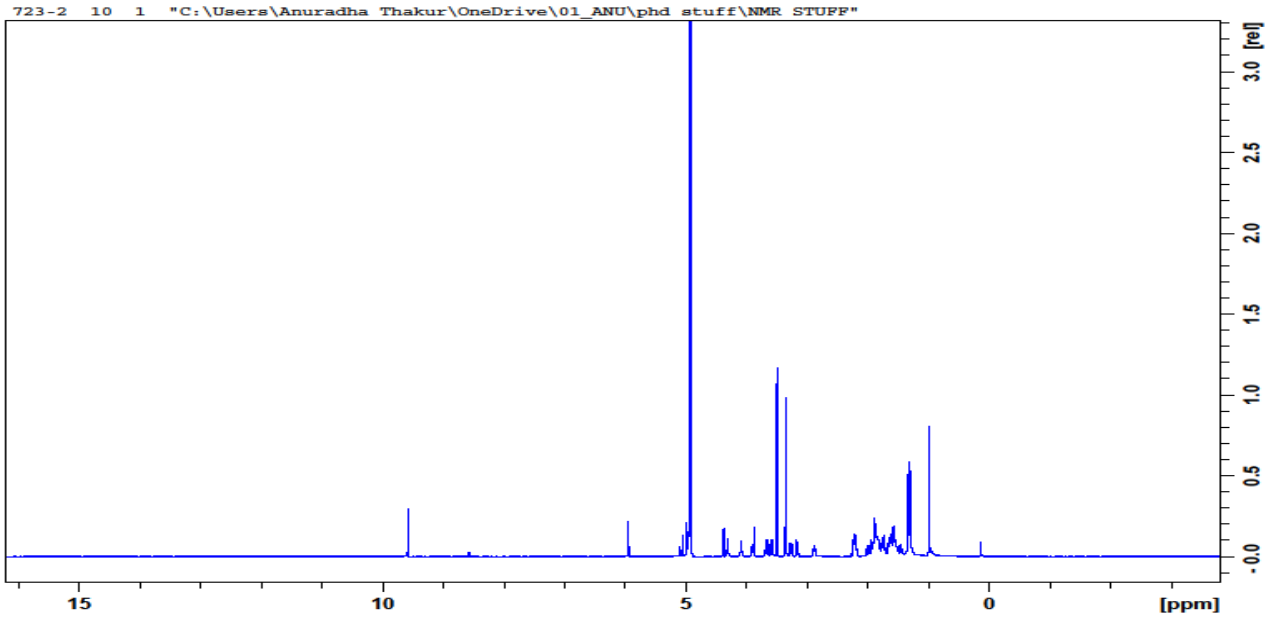
Supplementary data 48: COSY spectrum for Xysmalogenin-3, β-D-glucopyranoside **(57)** in MeOD-*d4*



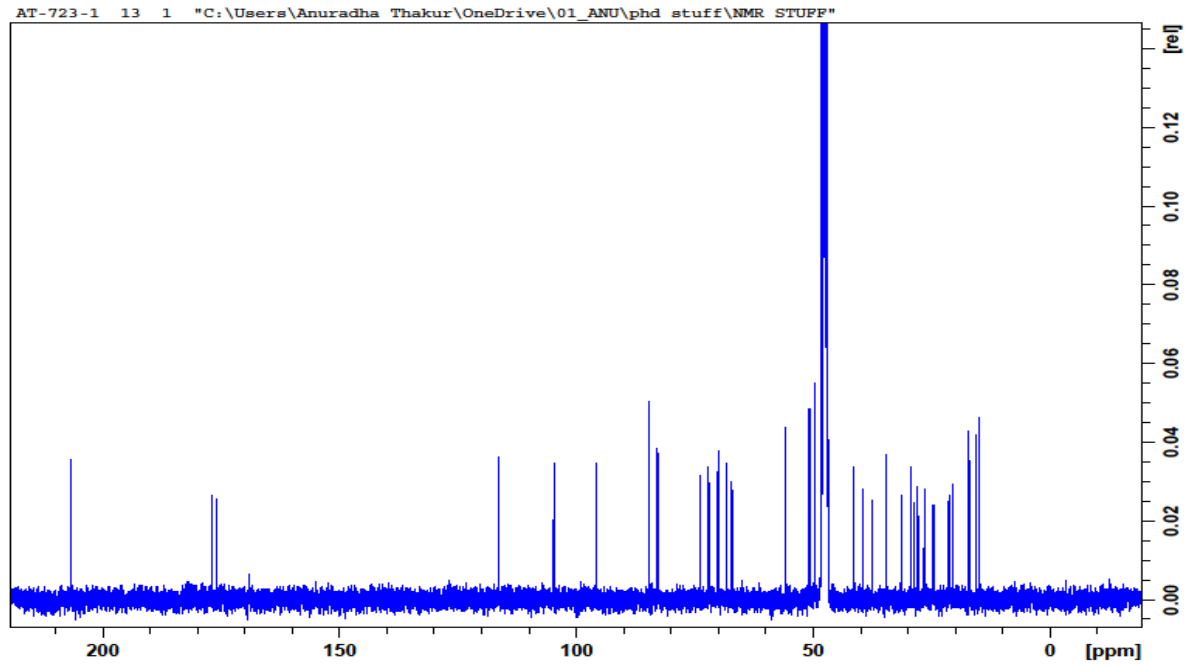
Supplementary data 49: COSY spectrum for Xysmalogenin-3, β-D-glucopyranoside **(57)** in MeOD-*d4*



