Characterization of an acetylcholinesterase inhibitor isolated from *Ammocharis coranica* (Amaryllidaceae)

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DECLARATION

I declare that the thesis hereby submitted to the University of Pretoria for the degree of Masters in Veterinary Science has not been previously submitted by me for a degree at this or any other University that it is my own work in design and execution, and all materials contained herein has been duly acknowledged.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEIs	Acetylcholinesterase inhibitors
ATCI	Acetylthiocholine iodide
AD	Alzheimer's disease
Ac	*Ammocharis coranica*
Aβ	Amyloid beta
APP	Amyloid precursor protein
ANS	Autonomic nervous system
BSA	Bovine serum albumin
BuOH	Butanol
BuChE	Butyrylcholinesterase
CNS	Central nervous system
ChEI	Cholinesterase inhibitors
DCM	Dichloromethane
DMSO	Dimethylsulphoxide
DRG	Dragendorff's reagent
DTNB	Ellman's dye or 5, 5´-dithiobis-2-nitro benzoic acid
EtOAc	Ethyl acetate
IC_{50}	Concentration that inhibits 50% of the enzyme activity
MeOH	Methanol
µg	Micro gram
µl	Micro litre
µM	Micro molar
MPA	Microtitre plate assay
mg	Milli gram
ml	Milli litre
NS	Nervous system
NP	Neural plaque

NFT	Neurofibrillary tangle
NMR	Nuclear magnetic resonance
R_f	Retardation factor
SNS	Somatic nervous system
TLC	Thin layer chromatography
TLCBA	Thin layer chromatography bioautographic assay
Tc	*Torpedo californica*
TcAChE	*Torpedo californica* acetylcholinesterase
UV	Ultra violet
US$	United States Dollar

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, with a mean duration of about 8.5 years between manifestation of clinical symptoms and death. The disorder is characterised by abnormal pathologic features, such as extracellular deposits of β-amyloid derived from amyloid precursor protein (APP) in senile plaques, intracellular formation of neurofibrillary tangles containing hyperphosphorylated form of a microtubule associated protein, tau, and the loss of neuronal synapses and pyramidal neurons, as well as a decrease in levels of the neurotransmitter acetylcholine (ACh) by nearly 90%. AD is the leading cause of dementia in elderly people; with the proportion of elderly people in the population increasing steadily, the burden of the disease, both to caregivers and national economies, is expected to become substantially greater over the next 2 to 3 decades. There are currently, 20 million people with the disorder world-wide. The "cholinergic hypothesis of AD", which states that cognitive impairment in AD are mainly due to decline in the level of ACh forms the neurobiological incentive for treatment aiming at the improvement of cholinergic function in AD.

Inhibition of acetylcholinesterase (AChE), the enzyme involved in the metabolic hydrolysis of acetylcholine (ACh) at the cholinergic synapses in central and peripheral nervous systems, promotes increase in the concentration, and duration of action of synaptic ACh, and serves as a strategy for the treatment of AD and discovery of active compounds. Drugs approved for the treatment of AD are mostly compounds with AChE inhibitory activity; these drugs have limitations to their therapeutic success, due to non selectivity, low efficacy, poor bioavailability, and adverse cholinergic side effects in the periphery, which include nausea, vomiting, diarrhoea, dizziness, and hepatotoxicity. This motivates the evaluation of more plants for AChE inhibitory compounds.

Bulbs of *Ammocharis coranica* were selected for AChE inhibitory assay because the plant is used in traditional medicine to treat people with mental illnesses. It also belongs to the Amaryllidaceae family known to contain compounds with AChE inhibitory activity. A 96% ethanol crude extract of the bulbs of *A. coranica* had good AChE inhibitory activity with an IC_{50} of 14.3 ± 0.50 µg/ml in a quantitative assay. In a qualitative bioautographic assay of the extract based on Ellman's method several white areas of inhibition against a yellow background were observed. No false positives were found. The minimum

concentration of the ethanolic crude of the plant extract visualised as a white area of inhibition of AChE enzyme on the TLC plates was 15 µg.

The crude extract was separated into several fraction of different polarity by solvent-solvent fractionation. The IC_{50} of the inhibition of the ethanolic crude extract of the bulbs of *A. coranica* was 14.3 ± 0.50 µg/ml. The intermediate polarity fractions (butanol and ethyl acetate) had IC_{50} of 0.05 ± 0.02 and 43.1 ± 1.22 µg/ml, respectively. Compound 1 was isolated from butanol fraction and compounds 1 and 2 were isolated from the ethyl acetate fraction using bioautographic assay guided fractionation. The structures of these compounds were elucidated using nuclear magnetic resonance (NMR) spectroscopy, and identified as lycorine (1) and 24-methylenecycloartan-3β-ol (2) respectively. Lycorine (1) had AChE inhibitory activity, with an IC_{50} of 102 ± 7.75 µM. This is equivalent to an IC_{50} of 29.4 µg/ml indicating that the butanol fraction was nearly 600 times more active. It is possible that the butanol fraction may have therapeutic uses if it is not toxic and the results imply that substantial synergism may have existed between different compounds in the butanol fraction. The 24-methylenecycloartan-3β-ol (2) was not active when tested using the microtitre plate assay although it did inhibit colour development in the bioautography assay.

CONFERENCES AND PROCEEDINGS 2009

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CHAPTER 1

GENERAL INTRODUCTION

Dementia is a clinical syndrome characterised by difficulties in speech, behavioural changes and impairment of activities of daily living (Burns and Iliffe, 2009). It is caused by damage to the neural network responsible for cognitive functions. Example of disorders with dementia includes: Lewy body disease, Pick's disease, cerebrovascular disease and Alzheimer's disease (AD), the latter being the most common form of cognitive and intellectual deterioration (Howes *et al*., 2003).

Alzheimer's disease is a progressive neurodegenerative disorder with mean duration of 8.5 years between manifestation of clinical symptoms and death (Francis *et al*., 1999). It is the leading cause of dementia in elderly people; with the proportion of elderly people in the population increasing steadily, the burden of the disease, both to caregivers and national economies, is expected to become substantially greater over the next 2 to 3 decades (Francis *et al*., 1999; Maslow, 2008). The current estimated financial burden of AD in the United States alone is US$148 billion (Maslow, 2008).

The brain regions associated with higher mental functions, such as the neocortex and hippocampus, are most affected by the characteristic pathology of AD. These abnormal pathologic features include, extracellular deposits of β-amyloid derived from amyloid precursor protein (APP) in senile plaques, intracellular formation of neurofibrillary tangles containing hyperphosphorylated form of a microtubule associated protein, tau, and the loss of neuronal synapses and pyramidal neurons, as well as a decrease in levels of the neurotransmitter acetylcholine (ACh) by nearly 90% (Cummings, 2004). These changes result in the development of the typical symptoms of Alzheimer's disease characterised by gross and progressive damage of cognitive function, often accompanied by behavioural disturbances such as memory loss, aggression, depression, and wandering. Caregivers find these features the most difficult to cope with and they often lead to the institutionalisation of the patient (Esiri, 1996).

The "cholinergic hypothesis of AD", formulated for the first time by Whitehouse *et al*. (1982), was postulated on the basis of the negative cognitive effect of cholinergic antagonists and the positive effect of muscarinic agonists on memory in humans (Guillou *et al*., 2000), and became the neurobiological incentive for a treatment aiming at the improvement of cholinergic function in AD.

Acetylcholinesterase (AChE) is the enzyme involved in the metabolic hydrolysis of ACh at cholinergic synapses in the central and peripheral nervous systems. Inhibitors of AChE activity (AChEIs) promote an increase in the concentration and duration of action of synaptic ACh (Rollinger *et al*., 2004), this

serves as a strategy for the treatment of Alzheimer's disease (AD) as well as senile dementia, ataxia, myasthenia gravis and Parkinson's disease (Houghton *et al*., 2006). Acetylcholinesterase enzyme (AChE) inhibitors from general chemical classes such as galanthamine (Razadyne®), an Amaryllidaceae alkaloid isolated from the plant *Galanthus woronowii*, the Caucasian snowdrop (Heinrich and Teoh, 2004), rivastigmine (Exelon®), a carbamate, tacrine (Cognex®) an acridine-based rapidly reversible cholinesterase inhibitor, and donepezil (Aricept®), a piperidine cholinesterase inhibitor have been tested for the symptomatic treatment of AD (Cummings, 2004). Clinical studies led to symptomatic improvements in some patients, resulting in the approval of these compounds for the treatment of AD (Nordberg and Svensson, 1998; Cummings, 2004). However, the non-selectivity of these drugs, their limited efficacy, and poor bioavailability, adverse cholinergic side effects in the periphery, such as nausea, vomiting, diarrhoea, dizziness, and hepatotoxicity are among the severe limitations to their therapeutic success (Burns and Iliffe, 2009). Beside the target organ brain, the heart is also rich in cholinesterases, and their inhibition may adversely affect cardiac function (Masuda, 2004), hence the continuous search for better AChE inhibitors from natural sources with fewer side-effects (Hostettmann *et al*., 2006; Houghton *et al*., 2006).

Millions of years of plant evolution led to the development of many plant metabolites with various and unique chemical structures. So far only 20% of all plant species have been evaluated chemically or biologically (Cordell and Colvard, 2005). Therefore, medicinal plants remain an important source for the search of new leads, chemical entities of drugs and new mechanism of drug action as well (Cordell and Colvard, 2005). There are about 250,000 species of higher plants on earth, which contain a much greater diversity of bioactive compounds than any chemical library (Raskin *et al*., 2002). South Africa, a country with a strong history of traditional healing, host a variety of around 24,000 of these plant species (McGaw and Eloff, 2008), which gives a wide option in the search for plant with medicinal values to man and animals. Indigenous bulbous plants of importance to South African traditional healers mainly belong to the Amaryllidaceae and Hyacinthaceae families (Stafford *et al*., 2008).

Cognitive disorders observe in AD and other memory-related illnesses are treated using plant products (Howes *et al*., 2003); long-established customs and beliefs reveal, very few plants in South African traditional medicine are used to improve memory and treat AD (Risa *et al*, 2004). Successful treatments of dementia using herbal preparations have been corroborated (Adams *et al*., 2007). Plants used by traditional healers to treat mental illnesses, function effectively because of the active secondary metabolites they contain instead of their actions as placebo or mystical symbols (Fennell *et al*., 2004).

Plants from the Amaryllidaceae family are a rich source of alkaloids (Lopez *et al*., 2002; Houghton *et al*., 2006). Hence, the continuous search from members of this family for alkaloids; in which the bulb of *A. coranica* was investigated for compounds with AChE inhibitory activities.

1.1. PROBLEM/ HYPOTHESIS

The bulbs of *A. coranica* contain active compounds that inhibit acetylcholinesterase and have the potential to be used as a novel template for the development of effective drugs for the treatment of Alzheimer's disease (AD).

1.2. AIMS AND OBJECTIVES

The aim of this project was to screen the bulb of *A. coranica* for AChE inhibitory activity, and to investigate the compound(s) responsible for this activity.

The objectives are:

1. To screen extracts of *A. coranica* for AChE inhibitory activity using TLC bioautographic and microplate assays.
2. To isolate and elucidate the structure of compounds.
3. To determine the biological properties of the isolated compounds.

