In vitro investigation of the potential of *Bidens pilosa* extracts for anti-Alzheimer activity

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by

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Colossians 3 vs 23

“And whatsoever ye do, do it heartily, as to the Lord, and not unto men...”

ABSTRACT

INTRODUCTION

Alzheimer's disease (AD) is a neurocognitive decline disease with no cure and has multiple aetiologies. Currently, available treatment is single-targeted, and disease management is through polypharmacy, which is costly and burdensome to both the patient and caregiver. *Bidens pilosa* (*B. pilosa*) is a widely distributed plant reported to have pharmacological effects on over 40 diseases, chiefly attributed to its flavonoid and polyacetylene phytochemicals.

AIM AND OBJECTIVES

The study aimed to investigate, *in vitro*, the potential of *Bidens pilosa* extracts for anti-Alzheimer activity by determining the phytochemical content, the antioxidant activity and the inhibitory effect of *Bidens pilosa* extracts on β-amyloid (Aβ) aggregation, the enzyme β-site amyloid precursor protein cleaving enzyme 1 (BACE 1) and acetylcholinesterase (AChE).

METHOD

The *B. pilosa* plant was extracted using hexane, methanol, water, dichloromethane (DCM), and acetone to get sample analytes. Qualitative phytochemical analysis was done, i.e., preliminary phytochemical screening and thin layer chromatography (TLC) to confirm the presence of secondary metabolites in the plant material. This was followed by quantitative phytochemical analysis, i.e., total polyphenol and total flavonoid content, with gallic acid and quercetin as the standards, respectively, to quantify the amount of the most active phytochemicals in the plant material.

B. pilosa's ability to quench reactive oxygen species was assessed through the *2,2*-diphenyl-*1*-picrylhydrazyl (DPPH) and the ferric reduction antioxidant power (FRAP) antioxidant assays with ascorbic acid as the standard. The BACE inhibition potential of the plant extracts was investigated using a BACE 1 fluorescence kit. The AChE inhibition potential of the plant extracts was

determined using the Ellman method. The Aβ-aggregation inhibition was investigated using the Thioflavin method.

RESULTS

Qualitative phytochemical analysis showed that *B. pilosa* contains alkaloids, saponins, phenols, tannins, flavonoids, quinones, anthraquinones, cardiac glycosides, steroids, and terpenoids. Most of the phytochemicals were present in the acetone and methanol extracts, as shown by the multiple fractions in the TLC profiles. Quantitative phytochemical analysis showed that the acetone extract had the highest phenolic (0.0331 mg GAE/g) and flavonoid (0.0380 mg QE/g) content. The acetone extract had the highest relative DPPH decolouration (78.51%) and FRAP value of 5.05 x 10^3 µM Fe (II) at 500 µg/mL compared to the other extract samples.

In the BACE 1 assay, the DCM extract gave the highest relative BACE inhibition (83.40%). In the AChE inhibition assay, the 5 µg/mL water extract exhibited the highest inhibition (16.71%). In the Aβ-aggregation inhibition assay, the 50 µg/mL water extract sampled after 120 hrs showed the highest inhibition (47.49%). Generally, there was an increase in relative inhibition with an increase in extract concentration for most samples in the inhibition assays. However, in at least one extract sample group, there was negative inhibition in the inhibition assays, which could indicate activation of the enzyme.

CONCLUSION

The findings reveal that *B. pilosa* possesses multiple phytochemicals that exhibit antioxidant activity and could have the potential for anti-Alzheimer activity. However, further investigations are necessary to confirm the pharmacological activity, such as toxicity and efficacy studies, to advocate for the plant's potential as a drug lead.

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LIST OF ABBREVIATIONS

ACh – Acetylcholine

AChE – Acetylcholinesterase

AChEI – Acetylcholinesterase inhibitor

ACTI – Acetylthiocholine iodide

ABTS – *2,2'*-azinobis (*3*-ethylbenzo thiazoline-*6*-sulphonic acid) diammonium salt

AD – Alzheimer's disease

ADI – Alzheimer's Disease International

APOE-ε4 – Apolipoprotein E-ε4

APP – Amyloid precursor proteins

BACE 1 – Beta-amyloid precursor cleaving enzyme 1

BBB – Blood-brain barrier

B. pilosa – Bidens pilosa

BChE – Butyrylcholinesterase

BChEI – Butyrylcholinesterase inhibitor

CNS – Central nervous system

CSF – Cerebral spinal fluid

DCM – Dichloromethane

DMSO – Dimethyl sulfoxide

DPPH – 2,2'-diphenyl-1-picrylhydrazyl

EC_{50} – Concentration of the extract that can scavenge 50% of oxidants

EOAD – Early-onset Alzheimer's disease

FDA – United States of America Food and Drug Administration

FRAP – Ferric reducing antioxidant power

HIV – Human Immunodeficiency Virus

HCL – Hydrochloric acid

IC_{50} – Concentration of the extract that can inhibit 50% of the enzyme's maximal activity

IL-(1α, 6, 8) – Interleukin (1α, 6, 8)

LOAD – Late-onset Alzheimer's disease

MRI – Magnetic resonance imaging

MMP – Metalloprotease protein

NaOH – Sodium hydroxide

NFTs – Neurofibrillary tangles

NMDA- N-methyl-D-aspartate

NO – Nitric oxide

PET – Positron electron transmission scanner

ROS – Reactive oxygen species

RSM – Response surface methodology

SAMF – South African Medicines Formulary

sTREM2 – Triggering receptor expressed on myeloid cells 2

TEAC – Trolox equivalent antioxidant capacity

ThT – Thioflavin T

THQ – Target hazard quotient

TNF-α – Tumour necrosis factor α

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Alzheimer's disease (AD) is a chronic, slow, and progressive neurodegenerative disease more prevalent in the elderly (Dai et al., 2022). Neurocognitive symptoms like speech impairment, long-term memory loss, difficulty in problem-solving and language problems characterise patients with AD (Dai et al., 2022; Xu et al., 2023). Patients with AD also develop neuropsychiatric symptoms like depression, apathy, aggression, and delusions, usually from the mid to the late stage of the disease (Lyketsos et al., 2011).

Alzheimer's disease currently has no cure (Hussein et al., 2018), and existing registered treatments are palliative or symptomatic. Currently, medicines approved by the United States of America Food and Drug Administration (FDA) fall under two categories: those that change disease progression in patients living with early-stage disease, i.e., lecanemab and the newly approved donanemab and those that mitigate symptoms, i.e., cholinesterase inhibitors and glutamate regulators, for example, donepezil and memantine (Mathew & Subramanian, 2014; Park et al., 2023; Sims et al., 2023). According to the South African Medicine Formulary (SAMF) 14th Edition, donepezil, galantamine and memantine are the only medications indicated for AD in South Africa (South African Medical Association & Division of Clinical Pharmacology: Faculty of Health Sciences, 2022). The existing AD treatments are not without side effects and are generally expensive and inaccessible to the common South African. For instance, lecanemab can cause amyloid-related imaging abnormality (ARIA) and vasogenic oedema, while cholinesterase inhibitors cause diarrhoea and headaches (de los Ríos & Marco-Contelles, 2019; Usman et al., 2021; Valiukas et al., 2022).

The exact causative agent of this neurodegenerative disease has yet to be successfully identified. Hence, there are various theories. Still, the primary five theories include the cholinergic, the tau protein, the β-amyloid (Aβ), the metal ion disorder and the neuroinflammation hypothesis. The predominant premises are the β-amyloid and cholinergic hypotheses (Dai et al., 2022).

More avenues still need to be explored to be considered for AD treatment. Indigenous African medicinal plants, as used often (or as commonly used) in traditional medicines, are a good start as they are usually less toxic, are readily available, cost-effective and possess better side effect profiles (Dai et al., 2022). Traditional medicine is wealthy in undiscovered drug leads, hence a direction worth investigating. The earliest known records of plant use in ailment treatment were in Egypt circa 1550 B.C (Peter et al., 2014). Approximately 80% of Africans depend on plants for primary healthcare treatment, and the market value of medicinal products is estimated to be at least USD100 billion a year (Bajin ba Ndob et al., 2016; Bitwell et al., 2023). Hence, this thesis is relevant in plant research as it will benefit humans financially and medically. *Bidens pilosa* (*B. pilosa*) is a conventional plant commonly used by people as medicine and food. Due to its ease of access, this plant is used as relish in some parts of Africa, tea in some parts of Asia and a 'fix most of our problems' medicine in different parts of the world (Silva et al., 2011).

B. pilosa (Figure 1), commonly known as blackjack, Spanish needles or hairy beggar ticks, is an Asteraceae family plant originating from the Southern American continent (Singh et al., 2017). In Southern Africa, it is referred to as '*inzala*' in IsiNdebele, '*mutsine*' in ChiShona, '*mokolonyana*' in Sepedi and '*ucadolo*' in IsiXhosa. It is also known as '*kichomma mguu*' in KiSwahili and '*chisoso*' in Chichewa. It is a plant with many variants, thriving best in the tropical and subtropical climates of the world. (Singh et al., 2017; Xuan & Khanh, 2016). It is commonly seen as a weed and can grow up to two metres in height if undisturbed, depending on the variant (Xuan & Khanh, 2016). *B. pilosa* was first recorded in the Kruger National Park, South Africa, as an invasive alien species in 1975 in a single camp, with evidence of naturalisation (Foxcroft et al., 2008). This plant grows quickly almost anywhere, provided that the environment is conducive, and it flourishes on cultivated land or waste areas (Mtenga & Ripanda, 2022).

Figure 1: Image showing the *Bidens pilosa* plant (on the left-hand side is a white petaled flower with a yellow centre, and on the right is a plant showing the black achenes with bristled ends once the flower dries up).

Studies have also shown that the *B. pilosa* plant exhibits antimicrobial, antidiabetic, antihypertensive, anti-inflammatory, hepatoprotective, antioxidant activity, and cytotoxic effects against specific cancer types (Cortés-Rojas et al., 2013; Dai et al., 2022; Rodríguez-Mesa et al., 2023; Wang et al., 2019; Xuan & Khanh, 2016). The pharmacological activity is owed to its main chemical components: flavonoids and polyacetylenes (Bartolome et al., 2013; Silva et al., 2011; Wu et al., 2013; Xuan & Khanh, 2016).

Previous studies have suggested neuroprotective action by *B. pilosa* (Xuan & Khanh, 2016). Since AD is a neurodegenerative disease that further progresses as neurons cease to function and die, a drug that has neuroprotective ability would be helpful in neuronal preservation, hence slowing disease progress and lowering mortality. Also, due to AD's complex interconnected disease causation, progression has been thought to involve the unending auto-oxidation of cells, which promotes their apoptosis; hence, a drug with antioxidation properties would benefit treatment. Furthermore, AD drugs tend to be single-target oriented, e.g., rivastigmine targets the cholinesterase

enzymes only. This has effectively treated AD by delaying memory loss by at least six months before the patient's further decline (Choi et al., 2014; Dai et al., 2022; Hussein et al., 2018). Due to the multifactorial standing of AD, the current treatment plans revolve around polypharmacy to address multiple targets. In contrast, a single drug with numerous targets should prove more efficacious than existing single-target drugs (de los Ríos & Marco-Contelles, 2019).

This knowledge gap gave rise to this research that assessed the enzyme dual inhibitory activity on acetylcholine esterase (AChE) and β-site amyloid precursor protein cleaving enzyme 1 (BACE 1) by *B. pilosa* phytochemicals. The investigation was performed with the hope that if inhibitory activity is established, this plant can be a potential lead compound in developing dual-target Alzheimer's medication drugs.

1.2 RATIONALE FOR STUDY

Currently, there is no cure for AD, and all the treatments available are either disease-modifying or symptom-managing (Hussein et al., 2018; Turcu et al., 2022). There are various theories on the causative mechanisms of the disease, with the predominant being the Aβ and the cholinergic hypotheses. Drugs for AD tend to be single-target oriented, e.g., rivastigmine targets the choline esterase enzyme. This has been effective in treating AD by delaying memory loss by at least six months before the patient starts to dilapidate further (Choi et al., 2014; Hussein et al., 2018).

Caring for a patient with AD is costly. The World Health Organisation's 2019 estimate of the global cost of dementia was US$1.3 trillion in high-income countries, projected to reach US$2.8 trillion in 2030, once corrected for cost increases. Patients with AD largely contribute to these costs since AD is a syndrome of dementia (World Health Organization, 2021). Low-income countries are bound to be experiencing way higher disease-incurred costs, hence the need for treatment that will be cheaper and easier to access for large populations of the world.

Multiple studies have identified *B. pilosa* as a worldwide distributed plant with pharmacological effects on over 40 diseases (Bartolome et al., 2013). Of interest to the above aetiologies are its anti-inflammatory, antioxidant, and immunomodulatory effects, which are said to be made possible by the flavonoids and polyacetylenes found in the plant. Therefore, this study aimed to investigate the inhibitory activity of *B. pilosa* on AChE and BACE 1. Based on existing literature, no study has been published investigating the inhibitory effect of *B. pilosa* extract on AChE and BACE 1 enzymes to identify a potential drug lead for a dual inhibitor drug for AD.

1.3 RESEARCH QUESTIONS

1. Do *Bidens pilosa* extracts have any inhibitory effect on acetylcholine esterase and β-site amyloid precursor protein cleaving enzyme 1?
2. What phytochemicals in *Bidens pilosa* may be responsible for the inhibitory effects?

1.4 AIM AND OBJECTIVES OF THE STUDY

The study aimed to investigate, *in vitro*, the potential of *Bidens pilosa* extracts for anti-Alzheimer activity. This was achieved by carrying out the following objectives:

1. The determination of the phytochemical content of the *Bidens pilosa* plant extracts.
2. The determination of the antioxidant activity of the *Bidens pilosa* plant extracts.
3. The assessment of the inhibitory effect of *Bidens pilosa* extracts of the enzyme - acetylcholinesterase (AChE).
4. The assessment of the inhibitory effect of *Bidens pilosa* extracts of the enzyme - β-site amyloid precursor protein cleaving enzyme 1(BACE 1).

CHAPTER 2: LITERATURE REVIEW

2.1 OVERVIEW OF THE *BIDENS PILOSA* PLANT

2.1.1 Morphology and Geographical Distribution

Bidens pilosa (*B. pilosa*) was first picked and named by Carl Linnaeus in 1753 (Bartolome et al., 2013). The *B. pilosa* species has numerous varieties like *pilosa*, *minor*, *radiata*, *odorata*, *alba*, *bimucronata*, *bisetosa*, *calcicola*, and *alausensis* (Figure. 2.1) (Bartolome et al., 2013; Chien et al., 2009; Xuan & Khanh, 2016). *B. pilosa* is an erect annual or perennial herb found in most parts of the world with tropical or subtropical temperatures, that is, Africa, Asia, South America, and Europe (Bartolome et al., 2013; Kuo et al., 2021; Mtenga & Ripanda, 2022; Yue et al., 2019). The average height of the plant is 30–150 cm, but it can reach two metres in good climate (Bartolome et al., 2013; Idris et al., 2023; Xuan & Khanh, 2016). The suitable climate for the *B. pilosa* plant is a temperature and rainfall range of 25–38°C and 500–800 mm, respectively (Mtenga & Ripanda, 2022). The leaves are green, opposite and petioled. Additionally, the leaves are slightly hairy, ovate-shaped, pinnately compound, lobed and with serrated leaf margins (Bartolome et al., 2013; Xuan & Khanh, 2016). Some varieties are flowered, and some are not. The flowers are either yellow or white on the flowered plant (Mtenga & Ripanda,2022).

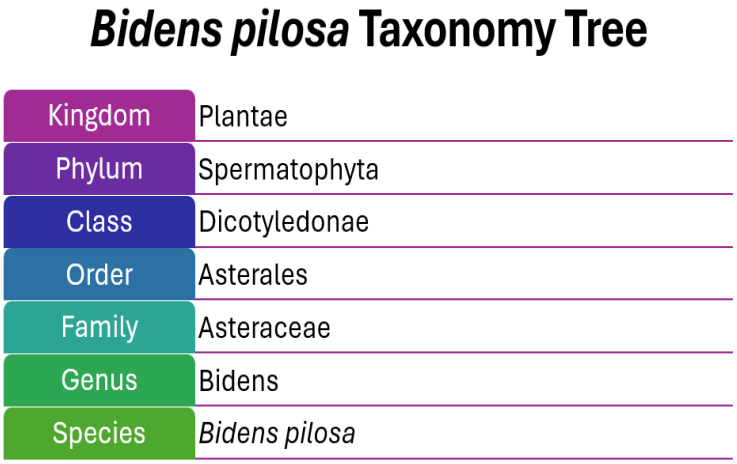


Figure 2.1: The scientific classification of B. pilosa (Rojas-Sandoval, 2022).

Ideal growth conditions include full sun and moderately dry soil, which produces up to 6000 seeds which are viable for at least three years before germination or being planted elsewhere (Bartolome et al., 2013). The optimum temperature for seed germination once in the soil is 15–40°C (Xuan & Khanh, 2016). The characteristic elongated black achenes with hooked bristled ends are the plant's tell-tale as they attach themselves to animals and humans (especially school children's socks). The achenes are easily blown away by the wind, ensuring vast dissipation. The sticky achenes are also the result of the various cultural nicknames the plant has earned over the years, like devil's needles and pitchforks in different locations (Bartolome et al., 2013; Xuan & Khanh, 2016). As alluded to before in the introduction, *B. pilosa* is a weed that can make it in moist, shady environments that can either be cultivated or uncultivated, thanks to its weedy nature (Xuan & Khanh, 2016).

2.1.2 Phytochemistry

A total of 301 phytochemical compounds of *B. pilosa* have been found thus far (Jayasundera et al., 2021). *B. pilosa*'s major active chemical constituents are flavonoids and polyacetylenes (Borges et al., 2013; Silva et al., 2011; Wu et al., 2013; Xuan & Khanh, 2016; Yan et al., 2022). Essential oils, phenylpropanoids and terpenes have also been found in the plant; however, their effects have not been as paramount as the former mentioned (Kumari et al., 2009; Silva et al., 2011; Xuan & Khanh, 2016). Approximately 37 polyacetylene and 60 flavonoid compounds have been isolated and identified in *B. pilosa* (Bartolome et al., 2013; Xuan & Khanh, 2016).

Most polyacetylenes in the *B. pilosa* plant are aliphatic acetylenes with triple or double bonds. The acetylenic compounds also have heterocyclic or glucoside ring end groups, which could also be aromatic (Xuan & Khanh, 2016). Polyacetylenes are reported to have antidiabetic effects on both common types of diabetes mellitus, while flavonoids are reported to possess anticancer, anti-inflammatory, antioxidant, and antibacterial activity

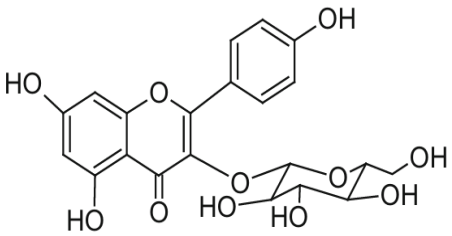
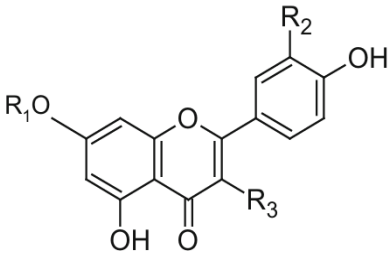
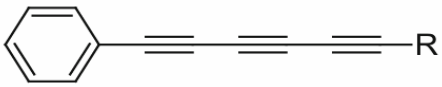
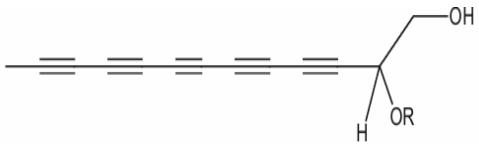
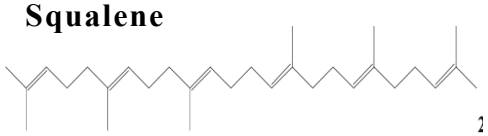
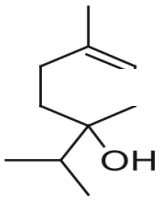
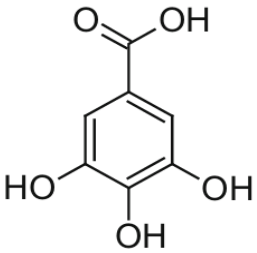
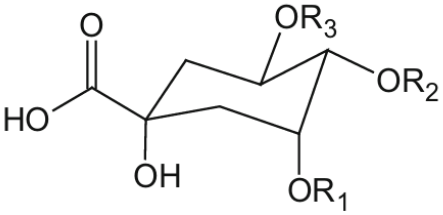
(Bartolome et al., 2013; Chien et al., 2009). Flavonoids are complex phenols. A phenolic compound is a plant secondary metabolite with an aromatic ring with at least one hydroxyl group (Tungmunnithum et al., 2018). The flavonoids, according to the *Bidens* genus, are further classified into derivatives of aglycones, okanin glycosides, aurones, chalcones, flavanones, flavones and flavonols, all with a wide variation of glycosylation and O-methylation patterns (Silva et al., 2011; Xuan & Khanh, 2016). The structures of these phytochemicals still need to be thoroughly researched to establish the full extent of their biological activity.

Table 2.1 shows the chemical structures of some of the common phytochemicals found in the *B. pilosa* plant.

2.1.3 Ethnobotany and Ethnopharmacology

As previously mentioned, *B. pilosa* has been reported to have various ethnobotanical and ethnopharmacological uses. Ethnobotany refers to the scientific study of the commensal relationship between plants and humans, in this context of *B. pilosa* and humans, focusing on human plant use and perception, especially in diverse cultures (Liengme, 1983; Shiracko et al., 2016). Ethnopharmacology studies how people use plants and folkloric medicine while confirming the agents responsible for their benefits outlined in the ethnobotany (Pirintsos et al., 2022). *B. pilosa* is a herb in teas, sauces, and medicines (Bartolome et al., 2013; Xuan & Khanh, 2016). The whole plant is reported to have pharmacological effects on the human body, e.g., antimicrobial, anti-inflammatory, immunomodulatory, antidiabetic and antimalarial effects. Usually, *B. pilosa* is dried and ground into a powder, then given orally or sometimes administered as a decoction, maceration or tincture in ailment treatment (Jayasundera et al., 2021). The rest of the chapter will briefly detail some of the plant's ethnobotany and ethnopharmacology, primarily focusing on AD's aetiology.

Table 2.1: Chemical structures of some of the common phytochemicals found in B. pilosa (Xuan & Khanh, 2016).

Phytochemical group	**Structural Example(s)**	
Flavonoids	**Astragalin** 	**Quercetin 3-O-glucoside**  R_1=H; R_2=OH; R_3=Oglu
Polyacetylenes	**1-phenylhepta-1,3,5-triyne** 	**1-phenylhepta-1,3,5-triyne**  R=Glu
Terpenes	**Squalene** 	**Terpinen-4-ol** 
Phenols	**Gallic acid** 	**4-O-Caffeoylquinic acid**  R_2=caffeoyl; R_1=R_3=H

2.1.3.1 Food/ Nutritional Value

In countries such as South Africa, Zimbabwe, Botswana and Congo, *B. pilosa* is consumed as an indigenous vegetable, given in stock feed and consumed by animals in the wild (Kampemba et al., 2017; Macheka et al., 2022; Mtenga & Ripanda, 2022). *B. pilosa* is rich in fibre, vitamins A and C and certain mineral elements like phosphorus, vanadium, calcium, potassium and iron (Ashafa & Afolayan, 2009; Kampemba et al., 2017; Nguyen et al., 2023; Nischwitz et al., 2017; Odhav et al., 2007; Tesfay et al., 2016; Xuan & Khanh, 2016). This information highlights that besides its medicinal value, the plant contributes to a healthy diet, promoting wholesome benefits.

2.1.3.2 Antidiabetic Effects

Diabetes and AD are closely related with their causations argued to be co-dependent or sometimes consequential. Insulin resistance, which is usually associated with type II diabetes mellitus, is a dementia and AD risk factor (Nguyen et al., 2020; Pathak & Kabra, 2024; Rojas et al., 2021). Brain insulin resistance, or as it is otherwise referred to, type III diabetes mellitus, will cause hyperinsulinemia, which will overwhelm insulin-degrading enzymes, leading to disrupted insulin metabolism and Aβ processing. Impaired brain glucose uptake further exacerbates tau hyperphosphorylation, Aβ accumulation, neuroinflammation and oxidative stress, resulting in neural apoptosis (Nguyen et al., 2020). This follows that a plant like *B. pilosa* that exhibits both anti-diabetic and anti-Alzheimer effects would be more attractive to patients with both or either disease.