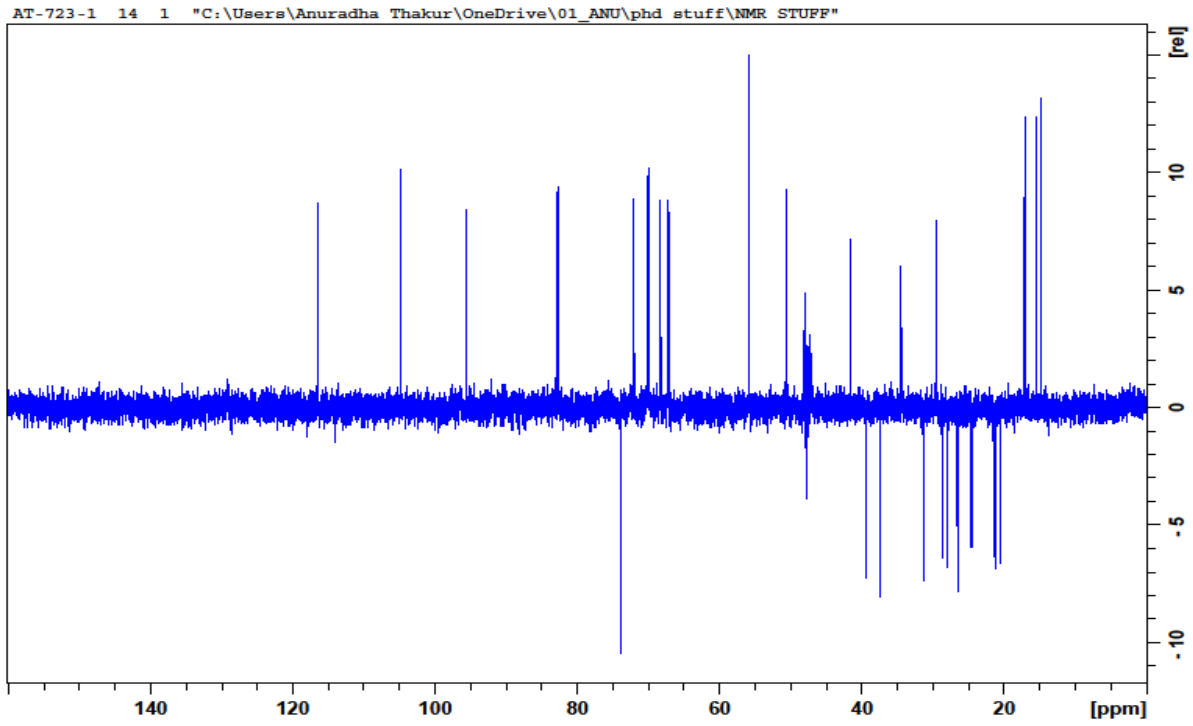
Supplementary data 50: iFit value of Crotoxigenin-3-*O*-β-digitalopyranosyl-(1-4)-*O*-β-digitoxopyanoside **(58)** in *X. undulatum* leaves extracted with DCM:MeOH



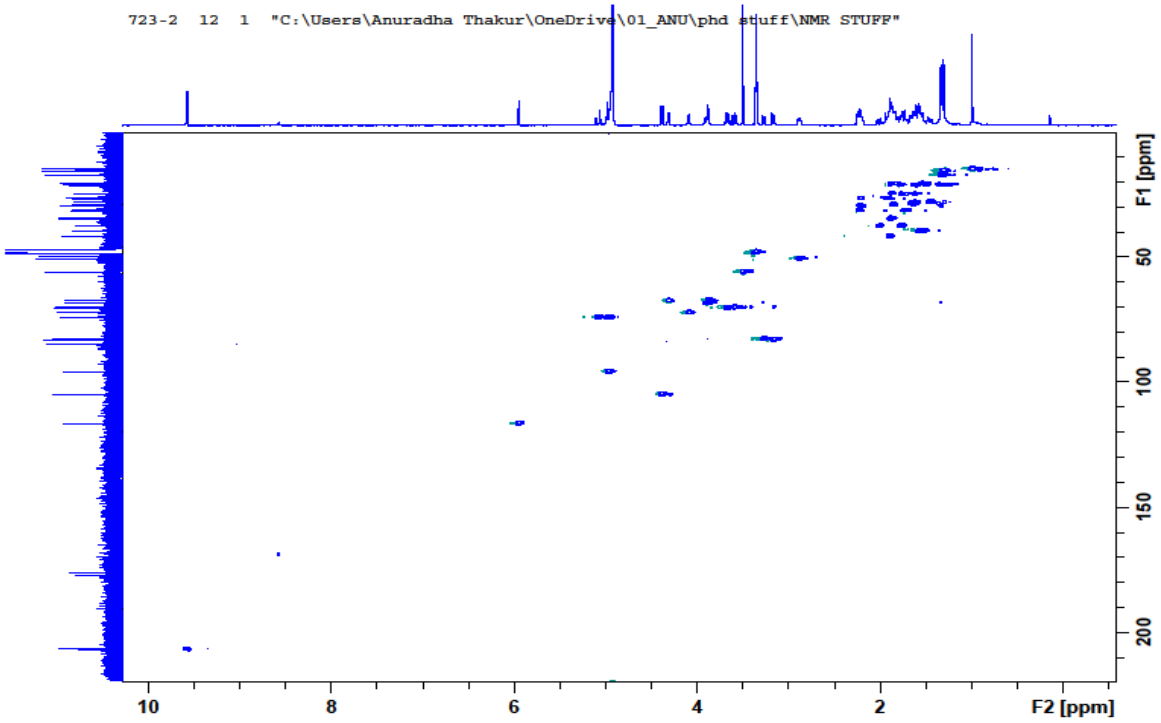
Supplementary data 51: ^{1}H NMR for Crotoxigenin-3-*O*-β-digitalopyranosyl-(1-4)-*O*-β-digitoxopyanoside **(58)** in MeOD-*d4*



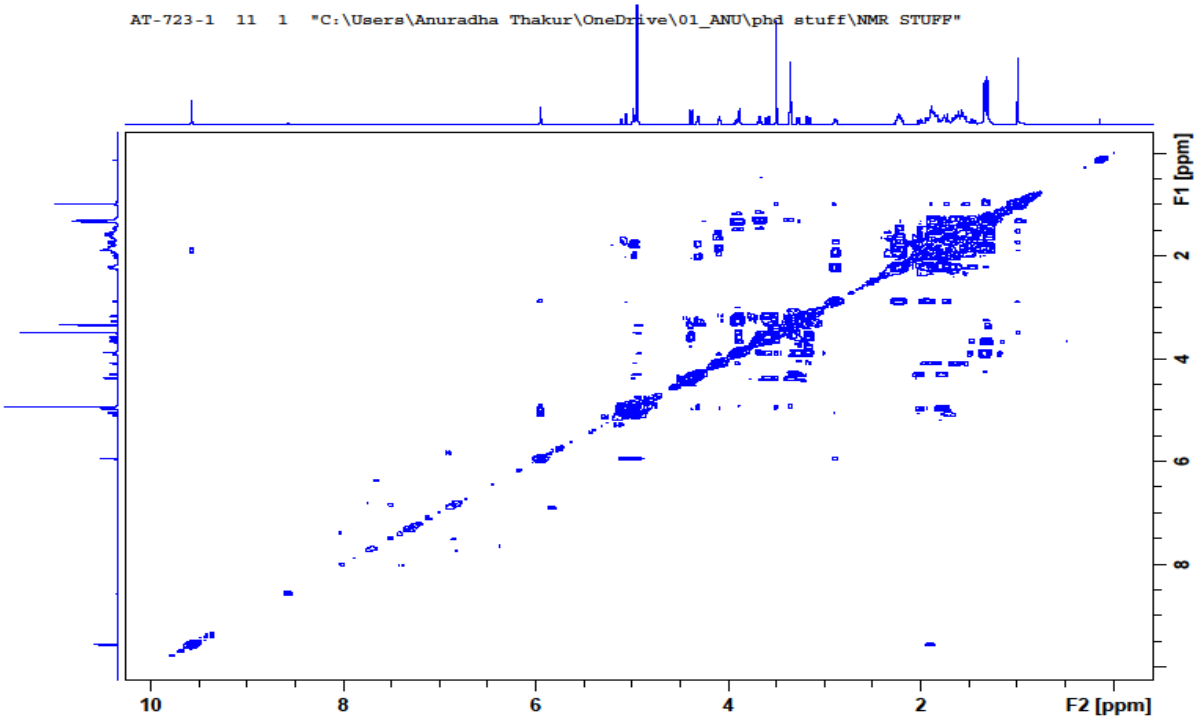
Supplementary data 52: ^{13}C NMR for Crotoxigenin-3-*O*-β-digitalopyranosyl-(1-4)-*O*-β-digitoxopyanoside **(58)** in MeOD-*d4*