CHAPTER 2

LITERATURE REVIEW

2.1. The Nervous System

The nervous system (NS) along with the endocrine system provides most of the control functions of the human body. The nervous system controls rapid activities, such as muscle contractions, visceral events, and the rate of secretion of some endocrine glands. The endocrine system, by contrast, regulates principally the metabolic functions. The NS is unique in the vast complexity of the control actions it can perform. It receives literally million bits of information from different sensory organs and integrates all these to determine bodily response (Thibodeau and Patton, 2003).

Anatomically, the human NS is divided into the central nervous system (CNS) and the peripheral nervous system (PNS); functionally into the somatic nervous system and the autonomic (visceral) nervous system (ANS) (Thibodeau and Patton, 2003; Noback *et al*., 2005). The CNS comprises the brain and spinal cord. Encapsulated within the skull is the brain and the spinal cord is lodged in the centre of the vertebral column. The peripheral nervous system (PNS) consists of the nerves emerging from the brain (called cranial nerves) and from the spinal cord (called spinal nerves). The peripheral nerves convey neural messages from the sense organs and sensory receptors in the organism inward to the CNS and from the CNS outward to the muscles and glands of the body (Noback *et al*., 2005). The somatic nervous system consists of neural structures of the CNS and PNS responsible for conveying and processing conscious and unconscious sensory (afferent) information, vision, pain, touch, from the head, body wall, and extremities to the CNS and motor (efferent) control of the voluntary (striated) muscles. The ANS is composed of the neural structures responsible for conveying and processing sensory input from the visceral organs (example, digestive system and cardiovascular system) and motor control of the involuntary (smooth) and cardiac musculature, and of glands of the viscera (Brown, 1991; Guyton and Hall, 1996; Thibodeau and Patton, 2003; Noback *et al*., 2005).

2.1.1. The Brain

The brain is the organ of behaviour and the centre of the NS in all vertebrates and invertebrates. Integrated into the structure and functional fabric of the brain are two broad classes of cells: neurons and glia (Kandel *et al*., 2000), they exhibit a wide variety of forms and sizes. The glia or neuroglia come in different types and do not usually conduct information themselves but perform critical functions such as structural support, metabolic support, insulation and guidance of neural development.

The human brain contains about 100 billion (10^{11}) neurons (Pelvig *et al*., 2008). These cells transmit signals to each other through 100 trillion synaptic connections (Toro *et al*., 2008). The synapse is the junction between a neuron and the next ; it is an advantageous site for control of signal transmission. Anatomically the brain is located above the spinal cord and it is divided into the brain stem (medulla oblongata, pons and midbrain), the cerebellum, the diencephalon (thalamus and hypothalamus) and the cerebral hemispheres (basal ganglia, amygdala, hippocampus and cerebral cortex). The medulla, the pons and the midbrain are a continuation of the spinal cord into the brain; hence they are called the "brain stem" (Thibodeau and Patton, 2003; Noback *et al*., 2005).

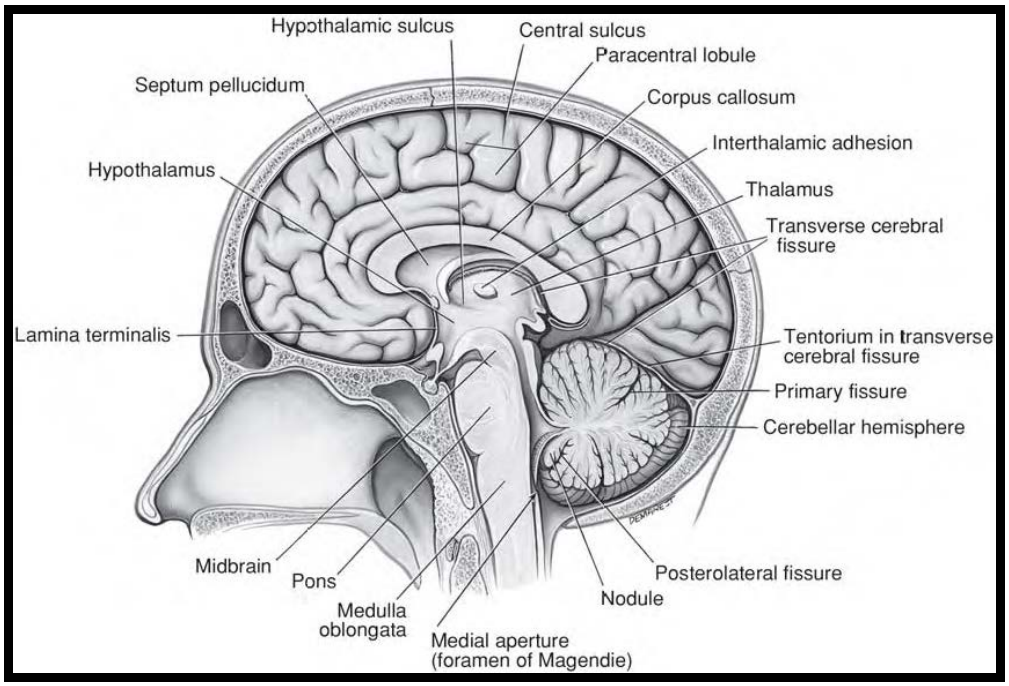

Figure 2-1: Midsagittal section of the brain (Noback *et al*., 2005)

The cerebrum accounts for 85 percent of the brain matter. It is the largest part of the human brain associated with higher function such as thought and action. The cerebral cortex is highly wrinkled; the convolutions make the brain more efficient, because of increase in the surface area and the amount of neurons present. The frontal lobe, the parietal lobe, occipital lobe, and the temporal lobe make up the four sections of the cerebrum. The frontal lobe located in the front of the brain, is associated with reasoning, planning, parts of speech, movement, emotions and problem solving abilities. Parietal lobe, which sits behind the frontal lobe, is associated with movement, orientation, recognition, and perception. The occipital lobe is at the back of the brain linked with visual processing. The temporal lobe runs along the side of the brain under the frontal and parietal lobes. It is associated with perception and recognition of auditory stimuli, memory and speech (Noback *et al*., 2005).

A deep furrow divides the cerebrum into the right and the left hemispheres. The right hemisphere is associated with creativity while the left hemisphere is associated with logic abilities. Corpus callosum, the neural bridge that connects the two hemispheres, is the main avenue of communication between the two hemispheres; it connects each point on the cortex to the mirror-image point in the opposite hemisphere, and also connects functionally related points in different cortical areas. The neocortex which occupies a large part of the cerebrum; is a six-layered structure found only in mammals associated with higher information processing. (Brown, 1991; Guyton and Hall, 1996; Thibodeau and Patton, 2003; Noback *et al*., 2005).

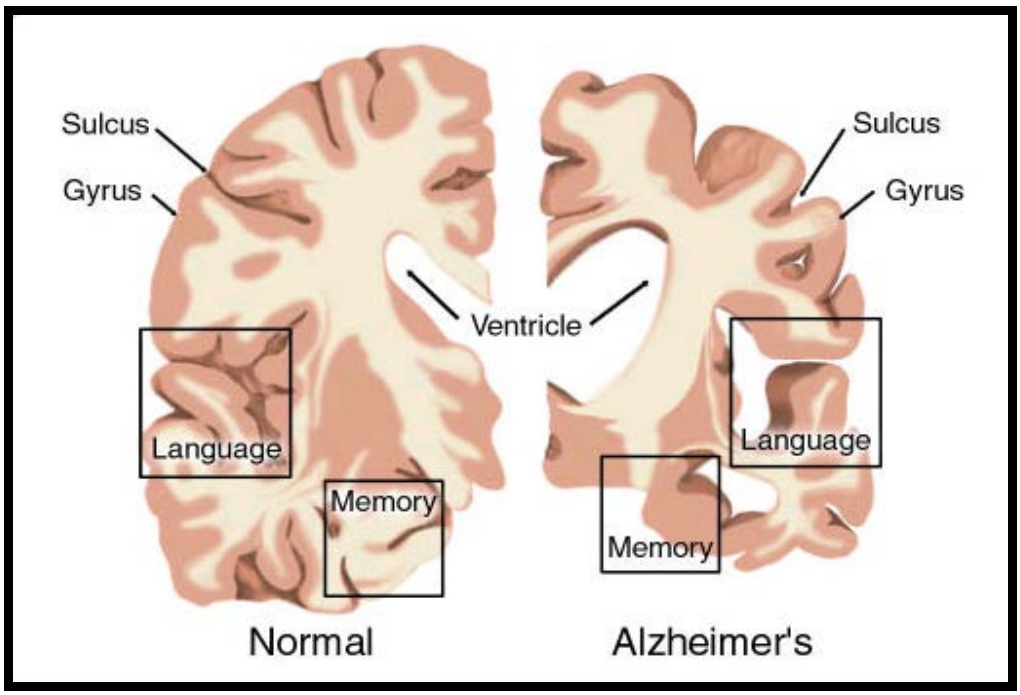

Figure 2-2: Comparison of a normal human brain and Alzheimer ravaged brain (Courtesy American Health Assistance Foundation, 2000-2009)

The cerebellum known as the little brain, forms 10 percent of the brain (Kandel *et al*., 2000). Anatomically it is located above the brain stem and underneath the occipital lobe. It is similar to the cerebrum with two hemispheres and a highly folded surface or cortex. The hemispheres receive information from the eyes, ears, muscles, and joints about body movement and position. It processes all the information and sends signal to the body through the rest of the brain and spinal cord. It is also involved in motor learning and remembering. The limbic system is where emotions reside and memory begins, referred to as the "emotional brain." It plays a role in spontaneity and creativity. Located in the limbic system are the thalamus, hypothalamus, amygdala and hippocampus (Brown, 1991; Guyton and Hall, 1996; Thibodeau and Patton, 2003; Noback *et al*., 2005).

The amygdala, an almond shaped structure helps in storing and classifying emotionally charged memories. It plays a large role in producing fear. It is located in the temporal lobe in front of the hippocampus (Brown, 1991; Guyton and Hall, 1996). The hippocampus, is located in the temporal lobe, it is responsible for learning and short–term memory formation, classifying information, long term memory. It is also involved in interpreting incoming nerve signals and spatial relationship. Hypothalamus is linked very closely to the pituitary gland; It monitors and controls the circadian rhythm (the sleep/ wake cycle), homeostasis, appetite, thirst, autonomic and motor functions (Brown, 1991; Guyton and Hall, 1996). The thalamus is located on top of the brain stem. It is the relay station of the brain. Most of the sensory signals, auditory, visual, somatosensory (skin and internal organs) go through this organ on their way to other parts of the brain for processing. Underneath the limbic system is the brain stem (Thibodeau and Patton, 2003; Noback *et al*., 2005). It is located at the base of the brain. It connects the spinal cord with the rest of the brain. Though the smallest of the three layers of the brain, its functions are crucial to survival. It is responsible for all basic vital life functions such as breathing, heart beat and blood pressure (Brown, 1991; Guyton and Hall, 1996; Thibodeau and Patton, 2003; Noback *et al*., 2005).