Ethnobotanical surveys and reports have revealed that *B. pilosa* is used in the form of food or as a product of decoction or maceration to manage diabetes (Beidokhti et al., 2018; Borges et al., 2013; Diallo et al., 2012; Ezuruike & Prieto, 2014; Katemo et al., 2012; Moyo et al., 2020). While on the laboratory front, Ubillas and team were among the first to investigate *B. pilosa* for anti-hyperglycaemic effects *in vivo*. The pronounced results were deduced to be from the polyacetylene glucoside mixture given at 500 mg/kg that exhibited reduced blood glucose, comparable to metformin when sampled 27 hours from the test start (Ubillas et al., 2000). In the following years, different *B. pilosa*

extracts were tested regarding insulin and glucose metabolism, and the results were mostly continuously promising (Chien et al., 2009; Hsu et al., 2009; Lee et al., 2013; Sin Oh, 2015; Waititu et al., 2024; Yang, 2014).

However, *B. pilosa* is yet to be considered a drug lead in diabetes treatment since some of these tests showed isolation problems like instability of the active compound (Ubillas et al., 2000). Also, the mechanism of action of crude plant extracts or isolated plant compounds has yet to be clearly outlined (Hsu et al., 2009). Despite their positive glycaemic control effects, the safety and efficacy of these isolated compounds or concoctions derived from *B. pilosa* remain in question (Sin Oh, 2015). A Taiwanese pilot study in 2015 tried to advocate for *B. pilosa* in diabetes treatment, and their results showed no obvious hepatic and nephrotic toxicity when *B. pilosa* was co-administered with typical diabetes medicine. The study also found negligible metal toxicity, which is important to assess since the *B. pilosa* plant is a hyperaccumulator (Lai et al., 2015). There has not been a follow-up full-scale participant-diversified clinical study to prove or disprove the pilot study and show if *B. pilosa* must be recommended in diabetes mellitus therapy. Filling this gap would benefit a good portion of the world, as diabetes is one of the most notorious diseases worldwide. It would also be beneficial on the AD front since a win in diabetes treatment means a reduction in AD risk factors, and it is one less comorbidity that would exacerbate AD progression in patients who are already diagnosed with both diseases.

2.1.3.3 Immunomodulatory Effects

Astroglia and microglia are the brain's chief immune cells in control of the central nervous system (CNS) inflammatory response. In AD pathogenesis, Aβ plaques lead to the over-activation of these cells, resulting in the release of pro-inflammatory cytokines like TNF-α, IL-6 and IL-1α (Munafò et al., 2020; Valiukas et al., 2022). Immune response medication that targets the removal of Aβ has been tried, but so far, very few have even managed to make it into stage III of clinical trials, with none succeeding at halting or significantly slowing disease progression (Valiukas et al., 2022). Only two passive immunomodulatory drugs, lecanemab and donanemab, are currently on the

market via the FDA fast track (FDA, 2023, 2024). On the other hand, scientists thought to try formulating a vaccine for AD. So far, about 184 reports on AD vaccines have been made, but none have been FDA-approved yet, with none having surpassed the third clinical stage (Usman et al., 2021). The vaccine immunotherapies are reported to only be useful in early AD disease. Some of these formulations are still preclinical and are yet to be tried for clinical efficacy and safety, so further investigation for more conclusive evidence is still required (Valiukas et al., 2022). Therefore, it shows that a plant-based therapy that also plays a part in immunomodulation would be attractive in patients with AD.

B. pilosa has immunomodulatory effects coupled with anti-inflammatory action, as shown by Pereira and friends when the ethanol extract of *B. pilosa* was used with *2*-O-*β*-glucosyltrideca-*11*(*E*)- en-*3,5,7,9*-tetrayn-*1*,*2*-diol polyene on T-lymphocytes and arthritis induced mice (Bartolome et al., 2013). In 2007, Chiang *et al*. found that the polyacetylene glycoside cytopiloyne extracted from *B. pilosa* can modulate T cell functions. Through the inhibition of naïve T helper cells (Th0) differentiation into type I T helper cells (Th1) but promoting differentiation into type II T helper cells (Th2), cytopiloyne could be the phytochemical whose mode of action is responsible for diabetes prevention or management (Chiang et al., 2007). That study exhibited the plant's immunomodulatory effects while suggesting treatment for a metabolic syndrome, which is also a huge factor in AD.

One of the major pitfalls of immunotherapies is the adverse reactions. Since the mode of action of these therapies is using the body’s own system to combat the disease, if there is an adverse reaction, it is also inbuilt (Valiukas et al., 2022). Hence, a natural plant-derived compound or extract with immune modulatory effects will be more easily metabolised or expelled from the body when such a reaction occurs, which is already rare, as evidenced by the ethnobotanical and ethnopharmacological reports (Anywar et al., 2020; Chiang et al., 2007; Chung et al., 2016; Gbashi et al., 2016; Rodríguez-Mesa et al., 2023).

2.1.3.4 Anti-inflammatory Effects

Microglial activation in inflammation causes the upregulation of soluble triggering receptors expressed on myeloid cells 2 (sTREM2). High sTREM2 quantities propagate neuroinflammation and amyloid plaque accumulation in early AD (Valiukas et al., 2022). Additionally, inflammation has been found to be related to psychiatric symptoms in patients with AD (Wadhwa et al., 2020). This follows that a plant that exhibits anti-inflammatory effects will be attractive in AD research, like *B. pilosa*. In 1996, *B. pilosa'*s anti-inflammatory effect was proved through prostaglandin inhibition by blocking COX-1 (Jäger et al., 1996). Later, a study by Yan *et al*. (2022) found that the compounds obtained after fractionation from *B. pilosa* had notable anti-inflammatory effects on IL-6 and nitric oxide (NO) secretion against LPS-induced RAW 264.7 cells *in vitro*. Compound **1** (*4*-O-b-*D*-glucopyranosyloxy-*1*-hydroxy-*6*-(*E*)-tetradecene-*8,10,12*-triyne) had more pronounced inhibition on IL-6 while it was outshined by compound **3** (*2*-O-b-*D*-glucopyranosyloxy-*1*-hydroxy-5-(*E*)-tetradecene-*7,9,11*-triyne) and **4** (icthyo- thereol acetate) on NO inhibition (Yan et al., 2022). These results further supported the relevance of the plant in anti-inflammatory research for potential drug leads in that sphere.

Another study extracted the whole *B. pilosa* plant using *n*-butanol, ethyl acetate, and water. These fractions were found to have notable NO inhibitory activity on LPS-stimulated macrophage RAW 264.7 cells. Ethyl acetate exhibited the highest results with an IC_{50} value of 36.2 µg/mL, followed by the butanol at 250.8 µg/mL. Despite having a high NO inhibition capacity, ethyl acetate was also highly cytotoxic, as shown by less than 30% cell viability within 24 hours of the MTT assay (Chiang et al., 2004). In China, an experiment was run on female Wistar rats to assess the anti-inflammatory effect of *B. pilosa* aqueous extract in finasteride-induced androgen deficiency dry eye syndrome. The rats fed *B. pilosa* extract showed statistically significant results suggesting condition management by the plant extract than the finasteride-only fed rats in the tear film break-up time, phenol red thread, and corneal epithelial fluorescein staining test. The finasteride-only rats' lacrimal glands and corneal histopathology showed a notable inflammatory

response and substantial lymphocyte infiltration. Twenty-two cytokines were expressed in the lacrimal glands of the finasteride-only rats compared to the control rats, while the *B. pilosa*-finasteride rats expressed only 15 (Zhang et al., 2016). These results showed the plant's capability to manage inflammation due to androgen deficiency dry eye. In ethnobotany surveys, the root juice of the *B. pilosa* plant was reported to help manage malaria and rheumatoid diseases in Brazil, China, Hong Kong and Africa (Bartolome et al., 2013; Kuo et al., 2021). The treatment management could have been made possible by the inflammation control effects that the plant exhibits.

It is important to note that *B. pilosa* can promote pro- or anti-inflammatory activity based on the extract used through different mechanisms of action (Rodríguez-Mesa et al., 2023). The continual neuroinflammation cascade not only deteriorates the neuronal system but has dire systemic effects due to the continual release of pro-inflammatory markers, which may manifest as uncontrolled oxidative damage (Munafò et al., 2020). That point further illustrates the interconnectedness of AD's pathogenesis with immunomodulation being closely related to inflammatory and oxidant issues that worsen the cognitive decline condition.

2.1.3.5 Antioxidant Effects

Normal CNS injury protocol follows that pro-inflammatory cytokines and reactive oxygen species (ROS) -like superoxide radicals (O_2^-), NO and many others are released. The cytotoxic ROS have a short biological half-life. However, due to the prolonged activation of glial cells in AD pathogenesis, there is prolonged release of ROS, leading to oxidative damage (Maccioni et al., 2018). It has been found that with age comes oxidative stress due to the reduction of the antioxidant defence mechanism in the body (Mathew & Subramanian, 2014). This is important to note since age is one of the most significant risk factors for AD.

The continual oxidative damage, either due to activation or age, is what makes plant-based treatments a good idea in AD because plants are known to be natural antioxidants attributed to their high phenolic content. Antioxidant activity is assessed *in vitro* through assays like the *2,2'*-diphenyl-*1*-

picrylhydrazyl (DPPH), Ferric reducing antioxidant power (FRAP) and the *2,2'*-azinobis (*3*-ethylbenzo thiazoline-*6*-sulphonic acid) diammonium salt (ABTS). These assays have their own respective pros and cons; for example, the DPPH is best for detecting hydrophobic antioxidants, whereas the FRAP is better for hydrophilic compounds, and the ABTS can assess both hydrophilic and hydrophobic antioxidants (Cortés-Rojas et al., 2011; Gulcin, 2020; Munteanu & Apetrei, 2021).

Phenolic compounds like caffeoylquinic acid and polyacetylene glucosides, alongside their derivatives, are found in abundance in the *B. pilosa* species, and they play a significant role in promoting the plant's antioxidant effects (Lee et al., 2013). Since extraction methods play a part in the amount of extracted antioxidants from the plant, Cortes-Rojas *et al.* (2013) investigated the Response Surface Methodology (RSM) in optimising the phenolic compound extraction. RSM was able to draw out antioxidants from the hydroalcoholic solutions of the *B. pilosa* plant, with the highest results noted at 62.7% ethanol in extraction at 66.2°C of 14.1±0.2 and 9.0±0.6 Trolox equivalent antioxidant capacity per gram of wet base (TEAC per gw.b.), in the DPPH and ABTS assay, respectively (Cortés-Rojas et al., 2013).

In another study of *B. pilosa* flower extracts, the FRAP value was 63.50±3.25 µmol Fe^{2+}/g, while the TEAC value was 30.79±2.43 µmol Trolox/g. The results suggested a strong correlation between antioxidant capability based on the different assays and phenolic content, with an R^2 value of 0.911 for FRAP and 0.954 for TEAC (Li et al., 2014). Nxumalo *et al.* (2023) tested the *B. pilosa*, *Lippia javanica*, *Syzygium cordatum*, and *Ximenia caffra* leaves sourced from Eswatini for antioxidant activity. The results from the tests showed that *B. pilosa* had the second-best DPPH EC_{50} (the concentration of the extract that can scavenge 50% of the available oxidants) value of 7.44±0.79 µg/mL compared to the other three plants. *B. pilosa* had the lowest FRAP EC_{50} value of 110.10±1.10 µg/mL, suggesting its FRAP assay superior potency, but was the least potent in the ABTS assay with an EC_{50} value of 69.20±1.12 µg/mL (Nxumalo et al., 2023). These results further supported the antioxidant effects of the *B. pilosa* plant.

2.1.3.6 Miscellaneous Effects

It is safe to say that the studies found that the whole plant positively influences metabolic syndromes like diabetes and hypertension, amongst other various ailments (Chang et al., 2004; Dimo et al., 2003, 2001; Hsu et al., 2009; Singh et al., 2017). In Africa, the entire plant manages morning sickness and menstrual irregularities (Bartolome et al., 2013; Mtenga & Ripanda, 2022). In Cuba, it has been reported that *B. pilosa* is used to treat the 'coolness' of the uterus, otherwise known as a cold womb, which manifests gynaecological symptoms such as dysmenorrhea and infertility (Bartolome et al., 2013).

Other than gynaecological and metabolic benefits, the plant has antimicrobial activity shown in Japan, Trinidad, and Tobago, especially because of the aerial parts (that is, flowers, leaves, stems, and seeds) (Bartolome et al., 2013; Cock et al., 2022; Deba et al., 2008). In Brazil, a study on postmenopausal women found evidence of the therapeutic antimicrobial activity of *B. pilosa* aqueous extracts against urinary tract infections. The highest activity from *B. pilosa* was noted against *Escherichia coli* compared to the other tested plants (Chiavari-Frederico et al., 2020). To further illustrate the plant's antimicrobial prowess, Ashafa and colleagues tested *B. pilosa*'s methanol, acetone and water root extracts for both antifungal and antibacterial activity. The study showed that the methanol and acetone extracts had comparable minimum inhibitory concentration (MIC) values of 10 mg/mL with the positive control chloramphenicol against *Pseudomonas aeruginosa*. All three extracts had notable *in vitro* results showing 100% suppression of the fungus *Penicillium notatum.* However, against *Candida albicans, Aspergillus niger* and *Aspergillus flavus*, the suppression was not as impressive (Ashafa & Afolayan, 2009). The information from this study supports the existence of antibacterial and antifungal activity, but the antifungal activity was not significant despite folkloric evidence of its use. The results are not definite, as the antifungal activity of the plant could be a result of the complete phytochemical profile in place of specific solvent extracts.

Additionally, *B. pilosa* leaves are used in treating eye infections, colds, renal infections and skin problems in Uganda, Middle America, Cuba, and China (Bartolome et al., 2013; Xuan & Khanh, 2016). Research on the plant's antimicrobial activity still proceeds *in vitro* and *in vivo*. For example, *B. pilosa*-extracted cytopiloyne was found to have macrophage-mediated *Candida* eradication when tested against mice and RAW 264.7 cells. The extracted isolate enhanced lysosome enzyme activation, phagolysosome fusion and acidification, and protein kinase C (PKC)-dependant phagocytosis (Chung et al., 2016). This study, among other *in vivo* designed investigations, shows that the plant's toxicity is negligible, supporting the plant's attractiveness in AD research.

The plant is also used in veterinary medicine. A study done on chickens to assess the plant's anthelminthic prowess against the gut parasite *Eimeria tenella* showed that co-administering the plant with probiotics is favourable in decreasing the infection severity with better mortality rates than the positive control group of the chickens (Memon et al., 2021). This further illustrated the plant's multipurpose benefits that are not only primarily for humans but for animals, too.

B. pilosa has also been reported to have antimalarial and anticancer activity. For example, phenyl-*1,3,5*-heptatriyne, a compound isolated from *B. pilosa* leaves, was found to have notable antimalarial activity against *P. falciparum* (NF54 strain) with an IC_{50} of 6 µg/mL compared to the hexane, methanol and chloroform crude extracts. The anticancer ability of the isolate was also significantly better with IC_{50} values that were below 1 µg/mL against the liver (HepG) and colon (CACO2) cells when viewed against the taxol control values (Kumari et al., 2009). Also, in the exhibition of anticancer activity, Kviecinski *et al*. (2008) tested *B. pilosa* crude extracts of chloroform, methanol, ethyl acetate and hydro alcohol for antitumour activity. This was achieved through performing *in vitro*, brine shrimp, haemolytic and cytotoxicity (Neutral red uptake- NRU and Tetrazolium salt- MTT) assays. Notable results were observed with the chloroform fraction where the NRU IC_{50} was 83.0±5.2 µg/mL, and the MTT IC_{50} was 97.0±7.2 µg/mL. In the *in vivo* studies on the

male isogenic mice, the chloroform fraction also showed the best results compared to all the other plant extracts (Kviecinski et al., 2008).

Furthermore, the Zulu people in South Africa are reported to use the plant in raising depressed fontanelles in infants (Mhlongo & Van Wyk, 2019). There are reports from Africa of the plant being used to treat colic, diarrhoea, dysentery, coughs and other respiratory infections (Muchuweti et al., 2007; Silva et al., 2011). Other than in paediatrics, the plant is also relevant in cosmetics. *B. pilosa* plant extract was found to be among three other Polynesian plants to have positive effects on hair proliferation when tested against dermal papilla cells (Hughes et al., 2020). The plant has also exhibited antiviral activity in an HIV study in 2016 and is used externally in treating snake bites and other wounds (Gbashi et al., 2016; Jayasundera et al., 2021).

Despite the rampant availability of the plant, Mtenga and Ripanda felt it was being underutilised, especially in the tropical Sub-Saharan region, due to the negative perception accompanied by its weed or wild plant status (Mtenga & Ripanda, 2022). However, some utilize the plant's invasive nature and capitalise on its insecticide capabilities, like in Tanzania and Malawi (Mkindi et al., 2017). The *B. pilosa* plant has a myriad of uses, both medical and non-medical; therefore, this abundant natural resource should be used without prejudice. With the increased plant uses being recorded and researched, the possibilities of finding medical cures are endless.

2.1.4 Toxicology

B. pilosa is a hyperaccumulator and excluder. This means it can absorb a significant number of major metals from the soil and hence must be cautiously harvested for medicinal purposes (Xuan & Khanh, 2016). This capability is used in agriculture as a plant biopesticide, weed control, and phytoremediation of heavy-metal polluted soils (Idris et al., 2023). The plant's ability to absorb heavy metals, which raises eyebrows in most places, could mean that it has a mechanism to metabolise and break the metals down or conjugate them such that the compounds left in the plant are not toxic for human consumption.

All parts of the *B. pilosa* plant are medicinally useful, from the roots, the stem, and leaves to the flowers (Dimo et al., 2001). The multiple benefits and uses of the *B. pilosa* plant raise the question of its toxicity to humans due to frequent consumption. A study in Sub-Saharan Africa, where people use the young leaves and shoots as food, suggested that the *B. pilosa* plant could be playing a part in the aetiology of human oesophageal cancer (Xuan & Khanh, 2016). In South Africa, another study reported that *B. pilosa* leaf consumption was an oesophageal cancer risk factor (Mokganya & Tshisikhawe, 2019). In a study by Mnisi and colleagues on three plants, namely *M. involucrate, B. pilosa and A. spinosus*, they found that when looking at the non-essential elements ingested because of eating these vegetables, there is a notable adverse health effect. The health effect was a carcinogen risk to children, as shown by the target hazard quotient (THQ) above 1 in *B. pilosa* (Mnisi et al., 2017). Where THQ is defined as 'the ratio of determined dose of a pollutant to a reference dose level' and a THQ<1 means that the population is unlikely to experience adverse effects from consumption or exposure (Mnisi et al., 2017; Zhuang et al., 2009).

In 2023, Idris and colleagues performed an investigation with the aim 'to determine the cytotoxicity of *B. pilosa* and to use chemical-based evidence to determine the health benefits and risks as well as correlate the identified metabolites with the biological activities,' where they concluded that no toxic compound was identified (Idris et al., 2023). Their study showed the relative safety of consuming the plant, hence its relevance in medical research. Also, it is important to note that the Food and Agricultural Organisation of the United Nations has reported *B. pilosa* as a staple food in Africa since 1975 (Yang, 2014), meaning that the plant has been recognised as edible for a long time.

B. pilosa extracts have not been reported to be toxic to rats or mice in the *in vivo* studies that have been done. Additionally, studies on chickens and reports on using the plant on horses and cows have been made without association with mortality or toxicity (Memon et al., 2021). For example, it was found that there was no statistically significant result suggesting harm or damage to the internal or external systems of mice and chickens tested over a period of 24 weeks post-treatment of food with ground *B. pilosa*. The following tests were

performed: toxicity, urinalysis, haematological and biochemical assay, histochemical and blood film staining, and a genotoxicity study. The results compared to the control groups did not suggest toxicity, leading to the paper's conclusion of high safety in veterinary use (Liang et al., 2020).

Though some studies have been done using *B. pilosa* extracts and other compounds, more research is needed to establish negligible toxicity in humans and show synergistic effects in disease treatment when used with other drugs. Assessing the plant's toxicity in synergistic use in this context is crucial. As previously stated, AD is a multifactorial causation disease currently managed by multiple drugs focusing on different aspects of the deterioration. Concurrent use of *B. pilosa* should be able to cut down on these drugs, given that dual activity is established, and be able to exist with the remaining medications in treating the patient. The positive effects of using both the existing treatment and the *B. pilosa* plant will hopefully include promoting improved quality of life post-AD diagnosis for both the patient and caregivers.

2.2 OVERVIEW OF ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a neurocognitive proteinopathy with the disease name coined by the German physician Alois Alzheimer in 1907 (Usman et al., 2021). According to the Diagnostic and Statistical Manual of Mental Disorders V (DSM-V) (American Psychiatric Association, 2013), AD is a dementia syndrome classified as either a major or minor neurocognitive disorder. AD is the most prevalent form of dementia (Moussa-Pacha et al., 2020). In 2011, Medeiros *et al.* described AD as a neurodegenerative disease resulting in spatial disorientation and in 2022, Dai and his colleagues echoed this definition by saying it is a chronic central nervous system breakdown that has become one of the most serious health threats worldwide (Dai et al., 2022; Medeiros et al., 2011). There are two forms of AD, classified according to the age of onset. 90% of the patients suffering from AD are above the age of 65, and the type of AD they have is called late-onset Alzheimer's disease (LOAD). The remaining 10% comprises those diagnosed, usually below the age of 65,

with the type of AD being called early-onset Alzheimer's disease (EOAD) (Kassab et al., 2024).

2.2.1 Common Hypotheses on the Causation of Alzheimer's Disease

AD is a complex disease with multiple aetiological components that pool in to cause the known signs and symptoms. Of interest to this research is the cholinergic and the Aβ theories part of the five major ones (Figure 2.2), including the neuroinflammation, metal ion imbalance and the tau hypotheses. The diagram model in Figure 2.2 highlights the interconnectedness of disease causations (Dai et al., 2022).

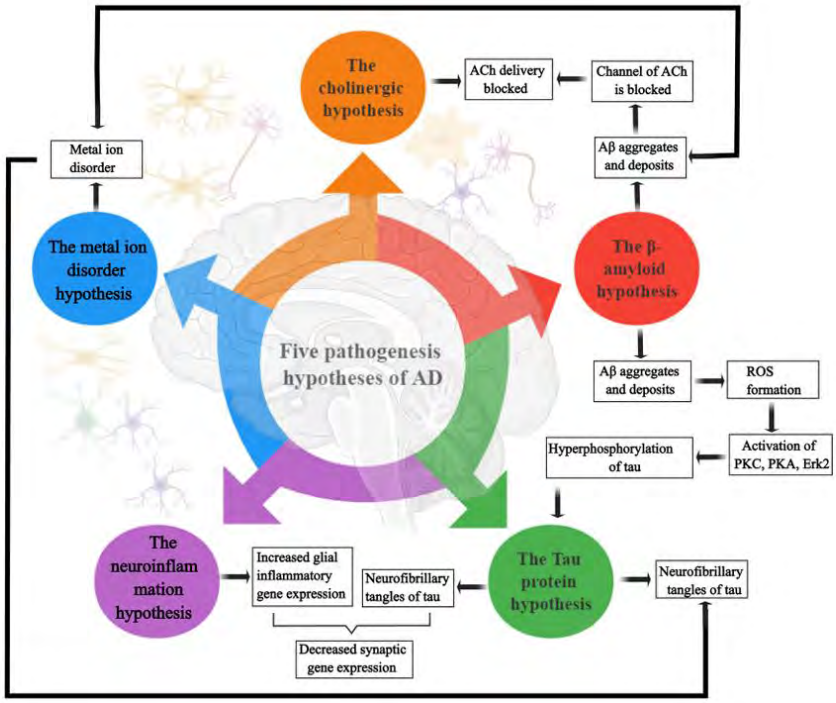


Figure 2.2: The diagram shows the five pathogenesis hypotheses of AD: the cholinergic, the Aβ, the tau protein, the neuroinflammation and the metal ion disorder hypothesis (Dai et al., 2022).

2.2.1.1 The Cholinergic Hypothesis

The cholinergic hypothesis was the first scientifically accepted theory as the cause of AD (Zang et al., 2024; Zhang et al., 2022). Two major enzymes in the cholinergic system responsible for the catalyses of acetylcholine (ACh) are AChE and butyrylcholinesterase (BChE). AChE is the more abundant enzyme

in the brain, which is why it is the most targeted in AD treatment. This is further evidenced by the existence of three out of six FDA-approved AD treatment drugs: AChE inhibitors (AChEIs) donepezil, galantamine and rivastigmine, still in use, as further explained below (Kassab et al., 2024; Pathak & Kabra, 2024). Tacrine was the first AChEI to be FDA-approved in 1993 but was recalled due to liver toxicity and low bioavailability (Kassab et al., 2024; Mitra et al., 2022; Zang et al., 2024).