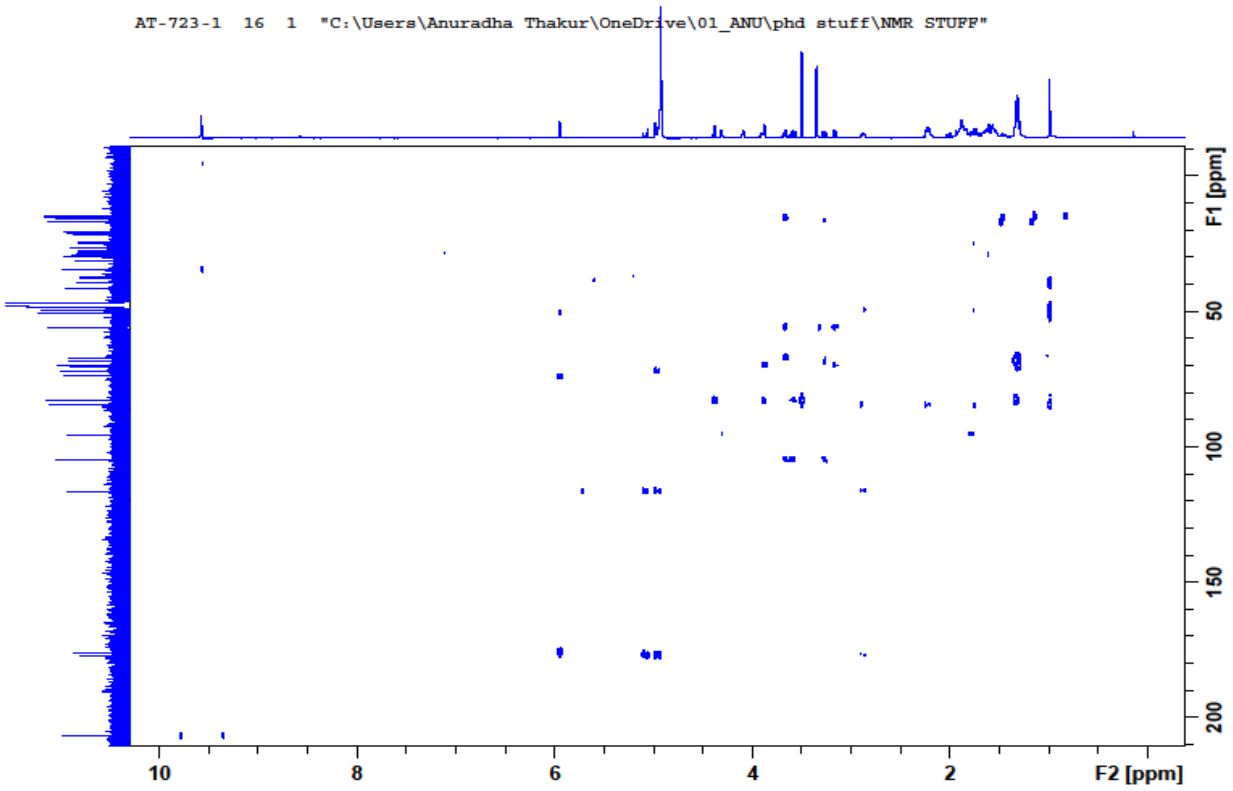
Supplementary data 53: DEPT 135 for Crotoxigenin-3-*O*-β-digitalopyranosyl-(1-4)-*O*-β-digitoxopyanoside **(58)** in MeOD-*d4*



Supplementary data 54: HSQC spectrum for Crotoxigenin-3-*O*-β-digitalopyranosyl-(1-4)-O-β-digitoxopyanoside **(58)** in MeOD-*d4*



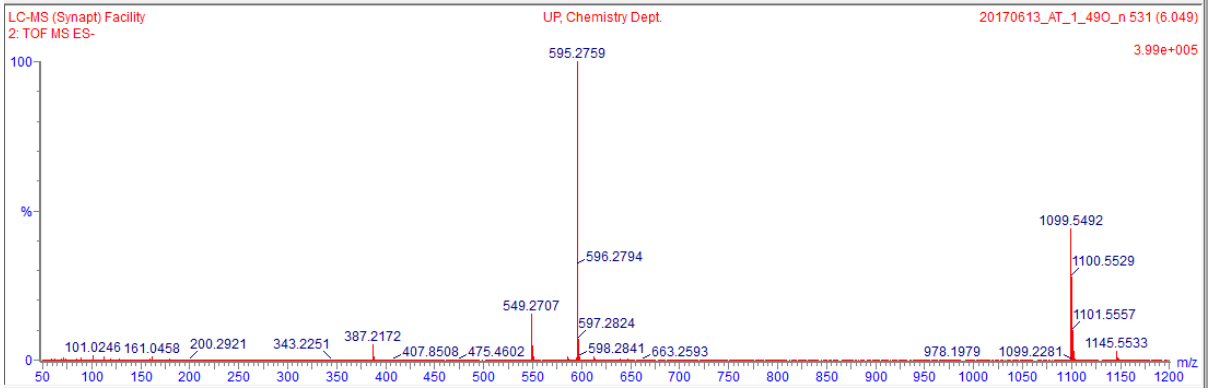
Supplementary data 55: COSY spectrum for Crotoxigenin-3-*O*-β-digitalopyranosyl-(1-4)-*O*-β-digitoxopyanoside **(58)** in MeOD-*d4*