2.1.2. The Neuron

Neurons are excitable cells that conduct the impulses, which makes it possible for all nervous system functions (Noback *et al*., 2005). They are specialised cells that can produce different actions because of their precise connections with other neurons, sensory receptors, and muscle cells. Interactions between neurons enable people to think, move, maintain homeostasis, and experience emotional sensations (Thibodeau and Patton, 2003; Noback *et al*., 2005). A typical neuron is composed of four structurally defined regions: a cell body (soma) that emits a single nerve process called an axon, which ends at presynaptic terminals, and a variable number of branching processes called dendrites (Brown, 1991; Guyton and Hall, 1996; Thibodeau and Patton, 2003; Noback *et al*., 2005). Each axon, including its collateral branches, usually terminates as an arbour of fine fibres; each fibre ends as an enlargement called a bouton, which is part of a synaptic junction. At the other end of the neuron, there is a three-dimensional dendritic field, formed by the branching of the dendrites. The cell body (soma) is the metabolic centre .It contains a nucleus which regulates protein synthesis and other genomic functions (Brown, 1991; Guyton and Hall, 1996). Dendrites are the main recipients of neural signals for communication between neurons and contain critical processing complexes. The axon is the conduit for conducting messages (action potentials) to the presynaptic terminals where each neuron is in synaptic contact with other neurons and, thus, is part of the network that constitutes the nervous system.

(Noback *et al*., 2005). Neurons are so specialised that most are incapable of reproducing themselves and they lose viability if denied oxygen supply for more than a few minutes (Brown, 1991; Guyton and Hall, 1996; Thibodeau and Patton, 2003; Noback *et al*., 2005).

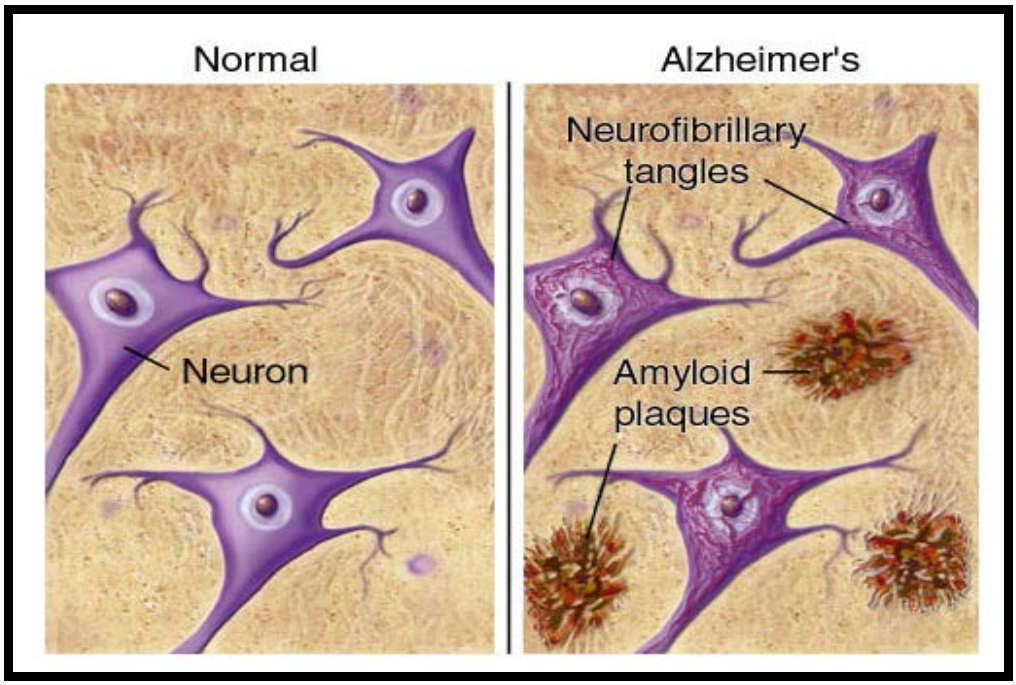

Figure 2-3: A comparison of a normal neuron and Alzheimer ravaged neuron (Courtesy American Health Assistance Foundation, 2000-2009).

2.1.3. Neurotransmitters

Neurotransmitters are molecules released from a neuron to relay information to another cell (Thibodeau and Patton, 2003). They are stored in membranous sacs called vesicles in the axon terminal. Each vesicle contains thousands of molecules of a neurotransmitter (Thibodeau and Patton, 2003; Noback *et al*., 2005). Neurons release their neurotransmitter into the synaptic space via exocytosis, as the vesicles fuse with the neuronal membrane. The neurotransmitter molecules released, diffuse across the synaptic space to the postsynaptic neuron. A neurotransmitter molecule then binds to a special receptor on the membrane of the postsynaptic neuron (Thibodeau and Patton, 2003; Noback *et al*., 2005). Receptors are membrane proteins able to bind to a specific chemical substance, such as a neurotransmitter. For example, dopamine receptor binds the neurotransmitter dopamine, but does not bind other neurotransmitters such as serotonin. The interaction between a receptor and neurotransmitter is like a lock-and-key for regulating neuronal function. Just as a key fits only a specific lock, a neurotransmitter binds only to a specific receptor (Brown, 1991; Guyton and Hall, 1996). The

chemical binding of neurotransmitter and receptor initiates changes in the postsynaptic neuron that may generate an action potential in the postsynaptic neuron. After a neurotransmitter molecule binds to its receptor on the postsynaptic neuron, it comes off the receptor and diffuses back into the synaptic space. The released neurotransmitter, as well as any neurotransmitter that did not bind to a receptor, is either degraded by enzymes in the synaptic cleft or it may be taken back up into the presynaptic axon terminal by active transport through a transporter or reuptake pump (Brown, 1991; Guyton and Hall, 1996), the neurotransmitter is either destroyed or repackaged into new vesicles released the next time the neuron is stimulated. Neurotransmitters have different functions in the brain, some are excitatory while others are inhibitory in action; the excitatory neurotransmitters act by stimulating the postsynaptic neuron, the inhibitory neurotransmitters block action potential generation in the responding cell (Brown, 1991; Guyton and Hall, 1996; Thibodeau and Patton, 2003; Noback *et al*., 2005). Table 2-1 lists major neurotransmitters used in the body and their functions. Each neuron generally synthesizes and releases a single type of neurotransmitter. The postsynaptic neuron often receives both excitatory and inhibitory messages. The response of the postsynaptic cell depends on which message is stronger (Brown, 1991; Guyton and Hall, 1996; Thibodeau and Patton, 2003; Noback *et al*., 2005).

2.2. Acetylcholine (ACh)

Choline acetyltransferase catalyses the synthesis of acetylcholine from choline and acetyl-CoA, in cholinergic neurons (Houghton *et al*., 2006). It acts by transmitting electrical impulses from one neuron to another and to muscle cells (Sussman *et al*., 1991). The two types of receptors sensitive to ACh are muscarinic and nicotinic. Muscarinic receptors are chiefly associated with the peripheral nervous system and with smooth and cardiac muscles (Tallarida and Jacob, 1979). Binding of ACh to the muscarinic receptors results in the stimulation of the parasympathetic nervous system. Classical symptoms of parasympathetic stimulation are decreased heart rate and blood pressure, constriction of bronchi, increased salivation (ptyalism), promotion of digestion and increase in intestinal peristalsis, release of fluids from the bladder and accommodation of the eyes for near vision, with contraction of the pupils. (Quinn, 1987). The nicotinic receptors are present in the central nervous system (CNS) and in the motor end plates (Brown, 1991; Guyton and Hall, 1996; Thibodeau and Patton, 2003; Noback *et al*., 2005). In the CNS, stimulation of the nicotinic receptors is associated with cognitive processes and memory, whilst in skeletal muscles it causes contraction. ACh is stored in the nerve terminals in structures called vesicles. Nerve terminal depolarization results in the release of vesicular contents; ACh released enters the synapse and binds to the receptor (Sussman *et al*., 1991).

Table 2-1. List of some neurotransmitters and their action

Neurotransmitter	Role in the body
Acetylcholine	A neurotransmitter used by spinal cord neurons to control muscles and by many neurons in the brain to regulate memory in most instances, acetylcholine is excitatory.
Dopamine	The neurotransmitter that produces feelings of pleasure when released by the brain reward system. Dopamine has multiple functions depending on where in the brain it acts. It is usually inhibitory.
GABA (gamma-amino butyric acid)	The major inhibitory neurotransmitter in the body
Glutamate	The most common excitatory neurotransmitter in the brain.
Glycine	A neurotransmitter used mainly by neurons in the spinal cord. It probably always acts as an inhibitory neurotransmitter.
Norepinephrine	Norepinephrine acts as a neurotransmitter and a hormone. In the peripheral nervous system, it is part of the fight–or–flight response. In the brain, it acts as a neurotransmitter regulating normal brain processes. Norepinephrine is usually excitatory, but inhibitory in a few brain areas.
Serotonin	A neurotransmitter involved in many functions including mood, appetite, and sensory perception in the spinal cord. Serotonin is inhibitory in pain pathway.

The ACh which is released has a very short half-life due to the presence of large amounts of acetylcholinesterase (AChE), an enzyme that hydrolyses the ester bond in the molecule, thus leading to loss of stimulatory activity (Quinn, 1987). AChE is a highly efficient catalyst with a turnover number in the order of 16,000-25,000 s^{-1} (Houghton *et al*., 2006), a rate approaching the limits imposed by the diffusion of substrate and an enhancement by a factor of 2 x 10^{13} over the hydrolysis rate in aqueous solution (Houghton *et al*., 2006).

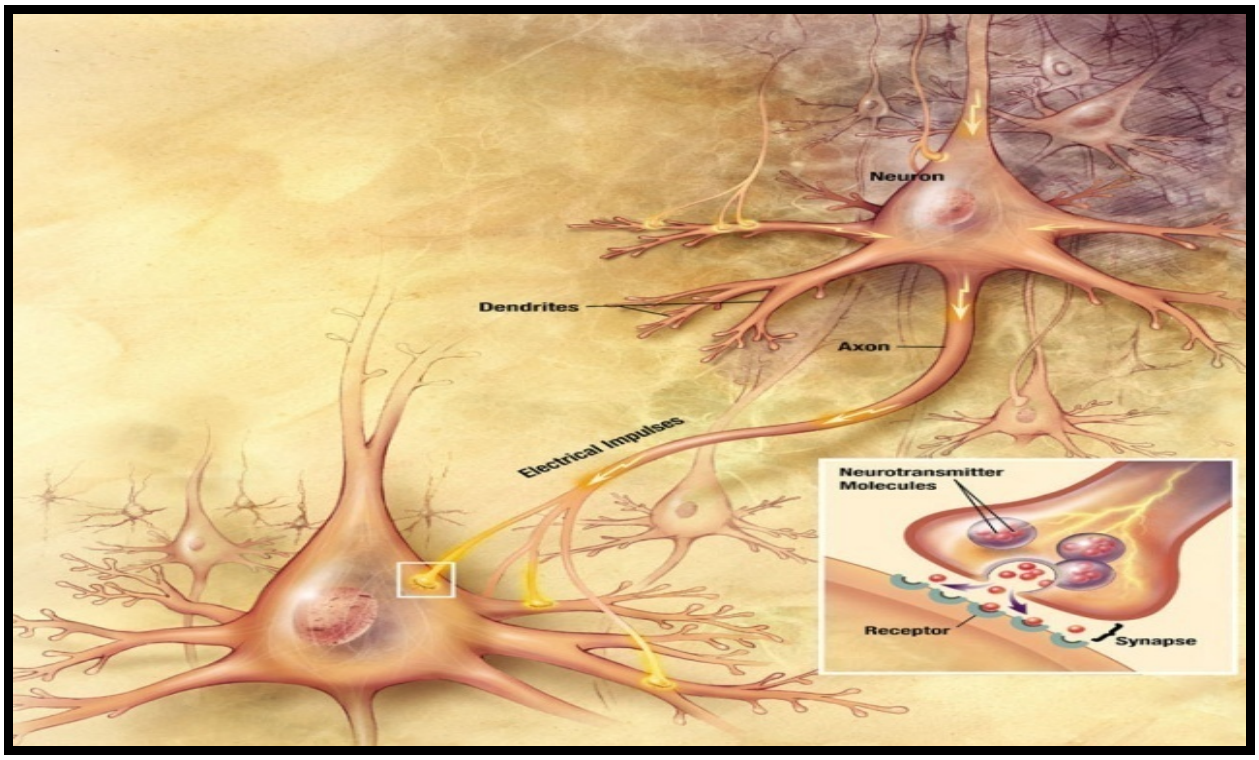

Figure 2-4: Cholinergic synapse (http://en.wikipedia.org/wiki/File:chemical synapse schema)