Acetylcholine (ACh) is a neurotransmitter that sends impulses to cholinergic synapses. ACh plays a vital role in learning and memory (Kassab et al., 2024). The cholinergic system is responsible for neurogenesis, neuroprotection, synaptic plasticity and neuronal differentiation. In AD pathogenesis, choline synaptic reuptake is significantly reduced on the nicotinic and muscarinic receptors (Zang et al., 2024). The reduction or imbalance has been found to be a large conspirator to neurofibrillary tangles (NFTs) formation, Aβ aggregation, reduced cerebral blood flow, radical metabolic ion disorder, neuroinflammation, neuronal death and other AD progression markers (Zhang et al., 2022). The function of the AChEIs would be to inhibit the catalyses of ACh such that there is enough in the synapses for neurotransmission, delaying AD progression.

2.2.1.2 The β-amyloid (Aβ) Hypothesis

Common knowledge is that AD pathogenesis rises from the amyloidogenic pathway, which constitutes amyloid precursor protein (APP) cleavage by β- and γ-secretase producing β-amyloid proteins (Das & Yan, 2019; Moussa-Pacha et al., 2020). The $A\beta_{1-42}$ is less abundant than $A\beta_{1-40}$ but is more prone to oligomerization (Das & Yan, 2019). The definitive function of APP is yet to be defined. However, there is a belief that it plays a part in the dendritic spine and neurotransmission maintenance (Hampel et al., 2021).

Normally, Aβ's physiological 'suggested' function in the body is to improve neuroplasticity, while other studies argue that high levels are responsible for toxicity in the body (Das & Yan, 2019). Aβ plaques are reportedly directly involved in producing ROS molecules, leading to extensive oxidative stress and induced memory loss (Jung et al., 2010).

In the amyloidogenic pathway, β-secretase out-competes α-secretase for APP cleavage. β-secretase cleavage products are membrane-bound C99 and the soluble ectodomain APPsβ. The membrane-bound C99 is further broken down to give $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, as shown in Figure 2.3 (Moussa-Pacha et al., 2020).

BACE 1 is a member of the aspartyl protease clan alongside others like BACE 2, pepsin, and cathepsin (CatD). It is, therefore, important to consider developing therapies that are highly specific to BACE 1 so that there are minimized side effects from off-target inhibition due to familiarity (Moussa-Pacha et al., 2020). BACE 1 is widely distributed in the brain, especially in neuronal cell types like astrocytes and oligodendrocytes (Hampel et al., 2021; Moussa-Pacha et al., 2020).

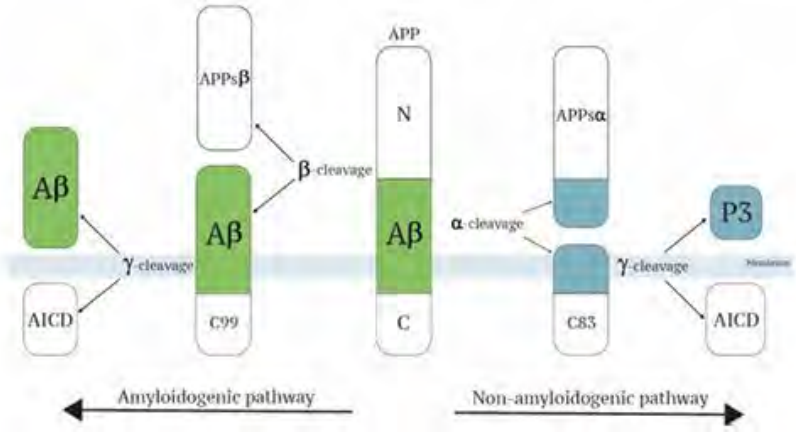


Figure 2.3: APP metabolism by secretase enzymes shows both the amyloidogenic and non-amyloidogenic pathways (Moussa-Pacha et al., 2020).

The rationale for targeting BACE 1 is that through the inhibition of the enzyme, there is decreased Aβ production. This further translates to a reduction in the likelihood of synaptic failure due to aggregation, among other hypotheses fed by toxic Aβ levels (Das & Yan, 2019). However, due to BACE 1's physiological function, inhibition treatment options must be able to shut down the toxic formulation of Aβ while leaving sufficient levels of BACE in the CNS. BACE 1 is responsible for neuroplasticity, regulating neurotransmission, synaptic function, tumour suppression, and axon guidance, among other vital functions. Treatment options that greatly suppress this enzyme will result in reduced Aβ levels in patients with AD and cognitive

impairment caused by senile plaques. However, without BACE 1, the CNS will also greatly suffer, so it is trading one toxin for another (Das & Yan, 2019; Hampel et al., 2021).

Studies have shown that the soluble forms of Aβ protein oligomers also cause neurotoxicity, leading to dysfunctional synapses like the insoluble Aβ that give the characteristic senile plaques (Moussa-Pacha et al., 2020). Moussa-Pacha *et al.* (2020) highlight that it may prove beneficial for more research to be directed towards clearly establishing which of the two, either insoluble Aβ plaques or soluble oligomers, should therapy be targeted for effective AD treatment. This need for more clarity is also supported by a study by He and associates, which suggested a severe neural decline in rats injected with oligomeric forms of Aβ compared to those with fibrillar forms (He et al., 2012).

Autosomal dominant AD is believed to be caused by a mutation in APP or presenilin, the catalytic subunit of γ-secretase. γ-secretase is involved in axon guidance, spine maturation, synaptic cell adhesion, and neural stem cell growth via Notch signaling (Carroll & Li, 2016). Therapies that will lead to complete inhibition of this enzyme in a bid to lower Aβ levels will likely result in reduced AD progression but will injure the CNS more as all other substrates that interact with it will also build up, halting their processes, especially in Notch signaling, which plays a vital role in neuronal stem cell differentiation (Das & Yan, 2019). Notch signaling is responsible for cell differentiation and proliferation during embryonic development and continues even in maturity (Moussa-Pacha et al., 2020). Since all four Notch receptors are γ-secretase substrates, inhibiting this enzyme is good for AD but will render neuronal stem cells inactive, as well as upending body tissue cell homeostasis, as Notch needs to be cleaved to be active (Carroll & Li, 2016; Zhou et al., 2022).

Drug therapies that focus on BACE 1 inhibition have been developed with exceptional results of lowered CSF Aβ levels facilitated through the prevention of formation at early APP processing. The results also show lowered cognitive decline viewed on MRI and PET scans (Carroll & Li, 2016;

Moussa-Pacha et al., 2020). However, most of the clinical studies have been discontinued due to adverse effects like liver toxicity, respiratory infections, and contact dermatitis (Asher & Priefer, 2022; Das & Yan, 2019; Hampel et al., 2021; Kim et al., 2022). Also, since the mechanism of action is BACE 1 elimination in the brain, this has led to other adverse effects like ophthalmic toxicity. The poor specificity of some of these drugs has led to side effects like hair colour change in the use of the clinical drug verubecestat (MK-8931), which was the first orally available BACE inhibitor to have reached the 3rd clinical trial stage (Moussa-Pacha et al., 2020). Verubecestat was reported to have a selective preference for targeting BACE 2 with an impressive over 45 000-fold selectivity over CatD, among other aspartyl proteases, yet it was discontinued because of lack of efficacy over the primary enzyme of interest – BACE 1 (Carroll & Li, 2016; Moussa-Pacha et al., 2020).

In vivo, BACE 1 inhibitors in mice have been successful, as shown by the marked progression markers of decreased Aβ and senile plaques. However, these therapies have not passed clinical trials, likely because of the difference in anatomy and physiology of mice compared to humans or because of BACE physiology as shown by the preferential inhibition of BACE 2 by the drug verubecestat, for example (Das & Yan, 2019; Moussa-Pacha et al., 2020). So, to possibly combat this, treatment options that target more than one pathology would be of great benefit, which aligns with this study's focus.

2.2.1.3 The Tau Protein Hypothesis

Tau neurofibrillary tangles (NFTs) are synonymous with neuronal and synaptic losses, which contribute to the characterization of patients with AD, such as memory loss, personality changes, and cognitive decline (Das & Yan, 2019).

In the National Institute on Aging and the Alzheimer's Association Research Framework article by Tang *et al*. in 2019, they suggested a system-based model in AD drug discovery and research. The model framework would use the ATN (β-amyloid [A], tau protein [T], and neurodegenerative disease [N]) classification system to group AD biomarkers by aetiological pathways to further understand their influence on the regular paths, for example, the

cholinergic, hyperexcitability, and neuroplasticity processes (Tang et al., 2019).

Post-mortem of brains from patients with AD have shown neuronal loss, oxidative damage, activated inflammatory cells, NFTs made up of hyperphosphorylated tau aggregates, and amyloid (senile) plaques made up of β-amyloid peptides (Medeiros et al., 2011). Medeiros *et al*. (2011) further stated that the hallmark pathologies are the NFTs and amyloid plaques. Maccioni *et al.* (2018) further supported this notion by focussing on the unified hypothesis of neuroimmunomodulation that centred on the vicious cycle of neuroinflammation promoting AD progression. They reported that the primary aetiology of AD was neuroinflammation facilitated by microglial cells due to hyperphosphorylation of tau protein forming NFTs in conjunction with the pathological misfolding of β-amyloid peptides forming senile plaques (Maccioni et al., 2018).

Literature supports that Aβ deposits could be what leads to tau aggregation and hyperphosphorylation (Gulisano et al., 2018; Maccioni et al., 2018; Muralidar et al., 2020; Roda et al., 2022). However, other studies suggest that tau oligomerization can occur despite any Aβ interaction via genetic mutations on the tau-coding gene (Das & Yan, 2019). Tau protein is usually an unfolded microtubule-associated protein primarily found in axons. Tau plays a role in regulating the assembly and maintaining the structural stability of microtubules. It also modulates axonal transport, such as kinesin anterograde transport by kinesins in axons. Its phosphorylation allows neurons to survive acute apoptotic death by stabilising β-catenin (Medeiros et al., 2011; Zang et al., 2024).

More progress has been made in researching and developing a treatment for AD based on the β-amyloid pathway. However, focusing on one causation is insufficient since AD is a multifactorial disease. Medeiros and colleagues pointed out that more focus should be on tau protein as statistics show that clinical trials concentrate more on β-amyloid (Medeiros et al., 2011). An extensive understanding of the role of tau in normal physiology would help identify target pathways to enhance AD, tauopathies, and other disease drug

research (Medeiros et al., 2011; Oddo et al., 2004). Oddo and associates found that when exerted early, β-amyloid immunotherapy via the proteasome results in hyperphosphorylated tau clearance in transgenic mice compared to when used at a later stage (Oddo et al., 2004). Their study further validated the interconnectedness of the β-amyloid and tau protein pathways in the causation and progress of AD. A shift from β-amyloid focus to tau protein was suggested as a more probable target. This may prove more effective since a disruption in the pathological pathway of tau, in turn, fails to feed the pathological state of β-amyloid. This will terminate the neuroinflammatory reactions occurring due to microglial activation, thus leading to neuronal degeneration and the accompanying symptoms (Maccioni et al., 2018; Medeiros et al., 2011).

Previous studies established that kinases play a pivotal role in stimulating tau phosphorylation, with Glycogen Synthase Kinase 3 (GSK3) showing the most prominent effect on promoting hyperphosphorylation (Medeiros et al., 2011; Wadhwa et al., 2020). GSK3 has two isoforms, alpha (α) and beta (β). GSK3-β is most prominently distributed throughout the brain and is reported to have been found in the hippocampal area of mice. It can play multiple roles in the pathogenesis of AD, namely in tau hyperphosphorylation, ACh reduction, apoptosis mediation, neuronal inflammation, axonal transportation, and synaptic effect (Wadhwa et al., 2020). Hence, concerns are raised when a discussion on kinase inhibitions is put to the floor due to their usual functions when zoning in on tau as a therapeutic target (Medeiros et al., 2011). Thus, a less toxic plant with selective inhibition on kinases will be most beneficial in its focus as a tau target since it will ultimately not harm the body.

Maccioni *et al*. (2018) further enforce that NFT formation due to tau hyperphosphorylation and oligomerization triggers inflammation, leading to memory loss. Memory is one of the most notable symptoms of AD and could result from tau pathology in the brain, known as an entorhinal and hippocampal formation. In addition, disrupting the cleaving of amyloid precursor protein results in the blockage of ACh channels, hindering memory retention and learning (Dai et al., 2022; Maccioni et al., 2018). Therefore, a multi-targeted therapeutic intervention like a natural plant will likely be a

more attractive option for dealing with AD than one focused on tau, Aβ, or the cholinesterase pathways.

2.2.1.4 The Neuroinflammation and Metal Ion Disorder Hypotheses

Inflammation is usually not a principal factor in the start of neurodegeneration. Persistent inflammation due to degeneration furthers disease progression, favouring cell injury and death (World Health Organisation, 2016). A correlation between ageing and pro-inflammatory signals/cytokines promotes psychiatric symptoms (Wadhwa et al., 2020). It has been found that with age, oxidative stress usually comes due to the reduction in metabolic processes that promote antioxidant activity. Because of oxidation, the activated pathways result in AD pathologies like Aβ aggregation and neuroinflammation (Kassab et al., 2024).

Uncontrolled ROS can drive biological destruction, leading to conditions like diabetes mellitus and neurodegenerative diseases because of accelerated ageing (Li et al., 2014). Homeostatic imbalance in metal ions may lead to free radicals promoting DNA, protein, and lipid peroxidation, furthering oxidative stress on the body and introducing Aβ toxicity (Kassab et al., 2024). Since plant lipoxygenases are known to be inhibited by antioxidants (Sankar Akula & Odhav, 2008), a plant with significant radical scavenging ability will be best suited to treating inflammatory-associated illnesses like AD.

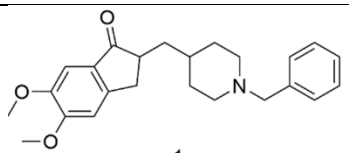
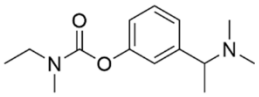
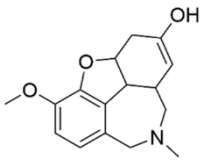
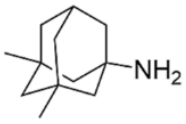
The Apolipoprotein E (APOE)-ε4 gene is part of the neuroinflammation theory. The APOE- ε4 was found to lower MMP-9 (Metalloproteinase protein 9), leading to a decline in MMP-9-induced astrocyte production and consequential Aβ clearance reduction with Aβ deposition promotion. MMPs play a role in inflammation, tau aggregation, and Aβ deposition. Because of their erratic side effects, they cannot be used as a diagnostic tool (Wang et al., 2014). AD is essentially a neurocognitive decline disease where there are a lot of synaptic failures, as shown by the varied hypotheses, due to impaired cellular homeostasis (Das & Yan, 2019; Ozadali-Sari et al., 2017).

2.2.2 Treatment Options

Six treatment options approved by the FDA are currently in use, including the three indicated for AD in South Africa. These are donepezil, galantamine,

rivastigmine, memantine, lecanemab, and donanemab, shown in Table 2.2 and Table 2.3 (Asher & Priefer, 2022; Kassab et al., 2024; Zhang et al., 2022). Passive immunotherapies, lecanemab and donanemab, have shown promising results related to Aβ protein decrease in early disease but are yet to reveal their significant effects on restoring cognitive function (FDA, 2024; Sims et al., 2023; Usman et al., 2021).

Table 2.2: Summary of FDA-approved AD treatment drugs

Drug Name	**Mechanism of action**	**Molecular structure**	**Reference**
Donepezil	AChEI		(Pathak & Kabra, 2024; Shin et al., 2018)
Rivastigmine	AChEI & BChEI		(Pathak & Kabra, 2024; Tuzimski & Petruczynik, 2022)
Galantamine	AChEI		(Pathak & Kabra, 2024; Tuzimski & Petruczynik, 2022)
Memantine	N-methyl-D-aspartate (NMDA) glutamate receptor antagonist		(Pathak & Kabra, 2024; Turcu et al., 2022)
Lecanemab	Aβ monoclonal antibody inhibitor	Monoclonal antibody structure* Chemical formula: $C_{6544}H_{10088}N_{1744}O_{2032}S_{46}$	(Park et al., 2023; Pathak & Kabra, 2024; https://go.drugbank.com/drugs/DB14580)
Donanemab	Aβ monoclonal antibody inhibitor	Monoclonal antibody structure* Chemical formula: $C_{6452}H_{10038}N_{1708}O_{2013}S_{42}$	(Decourt et al., 2021; FDA, 2024; Gueorguieva et al., 2023 ; https://go.drugbank.com/drugs/DB16647)

* The monoclonal antibody structure is too complex to depict in the table

Table 2.3: FDA-approved AD treatment drugs adapted from the Alzheimer's Association showing the approved indications and common side effects (Alzheimer's Association, 2024).

Change disease progression

Name (Generic/Brand)	Indicated For	Common Side Effects
Donanemab Kisunla™	Alzheimer's disease (MCI or mild dementia)	ARIA and headache
Lecanemab Leqembi®	Alzheimer's disease (MCI or mild dementia)	Infusion-related reactions, ARIA and headache

Treats cognitive symptoms (memory and thinking)

Name (Generic/Brand)	Indicated For	Common Side Effects
Donepezil Aricept®	Mild to severe dementia due to Alzheimer's	Nausea, vomiting, loss of appetite, muscle cramps and increased frequency of bowel movements.
Galantamine Razadyne®	Mild to moderate dementia due to Alzheimer's	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
Rivastigmine Exelon®	Mild to moderate dementia due to Alzheimer's or Parkinson's	Nausea, vomiting, loss of appetite and increased frequency of bowel movements.
Memantine Namenda®	Moderate to severe dementia due to Alzheimer's	Headache, constipation, confusion and dizziness.
Memantine + Donepezil Namzaric®	Moderate to severe dementia due to Alzheimer's	Nausea, vomiting, loss of appetite, increased frequency of bowel movements, headache, constipation, confusion and dizziness.

Aducanumab was the first FDA-approved Aβ monoclonal antibody inhibitor from Biogen Inc. for AD treatment in 2021 put on the market through the fast

track (Arndt et al., 2018; Dhillon, 2021; Pathak & Kabra, 2024). The drug was effective in selectively targeting the diseased forms of Aβ fibrils, resulting in a statistically significant reduction in oligomerisation and toxic Aβ accumulation (Wu et al., 2023). Yet, on the 31st of January 2024, Biogen Inc. announced the withdrawal of aducanumab, with their official reason being that they are redirecting their resources to the lecanemab research, which is their joint venture with Eisai Inc., not because of safety or efficacy concerns (Biogen Inc., n.d.). Others, however, still argue that, in truth, aducanumab's efficacy, in the long run, is still controversial (Decourt et al., 2021; Wu et al., 2023). Despite this setback to AD treatment research success, the FDA approved donanemab in July 2024, and it was also recommended for early disease (FDA, 2024; Gueorguieva et al., 2023).

In the past 10 years, more than 50 AD treatment drugs have failed to surpass clinical trial phase III despite having made it past phase II. Some have failed due to hepatoxicity and poor selectivity (Kassab et al., 2024), intense adverse effects (Merck Sharp & Dohme LLC (Responsible Party), 2019), and inefficacy (Kim et al., 2022; Neurotrope Bioscience, 2024). Hence, the search for anti-AD treatment continues.

Some scientists are focusing on novel muti-targeting chemical structures like fused thiophene scaffolds (Kassab et al., 2024), carbamate-based cholinesterase inhibitors (Zhang et al., 2022), edaravone *N*-benzyl pyridinium derivatives (Zondagh et al., 2024), and nitrogen-based heterocyclic scaffolds (Obaid et al., 2022), as it has been clearly shown that the one-target-one-drug system is not as effective in AD due to the complex pathogenesis. None of the multi-targeting chemical compounds has been clinically approved; however, there have been some promising *in vitro* results; hence, the exploration continues (Pathak & Kabra, 2024).

2.2.3 Risk Factors and Symptoms

Due to world development, age is the chief unmodifiable risk factor for AD (Kassab et al., 2024). One in 85 people is predicted to develop AD by 2050 (Zilberzwige-Tal & Gazit, 2018). Also, due to age, there is a natural increase

in pro-inflammation cytokine release, including IL-6, IL-8, and TNF-α as a protective mechanism. However, that promotes further microglia activation and neuropsychiatric symptoms, including clinical depression (Maccioni et al., 2018).

Additional significant unmodifiable factors include genetic mutations on key proteins like APP, presenilin 1, presenilin 2, β- and γ- enzymes (Das & Yan, 2019; Moussa-Pacha et al., 2020). Other risk factors for AD include environmental factors (e.g., moderate to severe brain trauma), family history (e.g., cardiovascular risk), lifestyle (e.g., lack of education and insulin resistance), and expression of the APOE-ε4 gene (Kassab et al., 2024; Moussa-Pacha et al., 2020; Nguyen et al., 2020; Pathak & Kabra, 2024).

As a result of metal ion imbalances, patients with AD present with increased senile plaque cores and rims where they have high levels of zinc and iron (Lin et al., 2008). As a result of ACh breakdown or shortage, patients with AD present with memory loss, decreased learning ability, and cognitive function decline (Dai et al., 2022; Lyketsos et al., 2011; Medeiros et al., 2011; Wadhwa et al., 2020).

Psychotic symptoms are most prominent in neurocognitive diseases in the mild to moderate stage due to AD degeneration (American Psychiatric Association, 2013). Visual hallucinations and mood disturbances, especially depression, apathy, and wandering, are common psychosocial symptoms (Lyketsos et al., 2011).

Continual neuronal inflammation leads to injury and, ultimately, neuronal death, promoting the secretion of corrupted tau, hence more NFTs (Maccioni et al., 2018). Long-standing activation of microglial cells occurs when they fail to return to their quiescent state. This leads to the sustained release of chemokines, cytokines, ROS, and nitrative stress, which eternalises the neuroinflammation cascade (World Health Organisation, 2016), detrimental to neural preservation. Microglial cells react according to the magnitude and nature of stimuli to produce either a neuroprotective or neurotoxic response (Maccioni et al., 2018). Inhibiting the β-amyloid pathway will cripple the tau

hyperphosphorylation and hopefully cut the continual cycle of inflammation due to microglial neurotoxic response.

Metabolic diseases like diabetes and hypertension also feed the neuroinflammation cascade. Stroke and atherosclerosis bump up the release of pro-inflammatory cytokines, fuelling inflammation and leading to further injury and death of the CNS neurons (Maccioni et al., 2018). Growth factors like insulin are responsible for the phosphorylation of GSK3-β. Obesity and diabetes affect insulin availability, preventing GSK3-β regulation and promoting AD pathogenesis (Wadhwa et al., 2020).

2.2.4 Prevalence of Alzheimer's Disease

According to WHO, in their 2019 report, more than 55 million people worldwide have dementia, and the number is exponentially growing and is predicted to be over 78 million by 2030. Dementia is the 7th leading cause of mortality according to the WHO Global Health Estimates 2019, released in December 2020. AD is the most common dementia syndrome, accounting for 60–70% of dementia cases reported (World Health Organization, 2021).

The Alzheimer's Disease International (ADI) reports that every three seconds, someone in the world is diagnosed with dementia. ADI projects that at least 139 million people globally will have dementia by 2050, of which 71% of those cases are estimated to be from low- and middle-income countries (Alzheimer's Disease International, n.d.). Africa will likely bear the brunt of most of those cases as the continent comprises most low and middle-income countries. However, African AD prevalence statistics are limited, with the available information quoting only a 2.3% – 20% prevalence (Akinyemi et al., 2022). Information on AD in South Africa is even more sparse, as the last large-scale AD prevalence study was done in the Eastern Cape in 2017. The study, conducted in a low income, rural, Xhosa-speaking population revealed the prevalence estimate of 11% for those aged 65 and over, indicating a higher than expected burden of dementia in South Africa(de Jager et al., 2017). In 2024, a South African situational analysis further revealed that South Africa is still lacking in healthcare policies, social care and support systems directed to

people living with dementia, mostly the geriatric population, who are already a vulnerable group (Jacobs et al., 2024). This prevalence statistic information further proves that this research aligns with the existing knowledge gap regarding addressing AD.

Females tend to suffer from AD more prevalently than males, and the exact causative reason is yet to be discovered. Some researchers theorise that it is a consequence of the neuroprotective decline that goes hand in hand with the reduction in steroidal hormones like oestrogens (Moussa-Pacha et al., 2020). Others believe that AD is more prevalent in women because females live longer due to a natural preference to engage in self-preservation lifestyle habits (American Psychiatric Association, 2013). In other research, the hypothesis that supports this gender bias of the disease is centred on the expression of the APOE-ε4 gene, which is more richly expressed in the fairer sex (Moussa-Pacha et al., 2020).

According to the 2022 Alzheimer's Disease International report, from the survey they conducted targeting surviving patients with dementia, 62% of the patients identified as female, and 37% identified as male (ADI, 2022). The high women prevalence is seen in the South African 2022 statistics, where approximately 9.2% of the population census were older people above 60 years, with 60,9% being female (www.statssa.gov.za). In the United States, AD is the 3rd leading cause of death for the elderly population (Wadhwa et al., 2020).