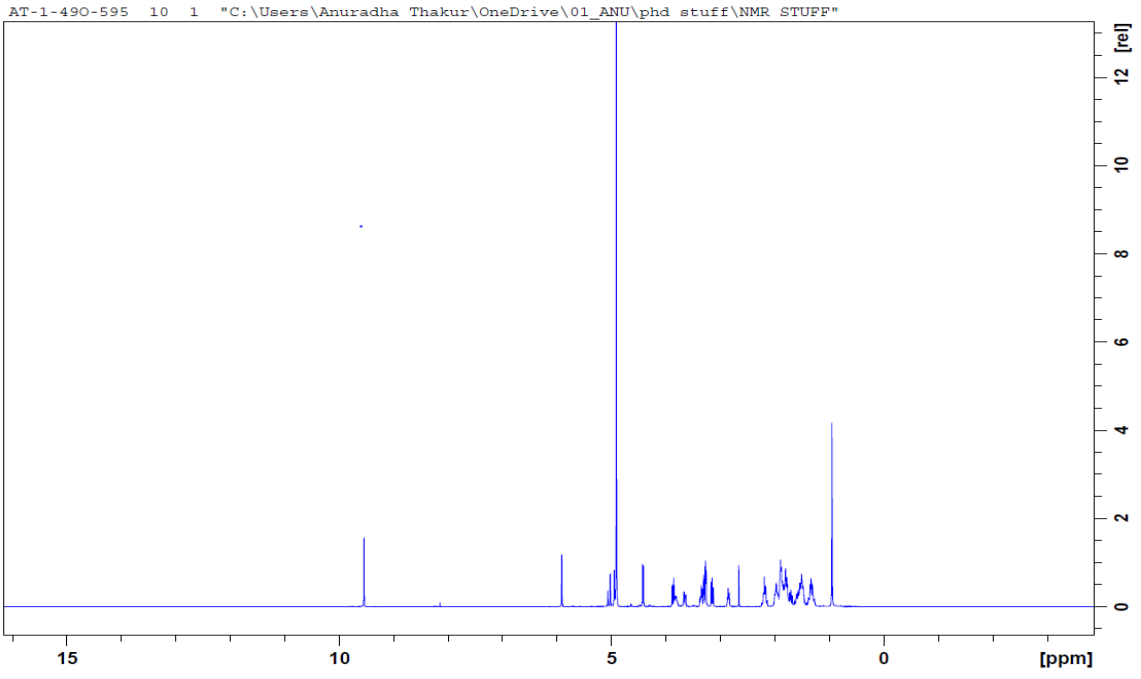
Supplementary data 56: HMBC for Crotoxigenin-3-*O*-β-digitalopyranosyl-(1-4)-*O*-β-digitoxopyanoside **(58)** in MeOD-*d4*

Single Mass Analysis
Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
148 formula(e) evaluated with 4 results within limits (all results (up to 1000) for each mass)
Elements Used:

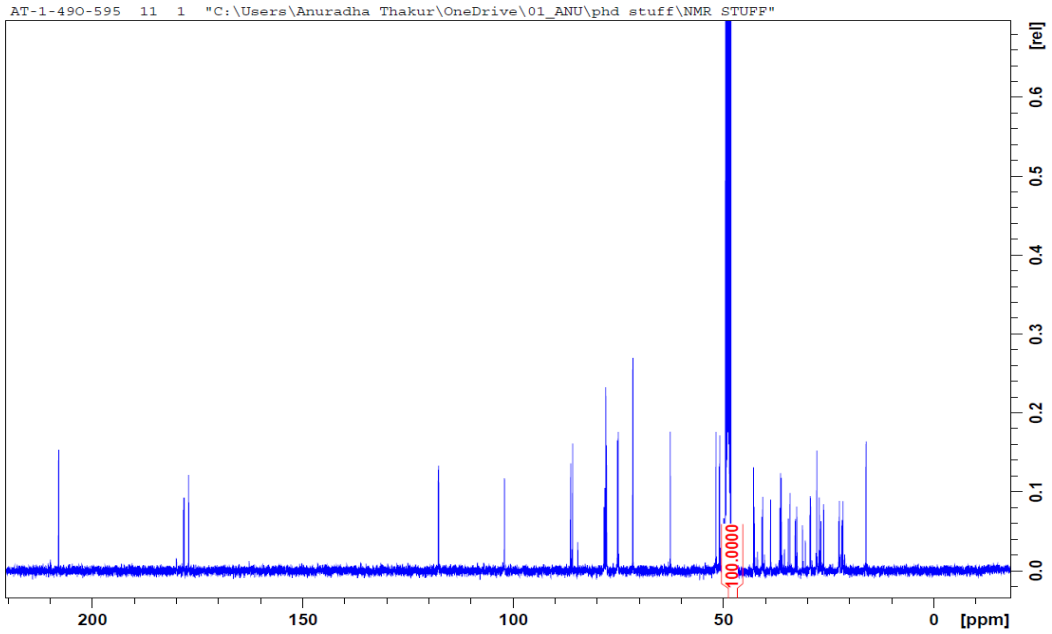
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
549.2707	549.2700	0.7	1.3	9.5	C29 H41 O10	218.4	0.005	99.46	29	41	10
	549.2758	-5.1	-9.3	0.5	C22 H45 O15	224.3	5.905	0.27	22	45	15
	549.2641	6.6	12.0	18.5	C36 H37 O5	224.5	6.028	0.24	36	37	5
	549.2794	-8.7	-15.8	22.5	C40 H37 O2	226.8	8.355	0.02	40	37	2