2.3. Neuropathology of Alzheimer's disease (AD)

Pathologically, AD is characterised by neuronal loss and by the hallmark features of senile plaques and neurofibrillary tangles (NFT) (Terry *et al*., 1994; Keefover, 1996). Degeneration occurs in a variety of neurotransmitter systems, including the cholinergic, noradrenergic, and serotonergic systems, (Court and Perry, 1991). Degeneration of cholinergic neurons occurs early in the disease and in greater proportion to cell loss in other neurotransmitter systems. Loss of cholinergic input from the basal forebrain affects the hippocampus and cortex, responsible for memory and cognitive reasoning, (Geula and Mesulam, 1994; Shadlen and Larson, 1999). Senile plaques and NFT are responsible for neuronal cell death in AD (Geula *et al*., 1998).

2.3.1. Neural Plaques (NP)

Plaques are extracellular, spherical deposits of amyloid-beta (Aβ); an insoluble protease cleavage product of the transmembrane region of amyloid precursor protein (APP) (Guillozet *et al*., 1997). APP is proteolytically processed by two enzymatic pathways: one cleaves APP within the Aβ sequence, producing a secreted non-amyloidogenic fragment of APP, whereas a second cuts APP at two sites, releasing the Aβ molecule and a truncated form of APP (Esch *et al*., 1990). In AD, the latter pathway is favoured, for reasons that are unclear. Plaques are present in the brains of non-demented elderly

individuals, but they are seen in greater number and in an altered form in the brains of patients with AD (Terry *et al*., 1994; Guillozet *et al*., 1997).

Plaques are not uniform entities, but undergo a process of maturation that reflects disease progression. The amyloid deposits found in preclinical AD are generally diffuse and are not associated with neurites (Guillozet *et al*., 1997). As AD advances, the plaques become compact as the amyloid assumes a highly structured β-pleated sheet conformation, and subsequently become associated with dystrophic neurites (Guillozet *et al*., 1997). In addition, they become associated with glial cells, reactive astrocytes and microglia in particular (Terry *et al*., 1994).

2.3.2. Neurofibrillary Tangle (NFT)

Neurofibrillary tangles are intracellular collections of highly insoluble paired helical filaments (PHFs) (Terry *et al*., 1994). Tangles are present in other neurodegenerative disorders, such as Parkinson's disease and progressive supranuclear palsy (Terry *et al*., 1994). PHFs are composed of proteins that normally make up the cytoskeleton of a neuron, including the microtubule-associated protein, tau (Terry *et al*., 1994). Abnormal phosphorylation of these proteins and subsequent collapse of the cellular cytoskeleton may directly result in the death of the neuron (Terry *et al*., 1994).

2.3.3. Disease Mechanism

Although the gross histological features of AD in the brain are well defined, three major hypotheses have been advanced regarding the primary cause. The oldest hypothesis suggests that deficiency in cholinergic signalling initiates the progression of the disease. Two alternative misfolding hypotheses instead, suggest that either tau protein or amyloid-beta initiates the cascade. While researchers have not identified a clear causative pathway originating from any of the three molecular hypotheses to explain the gross anatomical changes observed in advanced AD, variants of the amyloid beta hypothesis of molecular initiation have become dominant among the three possibilities (Cummings, 2004).

2.3.3.1. Cholinergic Hypothesis

The oldest hypothesis is the "cholinergic hypothesis". It states that Alzheimer's begins as a deficiency in the production of acetylcholine, a vital neurotransmitter (Shadlen and Larson, 1999). Much early therapeutic research was based on this hypothesis, including restoration of the "cholinergic nuclei". The possibility of cell-replacement therapy was investigated on the basis of this hypothesis (Mudher and

Lovestone, 2002). All of the first-generation anti-Alzheimer's medications are based on this hypothesis and work to preserve acetylcholine by inhibiting acetylcholinesterase (Mudher and Lovestone, 2002).

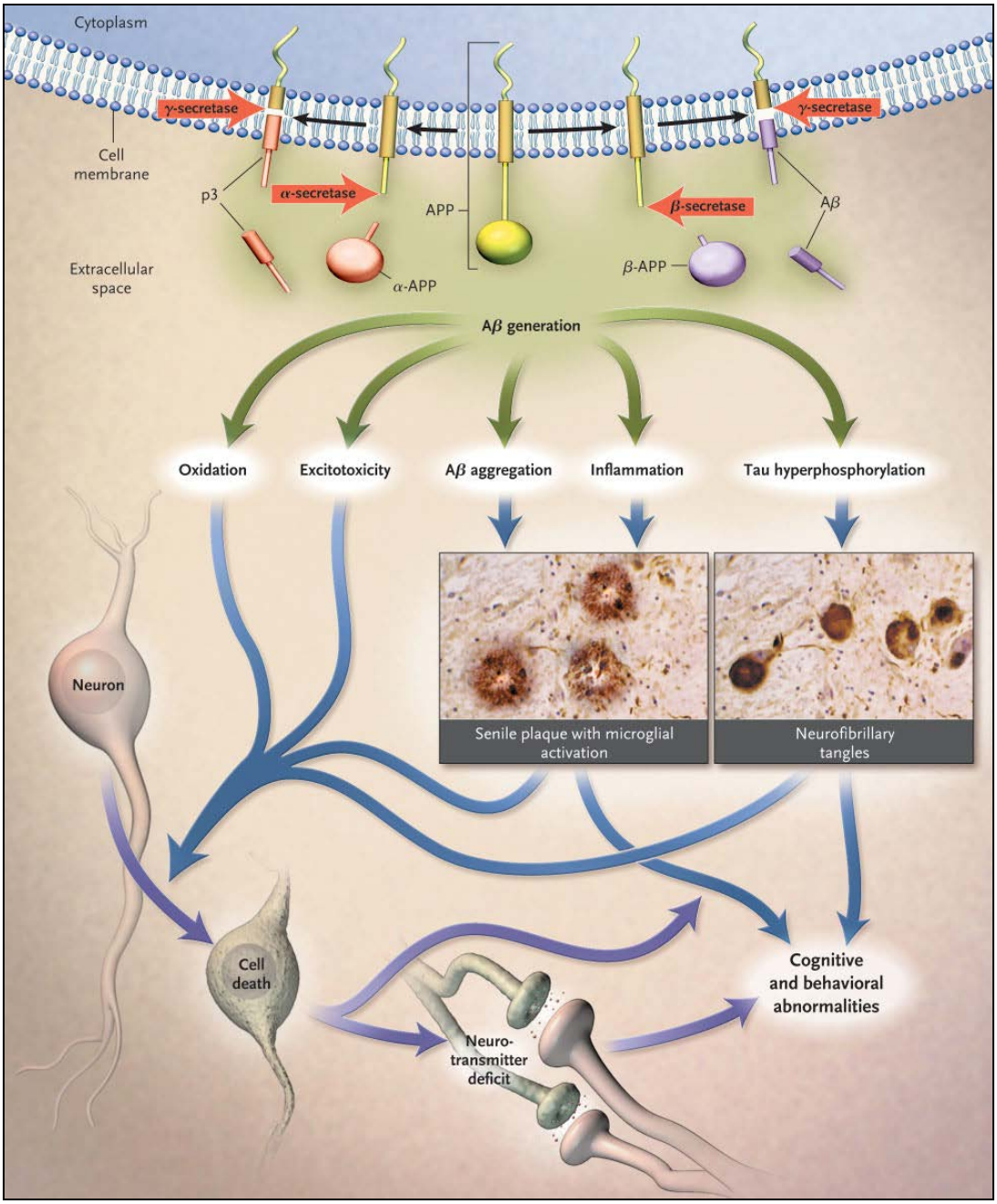

Figure 2-5: Putative Amyloid Cascade (Cummings , 2004).

These medications, though sometimes beneficial, have not led to a cure. In all cases, they have served to only treat symptoms of the disease and have neither halted nor reversed it (Mudher and Lovestone, 2002). These results and other research have led to the conclusion that acetylcholine deficiencies may not be directly causal, but are a result of widespread brain tissue damage, damage so widespread that cell-replacement therapies are likely to be impractical (Wenk, 2003). Cholinergic effects have been

proposed as a potential causative agent for the formation of plaques and tangles leading to generalised neuroinflammation (Shen, 2004).

More recent hypotheses centre on the effects of the misfolded and aggregated proteins, beta-amyloid and tau. The two positions are described as "ba-ptist" and "tau-ist" viewpoints in scientific publications by Alzheimer's disease researchers. "Tau-ists" believe that the tau protein abnormalities initiate the disease cascade, while "ba-ptists" believe that beta amyloid deposits are the causative factor in the disease (Mudher and Lovestone, 2002).

2.3.3.2. Tau Hypothesis

The hypothesis that tau is the primary causative factor has long been grounded in the observation that deposition of amyloid plaques does not correlate well with neuron loss (Schmitz *et al*., 2004). A mechanism for neurotoxicity has been proposed based on the loss of microtubule-stabilising tau protein that leads to the degradation of the cytoskeleton (Gray *et al*., 1987). However, consensus has not been reached on whether tau hyperphosphorylation precedes or is caused by the formation of the abnormal helical filament aggregates (Mudher and Lovestone, 2002). Support for the tau hypothesis also derives from the existence of other diseases known as tauopathies in which the same protein is identifiably misfolded (Williams, 2006). However, a majority of researchers support the alternative hypothesis that amyloid is the primary causative agent (Mudher and Lovestone, 2002).

2.3.3.3. Amyloid Hypothesis

The amyloid hypothesis is initially compelling because the gene for the amyloid beta precursor APP is located on chromosome 21, and patients with trisomy 21 - better known as Down syndrome - who thus have an extra gene copy almost universally exhibit AD-like disorders by 40 years of age (Nistor *et al*., 2007). The traditional formulation of the amyloid hypothesis points to the cytotoxicity of mature aggregated amyloid fibrils, which are believed to be the toxic form of the protein responsible for disrupting the cell's calcium ion homeostasis and thus inducing apoptosis (Yankner *et al*., 1990). This hypothesis is supported by the observation that higher levels of a variant of the beta amyloid protein known to form fibrils faster *in vitro* correlate with earlier onset and greater cognitive impairment in mouse models (Iijima *et al*., 2004) and with AD diagnosis in humans (Gregory and Halliday, 2005). However, mechanisms for the induced calcium influx, or proposals for alternative cytotoxic mechanisms, by mature fibrils are not obvious (Gregory and Halliday, 2005).