Dementia is a global health priority. This is evidenced by the disease management cost of AD, which now rivals that of cardiovascular disease, surpassing cancer (Kassab et al., 2024). Hence, there is relevance in researching possible effective drug therapies (Gauthier et al., 2022; World Health Organization, 2021). Existing drug therapies are directed at dealing with the cholinergic and β-amyloid pathways (Hussein et al., 2018; Wadhwa et al., 2020), hence the need to diversify research into looking at the dual activity by plant extracts.

Targeting one enzyme affects another enzyme theory on the causation of AD due to the interconnectedness of the disease pathologies, hence this study. Essentially, neutralising BACE 1 has the advantage of shutting down the tau protein winding and knotting, and inhibiting AChE disrupts the cholinergic and β-amyloid pathogenesis pathway (Dai et al., 2022; Jung et al., 2010). In this study, if the inhibitory effect on both enzymes is established, the successful venturing into developing it into a drug lead could potentially result in a drug that targets two sites and reduces polypharmacy, ultimately making it cost-effective and more conducive for patients with AD (Medeiros et al., 2011).

CHAPTER 3: MATERIALS AND METHODOLOGY

STUDY DESIGN

This experimental laboratory investigation examined *B. pilosa* extracts for anti-AD activity *in vitro,* as outlined in the flow diagram (Figure 3.1) below:

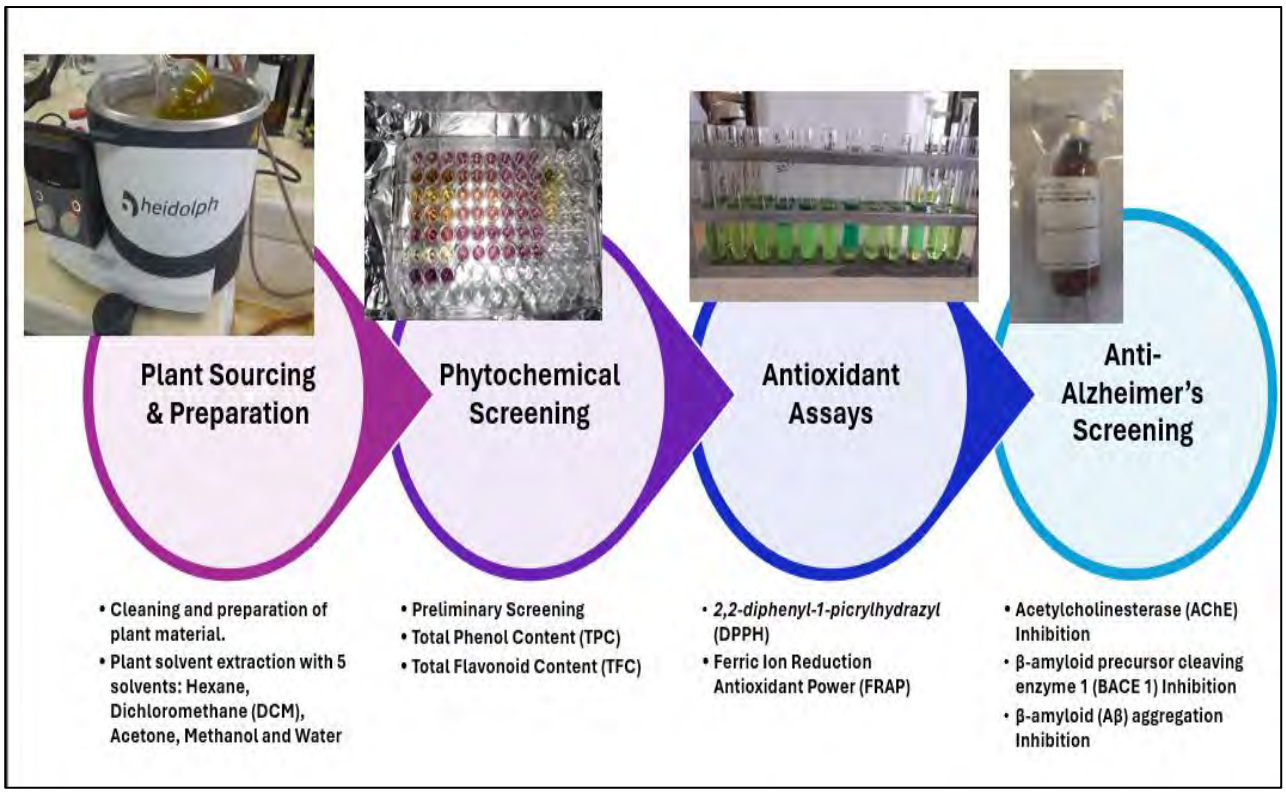


Figure 3.1: The flow diagram of the study.

3.1 SOURCING AND PREPARATION OF THE PLANT MATERIAL

Fresh whole flowered *B. pilosa* plants were collected in May 2023 at the herbarium in Johannesburg, Gauteng, South Africa. The species obtained were *Biden pilosa* var *radiata* and *Pilosa*, as authenticated by Emeritus Professor Roy A. Lubke from the Rhodes University Botany Department.

After rinsing the plant with cold running water to remove dust and other visible contamination, the leaves, flowers, and achenes were plucked off and shade-dried at room temperature (25℃ ±2°C) until completely dry. The plant material was pulverised using a blender. The resultant dry powder was

weighed on an analytical balance, and 100.12g was obtained. The dry powder was stored in the dark in an airtight bottle in a cupboard before maceration occurred.

3.1.1 Extraction

Dynamic maceration was employed to extract the powdered plant material using hexane, dichloromethane (DCM), acetone, methanol, and water. The solvent extraction order was done from the least to the most polar. All the accurately weighed dry powder material (100,12 g) was put into an Erlenmeyer flask and covered with (ten times) hexane. The flask was put in a sonicator (Ultrasonic cleaner, MRC, Israel) at 35°C for one hour and filtered out using Whatman filter paper (Grade 1, diameter 150 mm). The solid plant material was covered with hexane and sonicated at 35°C for another hour. After filtering out the solvent extract, the solid material was covered for the third time in hexane and left on an orbital shaker (OrbiShake, Labotec, South Africa), rotating at 180 rpm at 25°C overnight. The hexane was filtered out, and the solid plant material was dried at room temperature.

The same extraction process was followed using the solvents DCM, acetone, methanol, and water. However, due to DCM's low boiling point, the sonication temperature was decreased to 25°C.

The filtered hexane, DCM, acetone, and methanol solvent extracts were concentrated using a Heidolph rotary evaporator (Separations, Germany) at 40°C and 80 rpm to obtain the crude product extracted from the plant material by the respective solvents. The DCM was dried at 20°C, 80 rpm. The crude products were placed into sealable vials in preparation for the assays. The filtered water extract was lyophilized using a Labconco freeze drier (Labconco Corp, Alaska). The freeze-drying conditions were -50°C and a low vacuum pressure of 40 Pa. Once approximately 72 hours had lapsed, the lyophilized powder was collected and stored in a sealable vial at room temperature.

The weight of the remaining plant solid material after extraction was weighed using an analytical balance, and the recovery efficiency was 50% post-extraction.

3.2 PHYTOCHEMICAL ANALYSIS

3.2.1 Qualitative Phytochemical Analysis

3.2.1.1 Preliminary Phytochemical Screening

The presence of phytochemicals in *B. pilosa* extracts was determined through screening tests, and the results were assessed by visual observation. The powdered extract samples from their respective solvents were dissolved in DMSO. The tests were performed mainly according to Shaikh and Patil, alongside other references with minor modifications (Borokini, 2012; Ruiz-Reyes et al., 2022; Shaikh & Patil, 2020).

3.2.1.1.1 Alkaloids

To test for the presence of alkaloids, 1 mL of the plant extract was added to the test tube, and 3 drops of Wagner's reagent were slowly added to the sides of the test tube. The formation of a brown/ reddish precipitate indicated that alkaloids were present.

3.2.1.1.2 Flavonoids

To test for the presence of flavonoids, 1 mL of the plant extract was mixed with 10% sodium hydroxide, then a few drops of dilute hydrochloric acid. The formation of a yellow-red, coffee-orange, purple-red or blue solution colour that disappeared after the addition of hydrochloric acid indicated that flavonoids were present.

3.2.1.1.3 Saponins

To assess the presence of saponins, 1 mL of the plant extract was mixed with 1 mL of distilled water, and the mixture was shaken vigorously to froth. The mixture was allowed to stand for 10 minutes, and the presence of froth indicated the presence of saponins.

3.2.1.1.4 Quinones

To assess the presence of quinones, a drop of concentrated sulphuric acid was added to 1 mL of the plant extract. The formation of a red colour indicated the presence of quinones.

3.2.1.1.5 Anthraquinones

To test for the presence of anthraquinones, a drop of concentrated ammonium solution was added to 1 mL of the plant extract. The formation of a red colour observed after 2 minutes indicated the presence of anthraquinones.

3.2.1.1.6 Steroids

To test for the presence of steroids, the Salkowski test was used. A volume of 1 mL of the plant extract and 2 mL of chloroform were added to a test tube. Then, 1 mL of concentrated sulphuric acid was added slowly along the glass walls until a double phase formed. A dark-brown colour in the middle layer indicated a steroidal ring.

3.2.1.1.7 Terpenoids

The Lieberman-Bouchard test was used for the terpenoids test. A volume of 1 mL of anhydrous acetic acid and 3 drops of concentrated sulphuric acid were added to 1 mL of plant extract. The formation of a pink, red, magenta or violet colour indicated the presence of terpenoids.

3.2.1.1.8 Tannins

To assess the presence of tannins, 1 mL of plant extract was mixed with 2 mL of distilled water and 4 drops of 10% m/v ferric chloride aqueous solution. The formation of a blue or green colour proved the presence of tannins.

3.2.1.1.9 Cardiac glycosides

To test for the presence of cardiac glycosides, the Keller-Killani method was employed, where 1 mL of the plant extract, 1.5 mL of glacial acetic acid and 1 drop of 10% ferric chloride solution were added to a test tube. Then, 2 drops of concentrated sulphuric acid were added along the side of the tube. The

formation of a blue colour in the acetic acid layer indicated the presence of cardiac glycosides.

3.2.1.1.10 Phenols

In the phenol test, 1 mL of the plant extract was mixed with a few drops of the 10% ferric chloride solution. The formation of a dark green/ bluish-black colour indicated the presence of phenols.

3.2.1.2 Thin Layer Chromatography (TLC)

The powdered extract samples were dissolved in their respective extraction solvents (i.e., hexane, DCM, acetone, methanol and water). Then, they were analysed using aluminium-backed TLC silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) whose dimensions were 8 cm x 5 cm.

Concentrated extract samples were loaded onto the TLC plates and developed in three mobile phase systems of different polarities. The systems were composed of hexane/ethyl acetate 8.5:1.5 (v/v), acetone/ ethyl acetate 8:2 (v/v) and methanol/ toluene 8:2 (v/v). The developed plates were dried at room temperature and visualised under normal and ultraviolet light (254 and 365 nm) in a UV cabinet. The results were presented as images of the views.

3.2.2 Quantitative Phytochemical Analysis

3.2.2.1 Total Polyphenol Content (TPC) Determination

The TPC was determined using the Folin-Ciocalteu method with minor modifications (Idris et al., 2023). *B. pilosa* extracts were diluted in DMSO to 500 µg/mL, and a gallic acid standard curve was prepared in the range of 1-1000 µg/mL in 10% ethanol. A volume of 20 µL of the extract was mixed with 100 µL of Folin-Ciocalteu reagent (1:10 v/v in distilled water) and 80 µL of 7.5% m/v anhydrous sodium carbonate (Na_2CO_3), making a total volume of 200 µL in each well. The 96-well plate with the mixture was incubated at room

temperature for two hours, and the absorbance was assessed using an Epoch 2 microplate spectrophotometer (BioTek, USA) at 765 nm.

For the calibration curve, 20 µL of gallic acid replaced the extract, and all other conditions were kept the same. The assay was done in triplicate for each reading, and the results were expressed as mg gallic acid-equivalents (GAE) per gram of extract's total polyphenol (mg GAE/g) based on the gallic acid standard curve. The results were obtained using the following equation:

Equation 1

$$C = Cs\ x\ \frac{V}{m}$$

where C is TPC in mg/g, in GAE, Cs is the concentration of gallic acid established from the calibration curve in mg/ml, V is the volume of extract in ml, and m is the weight of the plant extract in g (Siddiqui et al., 2017).

3.2.2.2 Total Flavonoid Content (TFC) Determination

The TFC was determined using the quercetin standard with minor modifications (Idris et al., 2023). *B. pilosa* extracts were diluted in DMSO to 500 µg/mL. Then, 9 µL of extract and 9 µL of hydrochloric acid (HCl) (0.1% HCl, v/v in 95% ethanol) were added into the wells of the 96-well plate. This was followed by the addition of 162 µL of HCl (2% HCl, v/v in distilled water) to the wells to make up a total volume of 180 µL, and the mixture was incubated at room temperature for 30 minutes. After incubation, the plate was assessed in the dark using a spectrophotometer (Epoch 2, BioTek, USA) at 360 nm. Standard quercetin (95%) ethanolic solutions in the range of 5-1000 µg/mL were made, and for the calibration curve, 9 µL of quercetin ethanolic solution replaced the extract in the mixture. The assay was done in triplicate for each sample, and the resultant TFC was expressed as mg quercetin-equivalents per g dry mass of extracts (mg QE/g) using the linearity of the quercetin standard.

3.3 ANTIOXIDANT ASSAYS

3.3.1 The *2,2*-diphenyl-*1*-picrylhydrazyl Assay

The ability of *B. pilosa* extracts to scavenge free radicals was determined using the DPPH assay, according to Singh et al. (2017), with slight modifications. *B. pilosa* extracts from the respective extraction solvents (hexane, DCM, acetone, methanol and water) were diluted in DMSO at different concentrations (5, 50, 500 µg/mL). Volumes of 100 µL of the plant extracts were mixed with 200 µL of freshly prepared (0.01 Mm) DPPH methanolic solution in a 96-well plate and incubated in the dark for 30 minutes at room temperature. The absorbance was assessed using an Epoch 2 microplate spectrophotometer (BioTek, USA) at 517 nm. Ascorbic acid dissolved in DMSO was used as a positive control, and the alcoholic DPPH solution was used as a blank. Triplicate absorbance measurements were taken, and the ability to scavenge the DPPH radical was noted using the following formula:

Equation 2

$$Relative\ \%\ Inhibition = \left[1 - \left(\frac{Absorb.sample}{Absorb.control}\right)\right] x\ 100$$

Where control is the negative control with the alcoholic DPPH only, and the sample refers to wells with the ascorbic acid or the plant extracts.

3.3.2 Ferric Reducing Ability of Plasma Assay

The FRAP assay was done according to Sasikumar *et al*. (2012) with slight modifications. A volume of 1 mL of the extract samples was added to test tubes and mixed with 2.5 mL of (0.2 M, pH 6.6) phosphate buffer saline (PBS). 2.5 mL of 1% potassium ferricyanide was added to the mixture and vortexed for thorough mixing. The test tubes were covered with foil and incubated in a water bath at 50°C for 20 minutes. Then, 2.5 mL of 10% trichloroacetic acid (TCA) was added to terminate the reaction in each test tube, and the test tubes were centrifuged (Hermle, Lasec, South Africa) at

3000 rpm for 10 minutes. Thereafter, 2.5 mL of the mixture was collected into a different set of test tubes, where 2.5 mL of deionised water and 0.5 mL of 0.1% ferric chloride were added to give rise to the characteristic blue colour formation. Then, 150 µL of each respective mixture was added to a 96-well plate in triplicate, and absorbance was read in a microplate spectrophotometer (Epoch 2, BioTek, USA) at 700 nm.

3.3.2.1 Iron (II) standard.

Serial dilutions of ferrous sulphate in the 0-1000 µg/mL range were made. In test tubes, 1 mL of the respective concentrations was mixed with 2.5 mL of PBS and 1% potassium ferricyanide. The test tubes were incubated in a water bath at 50°C for 20 minutes, and the reaction terminated with 10% TCA. A volume of 150 µL of each respective mixture was added to a 96-well plate in triplicate, and absorbance was measured on the Epoch 2 spectrophotometer (BioTek, USA) at 593 nm. The FRAP value was expressed as µM Fe (II) equivalents.

3.4 ANTI-ALZHEIMER'S SCREENING

3.4.1 Acetylcholine Esterase Inhibition Assay

AChE inhibition assay was done according to Ellman's method in Uddin *et al*. (2021) with minor modifications. In a 96-well plate, 20µL of *B. pilosa* extracts in their respective concentrations (5, 50, 500 µg/mL) were mixed with 40 µL of 50 mM Tris-HCl buffer (pH 8.12), 100 µL of 3 mM *5,5′*-dithiobis [*2*-nitrobenzoic acid] (DTNB) and 20 µL of 1 U/mL AChE from *Electrophorus electricus* (electric eel) (Sigma-Aldrich, USA) dissolved in Tris-HCl buffer. The mixture was incubated at 37°C for 10 minutes. Then, a volume of 20 µL of acetylthiocholine iodide (ACTI) was added to start the reaction, and the absorbance was measured at 405 nm on a microplate spectrophotometer (Epoch 2, BioTek, USA).

Donepezil (Sigma-Aldrich, China) was used as the positive control, and the negative control was comprised of the buffer and all reactants except for the

standard drug or plant test sample. The assay was done in triplicate for each sample, and the inhibition was noted as a percentage inhibition using the following equation:

Equation. 3

$$Relative\ \%\ Inhibition = \left[\frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control}\right] x\ 100$$

Control is the negative control with the enzyme and substrate only, and the sample refers to wells with the enzyme, substrate, and standard drug or plant extracts.

The reaction was run for 6 minutes, but the 3-minute readings were selected to calculate the relative inhibition percentage.

3.4.2 β-Amyloid Precursor Protein Cleaving Enzyme 1 Inhibition Assay

The BACE 1 inhibition assay was done using the β-Secretase Activity Detection Kit (catalogue number: CS0010; product information: Sigma-Aldrich.com). Briefly, 20 µL of plant extracts (5,50,500 µg/mL) were added to the black 96-well plate and mixed with 58 µL of fluorescent assay buffer (F8303). In the negative control wells, 80 µL of buffer was added, and 78µL of buffer was added in the positive control wells. Then, 20 µL of 50 µM BACE 1 substrate (A1472) solution (prepared in fluorescent assay buffer) was added to all the wells. Lastly, 2 µL of ~0.3 unit/µL BACE 1 (B9059) solution was added to all the wells except the negative control wells. In summary, the positive control consisted of the buffer, substrate and enzyme, while the negative control consisted of the buffer and substrate with no enzyme. The fluorescence was immediately measured on the Varioskan Lux microplate multimode reader (Thermo Scientific, Singapore) at excitation 320 nm and emission 405 nm. Another fluorescence reading was taken after incubating the plate at 37°C for 2 hours. The assay was done in triplicate for each sample, and the inhibition was noted as a percentage inhibition using the following equation:

Equation 4

$$Relative\ \%\ Inhibition = \left[\frac{fluorescence\ of\ control - fluorescece\ of\ sample}{fluorescence\ of\ control}\right] x\ 100$$

The control is the enzymatic reaction in buffer only, and the sample is the enzymatic reaction within the standard drug or the extract samples.

3.4.3 β-Amyloid Peptide Aggregation Assay

3.4.3.1 Preparation of amyloid peptides.

To prepare the peptide solution, 1 mg of $A\beta_{1-42}$ (Sigma-Aldrich, USA) protein was dissolved in 200 µL of 50 mM sodium hydroxide and left at room temperature for 3 minutes. Thereafter, 700 µL of distilled water was added, and the solution was made up to 1 mL with (10 mM, pH 7.41) phosphate-buffered saline (PBS), bringing the peptide concentration to 25 µg/mL. The resultant mixture was sonicated for 3 minutes. Afterwards, 15 µL of the peptide solution was pipetted into separate test tubes and mixed with 200 µL of the extract samples and rifampicin (Sigma-Aldrich, USA) (positive control) or PBS (negative control). The test tubes were made up to 300 µL with PBS and incubated at 37°C. Aliquots were sampled twice (t=24 hrs and t=120 hrs) for the Thioflavin-T (ThT) assay.

3.4.3.2 Thioflavin-T method

B. pilosa extract inhibition of $A\beta_{1-42}$ was determined using the ThT method with slight modification (Ozadali-Sari et al., 2017). In a black 96-well plate, 200 µL of 30 µM ThT solution (prepared in 50 mM of glycine-NaOH buffer, pH 8.59) was added to all the wells. Afterwards, 20 µL aliquots of the peptide solutions taken at two intervals (t=24 hrs and t=120 hrs) were added to the wells. ThT fluorescence intensity was assessed using the Varioskan Lux multimode microplate reader (Thermo Scientific, Singapore) at excitation 450 nm and emission 480 nm. A graph of inhibition percentage versus time was plotted for the inhibitor compounds and compared with the control.

3.5 STATISTICAL ANALYSIS

Quantitative experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation. Analysis was performed on GraphPad Prism 10.4.0 and Microsoft Excel 365™. A two-way analysis of variance (ANOVA) trailed by a Dunnett's posthoc test was done on the DPPH, AChE inhibition, and Aβ aggregation inhibition assays to assess statistical significance between the solvent extracts and the positive controls. The statistical significance was acceptable at the level of $p<0.05$, and this was denoted by an asterisk (*).

The concentration needed to inhibit 50% of the enzyme's maximal activity (IC_{50}) is an important measure in pharmacology *in vitro* assays because it describes effectiveness. IC_{50} is one of the parameters that reveal the inhibiting power of a substance or compound against a specific biological body (Caldwell et al., 2012). IC_{50} in this research was estimated using non-linear regression.

CHAPTER 4: RESULTS

4.1 PHYTOCHEMICAL SCREENING

4.1.1 Preliminary Phytochemical Analysis

Table 4.1: Preliminary phytochemical analysis

Phytochemical	Extract Sample				
	Hexane	DCM	Acetone	Methanol	Water
Alkaloids	–	-	-	-	+/-
Flavonoids	+	+	+++	++	+/-
Saponins	+/-	+	+++	++	-
Quinones	-	-	-	-	+/-
Anthraquinones	-	+	+++	++	+/-
Steroids	-	-	+++	++	+
Terpenoids	-	-	-	-	+/-
Tannins	+	+++	+++	++	+
Phenols	+/-	++	+++	+	+/-
Cardiac glycosides	+/-	+++	+/-	+/-	+/-

[Not present (**-**), trace presence (**+/-**), moderate presence (**+**), present in appreciable quantity (**++**), impressive presence (**+++**)**]**

The acetone extract showed an impressive presence of most tested phytochemicals: flavonoids, saponins, anthraquinones, steroids, tannins and phenols. The hexane extract showed a moderate presence of flavonoids and tannins compared to the other extracts, with a trace presence of saponins, phenols and cardiac glycosides. The DCM extract showed an impressive tannin and cardiac glycoside presence but showed an appreciable quantity of phenols. The methanol extract showed an appreciable quantity of flavonoids, saponins, anthraquinones, steroids and tannins. The water extract showed a trace presence of most phytochemicals with appreciable quantities of steroids and tannins.

4.1.2 Thin Layer Chromatography

The TLC plates were set up such that the solvent extracts were assigned numbers on the plate, i.e., 1- hexane, 2- DCM, 3- acetone, 4- methanol and 5- water.