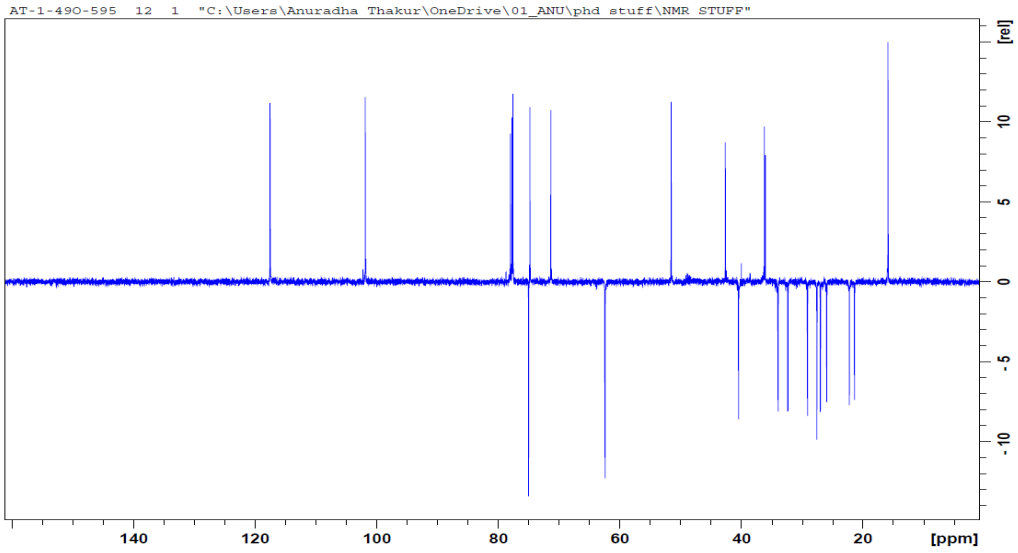
Supplementary data 57: iFit value of crotoxigenin 3-O-glucopyranoside **(59)** in *X. undulatum* leaves extracted with DCM:MeOH



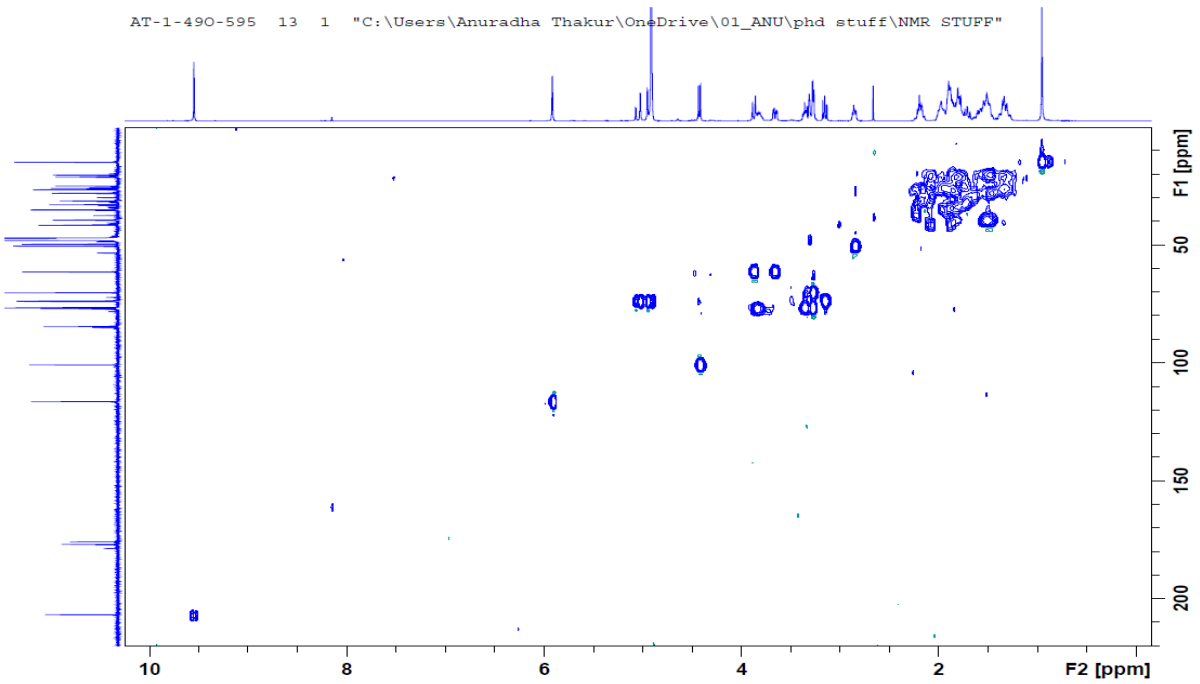
Supplementary data 58: ^{1}H NMR for crotoxigenin 3-O-glucopyranoside **(59)** in MeOD-*d4*



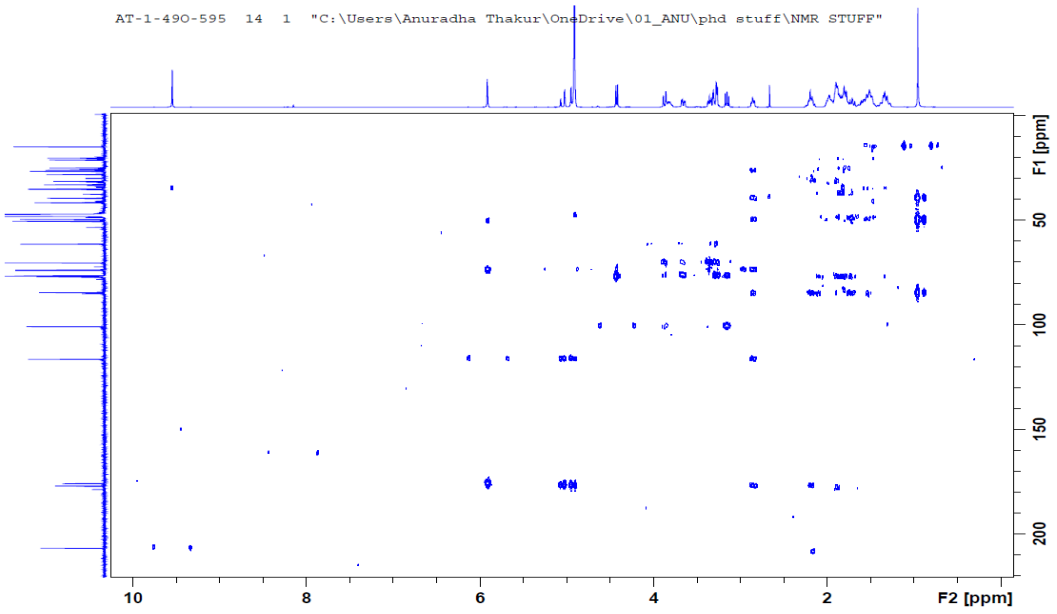
Supplementary data 59: ^{13}C NMR for crotoxigenin 3-O-glucopyranoside **(59)** in MeOD-*d4*