A more recent and broadly supported variation of the amyloid hypothesis identifies the cytotoxic species as an intermediate misfolded form of amyloid beta, neither a soluble monomer nor a mature aggregated polymer but an oligomeric species, possibly toroidal or star-shaped with a central channel (Blanchard *et al*., 2000) that may induce apoptosis by physically piercing the cell membrane (Abramov *et al*., 2004). A related alternative suggests that a globular oligomer localised to dendritic processes and axons in neurons is the cytotoxic species (Barghorn *et al*., 2005).

2.4. Acetylcholinesterase (AChE) and Its Inhibition

2.4.1. Structure and Mechanism of Action of Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE) is a complex protein of a/b hydrolase fold; ellipsoidal in shape, containing a deep groove, called the gorge about 20 A˚ deep (Houghton *et al*., 2006). Hydrolysis of ACh appears to take place at the bottom of the gorge. The mechanism is fairly complex. However, structural knowledge of AChE is mostly based on work carried out on the enzyme *TcAChE*, obtained from the electric eel (*Torpedo californica*.) The structure of the mammalian AChE is very similar to that of *TcAChE* (Bourne *et al*., 1995) and most *in vitro* work on AChE inhibitors has been carried out using the latter enzyme. All amino acid numbers are based on *TcAChE*. Although the hydrolysis process takes place in the base of the gorge, initial binding of ACh is thought to occur at its outer rim in a region called the “peripheral site”. There are four main subsites at the bottom of the gorge, where the actual hydrolysis occurs, these being the “esteratic site”, the “oxyanion hole”, the “anionic subsite” and the “acyl pocket” (Sussman *et al*., 1991).

The esteratic site contains the catalytic machinery of the enzyme, which is dependent on a catalytic triad of Ser200–His440–Glu327 (Sussman *et al*., 1991). Cholinesterases use this catalytic triad (Ser–His–Glu) to enhance the nucleophilicity of the catalytic serine, since the strong hydrogen bond between Histidine and Serine improves the ability of Serine to mount a nucleophilic attack on the substrate while Glutamate stabilises the histidinium cation of the transition state (Houghton *et al*., 2006). The “oxyanion hole” (OH) consists of Gly118, Gly119 and Ala201. These three peptide residues contain hydrogen bond donors and they stabilise the tetrahedral intermediate of ACh which is formed during the catalytic process (Houghton *et al*., 2006). The “anionic subsite” (choline-binding subsite or the hydrophobic subsite) is largely comprised of aromatic residues and contains Trp84, Phe330 and Glu199, which are believed to bind to quaternary ammonium ligands by p-cation interactions. The positive charge of the quaternary ammonium group of ACh can form a stable interaction with the electron-rich p systems of aromatic rings (Kua *et al*., 2003). Trp84 is an important residue for binding ACh. Substitution of Trp84

by an aliphatic residue (alanine) results in a large decrease in reactivity toward ACh, but no decrease for non-charged ligands, (Sussman *et al*., 1991). The “acyl pocket” (acyl binding pocket) consists of Phe288 and Phe290, which are believed to play a role in limiting the dimension of substrates which are able to enter the active site (Zhang *et al.,* 2002). The “peripheral anionic site” (PAS), which is remote from the catalytic site, has been identified by the use of inhibitor probes and X-ray diffraction (Bourne *et al*., 1995). The PAS varies among AChEs. In *TcAChE* it consists of aromatic and carboxylic acid residues, Asp72, Tyr70, Tyr121, Trp279, and Phe290 (Eichler *et al*., 1994). The AChEs from other living organisms differ in relatively minor details and biological properties. The AChE found in insects is an important target for some insecticides which are based on AChE inhibition (Houghton *et al.*, 2006).

2.4 2. Inhibitors of AChE

A number of studies have been conducted in order to investigate the interaction between inhibitors and AChE. These include X-ray diffraction of the crystal complex between AChE and inhibitors as well as site-directed mutagenesis (Greenblatt *et al*., 2003). Inhibitors can be divided between those that bind to the active site at the bottom of the gorge and those that bind to the PAS. As far as the alkaloidal inhibitors are concerned, binding takes place at the active site at the bottom of the gorge and the important features of an inhibitor appear to be a positively-charged nitrogen, which binds to the oxyanion hole area, especially the Trp84, and a region, separated by a lipophilic area from the positive charge, which can form hydrogen bonds with the serine200 residue and others such as His440 (Harel *et al*., 1995). There are indications that some inhibitors bind to both sites.

2.4.3. Uses of AChE Inhibitors (AChEIs)

AChE inhibitors (AChEIs) are used in two major ways, as pharmaceuticals and as pesticides, especially against insects and other arthropod vertebrates (Houghton *et al*., 2006). A more sinister use of these agents is their employment as toxic agents, either in traditional ritual killings and ordeal trials as practised in some societies or as chemical warfare agents (Houghton *et al*., 2006). The latter group consists of synthetic compounds with no chemical relationship to natural products, an example of which is the dangerous nerve gas sarin (Jann, 2000; Houghton *et al*., 2006).

In medicine, AChEIs are employed mostly for correcting the effects of insufficient levels of ACh. The oldest application in this respect is in ophthalmology for the treatment of glaucoma. Glaucoma is caused by a build-up of fluid in the eye, due to inadequate drainage. The vessels through which drainage occurs are dilated by cholinergic stimulation, so allowing faster drainage (Houghton *et al*., 2006). Physostigmine an alkaloid isolated from the plant *Physostigma venenosum* was formerly used in

this way but has now been largely replaced by the use of direct cholinomimetics, such as pilocarpine. Another major use for naturally-occurring AChEIs is for the treatment of myasthenia gravis, a genetic condition characterised by muscle weakness due to insufficient ACh at the neuromuscular junction (Houghton *et al*., 2006).

2.4.4. Cholinesterase Inhibitors (ChEI) in the Treatment of Alzheimer's disease

The advent of the cholinergic hypothesis in Alzheimer's disease states that degeneration of cholinergic neurons in the basal forebrain nuclei causes disturbances in presynaptic cholinergic terminals in the hippocampus and neocortex, which is important for memory disturbances and other cognitive symptoms (Robertson and Harrell, 1997). One therapeutic approach to enhance cholinergic neurotransmission is to increase the availability of acetylcholine by inhibiting acetylcholinesterase, the enzyme that degrades acetylcholine in the synaptic cleft (Houghton *et al*., 2006). The acetylcholinesterase inhibitors tacrine donepezil, rivastigmine, and galanthamine are approved for clinical use in Alzheimer's disease treatment (Jann and Grossberg, 2000).

Tacrine (Cognex®), approved in 1993, was the first ChEI available and represented a major breakthrough for a disease previously considered largely untreatable. However, its use is now limited due to hepatotoxicity (Madden *et al*., 1995; Shadlen and Larson, 1999). It is an acridine-based, rapidly reversible inhibitor of both AChE and BuChE (Nordberg and Svensson, 1998). The adverse effects of tacrine are predictable cholinergic side-effects such as nausea, vomiting, diarrhoea, headaches, dizziness and elevated levels of alanine aminotransferase (ALT) indicating liver toxicity (Knapp *et al*., 1994).

Donepezil (Aricept®), a piperidine-based ChEI, was approved in 1997 (Nordberg and Svensson, 1998). It is selective for AChE with negligible inhibition of Butyrylcholinesterase (BuChE) (Nordberg and Svensson, 1998). In preclinical trials, donepezil demonstrated a greater specificity for brain tissue than tacrine (Small, 1998). Donepezil is not associated with hepatotoxicity and has, until lately, been the mainstay treatment of AD. Adverse side-effect associated with the use of Donepezil is cholinergic related such as nausea, vomiting, anorexia, dizziness and fatigue (Rogers *et al*., 1998).

Rivastigmine (Exelon®), an analogue of Physostigmine, was approved by the FDA in 2000 (Exelon®, 2000). Like tacrine, it inhibits both AChE and BuChE (Nordberg and Svensson, 1998). In preclinical trials, rivastigmine preferentially blocked the G1 form of AChE (Nordberg and Svensson, 1998), which is the predominant molecular form in the AD cortex and hippocampus (Siek *et al*., 1990; Weinstock, 1999). Rivastigmine, therefore, selectively inhibits the cholinesterases in the areas of the brain most

severely affected by cholinergic deficits in AD. Adverse effects of this drug include nausea and vomiting (Corey-Bloom *et al*., 1998).

Galanthamine (sometimes referred to as galantamine) was first isolated from *Galanthus* spp., the Caucasian snowdrop, by Bulgarian scientists in the mid twentieth century (Heinrich and Teoh, 2004). It displays fairly strong *in vitro* AChE inhibitory activity with IC_{50} of 1.07μM (Lopez *et al*., 2002). Its cholinesterase inhibitory properties were first exploited in Bulgaria for the treatment of muscle contraction in polio victims but wide spread interest was aroused by its potential as a treatment for AD. Recently, the alkaloid galanthamine (Reminyl ^{R}) was also approved and licensed in many European countries for the treatment of early stages of Alzheimer's disease (Sramek *et al*., 2000). In addition to the long acting, selective, reversible, and competitive AChE inhibitory properties of galanthamine; it has nicotinergic effect in the CNS which makes it very effective in AD therapy (Woodruff-Pak *et al*., 2001). These unique properties of galanthamine led to the search for other AChE inhibitors from the family Amaryllidaceae (Houghton *et al*., 2006). The research focused mainly on galanthamine-type alkaloids. Significant AChE inhibitory activity for Amaryllidaceae alkaloids other than galanthamine-type alkaloids such as the lycorine-type alkaloids, assoanine, oxoassoanine, and 1-O acetyllycorine has also been reported (Lopez *et al*., 2002; Elgorashi *et al*., 2004).

Another natural cholinesterase inhibitor, huperzine A has been introduced in China for clinical treatment of Alzheimer's disease. Huperzine A is one of the alkaloids found in the club moss *Huperzia serrata* (Lycopodiaceae) which is used in various formulae in traditional Chinese medicine to alleviate problems of memory loss, promote circulation and for fever and inflammation.(Skolnick, 1997). Huperzine A is related to the quinolizidine alkaloids and it reversibly inhibits AChE *in vitro* and *in vivo* (Bai *et al*., 2000). Huperzine A has been found in other species of Huperzia and the related genus Lycopodium, together with huperzine B and sieboldine (Houghton *et al*., 2006).

2.5. Epidemiology of Alzheimer's disease (AD).

CNS disorders associated with old age, such as Alzheimer's (AD) and Parkinson's disease (PD), and other 'senile' dementia will have spectacular societal and economic impact in the next decades due to increasing life expectancy worldwide (World Health Report, 1998). The number of elderly people in Africa is increasing very rapidly; figures from the United Nations (UN), project life expectancy in Africa to increase from under 40 years in 1950 to almost 60 years in 2010 (Ineichen, 2000; United Nations, 2003). The prevalence of AD among indigenous South Africans is not known (De Villiers and Louw, 1996); cultural attitudes in Africa surrounding dementia may have contributed to this apparent low prevalence, the demented may not even be seen as ill (Stafford *et al*., 2008), given that rural

communities still have difficulties accessing tertiary health care, patients affected are not likely to consult Orthodox doctors; most of the elderly persons affected with AD die quickly from complications of pneumonia and diarrhoea (Chandra *et al*., 1994; Levkoff *et al.,* 1995; De Villiers and Louw, 1996). Out of the approximately 100 studies of the prevalence of dementia that have been carried out worldwide, only a small number have been conducted in Africa (Ineichen, 2000). There are very few epidemiological studies of mental illness in Africa centred on dementia or general psychogeriatric problems (Neuwinger, 2000; Adams *et al*., 2007).