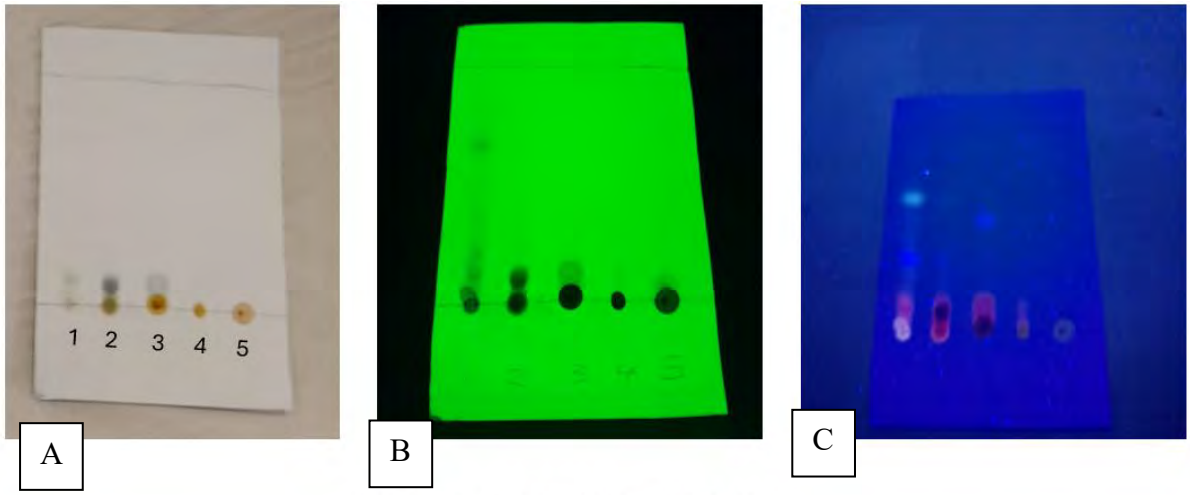
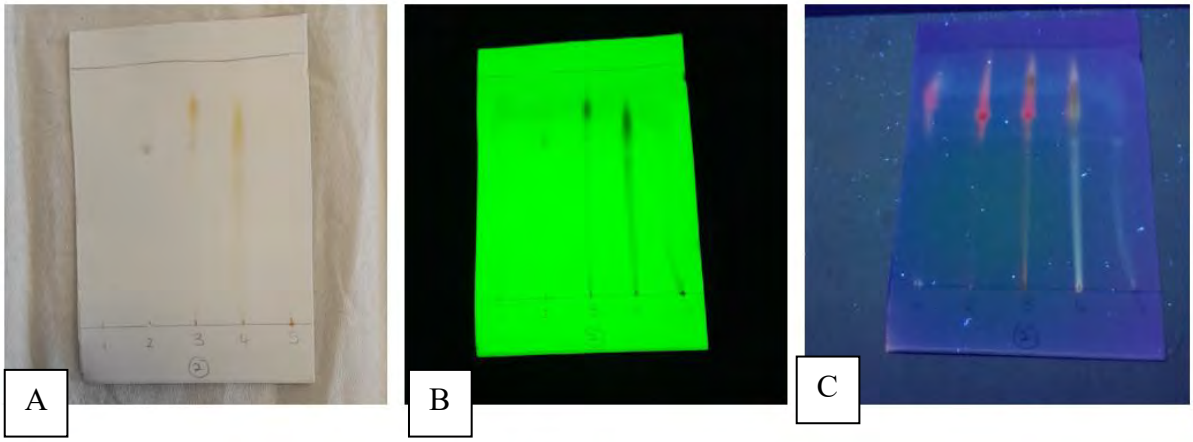


Figure 4.1: Chromatograms of B. pilosa extracts in the hexane/ ethyl acetate system. The views from the left to the right are A – normal view, visible to the naked eye; B – view under short wavelength (254 nm); and C – view under long wavelength (365 nm). 1- hexane, 2- DCM, 3- acetone, 4- methanol and 5- water extracts.

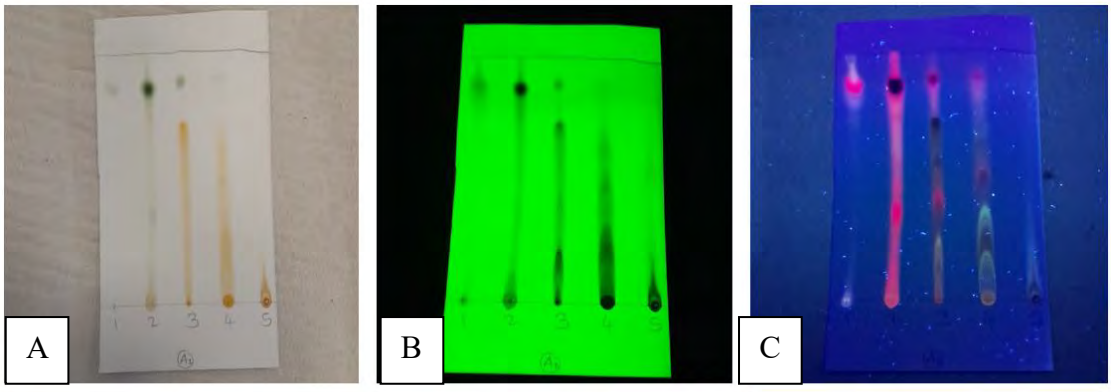
Figure 4.1 shows more fractions under the long wavelength than the other views in the developed chromatograms. There are at least 4 fractions in the hexane extract. In the DCM, methanol and acetone sample extracts, there are at least 2 fractions, and there was barely any separation in the water extract.



METHANOL: TOLUENE

Figure 4.2: Chromatograms of B. pilosa extracts in the methanol/toluene system. The views from the left to the right are A – normal view, visible to the naked eye; B – view under short wavelength (254 nm); and C – view under long wavelength (365 nm). 1- hexane, 2- DCM, 3- acetone, 4- methanol and 5- water extracts.

Figure 4.2 shows more fractions under the long wavelength than the other views in the developed chromatograms. There were at least 2 fractions in the acetone and methanol extract. The fractionation in the hexane, DCM, and water sample extracts was unclear.



ACETONE: ETHYL ACETATE

Figure 4.3: Chromatograms of *B. pilosa* extracts in the acetone/ethyl acetate system. The views from the left to the right are A – normal view, visible to the naked eye; B – view under short wavelength (254 nm); and C – view under long wavelength (365 nm). 1- hexane, 2- DCM, 3- acetone, 4- methanol and 5- water extracts.

Figure 4.3 shows more fractions under the long wavelength than the other views in the developed chromatograms. There are at least 5 fractions in the acetone and methanol extracts. The hexane extract has a single fraction and at least 2 fractions in the DCM extracts. The water sample extracts did not exhibit clear fractionation.

4.1.3 Total Phenol Content

Table 4.2: TPC of B. pilosa extracts in mg gallic acid equivalents (GAE)/g of dry sample mass

Extract sample	Hexane	DCM	Acetone	Methanol	Water
Average TPC	0.0035 ± 0.0009	0.0107 ± 0.0005	0.0331 ± 0.0005	0.0223 ± 0.0001	0.0042 ± 0.0003

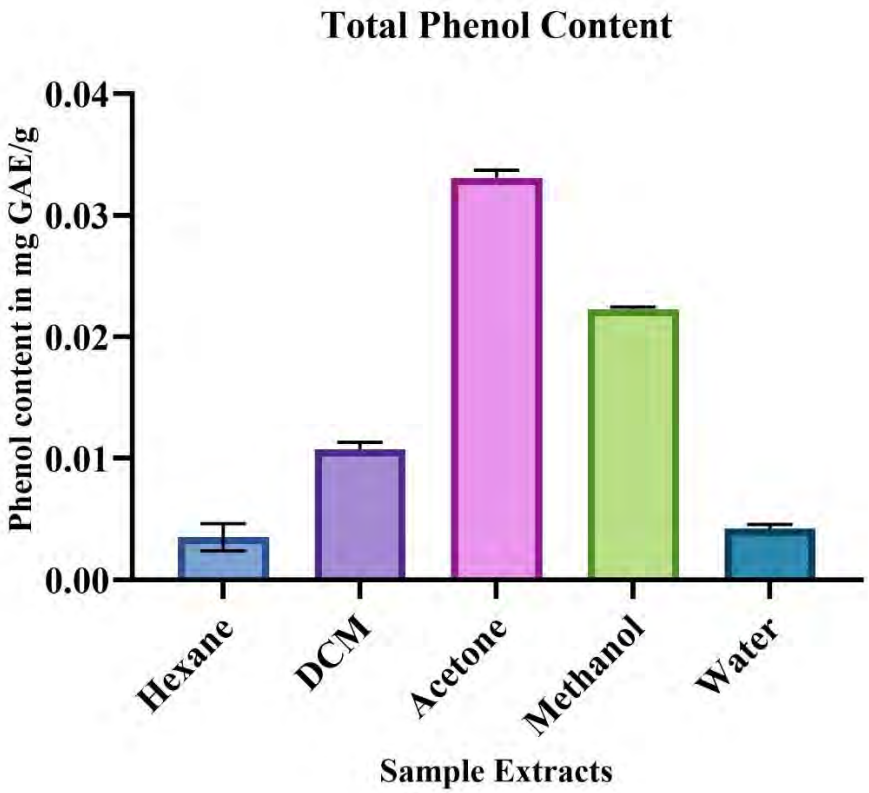


Figure 4.4: Total polyphenol content of extracts at 500 µg/mL concentration in gallic acid equivalents (GAE) per g of dry sample. The calibration curve can be found in Appendix 1: Gallic Acid Calibration Curve.

The acetone extract had the highest TPC value (0.0331 ± 0.0005 mg GAE/g), followed by methanol and DCM, respectively. The water and the hexane extracts showed similar phenol content measures of <0.01 mg GAE/g.

4.1.4 Total Flavonoid Content

Table 4.3: TFC of B. pilosa extracts in mg quercetin equivalents (QE)/g of dry sample mass

Extract sample	Hexane	DCM	Acetone	Methanol	Water
Average TFC	0.0157 ± 0.0033	0.0281 ± 0.0068	0.0380 ± 0.0020	0.0150 ± 0.0014	0.0024 ± 0.0002

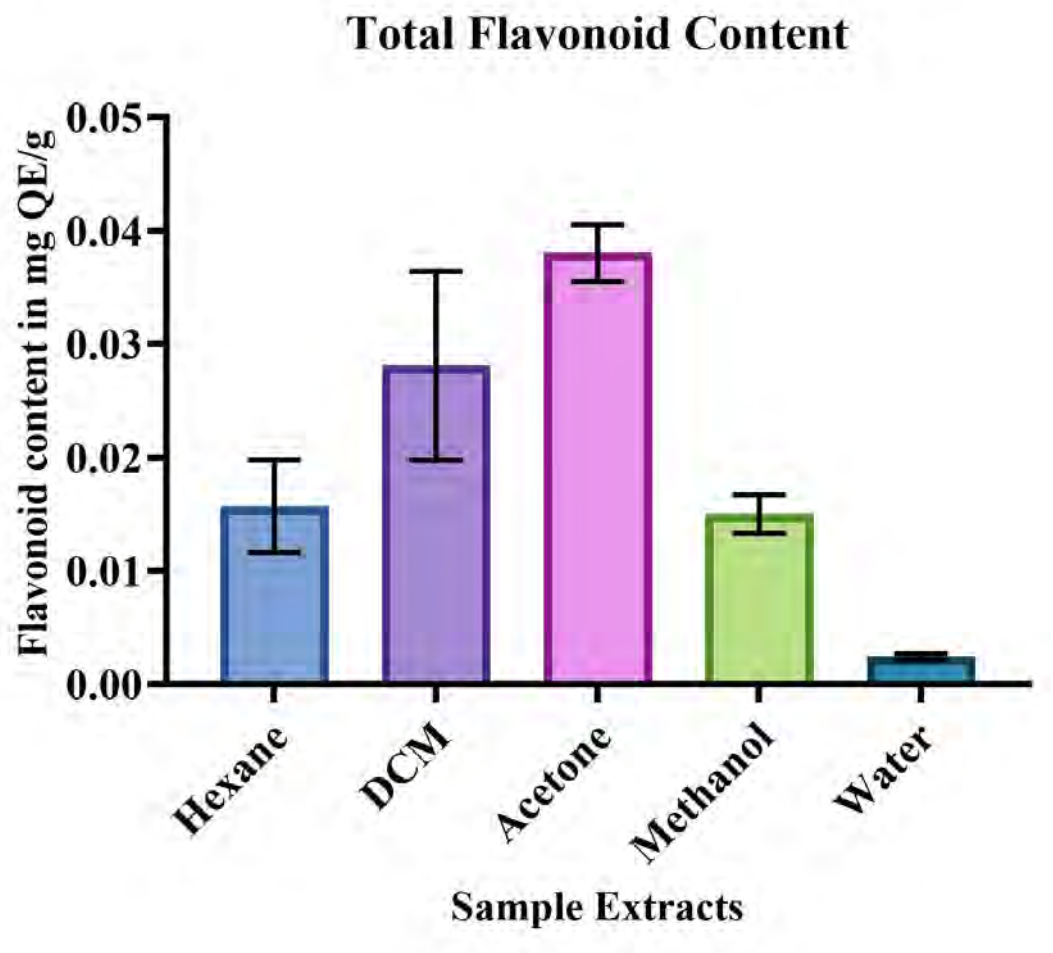


Figure 4.5: Total flavonoid content of extracts at 500 µg/mL concentration in quercetin equivalents (QE) per g of dry sample. The calibration curve can be found in Appendix 2: Quercetin Calibration Curve.

The acetone extract had the highest TFC value (0.0380 ± 0.0020 mg QE/g), followed by DCM, hexane and methanol. The water extract showed a very small flavonoid content measure of <0.01 mg QE/g.

4.2 ANTIOXIDANT ASSAYS

4.2.1 DPPH Radical Scavenging Assay

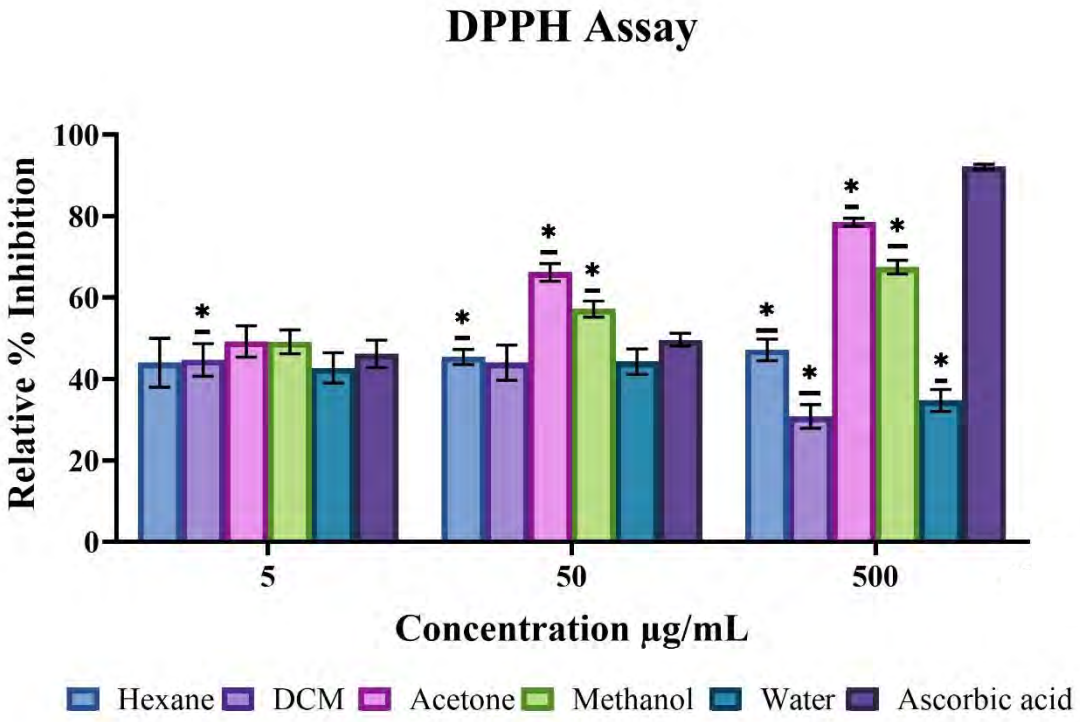


Figure 4.6.1: DPPH free scavenging activity of extract samples and ascorbic acid (compared in their concentration groups) at different concentrations (5, 50, 500 µg/mL).

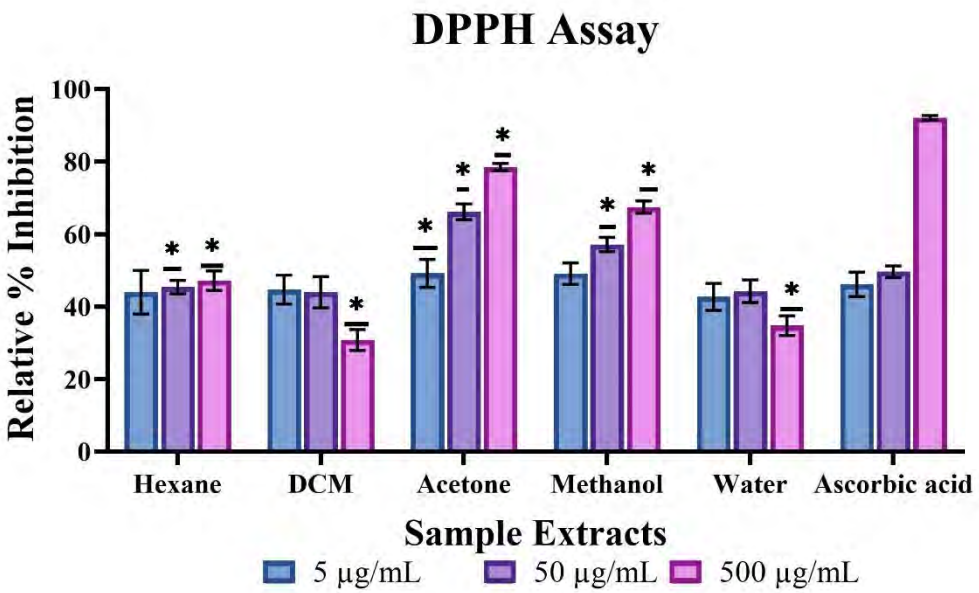


Figure 4.6.2: DPPH free scavenging activity of extract samples and ascorbic acid (compared in their solvent name groups) at different concentrations (5, 50, 500 µg/mL).

The DPPH-free scavenging activity of extract samples and ascorbic acid is depicted in relative percentage inhibition. The existence of an asterisk (*) above the bars means a significant statistical difference relative to ascorbic acid, the positive control experiment, according to the Two-way ANOVA followed by Dunnett's posthoc test.

The acetone extract had the highest scavenging ability compared to the other plant extracts, as shown by its high inhibition percentages in Figure 4.6.1 and Figure 4.6.2. The hexane and methanol extracts showed statistically significant inhibition percentages in their 50 and 500 µg/mL samples. Only the highest concentration of the water and DCM extract were statistically significant compared to the ascorbic acid standard.

The methanol extract had the lowest IC_{50} compared to the acetone extract and was 23% less than the assay standard, shown in Table 4.4. The IC_{50} for the hexane, DCM, and water extracts was not determined because of unstable values from the linear regression calculation.

Table 4.4: DPPH IC_{50} values of the B. pilosa extracts and the standard ascorbic acid

Sample Extract	**IC_{50} value (µg/mL)**
Hexane	ND
DCM	ND
Acetone	1299
Methanol	641.9
Water	ND
Assay Standard	
Ascorbic acid	2806

[ND = unstable IC_{50} value from the linear regression calculation]

4.2.2 FRAP Assay

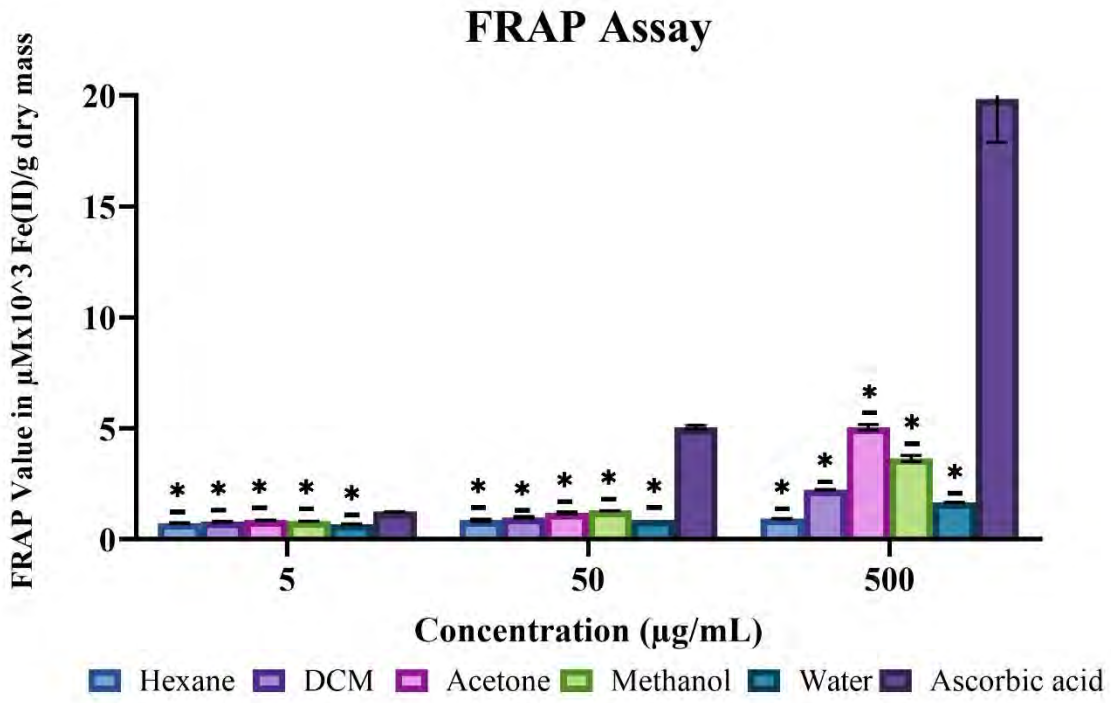


Figure 4.7.1: FRAP values of the *B. pilosa* extract samples and ascorbic acid (compared in their concentration groups) at different concentrations (5, 50, 500 µg/mL).

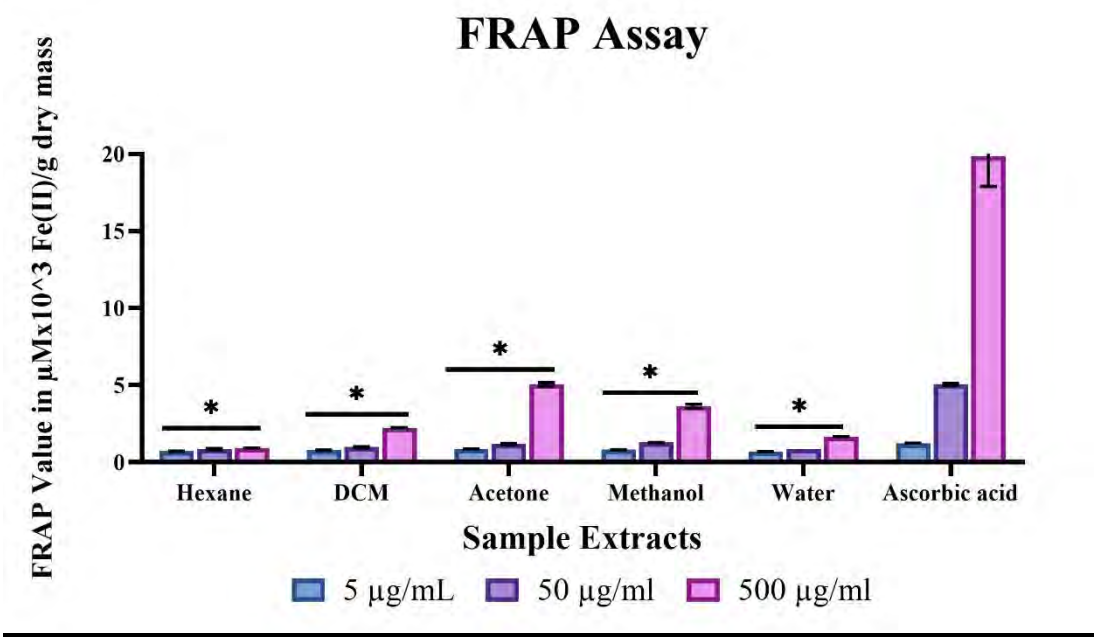


Figure 4.7.2: FRAP values of the *B. pilosa* extract samples and ascorbic acid (compared in their solvent name groups) at different concentrations (5, 50, 500 µg/mL).

The ferric-reducing antioxidant power of the extract samples and ascorbic acid is depicted as a FRAP value of Iron (II) equivalents. The Iron (II) standard curve is shown in Appendix 4: $FeSO_4$ Calibration Curve.

The existence of an asterisk (*) above the bars means a significant statistical difference relative to ascorbic acid, the positive control experiment, according to the Two-way ANOVA followed by Dunnett's posthoc test.

The acetone extract, particularly the 500 µg/mL sample, had the highest FRAP value compared to the other plant extracts in Figure 4.7.1 and Figure 4.7.2. All the sample extracts, irrespective of the concentration, showed statistically significant FRAP values compared to the ascorbic acid standard.

4.3 ANTI-ALZHEIMER ASSAYS

4.3.1 AChE Inhibition Assay

The AChE inhibitory activity of extract samples and donepezil is depicted in relative percentage inhibition. The existence of an asterisk (*) above the bars means a significant statistical difference relative to donepezil, the positive control experiment, according to the Two-way ANOVA followed by Dunnett's posthoc test.