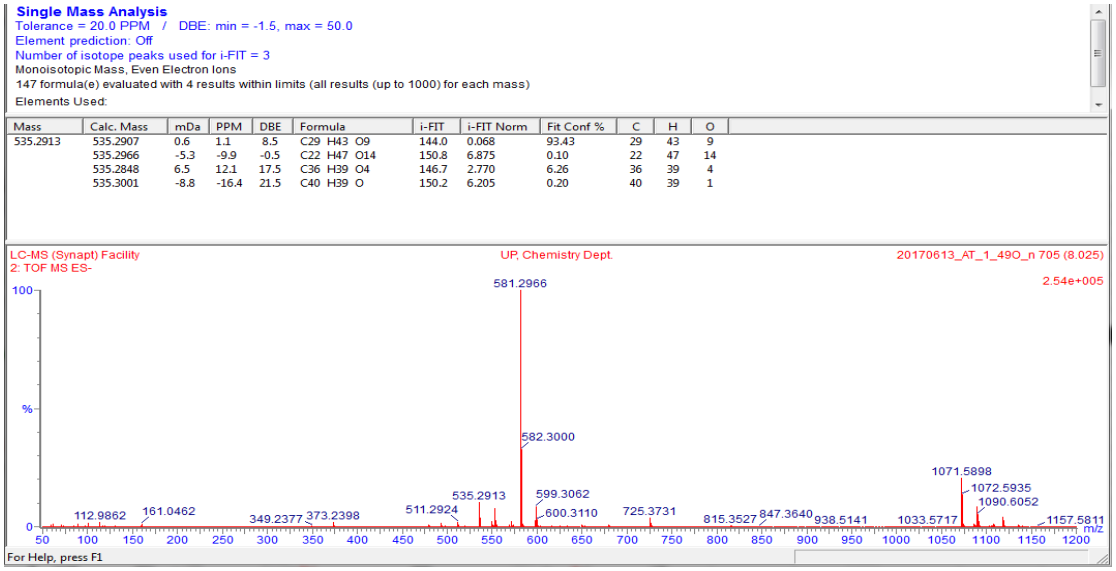
Supplementary data 60: DEPT 135 for crotoxigenin 3-O-glucopyranoside **(59)** in MeOD-*d4*



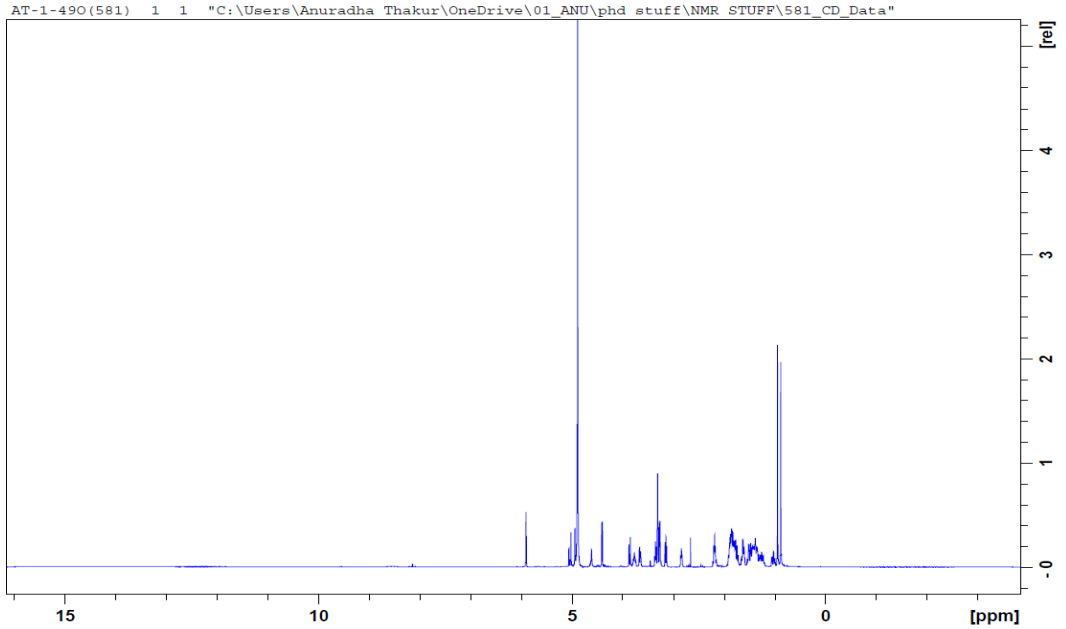
Supplementary data 61: HSQC spectrum for crotoxigenin 3-O-glucopyranoside **(59)** in MeOD-*d4*



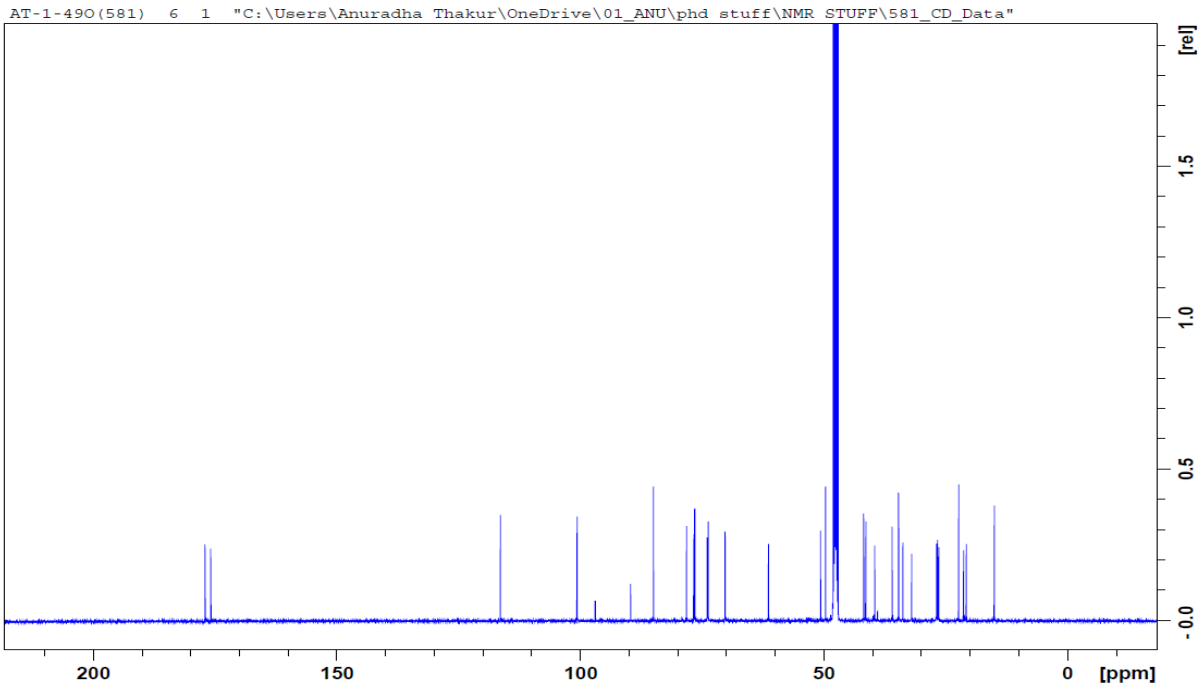
Supplementary data 62: HMBC spectrum for crotoxigenin 3-O-glucopyranoside **(59)** in MeOD-*d4*