However, in the United States Alzheimer's disease (AD) is the seventh leading cause of all deaths and the fifth leading cause of death in persons older than the age of 65 years. More than 5 million Americans are estimated to have AD. Every 71 seconds someone in America develops the disease, and by 2050 it is expected to occur every 33 seconds. During the coming decades, 'baby boomers' are projected to add 10 million people to these numbers. By 2050, the incidence of AD is expected to approach nearly a million people per year, with a total estimated prevalence of 11 to 16 million persons. Significant cost implications related to AD and other dementias include an estimated US$148 billion annually in direct (Medicare/Medicaid) and indirect (example, caregiver lost wages and out-of-pocket expenses, and decreased business productivity) costs. Not included in these figures are the estimated 10 million caregivers who annually provide US$ 89 billion in unpaid services to individuals with AD (Maslow, 2008).

2.6 The medicinal uses of *Ammocharis coranica* and its potential as an acetylcholinesterase inhibitor.

Ammocharis coranica (Ker-Gawl.) Herb., a member of the Amaryllidaceae family belongs to the subtribe-Crininae. The bulbous plant is widely found in the summer-rainfall regions of southern Africa, and consists of approximately five species (Snijman and Linder, 1996), three of which occur in southern Africa (Koorbanally *et al*., 2000). *A. coranica* also known as Berglelie, Gifbol, Seeroogblom (Afrikaans); boka (South Sotho); Icukudo, Incukudwane, Isidiya, Umbhodiya (Zulu), and Ground lily (English); develops into a large bulb with a hard papery outer sheath covering and fleshy roots that persist all year (Snijman and Linder, 1996).

Partially burned outer scales of *A. coranica*, form pitch-like substances transformed into ornamental items (Pole-Evans, 1938; Gerstner, 1941), or used as adhesive mixtures (Jacot-Guillarmod, 1971; Giess and Snyman, 1972). Medicinally, the bulbs of *A. coranica* serve as cure to certain conditions resulting from witchcraft and diabolic spells (Hulme, 1954). Its use as remedy for mentally ill patients

(Page, 1998; Louw *et al*., 2002) was corroborated when the alkaloid- buphanidrine, which exhibit affinity to the serotonin transporter (SERT) protein, was isolated (Stafford *et al*., 2008).

Previous, isolation work on *A. coranica* yielded lycorine, caranine, acetylcaranine, buphanisine, epibuphanisine, buphanidrine, ambelline, crinamine, 6-hydroxycrinamine, epivittatine and coranicine (Mason *et al*., 1955; Hauth and Stauffacher, 1962). Recently, eight alkaloids: lycorine, acetylcaranine, crinamine, 1-*O*-acetyllycorine, hippadine, 6α-hydroxypowelline, hamayne, 1-*O*-acetyl-9-*O*-demethylpluviine, and the cycloartane compounds: 24-methylenecycloartan-3β-ol, cycloeucalenol, cycloeucalenone and 24- methylenepollinastanone were isolated (Koorbanally *et al*., 2000). The following Amaryllidaceae alkaloids: Lycorine, buphanisine, epibuphanisine, 6- hydroxycrinamine and epivittatine were tested for acetylcholinesterase inhibitory activity (Elgorashi *et al*., 2004; Elgorashi *et al*., 2006), and found to have different degrees of acetylcholinesterase inhibitory activity.

Ammocharis coranica selected for AChE inhibitory activity screening belongs to Amaryllidaceae family, known to produce alkaloids with the potential of acting on the central nervous system (CNS). Galanthamine is example of an Amaryllidaceae alkaloid licensed for use in the treatment of mild to moderate AD (Heinrich and Teoh, 2004). Despite the extensive isolation work by Mason *et al*., (1952); Hauth and Stauffacher, (1962), and Koorbanally *et al*. (2000), careful search of literatures and other scientific publications indicated no report of the investigations on the crude extract or fractions of the bulb of *Ammocharis coranica* for AChE inhibitory activity. In addition, results of preliminary work on AChE inhibition screening will provide impetus to the Phytomedicine group in embarking on extensive search for alternatives to the current drugs use in the treatment of AD. By using a bioassay guided approach the compounds with AChE inhibitory activity can be identified and characterised.

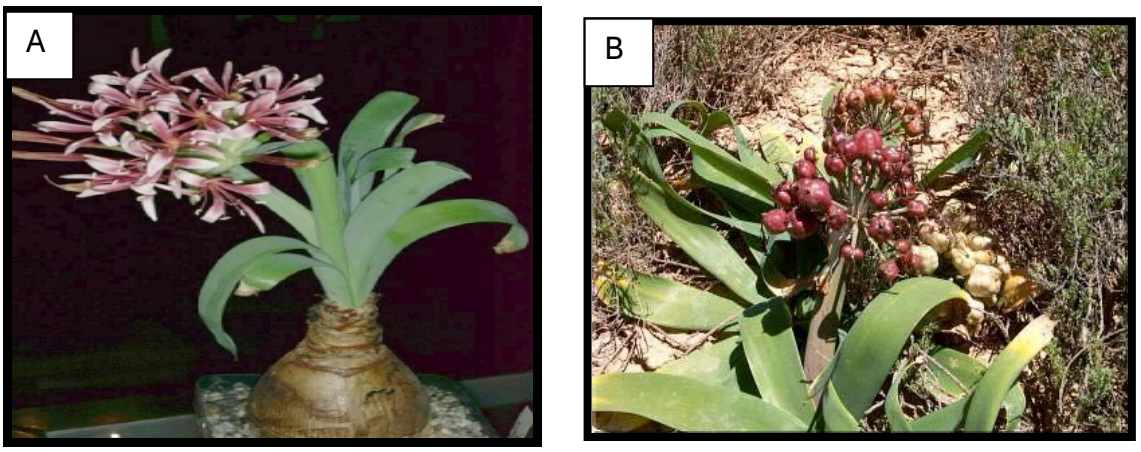

Figure 2-6: Bulb (A) and fruits (B) of *Ammocharis coranica* (Courtesy Caudiform Birhmann, 28 June 2005).

CHAPTER 3

Preliminary screening of ethanolic crude extract of the bulbs of *Ammocharis coranica* (Ker-Gawl.) Herb., for acetylcholinesterase (AChE) inhibitory activity using TLC bioautographic and microplate assays.

3.1. Introduction.

Natural products provide mankind with environment-friendly alternatives to synthetic remedies (Bai *et al.*, 2000). Bulbous plants, though less investigated than herbs and trees, regarding their medicinal potentials, contain a wide range of unique biologically active compounds (Louw *et al*., 2002). Important applications from bulbous plants include: Anticholinesterase, analgesia, anticancer, antimutagenic, immune stimulating, anti-infective, antimalarial, cardiovascular and respiratory system effects (Hutchings *et al*., 1996; Fennell and Van Staden, 2001; Howes *et al*., 2003).

The preliminary investigation of acetylcholinesterase (AChE) inhibitory activity of the ethanolic crude extract of the bulbs of *Ammocharis coranica* were determined qualitatively using thin layer chromatography (TLC) bioautographic assay and quantitatively by the microplate assay (Rhee *et al*., 2001). The TLC bioautographic and the microplate assays are colorimetric tests (Ellman *et al*., 1961); their reaction is based on the quantity of thiocholine released when acetylthiocholine iodide (ATCI) is hydrolysed by AChE (Ellman *et al*., 1961). The thiocholine released in this reaction is quantified spectrophotometrically, by its reaction with the chemical dye- 5, 5´-dithiobis-2-nitrobenzoic acid (DTNB) also known as the Ellman's reagent. The product of the reaction is a yellow-coloured compound (5-thio-2-nitrobenzoate) detected by absorbance at 405 nm (Rhee *et al*., 2001; Brühlmann *et al*., 2004). A reduction in the yellow compound (5-thio-2-nitrobenzoate) indicates an inhibitory activity of the substance added. The colorimetric method is a high-throughput anti-acetylcholinesterase technique that can be used on a small scale using a microtitre plate. The possibility of running several replicates for each test has also enabled improved statistical analysis of results (Brühlmann *et al*., 2004; Giovanni *et al*., 2008).

The Ellman reaction for detecting AChE inhibitory activity has also been adapted for TLC plates (Rhee *et al*., 2001; Marston *et al*., 2002). This method is considered more sensitive than the microplate assay (Houghton *et al*., 2006), and used for the rapid screening of natural and synthetic products (Houghton *et al*., 2006). It gives quick access to information about inhibitory activity and localisation of constituents in plant matrices (Marston *et al*., 2002). Active crude plant extracts, synthetic and other natural products (Howes *et al*., 2003; Hostettmann *et al*., 2006; Houghton *et al.,* 2006) developed on TLC plates, when sprayed with the reagents (ATCI/DTNB mixture followed by AChE) produces a white area of inhibition

against a yellow background (Rhee *et al*., 2001; Marston *et al*., 2002). The TLC bioautographic assay is simple to use (Marston *et al*., 2002), and allows screening of several plant extracts at the same time (Giovanni *et al*., 2008).

3.2. Materials and methods.

3.2.1. Plant collection and preparation.

The bulbs of *A. coranica* used in this work were kind donations by Mr Graham Duncan of the Kirstenbosch National Botanical Garden, Cape Town. The plant bulbs were collected from Bethelsdorp in the Eastern Cape region of South Africa, and authenticated by the donor. A voucher specimen, No. 39/69 was kept in the herbarium of the Kirstenbosch National Botanical Garden.

The scales of the bulb of *A. coranica* were separated and dried at 37 ^{o}C for several days to a constant mass (210 g). Dried plant material were ground to powder using the Masalab mill (Model 200 Lab). The bulb powders (200 g) were stored in closed bottles in the dark at room temperature until used.

3.2.2. Preliminary extraction of the bulb of *A. coranica*.

Approximately 1 g of the powdered plant material was extracted with 20 ml of 96% ethanol in a centrifuge tube, with vigorous shaking on a Labotec® shaking machine at least six hours daily. The extraction lasted 48 hours, after which the supernatant was filtered through Whatman No.1 filter paper into a pre-weighed vial and kept under fan to dry.

3.2.3. Thin layer chromatography (TLC) fingerprinting.

The dried crude extract of the bulbs of *A. coranica* from section 3.2.2 was dissolved in methanol (MeOH) to a concentration of 10 mg/ml, of which 3 µl were spotted on TLC plate (Silica gel 60 F_{254} Merck) and developed using chloroform: methanol (4:1) as the eluent. The developed TLC plate was visualised under ultraviolet (UV) light at 254 nm and 365 nm respectively, using Camac Universal lamp TL-600. Thereafter, the plate was sprayed with Dragendorff's reagent to examine the presence and location of alkaloids.