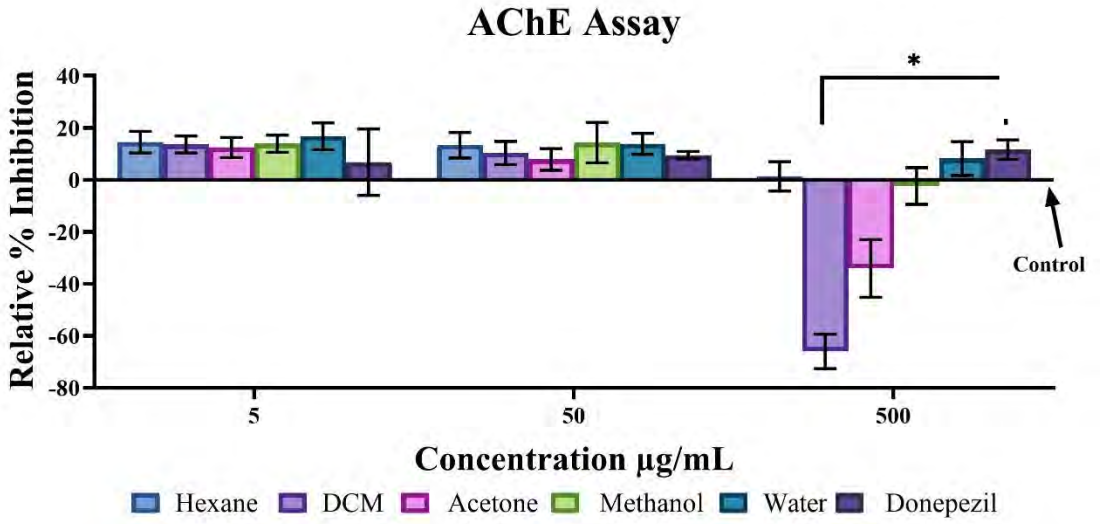


Figure 4.8.1: AChE Inhibition by *B. pilosa* plant extracts and donepezil (compared in their concentration groups) at different concentrations (5, 50, 500 µg/mL).

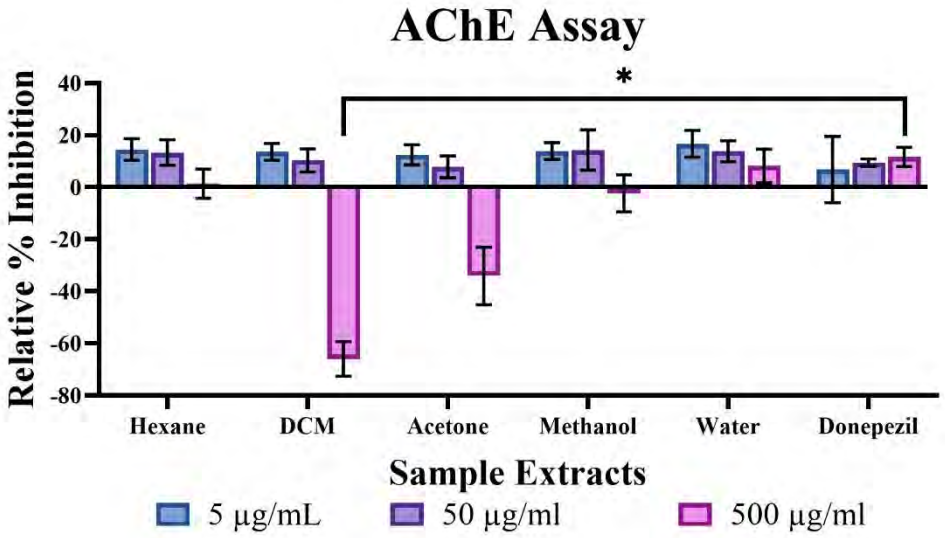


Figure 4.8.2: AChE Inhibition by *B. pilosa* plant extracts and donepezil (compared in their solvent name groups) at different concentrations (5, 50, 500 µg/mL).

The water extract, particularly the 5 µg/mL sample, had a slightly higher AChE inhibition ability than the other plant extracts in Figure 4.8.1 and Figure 4.8.2. The DCM, acetone, and methanol 500 µg/mL extracts showed negative inhibition, which could indicate activation. Only the highest concentration of the DCM extract was statistically significant compared to the donepezil standard.

Table 4.5: AChE inhibition IC_{50} values of the B. pilosa extracts and the standard donepezil

Sample Extract	**IC_{50} value (µg/mL)**
Hexane	0.958
DCM	0.804
Acetone	0.737
Methanol	0.921
Water	1.135
Assay Standard	
Donepezil	0.431

The acetone extract had the lowest IC_{50} value compared to all the other extract samples. However, it was 1.7 times slightly higher than the standard drug, donepezil.

4.3.2 BACE 1 Inhibition Assay

The BACE 1 inhibitory activity of extract samples is depicted in relative percentage inhibition (Figure 4.9.1 and Figure 4.9.2).

The DCM extract showed the highest relative inhibition compared to the other plant extracts, as shown by its high inhibition percentages in Figure 4.9.1 and Figure 4.9.2. Generally, there was an increase in relative inhibition with an increase in solvent extract concentration. However, the lowest concentrations (5 µg/mL) of the DCM and methanol extracts exhibited negative inhibition, which could indicate activation.

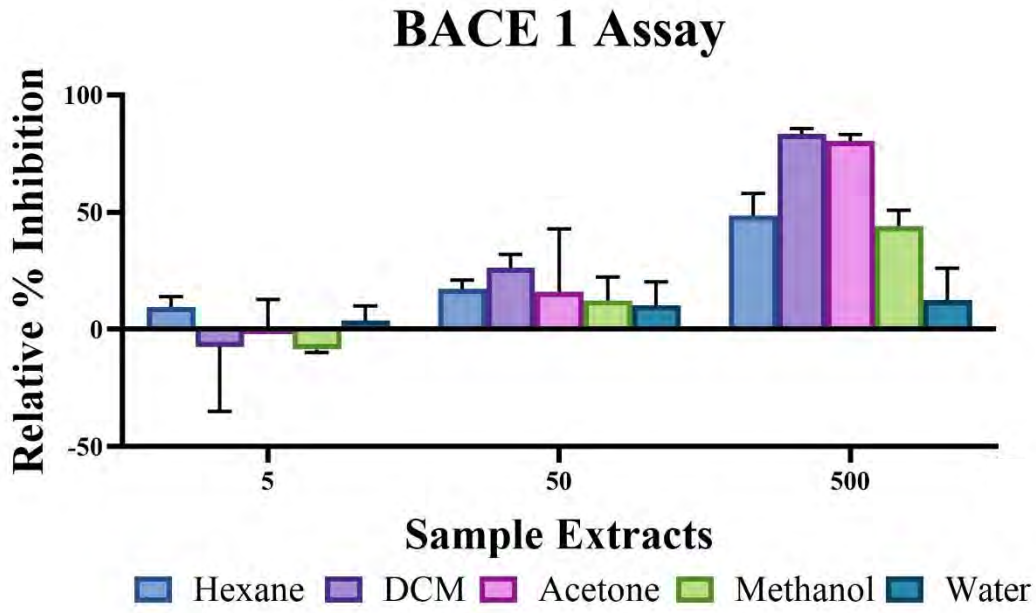


Figure 4.9.1: BACE 1 Inhibition by *B. pilosa* plant extracts (compared in their concentration groups) at different concentrations (5, 50, 500 µg/mL).

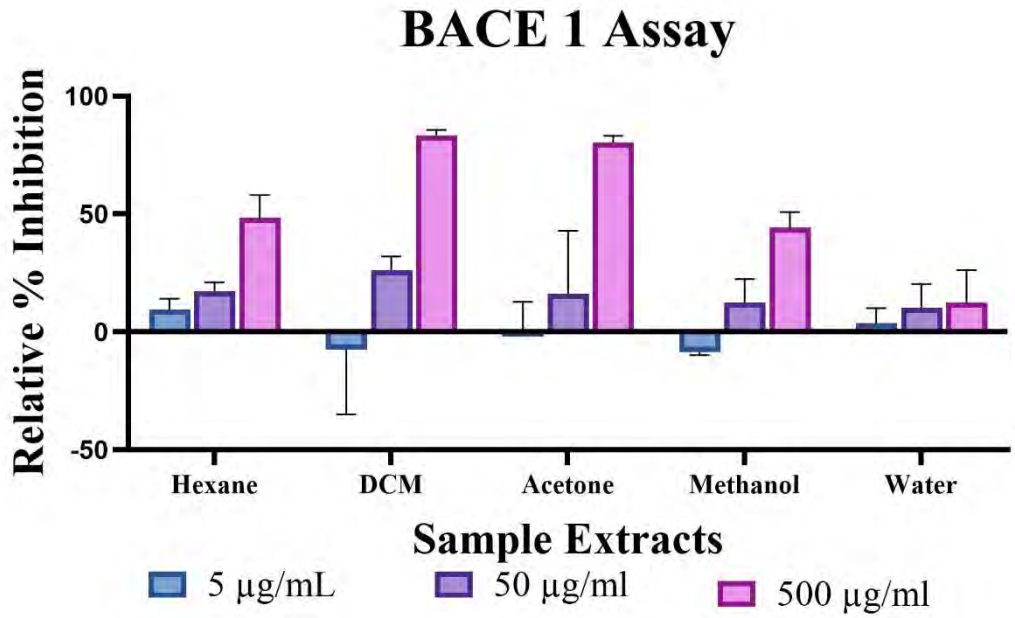


Figure 4.9.2: BACE 1 inhibition by *B. pilosa* plant extracts (compared in their solvent name groups) at different concentrations (5, 50, 500 µg/mL).

Table 4.6: BACE 1 inhibition IC_{50} values of the B. pilosa extracts

Sample Extract	**IC_{50} value (µg/mL)**
Hexane	0.698
DCM	2.72 E-10
Acetone	0.146
Methanol	2.86 E-11
Water	0.258

The DCM extract had the lowest IC_{50} value compared to all the other extract samples. The IC_{50} value trend of the extracts was DCM > methanol > acetone > water > hexane.

4.3.3 Aβ Aggregation Inhibition Assay

Figures 4.10.1, 4.10.2, and 4.10.3 show a decrease in fluorescence intensity (left-hand-side), indicating the absence of protein aggregation. The rifampicin was a positive control, and the negative contained a buffer in place of plant test extract. The relative inhibition percentages obtained from the decrease in

fluorescence are on the right-hand side of these figures. Figure 4.10.4 shows the grouped data on the relative percentage inhibition of Aβ aggregation from the sampling times (t = 24 hr and 120 hrs).

At 5 µg/mL (Figure 4.10.1), the methanol extract had the highest inhibition of 24.07 ± 15.40%, and the hexane extract had the lowest inhibition, which was also negative, suggesting activation at that concentration. At 50 µg/mL (Figure 4.10.2), the water extract had the highest inhibition of 42.80 ± 10.67%, while the methanol extract had the lowest inhibition. At 500 µg/mL (Figure 4), the acetone extract had the highest inhibition of 35.03 ± 8.62%, and the methanol extract had the lowest inhibition of 9.06 ± 22.57%.

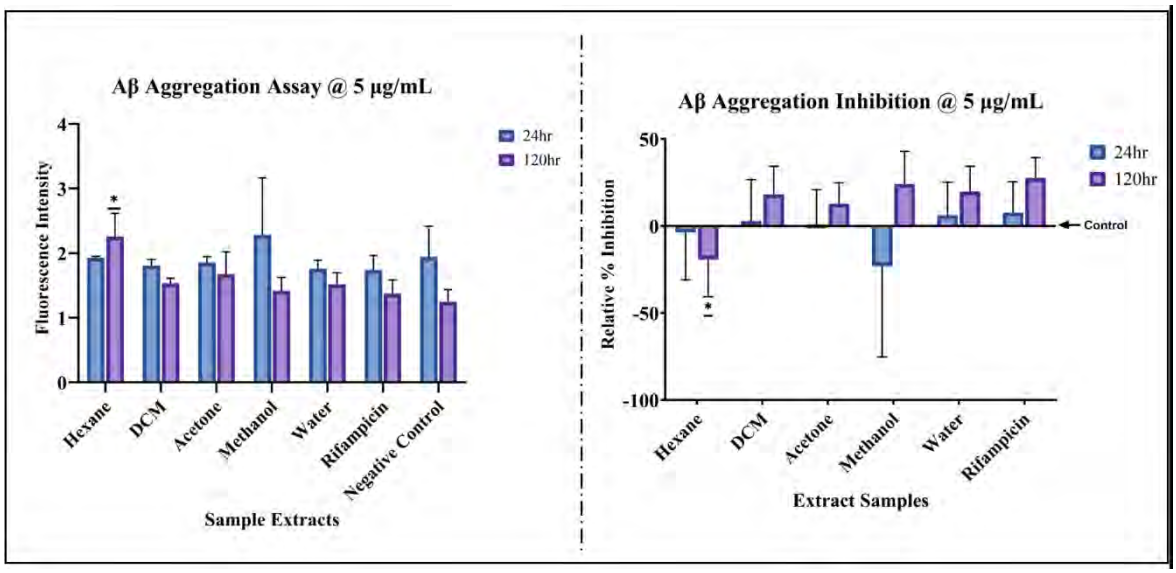


Figure 4.10.1: Aβ aggregation inhibition by 5 µg/mL *B. pilosa* plant extracts sampled at t = 24 hrs and 120 hrs, shown by fluorescence intensity (left-hand-side) and relative inhibition percentage (right-hand-side).

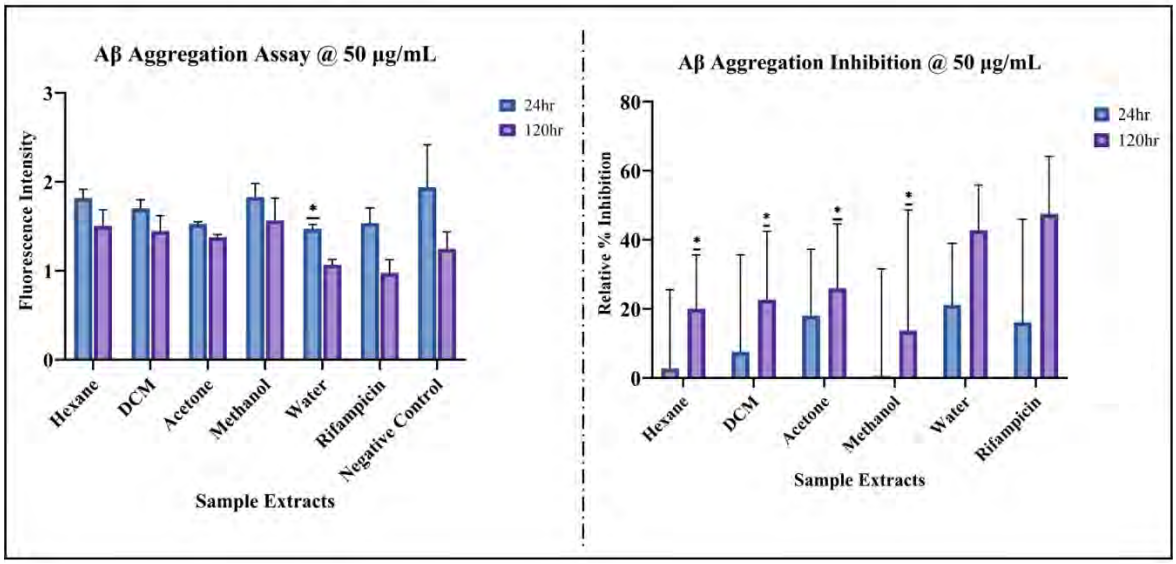


Figure 4.10.2: Aβ aggregation inhibition by 50 µg/mL *B. pilosa* plant extracts sampled at t = 24 hrs and 120 hrs, shown by fluorescence intensity (left-hand-side) and relative inhibition percentage (right-hand-side).

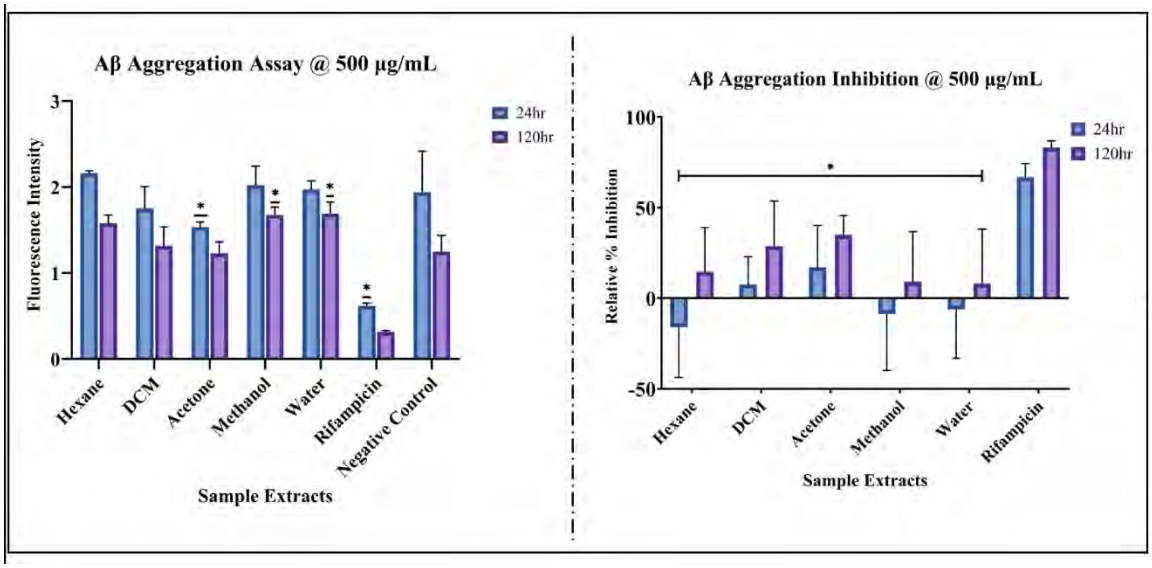


Figure 4.10.3: Aβ aggregation inhibition by 500 µg/mL *B. pilosa* plant extracts sampled at t = 24 hrs and 120 hrs, shown by fluorescence intensity and relative inhibition percentage.

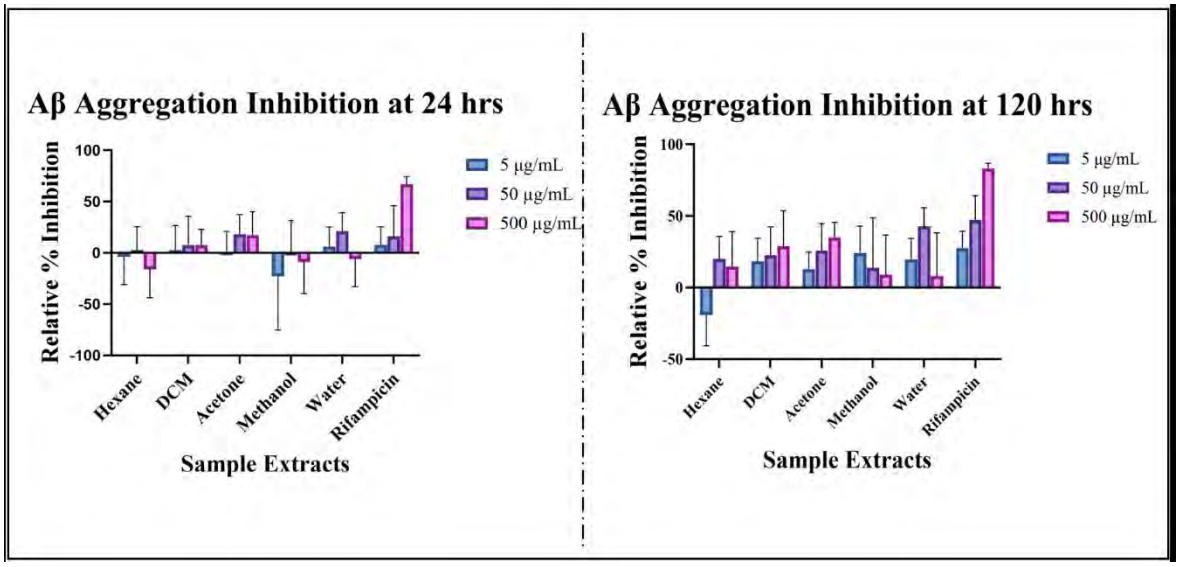


Figure 4.10.4: Aβ aggregation inhibition of grouped *B. pilosa* plant extracts sampled at t = 24 hrs and 120 hrs, shown by relative inhibition percentage.

Figure 4.10.4, at t = 120 hr, shows a positive dose-dependent relationship for the DCM, acetone, and rifampicin samples. The methanol extract showed a negative dose-dependent relationship, while the hexane and water extracts showed no discernible trend.

The existence of an asterisk (*) above the bars means a significant statistical difference relative to rifampicin, the positive control experiment, according to the Two-way ANOVA followed by Dunnett's posthoc test.

Table 4.7: Aβ aggregation inhibition IC_{50} values of the B. pilosa extracts and the standard rifampicin obtained at t=120 hrs.

Sample Extract	**IC_{50} value (µg/mL)**
Hexane	15.29
DCM	51.54
Acetone	48.09
Methanol	46.02
Water	157.20
Assay Standard	
Rifampicin	53.33

The hexane extract had the lowest IC_{50} value compared to all the other extract samples and was 29% lower than the standard rifampicin drug despite showing poor relative inhibition at t = 120 hr.

CHAPTER 5: DISCUSSION

As alluded to in the literature review, AD is the most common dementia syndrome whose management cost now competes with cardiovascular disease and is outshining cancer (Kassab et al., 2024). Available treatment options like donepezil and lecanemab are mostly single-target-oriented, but due to the multifactorial disease pathology, treatment options that eliminate polypharmacy and have better side effect profiles would be more attractive to patients with the disease (Pathak & Kabra, 2024). *B. pilosa* is a known plant with multiple pharmacological effects (Bartolome et al., 2013; Xuan & Khanh, 2016), but there has not been any record of it being associated with anti-AD activity. Based on the need to fill this knowledge gap, the plant was assessed in this thesis.

5.1 Qualitative Phytochemical Screening

Thin Layer Chromatography

After plant extraction, TLC was performed. TLC is an identification technique where a sample adsorbed onto a stationary phase is separated into fractions by the mobile phase. The separation is enabled by a capillary motion of the mobile phase and the competition for binding spaces on the stationary phase between the sample fractions and the mobile phase (Bele & Khale, 2011; Santiago & Strobel, 2013). Adsorption TLC separates compounds based on polarity differences (Bele & Khale, 2011). For this thesis, the TLC test conducted on the crude extracts was to see how many fractions can be obtained from the respective solvents. The retardation factor (RF value) was not calculated because since the separation was of crude extracts, it would not be possible to compare this to known standard compounds. Also, there was tailing seen in most of the TLC plates, suggesting poor separation in the two-solvent systems. However, these results will still be interpreted as the main aim was to show that multiple fractions are present in the crude extracts. In

the future, a three-solvent system will be considered to improve the degree of separation.

In Figure 4.1, the hexane extract had the most fractions compared to the other extracts, meaning it was the solvent that contained the most polar solutes in that solvent system of hexane/ethyl acetate. In Figure 4.2 and Figure 4.3, the acetone and methanol extracts were the most fractionated than the other extract samples, meaning they possessed the most polar solutes in the respective solvent systems (acetone/ ethyl acetate and methanol/ toluene).

Also, the high phytochemical content, evidenced by the high fractionation of the acetone and methanol extracts in the solvent systems, revealed considerable extraction solvent compatibility with *B. pilosa*.

In other studies, they performed *B. pilosa* TLC and combined it with higher power identification tests like the high-resolution electrospray ionisation mass spectrometry (HR-ESI MS) (Xin et al., 2021), gas chromatography (GC) (Cázares-Jaramillo et al., 2023) and high-performance liquid chromatography (HPLC) (Lee et al., 2024a) to obtain the chemical identities and names of the compounds within their extracts. Most fractions were observed from methanol and ethyl acetate, the most active polar solvent system used in their extraction methods.

Since more fractions were seen in the more polar solvents used in the extraction process of this study, it could be inferred that the plant extracts contained more polar compounds based on the phytochemical TLC profiles. The TLC profiles proved the existence of different phytochemicals, so the next step was to perform phytochemical screening to identify present compounds.

Preliminary Phytochemical Screening

In the preliminary phytochemical screening (Table 4.1), the plant possessed all the phytochemicals tested in at least one solvent extract. This was an expected result since *B. pilosa* is known to have at least 301 phytochemicals (Jayasundera et al., 2021). Extraction was performed on the aerial parts of the plant; hence, it was expected that the screening would turn up positive for the phytochemicals screened, as other studies have proved (Mwesigwa et al.,

2024; Nakpane et al., 2020; Ruiz-Reyes et al., 2022; Shandukani et al., 2018; Son et al., 2022). These phytochemicals have been found to be responsible for the pharmacological effects of the plant.