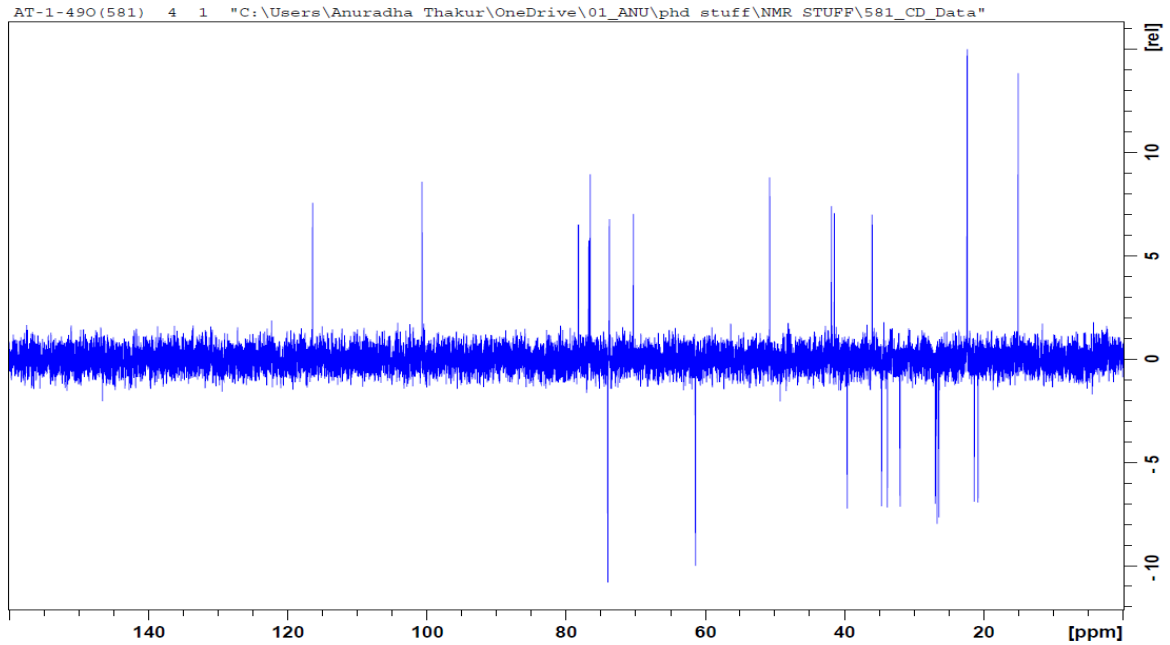
Supplementary data 63: iFit value of desglucouzarin **(60)** in *X. undulatum* leaves extracted with DCM:MeOH



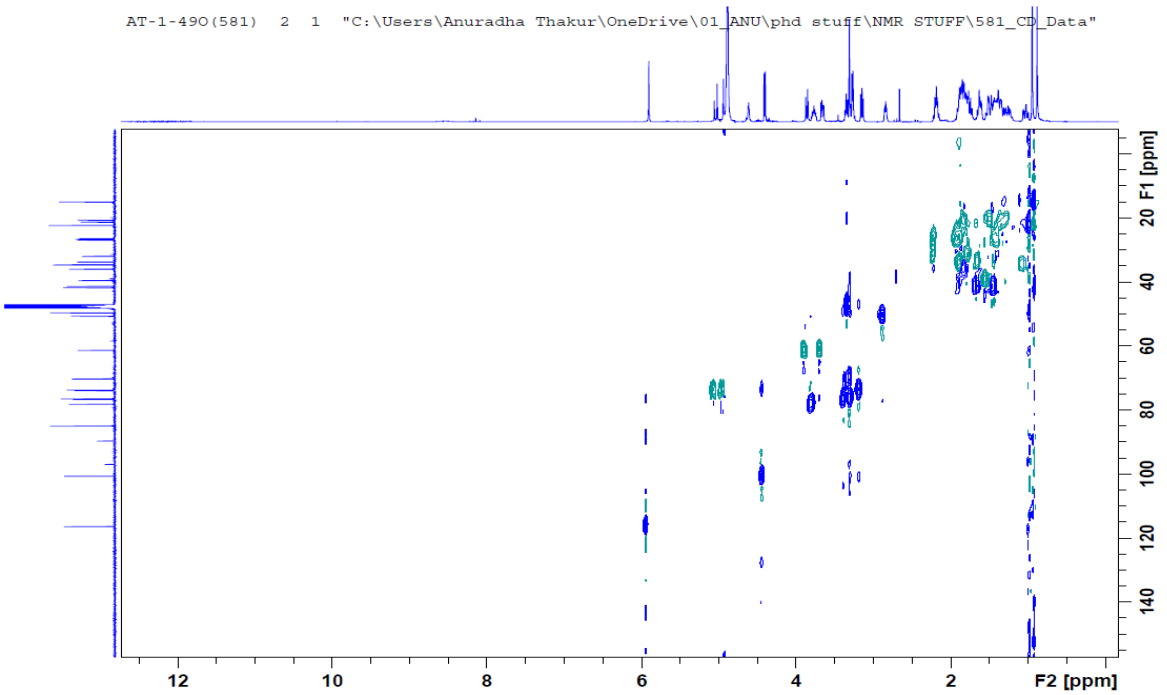
Supplementary data 64: ^{1}H NMR for desglucouzarin **(60)** in MeOD-*d4*