3.2.4. Preliminary determination of acetylcholinesterase (AChE) inhibitory activity.

The TLC bioautographic assay, including false positive detection, and microplate assays were based on this principle (Ellman *et al*., 1961):

Acetylthiocholine iodide (ATCI) + H_2O $\xrightarrow{\text{AChE}}$ Acetate + Thiocholine

Thiocholine + DTNB ⟷ 5-thio-2-nitrobenzoate (yellow colour)* + 2-nitrobenzoate-5-mercapto-thiocholine

* The yellow colour is detected at 405 nm in microplate assay. Inhibition of the enzyme by the plant product produces a white spot on the TLC plate against a yellow background.

3.2.4.1. Chemicals and equipments.

All the solvents used in this work, such as methanol, chloroform, ethyl acetate, dichloromethane, and ethanol were analytical grade, purchased from Saarchem, South Africa.

3.2.4.2. Buffers and their preparation.

Several buffers were used in the screening of ethanolic crude extract of the bulbs of *A. coranica* for AChE inhibitory activity using TLC bioautographic and microplate assays, see their preparations in (Table 3.1).

Table 3-1: Lists of buffers used and their preparation.

Types of buffer and their preparations
1. Buffer A- 50 *mM* Tris-HCl, pH 8 6.05 g Tris-HCl was dissolved in 700 ml of sterile distilled water, 0.2 N HCl was then added to the solution, while stirring and monitoring the pH with a pH meter, until pH 8. Distilled water was added to make 1000 ml. 2. Buffer B- 50 *mM* Tris-HCl, pH 8, containing 0.1% bovine serum albumin (BSA) 0.5 g of BSA was added to 500 ml of buffer A to produce buffer B. 3. Buffer C- 50 *mM* Tris-HCl, pH 8, containing 0.1 *M* NaCl and 0.02 *M* $MgCl_2.6H_2O$ 2.92 g NaCl, and 2.03 g $MgCl_2.H_2O$ were added to 500 ml of buffer A to produce buffer C.

3.2.4.3. Enzyme.

AChE from electric eel (type VI-s, lyophilised powder) was purchased from Sigma (South Africa). The lyophilised enzyme was dissolved in buffer A to make a 1000 units/ml stock solution. It was further diluted with buffer B to get 0.22 unit/ml enzyme solutions for the microplate assay, or diluted with buffer A to get 3 units/ml for TLC assay. The enzyme stock solution was kept in -80 ^{o}C freezer.

3.2.4.4. Substrate.

Acetylthiocholine iodide (ATCI) was purchased from Sigma, (South Africa). One *mM* ATCI in buffer A and 15 *mM* in Millipore water were used for TLC bioautographic and microplate assay respectively.

3.2.4.5. Ellman's reagent (Dye).

5.5´-Dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from Sigma (South Africa). For the TLC bioautographic assay, 1 *mM* of DTNB in buffer A was used, while 3 mM DTNB was used for the microplate assay.

3.2.4.6. Dragendorff's reagent (DRG).

Basic bismuth nitrate (1.7 g) was dissolved in a mixture of 20 ml acetic acid and 80 ml distilled water to form solution A. 16 g of potassium iodide was then dissolved in 40 ml of distilled water to form solution B. Solutions A and B are then mixed 1:1 (*V/V*) to make the stock solution. The stock solution was kept in the refrigerator until needed. For spraying of the TLC plates, 1 ml of the stock solution in 2 ml acetic acid and 10 ml of distilled water was used (Harborne, 1980).

3.2.4.7. Reference AChE inhibitor.

Physostigmine hemisulfate (Eserine hemisulfate) was purchased from Sigma (South Africa). A stock solution was prepared for both TLC bioautographic and microplate assays.

3.2.4.8. TLC plate.

Silica gel 60 F_{254}, 0.25 mm thick TLC plates were purchased from Merck (South Africa).

3.2.4.9. The microplate reader.

Bio-Rad microplate reader model 3550 UV was used to measure the absorbance at 405 nm for the enzyme reaction in the microplate assay.

3.2.4 10. TLC Bioautographic assay.

The TLC bioautographic assay was carried out as adopted by Rhee *et al*. (2001). TLC plates were developed as described in section 3.2.3. An exception was that 3 µl of 0.01 *M* physostigmine hemisulfate (reference sample) were spotted on TLC plates. Thereafter, enzyme inhibiting activity of the samples was detected by spraying a mixture of the substrate (1 *mM* ATCI) and the dye (1 *mM* DTNB)

until the silica was saturated with the solution. The plates were allowed to dry for 3-5 minutes, before 3 units/ml enzyme solutions were sprayed on the plates. A yellow background appeared, while white spots for inhibiting compounds became visible after 5 minutes. The TLC plates were scanned or photographed within 15 minutes before the reaction colour disappeared.

3. 2.4.11. Acetylcholinesterase (AChE) inhibition detection limit of ethanolic crude extract of the bulbs of *A. coranica*.

The detection limit of the AChE inhibitory activity of the crude extract of *A. coranica* was determined by spotting different concentration of the extract on the TLC plate. Ten mg/ml of the crude extract was reconstituted using methanol, after which 50 µg, 40 µg, 30 µg, 25 µg, 20 µg, 15 µg, 10 µg, and 5 µg were spotted. The TLC bioautographic assay was carried out as described in section 3.2.4. The lowest amount of the ethanolic crude extract, able to produce observable white spots of inhibition with the naked eyes was recorded.

3.2.4.12. Test for false positive results in the Bioautographic method.

The false positive reactions were eliminated using a method developed by Rhee *et al*. (2003). The TLC plate was prepared as described in section 3.2.4, and sprayed with 1 *mM* DTNB in buffer A. The plate was allowed to dry for about 3-5 minutes. After which it was sprayed with thiocholine, the product formed when 1 *mM* ATCI in 3 units/ml enzyme stock solution was incubated at 37^{o}C for 20 minutes. The appearance of a yellow background or white spot were scanned or photographed and saved.

3.2.4.13. Microplate assay.

The assay was carried as outlined by Rhee *et al*. (2001). The ethanolic crude extract of the bulbs of *A. coranica* and physostigmine hemisulfate (positive reference compound) were prepared in eight different concentrations using twofold serial dilutions of the plant extract and tenfold dilutions of the positive reference compound (Figure 3-2). In the 96 well plates, 25 µl of 15 *mM* ATCI, 125 µl DTNB (3 *mM*), 50 µl buffer B [50 *mM* Tris-HCl, pH 8, containing 0.1% bovine serum albumin (BSA), and 25 µl of samples were added and the absorbance was measured at 405 nm every 30 seconds three times, at 30 ^{o}C. Finally, 25 µl of 0.2 U/ml of enzyme solution was added in and the absorbance was measured again after 3, 5, and 10 minutes respectively. The assays were conducted in triplicate.

3.2.4.14. Calculation of the percentage inhibition and the inhibitory concentration 50 (IC_{50}) of the ethanolic crude extract of the bulb of *A. coranica* on AChE: -

Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the average absorbance before adding the enzyme from the absorbance after adding the enzyme. The percentage inhibition was calculated by comparing the inhibition of the sample to the blank (10 % methanol in buffer A). The IC_{50} value was calculated from at least four different concentrations of the sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000						1000					
B	100						500					
C	10						250					
D	1						125					
E	0.1						62.5					
F	0.01						31.25					
G	0.001						15.625					
H	0.0001						7.8165					

+ve control Crude extract and fractions -ve control

Figure 3-1: Illustration of the microtitre plate made up of 12 columns (1-12) and 8 rows (A-H), with the different concentrations (µg/ml) of plant extracts [1/2 dilution] - represented by light green shaded area, the positive control- physostigmine hemisulfate (1/10 dilution) - represented by red shaded area and the negative control-methanol, represented by orange shaded area.

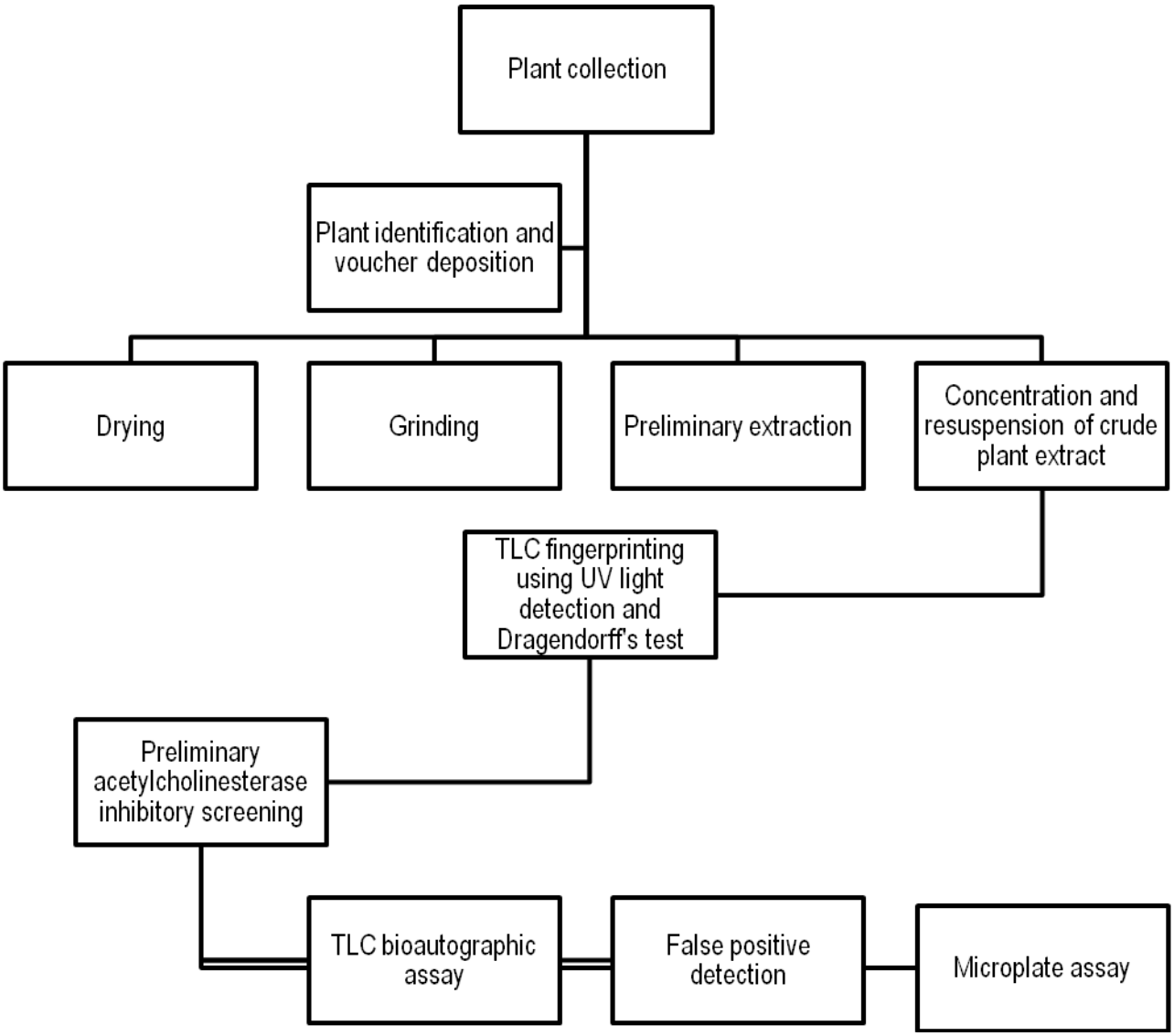

Figure 3-2: Flow diagram showing the layout of procedures during the preliminary screening of the ethanolic crude extract of the bulbs of *Ammocharis coranica* using TLC bioautographic and microplate assays.