In the alkaloid test, only the water extract showed a trace presence of alkaloids, and the other extracts were negative. This information did not match the results of Mashinini *et al.* (2023), who had a positive result for the Wagner test on the acetone and ethanol dry plant material extracts. However, the negative results obtained for the hexane and the methanol extracts matched, as well as the positive results for the water extract. Alkaloids are heterogenous nitrogen-based compounds that act as plant protectors against microbes and herbivores (Alamgeer et al., 2020). They have been reported to play a part in anti-inflammation, nociceptive management, and AChE inhibition when their natural protective mechanisms are translated onto humans (Alamgeer et al., 2020; Tuzimski & Petruczynik, 2022). Some AD drugs, galantamine and rivastigmine, are plant alkaloid derivatives that offer symptomatic relief (Mathew & Subramanian, 2014).

In the flavonoid test, all the extracts tested positive for these phytochemicals. Mashinini *et al*. (2023) also had positive results for all their extract solvents: water, acetone, ethanol, methanol and hexane. Their dry plant material extracts all had an equally strong presence in contrast to the current study, where the acetone extract showed the most impressive presence, closely followed by the methanol extract, with an appreciable quantity. Flavonoids are polyphenols with a common C6-C3-C6 skeleton, where a three-carbon bridge joins two aromatic rings. The glycosylation, hydroxyl groups and methoxy groups affect the phytochemicals' polarity depending on the molecular structure's richness (Santos-Buelga & Feliciano, 2017; Zhuang et al., 2023). The variability of the structure could be why flavonoids were found in all the extracts. There have been over 50 flavonoids reported in *B. pilosa*. These phytochemicals in other plants have been reported to have neuroprotective effects and the ability to protect the brain during experimental haemorrhage in rats (Wang et al., 2019). Flavonoids are responsible for many other pharmacological effects, like astragalin found in *B. pilosa,* which has been shown to have antifungal activity (Mtenga & Ripanda, 2022).

In the test for saponins, all the extracts were positive except for the water extract. Mashinini *et al.* (2023) obtained a positive result even in their water extract. Contrary to the study's results, Ajanaku *et al.* (2018) found no saponins in the *B. pilosa* leaf hexane, DCM, ethyl acetate and methanol extracts. Saponins possess one or more sugar moieties combined with an aglycone (Ashour et al., 2019). Saponins are classified as steroid or triterpenoid glycosides with antioxidant, analgesic, AChE inhibition, and immunostimulant properties (Tuzimski & Petruczynik, 2022). By extension, it would be reasonable to expect *B. pilosa* to exhibit these pharmacological effects as well.

In the test for quinones, all the extracts tested negative except for the water extract, which exhibited a trace presence of the phytochemicals. Quinones are characterised by a conjugated cyclic dione in their aromatic structure. Due to the presence of the aromatic ring, quinones are generally hydrophobic; however, the attached groups can alter the compound's solubility, increasing its hydrophilicity degree by the presence of polar groups (Kato & Suga, 2018). This could have explained the trace presence only in the water extract. They are known for their hepatoprotective, antioxidant and anti-inflammatory effects, like thymoquinone, which has also exhibited AChE inhibition activity (Kato & Suga, 2018; Tuzimski & Petruczynik, 2022). Anthraquinones, which are complex quinones (Kato & Suga, 2018), tested positive in all extracts except for the hexane. This could have been a result of the specificity of the phytochemical test on the solvent extracts.

In the test for steroids, hexane and DCM tested negative, while the acetone, methanol and water extracts tested positive. Mashinini *et al*. (2023) found a negative result in their hexane and water extracts and a positive in their methanol and ethanolic extracts. The steroid core structure comprises four fused rings: three cyclohexane rings and one cyclopentane ring. Attached to these rings are various groups like the hydroxyl and ketone moiety, which affect the phytochemical's polarity, solubility and pharmacological activity (Hajdaś et al., 2025). Steroids obtained from *B. pilosa* are reported to have anti-inflammatory and antioxidant activities (Mwesigwa et al., 2024). By

extension, the presence of these phytochemicals would benefit the therapeutic management of AD.

Terpenoids have been found to have AChE and BChE inhibition when tested *in vitro* in other plants (Tuzimski & Petruczynik, 2022). This unveiled the relevance of confirming the presence of terpenoids, which literature already supports based on a study that published positive results when testing with similar solvents as in this research (Mashinini et al., 2023). The terpenoid results (Table 4.1) were negative for all extracts except the water extract, which showed a trace presence. This contrasted with the reported findings in other studies (Jayasundera et al., 2021; Mwesigwa et al., 2024). Terpenoids, or isoprenoids, are products of isoprene unit repetitions that would have formed linear, cyclic or polycyclic structures. The attached functional groups to the isoprenoids, such as the carbonyl group, are responsible for these phytochemicals' biological activity, polarity and solubility (Câmara et al., 2024). Some *B. pilosa* studies found that triterpene squalene, a terpenoid, has shown anti-inflammatory and antioxidant effects (Mtenga & Ripanda, 2022).

In the tests for tannins and phenols, all extracts were positive for these phytochemicals. However, the hexane and water extracts only confirmed the presence of trace quantities of phenol. Mashinini *et al*. (2023) also had similar results, with all their samples testing positive but with intense positives for all their readings. Tannins, phenols and flavonoids are polyphenolic compounds known for their antioxidant, antimicrobial, endothelial function improvement and cardiovascular protective effects, among other benefits (Mashinini et al., 2023; Muchuweti et al., 2007). Polyphenolic compounds are aromatic amino acids expected to be present in the *B. pilosa* plant as they are thought to be the major contributors to its pharmacological activity (Bartolome et al., 2013; Shraim et al., 2021; Xuan & Khanh, 2016).

All extract samples tested positive for cardiac glycosides, with the DCM extract exhibiting impressive presence and the rest trace presence. *B. pilosa* methanol, water, and hexane extracts have been found to possess cardiac glycosides (Mashinini et al., 2023). Hence, cardiac glycosides were expected in the researched *B. pilosa* plant material. Cardiac glycosides are steroid

compounds chemically composed of a steroid core, a lactone ring and a sugar moiety. The general function of cardiac glycosides is modulating heart contractility and rhythm by inhibiting the Na^+/K^+-ATPase signalosome. Cardiac glycosides also have an immunomodulatory function, which would, therefore, be projected to play a part in the management of AD (Škubník et al., 2021).

The differences in the results obtained in this study and those by others on the *B. pilosa* plant could result from variable phytochemical extraction. Phytochemical extraction is affected by pre-extraction factors (e.g., plant part used, degree of processing, plant harvesting conditions and plant origin) and extraction-related factors (e.g., extraction method, pH and temperature of solvent) (Ruiz-Reyes et al., 2022; Shaikh & Patil, 2020). Under pre-extraction factors, the plant's origin environment plays a pivotal role in secondary metabolite production, which could result in negative results because of insufficient compound for chemical detection (Cortés-Rojas et al., 2013). The differences in these conditions could be a source of the difference in results, but it is important to note that qualitative test results are not absolute (Shaikh & Patil, 2020), hence the importance of performing quantitative tests like the TPC and TFC.

5.2 Quantitative Phytochemical Screening

In the TPC assay, the acetone extract had the highest phenolic content of 0.0331 ± 0.0005 mg GAE/g. The methanol extract was a close second with a TPC value of 0.0223 ± 0.0001 mg GAE/g, as shown in Table 4.4 and Figure 4.2. The rest of the extracts had a phenolic content below 0.015 mg GAE/g. Other studies have found significantly higher *B. pilosa* TPC values. For example, Muchuweti and colleagues found a TPC value of 1102.797 ± 2.239 mg g^{-1} for their tested methanolic extract in Zimbabwe (Muchuweti et al., 2007). Nguyen *et al.* (2023) revealed a *B. pilosa* methanol flower extract TPC value of 128.19 ± 3.94 mg GAE/g, 80.69 ±6.47 mg GAE/g for the leaf water extract and 20.42 ± 0.00 mg GAE/g for the acetone extract. Li *et al.* (2014) obtained a TPC value of 8.12 ± 0.41 mg GAE/g on *B. pilosa* flower extracts. Furthermore, in GAE, another methanol and water extract TPC value of 38.1 ±

0.08 and 24.4 ± 0.32 mg/g were published by Akula and Odhav (Sankar Akula & Odhav, 2008). This study's results are not so far-fetched as some have reported *B. pilosa* aqueous-ethanolic extract TPC values that ranged from 0.40 and 1.24 mg/g of wet base (Cortés-Rojas et al., 2011).

On the other hand, Shandukani *et al*. (2018) observed decreased phenolic content with increased extraction solvent polarity, i.e., ethyl acetate < acetone < methanol on the *B. pilosa* plant. This was true from the results of the current study's acetone-to-water extract (Figure 4.2). However, the trend does not apply to the hexane and DCM extracts. Nguyen *et al*.'s (2023) study found that the highest TPC value was from methanol, and the lowest was from the acetone extract, whereas, in this study, the acetone extract had the highest TPC value.

The Folin-Ciocalteu method employed in the TPC assay has limitations. The efficacy of the assay is dependent on the antioxidants being hydrophilic, and since multiple standards like ascorbic acid and rutin can be used, this suggests reduced specificity. Also, the threat of these standards reacting with the reagent if present in place of the phenols in question is considerable (Bessada et al., 2015; Cortés-Rojas et al., 2013; Li et al., 2014). Additionally, the absorbance is proportional to the present HO•, which means the quantity shown relies on the molecular structure of the phytochemicals (Bessada et al., 2015). These limitations could be why this study's results are lower than those of other published records.

In the TFC assay, the acetone extract had the highest flavonoid content of 0.0380 ± 0.0020 mg QE/g, closely followed by the DCM at 0.0281 ± 0.0068 mg QE/g, as shown in Table 4.5 and Figure 4.3. The hexane extract had a flavonoid content of 0.0157 ± 0.0033 mg QE/g, and the remaining samples were below 0.015 mg QE/g in flavonoid content.

Some studies have found significantly higher TPC and TFC values for the different extracts of *B. pilosa* (Hughes et al., 2020; Moyo et al., 2020; Ruiz-Reyes et al., 2022). Others have reported similar quantities to this study, like Cortés-Rojas *et al*. (2011), who reported TFC values that ranged between 0.06 and 0.34 mg/g of wet base.

TPC and TFC results depend on many factors, like the phytochemical structure, extraction method and solvent type (Cortés-Rojas et al., 2011; Iloki-Assanga et al., 2015). Phytochemical structure properties like molecular structure, size and polarity play a part in the quantities observed in the TFC and TPC assays. For instance, large-sized phenols and flavonoids, such as tannins and polymeric flavonoids, contribute more to TPC and TFC content. However, the large molecule sizes affect extraction efficiency and solubility compared to the smaller molecules, reiterating that TFC and TPC results largely depend on the extraction method and solvent type (Azahar et al., 2017; Iloki-Assanga et al., 2015; Mehmood et al., 2022).

Regarding the extraction method, some studies, like Moyo *et al.*(2020), have found that by boiling the *B. pilosa* methanolic extract, there was an increase in TPC, TFC and FRAP values. This could have been attributed to the fact that since phenols are usually found in the plant cell vacuoles, boiling splits cell walls, increasing phenols in the solvent. Also, solvent permeability with the split cell wall becomes increased, promoting further leaching of the phytochemicals out of the plant cells.

Regarding the solvents, the laws of intermiscibility and similarity, ‘like will dissolve like,’ apply (Lee et al., 2024b). The acetone extract was seen to have the most phytochemicals compared to the other extract samples in the phytochemical screening. This agrees with the literature that the acetone solvent normally has high recovery rates in most extraction methods (Kumar, 2023). Acetone has an intermediate polarity, leaning in more on the lipophilic side. It is well known for its ability to extract phenols and flavonoids due to the presence of the hydroxyl group (Lee et al., 2024b; Ruiz-Reyes et al., 2022). Compared to this study’s solvents, acetone's intermediate polarity property most likely resulted in the high TPC and TFC values since it could extract a considerable amount of hydrophilic and hydrophobic compounds from the plant. Water is the most polar, environmentally friendly solvent used in extraction, but the extraction efficiency is usually low (Lee et al., 2024b). This agrees with the results obtained in the phytochemical screening, as the water extract showed the least presence of phytochemicals qualitatively and quantitatively. However, the reason for proceeding with the water extracts in

this research is that the trigger was based on anecdotal evidence. The general populace, most notably traditional medicine practitioners, daily use water as their extraction solvent instead of the expensive, mostly toxic organic solvents. Hence, the importance of proving the ethnopharmacology.

5.3 Antioxidant Assays

One of the theories of the pathology of AD is the neuroinflammation hypothesis, as explained in the literature review above (2.2.1.4). The neuroinflammation hypothesis is fed largely by the continual activation of microglia leading to the uncontrolled formation of ROS (Das & Yan, 2019; Wang et al., 2014). Antioxidants have long been said to reduce inflammatory damage (Bessada et al., 2015; Nguyen et al., 2020). Therefore, the plant's ability to quench uncontrolled oxidants is an attractive trait in treating AD. This study assessed the plant's antioxidant ability using the DPPH and FRAP assays.

DPPH is a free radical scavenging method where there is the reduction of the purple DPPH radical to the yellow stable diamagnetic molecule hydrazine in the presence of hydrogen-donating antioxidants (Munteanu & Apetrei, 2021; Noreen et al., 2017). Ideally, the lower the absorption recorded, the higher the antioxidant scavenging ability. This means the extent of decolouration corresponds to the radical scavenging ability of the antioxidant (Bessada et al., 2015; Munteanu & Apetrei, 2021).

Figure 4.6.1 and Figure 4.6.2. show that the 500 µg/mL acetone extract had the highest relative DPPH decolouration (78.51 ± 0.82%), followed by the methanol extract (64.47 ± 1.39%), and the rest of the plant extracts had a relative inhibition of less than 50%. The acetone extract's high inhibition capability could have resulted from the solvent's ability to extract more lipophilic antioxidants due to its intermediate polarity compared to the other solvents. In another study, the *B. pilosa* methanolic extract exhibited 88.7 ± 0.34%, and the aqueous extract showed 40.5 ± 0.38% DPPH scavenging ability (Sankar Akula & Odhav, 2008). The methanol extract readings were marginally higher than those obtained in the study, while the water extract results matched the current study findings.

In the study done by Nguyen *et al.* (2023), the DPPH radical scavenging (depicted through inhibition percentage) trend they observed in the flower extracts was ethyl acetate fraction > water fraction > acetone > methanol and in leaf extracts was ethyl acetate > methanol > water > acetone. In this study, the trend at 500 µg/mL extracts was acetone > methanol > hexane > water > DCM. Since DPPH favours hydrophobic antioxidants (Berker et al., 2012; Cortés-Rojas et al., 2011), the expected results were higher inhibition percentages from the hexane and DCM extracts. However, there was no clear depiction of this in the results obtained in this study, and the hydrophobic preference trend was also not shown by Nguyen *et al.* (2023). The non-conformity of the results could be attributed to the difference in extraction methods, collection sites, or plant parts used. This shows that not only does the type of solvent play a part in the scavenging ability, but the plant's source (origin) determines the phytochemicals available for extraction by each solvent, which cascades down to the extent of protective ability, like antioxidant ability.

According to published literature, the expected trend was a dose-dependent relationship (Son et al., 2022), which, according to Figure 4.6.2, is shown by the ascorbic acid, hexane, acetone, and methanol. However, the DCM and water extract did not conform to the expected trend.

The IC_{50} value of the methanol extract (641.9 ± 556.5 µg/mL) suggested greater efficiency than the acetone extract (1299 ± 1332 µg/mL), shown in Table 4.4 and was even more efficient than the ascorbic acid standard. In the study by Lee *et al*. (2013), the methanolic, water, and hexane *B. pilosa* extracts had DPPH IC_{50} values of 207.3 ± 43.5 µg/mL, 1045.4 ± 63.6 µg/mL, and 260.2 ± 5.0 µg/mL, respectively when compared to a Trolox standard. Lee and colleagues obtained DPPH inhibition greater than 50% for the methanol, water, and hexane extracts, where the methanolic extract had a significantly higher inhibition efficiency than the results obtained in this study (Lee et al., 2013). The higher inhibition efficiency seen in their study could result from a different extraction method since the fractions they used were obtained from methanolic extracts that were further partitioned, whereas the crude extracts in this study were of pure solvents.

In another study, their *B. pilosa* ethanol extract had a DPPH IC_{50} value of 455.78 ± 3.28 µg/mL (Son et al., 2022). This study's methanol extract IC_{50} value was 1.4 times more than what was obtained by Son *et al*. (2022). This suggests that the alcoholic extract's efficiency in scavenging the DPPH radical was significantly less.

In the FRAP assay, the ferric ion is reduced to the ferrous ion in an acidic medium, forming the coloured ferrous-tripyridyl triazine complex (Benzie & Strain, 1996; Bessada et al., 2015) or the Prussian blue ferrocyanide complex as in this study. The ferric–ferricyanide FRAP method has the capability to assess both lipophilic and hydrophilic antioxidants compared to the one-sided DPPH assay but has a greater affinity for the hydrophilic (Berker et al., 2012; Munteanu & Apetrei, 2021). The FRAP value is obtained by assessing the absorbance change of the test samples versus a standard curve of known Fe (II) concentrations (Benzie & Strain, 1996; Munteanu & Apetrei, 2021) like the one shown in Appendix 4.

In the current study, the FRAP assay results (Figure 4.7.1 and Figure 4.7.2) show a dose-dependent relationship between Fe (II) equivalents concerning the tested samples and the standard. The highest acetone extract concentration of 500 µg/mL gave the highest FRAP value of $5.05x10^3$ ± 0.10 µM Fe (II)/g (Figure 4.7.1). Acetone's intermediate polarity, when compared with the other extraction solvents, allowed it to extract a larger number of hydrophilic antioxidants, which could be why it showed a higher FRAP value. FRAP's hydrophilic preference is supported by the higher values of the 500 µg/mL extracts of methanol ($3.65x10^3$ ± 0.11 µM Fe (II)/g) and water ($1.66x10^3$ ± 0.01 µM Fe (II)/g) compared to hexane ($0.94x10^3$ ± 0.003 µM Fe (II)/g) and DCM ($2.24x10^3$ ± 0.01 µM Fe (II)/g).

Despite the lack of outstanding FRAP value differences of the 5 and 50 µg/mL samples of the extracts (Figure 4.7.2), all the FRAP results were statistically significant compared to the ascorbic acid standard.

Furthermore, methanol is usually the recommended solvent for antioxidant extraction (Lee et al., 2013). However, based on the results in Figures 4.7.1 to 4.8.2, the acetone extract showed the highest antioxidant activities in the

DPPH and FRAP assay. This could have been because the intermediate polarity allowed it to draw out considerable quantities of antioxidants from the plant.

The FRAP values obtained in this study were higher than those obtained by Tesfay *et al.* (2016). The highest FRAP value obtained in their study for freeze-dried *B. pilosa* leaves was 1651.45 ± 339.5 µmol.g^{-1} at 10 weeks after transplanting. After 12 weeks, the value reduced to 1000.2 ± 289.2 µmol.g^{-1}, which could have been signaling the dipping trend with maturity where their theorized reasoning was that the reduction in abiotic and biotic stressors led to reduced antioxidant production (Tesfay et al., 2016). This could mean that the high results that were obtained from the plant material used in this study were likely due to harvesting young plants that were still at their antioxidant production peak.

Li *et al.* (2014) performed a FRAP assay on *B. pilosa* flowers and found the value to be 63.50 ± 3.25 µmol Fe (II)/g. Compared to the current study results, this value was significantly lower, and this could be attributed to the fact that they performed the study only on *B. pilosa* flowers while the aerial parts of the plant were tested in this research. Additionally, the difference in FRAP values obtained in this study and those published could be due to the difference in observation times since different antioxidants have different reaction times for detection (Munteanu & Apetrei, 2021).

The bulk of a plant's antioxidant activity is attributed to its polyphenols (Li et al., 2014; Muchuweti et al., 2007). This was proven in a study establishing the inhibition of lipid peroxidation (Chiang et al., 2004). It is safe to assume that the higher the number of hydroxyl groups in the structure, the higher the scavenging ability (Chiang et al., 2004; Cortés-Rojas et al., 2011; Li et al., 2014). However, Muchuweti *et al.*'s (2007) results reported the lack of a direct relationship between methanol extract TPC and antioxidant ability, with the highest TPC being from *Cleome gynandra* while it had the lowest DPPH scavenging ability (61%) compared to *B. pilosa* and *Vigna ungiculata,* whose DPPH scavenging ability was above 70%. One could also argue that the methanolic extracts of the plant in Muchuweti *et al.*'s (2007) study would have extracted only the hydrophilic phenols, hence the low antioxidant values. This

current study's findings support a direct relationship between phenolic content and antioxidant ability, with the acetone extract that exhibited the highest phenol and flavonoid content also showing the highest DPPH result and FRAP value.

5.4 Anti-Alzheimer's Screening

5.4.1 AChE Inhibition

In the AChE inhibition assay, ACTI was hydrolysed by the enzyme to give thiocholine that reacted with the Ellman reagent (DTNB), resulting in the yellow-coloured spectrophotometrically detected *2*-nitrobenzoate-*5*-mercaptothiocholine and *5*-thio-*2*-nitrobenzoate. AChE is a serine hydrolase that catalyses ACh in the body. ACh preservation in AD is currently being achieved by AChEIs like donepezil, galantamine, and rivastigmine (Halder & Lal, 2021). The relative inhibition percentage of all the extracts and the donepezil standard on AChE was below 20%, as shown in Figure 4.8.1 and Figure 4.8.2. Interestingly, the water 5 µg/mL extract exhibited the highest inhibition of 16.71 ± 4.22%, followed by the hexane extract (14.50 ± 3.38%). These extracts contained the least phytochemical presence in the preliminary screening and antioxidant activity tests.

Generally, inhibition potential decreased with increased plant extract concentration. The expected trend would likely be that of donepezil, as seen in Figure 4.8.2. However, this decrease raises the possibility that the lower the concentration of plant extracts, the higher the inhibition potential. Low doses with high positive outcomes are usually associated with lower toxicity, so the plant should be considered for more in-depth research in AD treatment. With the decrease in inhibition, most of the 500 µg/mL samples (i.e., DCM, acetone, and methanol) exhibited negative inhibition of AChE, which could have been suggestive of activation. The activation could mean that at that concentration, the plant extracts could not inhibit AChE, with the possibility that samples taken from these extracts could further neurodegeneration at high concentrations by worsening the cholinergic deficit.

Phytochemicals like flavonoids, alkaloids, and terpenoids are known to have AChE inhibition effects (Cao et al., 2013). Since the phytochemicals were

proven to be present in the screening of the plant material, there is expected to be inhibition of AChE. Also, according to the literature, plants that possess flavonoids (e.g., luteolin-7-glucoside), like *B. pilosa,* tend to have AChE inhibition effects (Jayasundera et al., 2021; Uysal et al., 2018). That would explain the inhibition exhibited though miniscule from all solvent extracts. The inhibition levels could have been below 20% because the flavonoid content values obtained in this study were lower than a good number of other published results.

In a study by Wang *et al.* (2019) using *B. pilosa* ethanol extracts, they speculated that the neuroprotective effects, improving learning and memory in aged rats could have been a consequence of AChE inhibition thanks to the high flavonoid content, among other possible mechanisms of action brought about by the plant extracts ingested by the test subjects. Based on this thesis' results, the hope would be that if researched further, *B. pilosa* flavonoids would result in clinically effective AChE inhibition.

As the literature review explains, inhibition of AChE would mean increased ACh in the CNS, promoting neuronal function. Uysal *et al.* (2018) reported the AChE inhibition potential of *Bidens tripartite* ethyl acetate, methanol, and water extracts as galantamine equivalents. Interestingly, the highest enzyme inhibitory effect they observed was ethyl acetate, followed by the water extract and the methanol extract (Uysal et al., 2018). As in this study, though investigating a different plant species, the results showed better inhibition from the water extract compared to the methanol extract. Other than the study by Uysal *et al.* (2018), there is no literature on studies involving the *Bidens* species, particularly *B. pilosa,* in AChE inhibition.

The acetone extract had the lowest IC_{50} value (0.737 µg/mL) compared to the other plant extracts, as shown in Table 4.5. However, it was 1.7 times greater than the donepezil standard. This meant that compared to the other plant extracts, the acetone extract exhibited the best AChE inhibitory efficiency. Intriguingly, the acetone extract exhibited the least relative inhibition (12.42 ± 3.18%) compared to the other extract samples at 5 µg/mL. The acetone extract's high inhibition efficacy would have been in line with the above-

obtained results, as it had the highest TPC and TFC values, largely associated with AChE inhibition, implying that *B. pilosa* extracts may reduce the cholinergic deficit in AD.