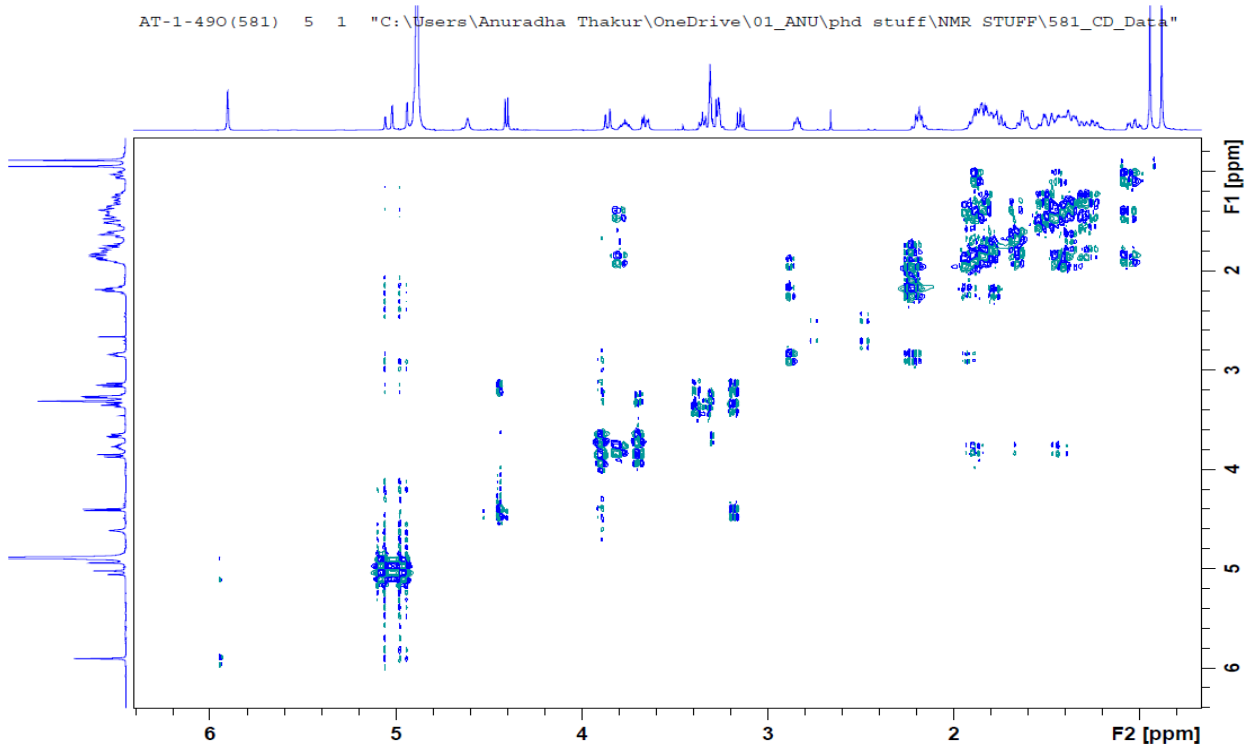
Supplementary data 65: ^{13}C NMR for desglucouzarin **(60)** in MeOD-*d4*



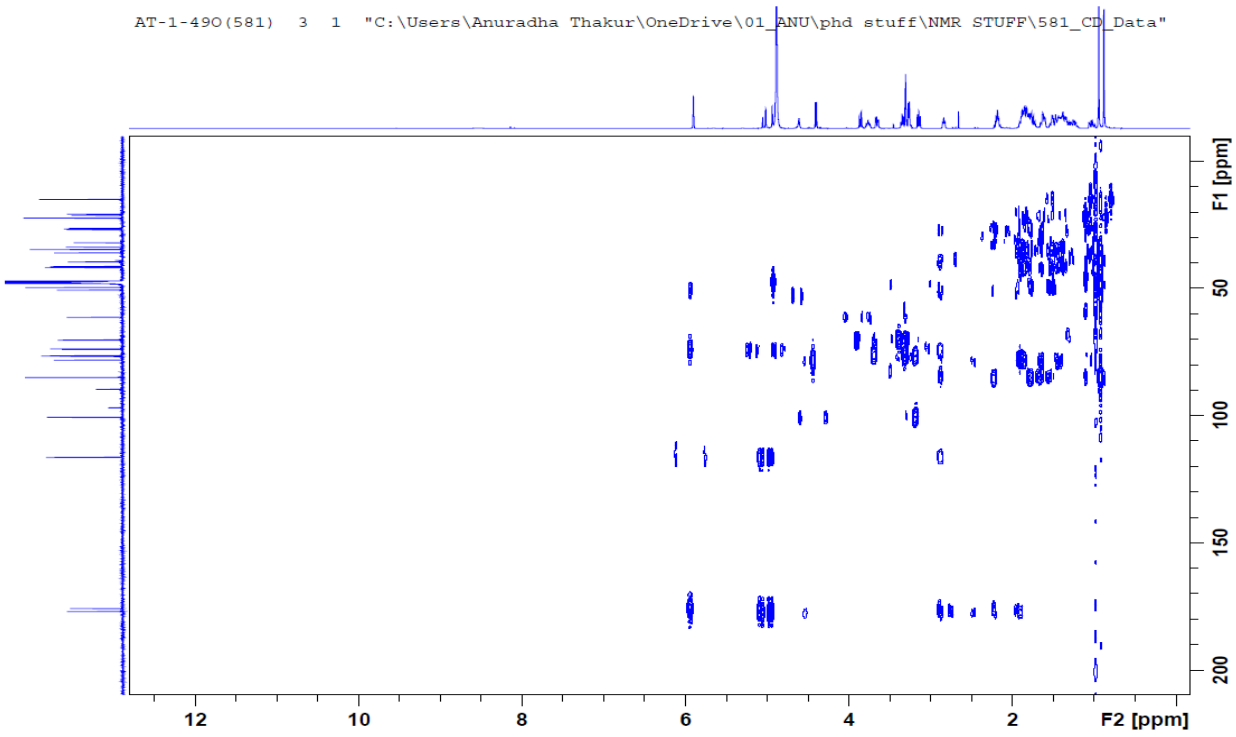
Supplementary data 66: DEPT 135 for desglucouzarin **(60)** in MeOD-*d4*



Supplementary data 67: HSQC spectrum for desglucouzarin **(60)** in MeOD-*d4*



Supplementary data 68: ^{1}H NMR for desglucouzarin **(60)** in MeOD-*d4*



Supplementary data 69: ^{1}H NMR desglucouzarin **(60)** in MeOD-*d4*