5.4.2 BACE 1 Inhibition

BACE 1 has been shown to have increased concentrations in AD patients, hence its relevance as an AD target (Valiukas et al., 2022). A dose-dependent relationship was depicted in the BACE 1 inhibition assay, as shown in Figures 4.9.1 and 4.9.2. The highest inhibition observed was from the 500 µg/mL DCM (83.40 ± 1.84%) and acetone (80.33 ± 2.32%) extracts. The remaining plant extract samples exhibited an inhibition percentage of less than 50%. DCM's IC_{50} value was also the lowest compared to the other plant extract, which agreed with the inhibition results, unlike in the AChE inhibition assay with the water and acetone extracts. However, the lowest concentrations (5 µg/mL) of the DCM and methanol extracts exhibited negative inhibition, which could indicate activation. Like AChE, the activation could mean that at that concentration, the plant extracts were unable to inhibit BACE 1, with the possibility that samples taken from these extracts could potentiate neurodegeneration at low concentrations by promoting uncontrolled APP processing.

It is important to note that in this experiment, the concept tested was a comparison between normal functioning [Enzyme + Substrate ⇌ Product(s)], serving as the positive control, whereas the tested variable [Enzyme + Substrate + Inhibitor ⇌ Product(s)] was where there were plant extracts. This form of experimentation allowed for the investigation of inhibition potential only and not the degree of inhibition which would have been obtained by including a known protein inhibitor in the positive control wells. Since this dissertation served as foundational work in relation to *B. pilosa* and AD, further studies will investigate the degree of inhibition.

As aforementioned, this is the first study to report on BACE 1 inhibition by *B. pilosa* extracts so there is no literature to compare the obtained data with. However, based on the existing BACE 1 literature, the ideal BACE 1 inhibitor

must be a small, highly lipophilic molecule to penetrate through the blood-brain barrier (BBB) to enact the inhibitory effect (Jung et al., 2010). The proteolytic pocket of BACE 1 is less hydrophobic and relatively large (Das & Yan, 2019), which could explain the high activity of inhibition by the acetone and DCM fractions compared to water, methanol, and hexane. However, it is paramount to note that this depends on the plant extract's inhibition mechanism, which is yet to be investigated.

Additionally, BACE 1 active site is made of aspartate residues, so it is acidic (Ahmed et al., 2010; Moussa-Pacha et al., 2020). This means a basic inhibitor will have better binding affinity to the active site, or the performance of the inhibition assay in an acidic environment will yield better results once the mode of inhibition by the extracts is determined.

It is without question that BACE 1 is a good pharmacological target in the treatment or management of AD. However, inhibition strength and selectivity are among the chief problems surrounding the existing synthetic BACE 1 inhibitors (Hampel et al., 2021). Due to the plant material being organic, perhaps when researched in depth, the selectivity and inhibition strength would be favourable for normal physiological function while curbing toxic Aβ synthesis, as can already be evidenced by the plant's inhibitory activity from the results.

BACE 1 inhibition's limitation is the inability to clear out existing Aβ aggregates (Olasehinde et al., 2019), hence the importance of a clearing mechanism to improve cognitive function and halt neurodegeneration.

5.4.3 Aβ Aggregation Inhibition

Amyloid fibrils form from the α-helical protein structure misfolding to β-sheets. The β-sheets stack and have outsticking side chains. ThT dye from the Aβ aggregation inhibition test reacts with these side chains and fluoresces (Kumar & Walter, 2011; Sulatskaya et al., 2017; Xue et al., 2017). This would mean that the higher the fluorescence observed, the higher the aggregation and, consequently, the lower the inhibition potential of the plant extracts put into the reaction mixture. Rifampicin is a natural compound typically used as

an antibiotic, but it also has Aβ aggregation and neurotoxicity inhibition capabilities *in vitro* (Umeda et al., 2016). Rifampicin is not used in AD treatment because of its adverse effects, e.g., hepatic induction and hepatotoxicity, and lack of AD clinical efficacy in late-stage disease (Tomiyama et al., 1996; Umeda et al., 2016). However, in this investigation, it was the preferred standard to polyphenol resveratrol because of its broad spectrum and the ability to reduce both Aβ and tau hyperphosphorylation (Hudson et al., 2009; Umeda et al., 2016; Vikal et al., 2024).

ThT fluorescence sampling was performed at t=24 hr and t=120 hrs to give the $A\beta_{1-42}$ protein time to aggregate without an inhibitor. Figures 4.10.1, 4.10.2, and 4.10.3 show that the fluorescence intensity decreased over time for most extract samples, including the standard drug rifampicin, except for the hexane 5 µg/mL extract. The relative inhibition percentage increased with the increase in the sample concentrations seen in Figure 4.10.4. At t=24 hr, there was negative inhibition seen in the hexane (5 and 500 µg/mL), methanol (5 and 500 µg/mL) and water (500 µg/mL) extracts. While at t=120 hr, the negative inhibition was only seen in the hexane 5 µg/mL extract.

The expected inhibition percentage trend was a dose-dependent relationship, as shown by the rifampicin positive standard also seen in Umeda *et al.*'s (2016) study. This trend was more prominent at t=120 hr compared to t=24 hr, and this could have been because, in the latter time, the reaction system was still maturing. Hence, the results obtained at t=120 hr are what will be discussed further.

The DCM and acetone extracts showed a positive dose-dependent relationship, while the methanol was negative. The methanol extract results suggested that the lower the extracted sample, the higher the inhibition potential, which is an appreciable quality in pharmacology as that usually signals lower toxicity, but more studies would be required to support this possible conclusion.

The hexane and water extracts were erratic, with no discernible trend. However, the water 50 µg/mL extract gave the highest sample extract inhibition percentage of 42.80 ± 10.67%. The rest of the extract samples were below 40%, half the inhibition shown by the positive standard, as shown in

Figure 4.10.4. It is also interesting to note that the water extract was the least expected extract to give noteworthy inhibition results compared to the other extracts since the phytochemical profile and antioxidant results obtained earlier for the extract were not impressive.

Contrary to the relative inhibition results, the hexane extract had an IC_{50} value 29% less than rifampicin's (Table 4.7), suggesting that it had better efficacy than the other extracts. On the other hand, the water extract's IC_{50} was three times that of rifampicin, suggesting poor inhibition efficacy despite exhibiting the highest relative inhibition.

No literature has been published on the effects of *B. pilosa* extracts on Aβ aggregation inhibition. However, in another study on the aerial parts of *Agrimonia pilosa*, they found that the ethyl acetate pure compound subfractions had notable aggregation inhibition potential at 100 µg/mL concentrations. The most effective compound exhibited 43% inhibition after a 24-hour pre-aggregation period compared to the Aβ-only group in the study (Lee et al., 2024a). Lee *et al.*'s (2024a) results exhibited a dose-dependent relationship for all compounds, which further solidifies that concentration plays a vital role in the inhibition potential of an extract.

Since ThT fluorescence intensity linearly correlates with amyloid fibril concentration (Xue et al., 2017), it is safe to say that according to the obtained results, amyloid fibril concentration decreased with an increase in sample concentration for the acetone and DCM plant extracts, while it increased in the presence of the methanol extract. This allows speculation that in *B. pilosa*, polar phytochemicals from the methanol extract promote aggregation, while the less polar ones from the DCM and acetone extracts promote disaggregation.

The reduction in fluorescence intensity after 120 hr suggests that the extract samples were able to disaggregate the matured $A\beta_{1-42}$. Since Aβ aggregation is associated with ROS formation, NFT formation, and cerebral toxicity (Lee et al., 2024a), a multi-target treatment option that addresses these would eventually bring therapeutic relief to patients with AD.

CHAPTER 6: SUMMARY AND CONCLUSION

AD is a non-communicable neurological brain depreciation disorder whose pathology is multifaceted and currently has no cure. With technological advancement because of urbanisation and an increase in unhealthy lifestyle practices, it is an emerging global health challenge. The existing treatment protocols are expensive, rivalling cancer and cardiovascular treatment, and single-targeted, with unruly side effects. The single-targeted management options offer palliative care that promotes polypharmacy, which is tedious to both the patient and caregiver. Despite the limited understanding of the disease causation, there is a need for new therapies that are multi-targeting with friendlier side effect profiles.

Traditional medicine is a wealthy source of biodegradable, sustainable drug leads that have the potential to be multi-targeting with fewer side effects. This thesis researched the potential of *B. pilosa*, an Asteraceae plant's potential to inhibit BACE 1 and AChE *in vitro*. AChE and BACE 1 are key players in the cholinesterase and Aβ hypothesis in the causation of AD.

In the preliminary phytochemical screening, the *BP* plant showed the presence of alkaloids, flavonoids, saponins, quinones, anthraquinones, steroids, terpenoids, tannins, phenols and cardiac glycosides. The acetone extract had the most impressive presence of a good number of phytochemicals, followed by the methanol and DCM extracts. The DCM, acetone, and methanol extracts showed the most fractions in the TLC profiles

Quantitatively, the acetone extract had the highest TPC and TFC value, which was expected due to the impressive qualitative results. Also, the acetone extract could have exhibited higher yields than the other extraction samples due to its intermediate polarity, allowing it to draw notable quantities of lipophilic and hydrophilic phytochemicals during plant extraction.

All the plant extracts exhibited antioxidant ability, but the acetone extract had the highest DPPH radical scavenging ability and FRAP value compared to the other extraction samples. This was expected since the same solvent exhibited the best results during the phytochemical screening. A dose-dependent

relationship was seen in the FRAP but not in all the samples in the DPPH; further testing would have to be done to determine the reason for the lack of trend.

In the anti-AD screening, *B. pilosa* plant extracts exhibited AChE, BACE 1, and Aβ aggregation inhibition potential. The acetone extract had the most efficient AChE IC_{50} compared to the other extract samples, 1.4 times that of the donepezil standard. The DCM extract had the highest BACE 1 inhibition percentage compared to the other extract samples. Interestingly, the water extract exhibited the greatest inhibition percentage compared to the other extract samples in the AChE inhibition and Aβ aggregation inhibition assay. Negative inhibition exists in at least one sample concentration in all the anti-AD screening assays. This speaks to the importance of the correct dose in pharmacological activity, as too little or too much can cause a subtherapeutic effect or toxicity.

Based on the Western medicine model, identifying a particular phytoconstituent in plants is the preferred method for drug-lead identification. However, it is important to note that these phytoconstituents exist in tandem with others inside the plant, bringing about the actions scientists wish to replicate in humans and animals, pharmacologically like fighting disease and protecting from stress and UV rays. It could mean that perhaps consideration should be put into researching combined plant use as most of the evidence that gives a basis for plant research is indigenous knowledge. In traditional medicine practice, they do not strip the plant to try and find the chemical in charge of the healing property but use it in its entirety.

The inhibition of AChE, BACE 1, and Aβ aggregation, alongside antioxidant activity, shows that the *B. pilosa* plant is a promising source for AD drug treatment. A plant with multifaceted benefits might be best for a multifaceted disease.

At the time of the study, there were no reports on the potential of *BP* in AChE and BACE 1 inhibition. This investigation details the first report with this focus. More research needs to be done to determine whether *BP* can positively affect other AD pathologies.

Limitations

- Budget constraints made optimizing investigative methods difficult due to resource limitations. It would be ideal to conduct multiple assay runs to further validate the results. Also, for more detailed information, modern environmentally friendly extraction techniques like enzyme-assisted extraction, microwave-assisted extraction, ultrasound-assisted extraction, and supercritical fluid should be employed instead of the archaic ones used.
- Plant phytochemical content depends on multiple factors, such as harvesting time and geographical location. The plant material used in this study was only from one location. Future studies could use material from different locations after assessing the best harvest time, guided by ethnobotanical use.

Recommendations

- Although *B. pilosa* has been proven to have negligible toxicity in humans, toxicity studies focusing on neuronal cells would be necessary if the material is to be developed as a drug lead in AD treatment.
- Further studies investigating the extent of inhibition from crude extracts and isolated compounds are required to clearly distinguish which will give the best results. The study results suggest that the most active compounds were from the acetone extract.
- Since the *B. pilosa* potential of enzyme inhibition has been confirmed, enzyme inhibition kinetics are required to establish *B. pilosa*'s inhibition mechanism of action.
- Since *in vitro* results are not an absolute reflection of what would happen to the human body, in vivo studies and potential variability in the plant extract composition should be done in the future for a more detailed picture of *B. pilosa's* pharmacological effects on AD.

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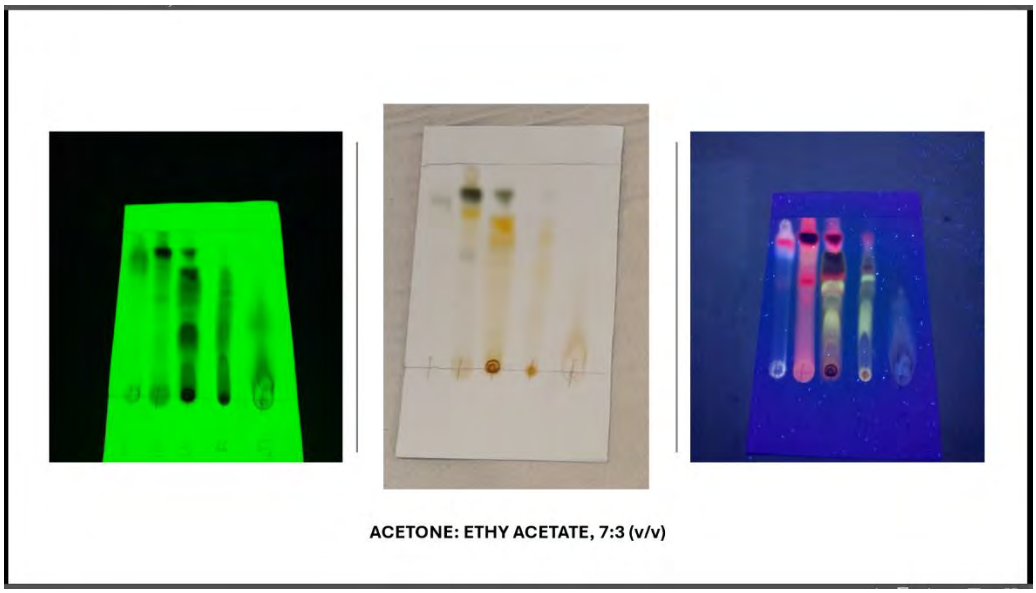
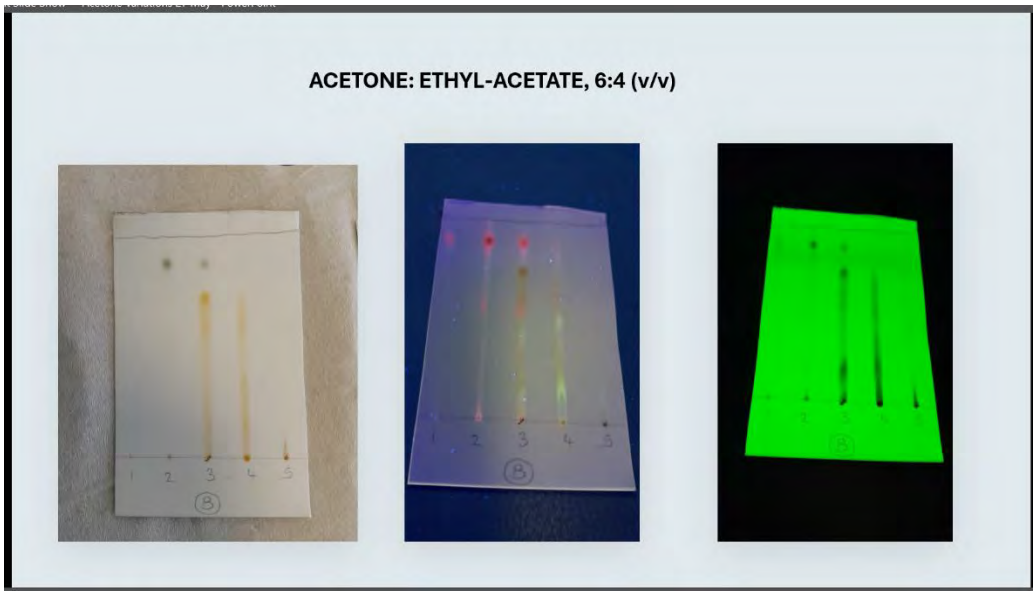
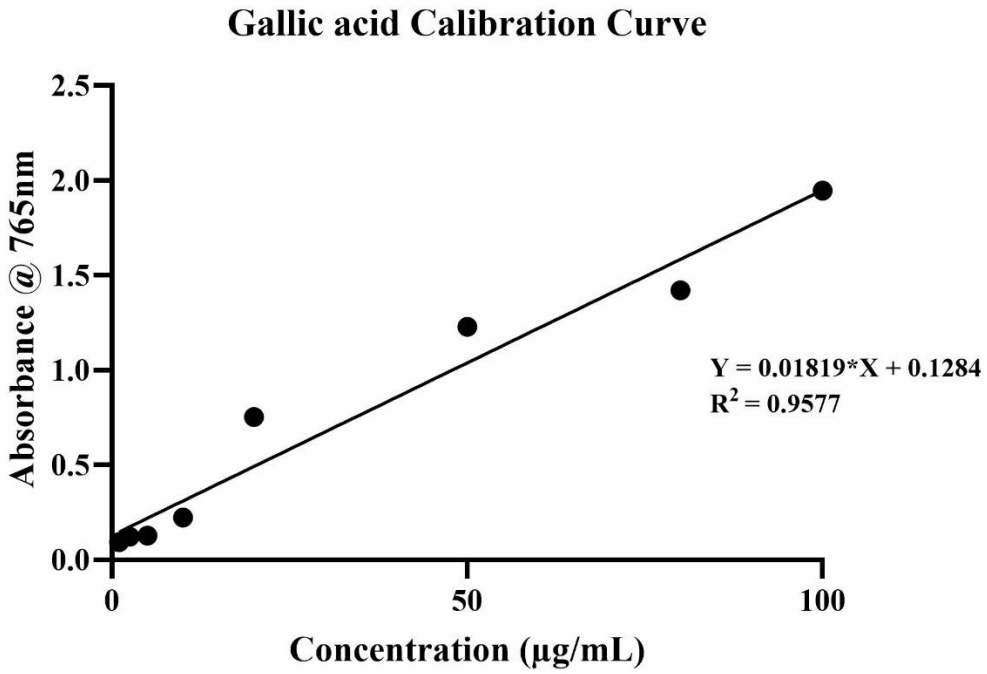
APPENDICES

APPENDIX A: TLC plates from the acetone: ethyl acetate system. These plates are examples of the earlier chromatograms obtained in the investigation depicting separation; however, with bunching up or less clear fractionation. This resulted in the change in system composition to get Figure 4.3 in the results section.

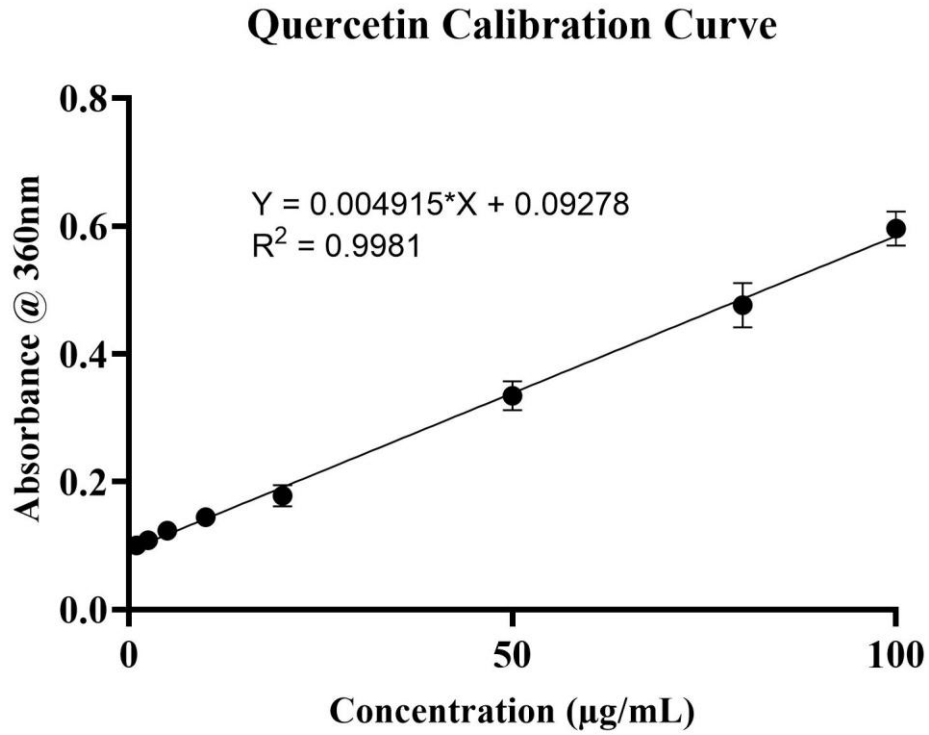




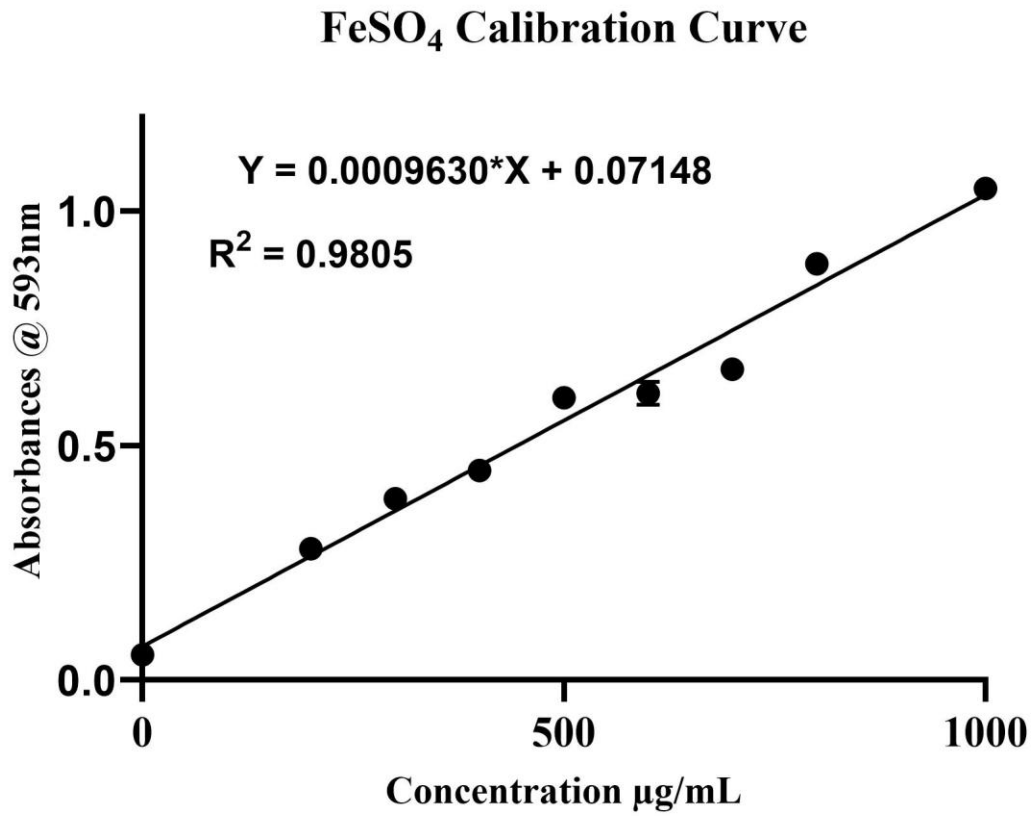
APPENDIX B: Gallic Acid Standard Curve used to determine the total polyphenol content of the extract samples.



APPENDIX C: Quercetin Standard Curve used to determine the total flavanol content of the extract samples.



APPENDIX D: Iron (II) Standard Curve used to determine the ferric-reducing antioxidant power value of the extract samples.



APPENDIX E: Academic Conferences

CERTIFICATE
OF PRESENTATION

This is to proudly acknowledge and honour

Leseli Chipomho

for presenting at the 6th Annual

Rhodes University Postgraduate Conference

Building Bridges: Unleashing the Potential of Scholarship

13-14 September 2024

Professor Sybert Mutereko
Director: Centre for Postgraduate Studies

Dr Nomakwezi Mzilikazi
DVC: Research, Innovation & Strategic Partnerships

- **South African Society for Basic and Clinical Pharmacology (SASBCP), Grahamstown/ Makhanda, 2023 (Attendee).**
- **Rhodes University Faculty of Pharmacy Postgraduate Research Day, Grahamstown/ Makhanda, 2023 (Presenter).**
- **International Interdisciplinary Conference: Indigenous Knowledge Systems, Gqeberha/ Port Elizabeth, 2024 (Presenter).**
- **Rhodes University Center for Postgraduate Studies Conference (CPGS), Grahamstown/ Makhanda, 2024 (Presenter) ~ certificate image shown above.**
- **South African Society for Basic and Clinical Pharmacology (SASBCP), Pretoria, 2024 (Presenter).**
- **Rhodes University Faculty of Pharmacy Research Symposium, Grahamstown/ Makhanda, 2024 (Presenter).**
- **The Border and Eastern Districts (BED) Branch of the Pharmaceutical Society of South Africa (PSSA) – Annual Research Day, 2024 (Presenter).**