3.3. Results and discussion.

3.3.1. Phytochemical screening.

The primary target of this investigation was to screen the ethanolic crude extract of the bulbs of *A. coranica* for secondary plant metabolites with acetylcholinesterase inhibitory activity.

3.3.1.1 Ultraviolet light detection of chromatogram at 254 nm and 365 nm.

Ultraviolet (UV) light detection at 254 nm of the crude extract produced a faint blue fluorescence against a green background; UV detection at 365 nm produced a blue fluorescence. Physostigmine appeared as a black spot at 254 nm but did not produce fluorescence at 365 nm (Figure 3-3 A and B).

3.3.1.2. Dragendorff's test.

The chromatogram developed in chloroform: methanol (4:1) produced several spots which appeared orange-brown in colour after spraying with Dragendorff's reagent (Figure 3-4 A). The orange-brown precipitates confirm the presence of alkaloidal compounds in the crude extract.

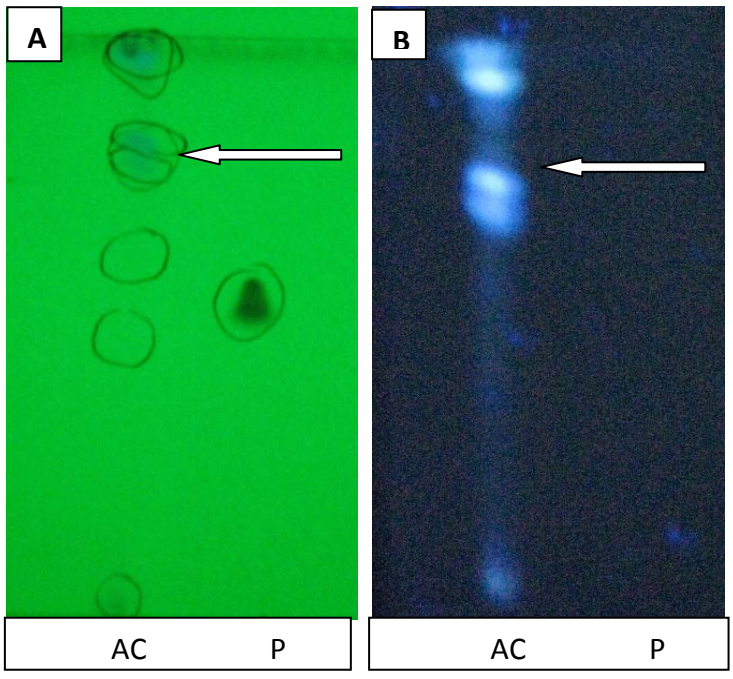

Figure 3-3: (A) Chemical profile of the ethanolic crude extract (30 µg) of the bulbs of *Ammocharis coranica* (AC) and physostigmine (P) on the TLC plate after elution with chloroform: methanol (4:1) visualised at 254 nm and (B) The arrow showing fluorescent compounds present in the crude extract visualised at 365 nm.

3.3.1.3. TLC bioautographic assay of the crude extract of *A. coranica*.

Several white spots of inhibition with similar R_f values to the alkaloids were observed after spraying with substrate, dye and enzyme (Figure 3-4 A and B). This indicates that the crude ethanol extract of *A. coranica* contains compounds with AChE inhibitory activity. This conclusion was confirmed by the absence of white spots on the TLC plate when false positive bioautographic assay was carried out (Fig 3-4C). AChE inhibitory activity detection using the TLC bioautographic assay described in section 3.4 may produce false positive results. Thiocholine, a product of the reaction between the enzyme acetylcholinesterase and ATCI, reacts with DTNB to produce 5-thio-2-nitrobenzoate; the product gives a yellow background on the TLC plate. The development of a white spot on the plate may be due to inhibition of the chemical reaction between DTNB and thiocholine and not due to inhibition of the enzyme which had already been used up in the initial reaction with the substrate (ATCI); confirming that the compound or fractions are not inhibiting on the enzyme (Rhee *et al*., 2003). The results of the detection limit of the inhibitory activity of crude extract on TLC plate are presented in Figure 3-5. The minimum concentration of the ethanolic crude plant extract which can be conveniently visualised as white area of the inhibition of AChE enzyme on the TLC plate was 15 µg; this is far less than the detection limit of physostigmine found to be 0.01 µg (Rhee *et al.*, 2001).

3.3.1.4. Quantitative determination of AChE inhibitory activity of the ethanolic crude extract of *A. coranica* using the microplate assay.

AChE inhibitory activity of the crude extract of *A. coranica* and physostigmine (positive control) expressed as IC_{50} are presented in Table 3-2. Ethanolic crude extract of the bulbs of *A. coranica* inhibited acetylcholinesterase (AChE) in a dose-dependent manner. The minimum concentration of the crude extract able to inhibit 50 % of the enzyme (IC_{50}) was 14.3 ± 0.50 µg/ml. The correlation coefficient to determine the IC_{50} had an $r^2 > 0.9$. The IC_{50} of physostigmine measured using the microplate reader was 5.5 ± 0.78 µM (1.51 µg/ml). Physostigmine is about nine times more active than the ethanolic crude extract of *A. coranica*. This is the first report of the IC_{50} of the ethanolic crude extract of the *A. coranica.* Risa et al.(2004) investigated plants used in southern Africa for treating age-related dementia and debilitating mental disorders using the TLC and microplate assays for AChE inhibitory activity based on the Ellman's method: aqueous and ethanol extracts of *Malva parviflora* L. (leaves), *Boophone disticha* (L.f.) Herb. (leaves and bulbs), *Albizia adianthifolia* (Schumach.) W. Wright (stem bark), *Albizia suluensis* Gerstner (root bark) and *Crinum moorei* Hook.f. (bulbs) were tested .Promising results were obtained with Amaryllidaceae bulbs of *Boophone disticha* and *Crinum moorei*.

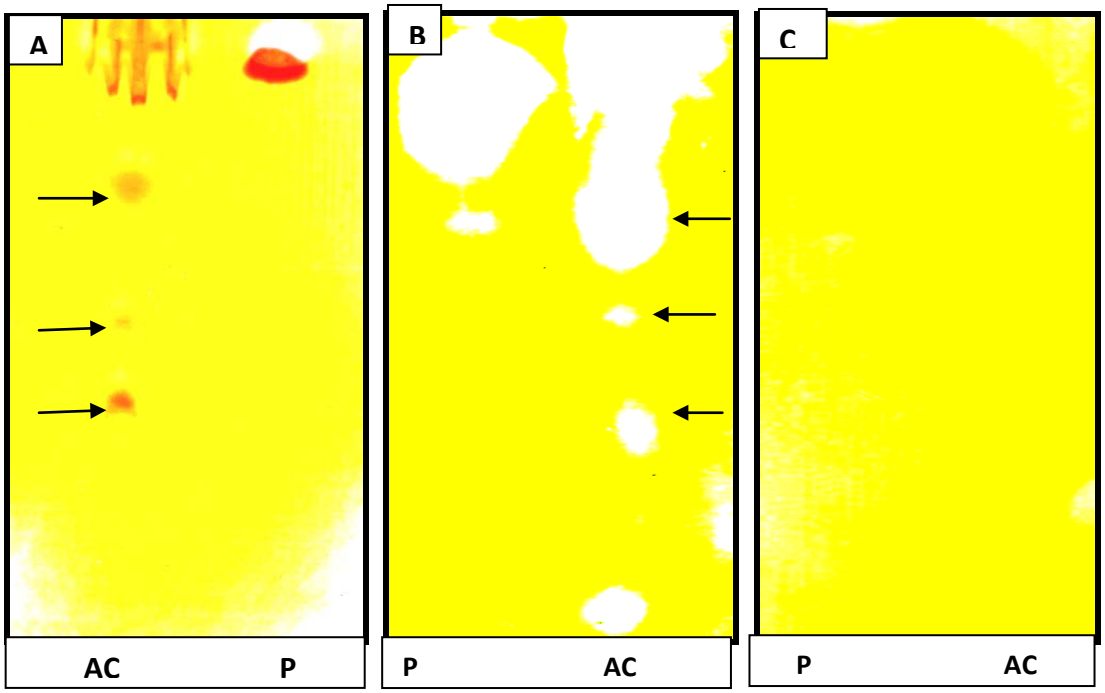

Figure 3-4 TLC fingerprint: (A) Orange spots indicate the presence of alkaloids from crude extract of *Ammocharis coranica* after spraying with Dragendorff's reagent. (B) Results of bioautographic assays: White spots on the TLC plate with similar R_f to the alkaloids indicate areas of inhibition to acetylcholinesterase (AChE). (C) Bioautographic false positive result: All yellow background indicates absence of false positives. AC represents *A. coranica* and P represents Physostigmine.

Aqueous and ethanol extracts of *Crinum moorei* and *Boophone disticha* also showed AChE inhibiting activity in the TLC assay. The aqueous and ethanol extracts of *B. disticha* 100 µg/ml yielded 38% and 27% inhibition in the microplate assay, respectively, while the ethanol extract of *C. moorei* had good dose-dependent inhibiting activity with 67% inhibition at the highest concentration. Aqueous and ethanol extracts of *C. moorei* and *B. disticha* showed AChE inhibiting activity in the TLC assay. Rhee et al. (2001) screened methanolic extracts of 15 different Amaryllidaceae: (*Hymenocallis* X *festalis* 'Zwanenburg'; *Chlidanthus fragrans* Herb.; *Narcissus* 'Avalanche' (Tazetta); Nerine *bowdenii*; *Narcissus* 'Grand Soleil d'Or' (Tazetta); Zephyranthes *candida* (Lindl.) Herb.; *Crinum* X *powellii* Baker; *Polianthes tuberosa* L.; *Amaryllis belladonna* L.; *Eucharis amazonica* Linden ex Planch.; X *Hippeastrelia*; *Habranthus robustus* Herb. ex Sweet; *Rodophilia bifida* (Herb.) Traub; *Hymenocallis* 'Sulphur Queen';*Sprekelia formosissima* (L.) Herb.) using TLC method, from which candidates for acetylcholinesterase inhibitors were selected. All plant extracts showed white spots except *Polianthes tuberosa* L, on the yellow background representing inhibition, when 1 m*M* DTNB and 1 m*M* ATCI in 50 m*M* Tris–HCl, pH 8.0, was sprayed, followed by 3 U/ml of AChE spray. *Crinum X powelli* Baker and

Amaryllis belladonna which had several inhibiting spots on the TLC plates showed strong inhibition when tested using the microplate assay. The TLC and microplate assays provide valuable information for bioguided isolation of active compounds. The TLC assay is simple, sensitive and very effective and active compounds can be detected early using this assay.

Table 3-2: Inhibition of acetylcholinesterase by the ethanolic crude extract of the bulbs of *A. coranica* as determined by microplate assay.

Sample	IC_{50} (µg/ml)
Ethanolic crude extract of the bulbs of *A. coranica*	14.3 ± 0.50
Physostigmine (reference sample)	1.51

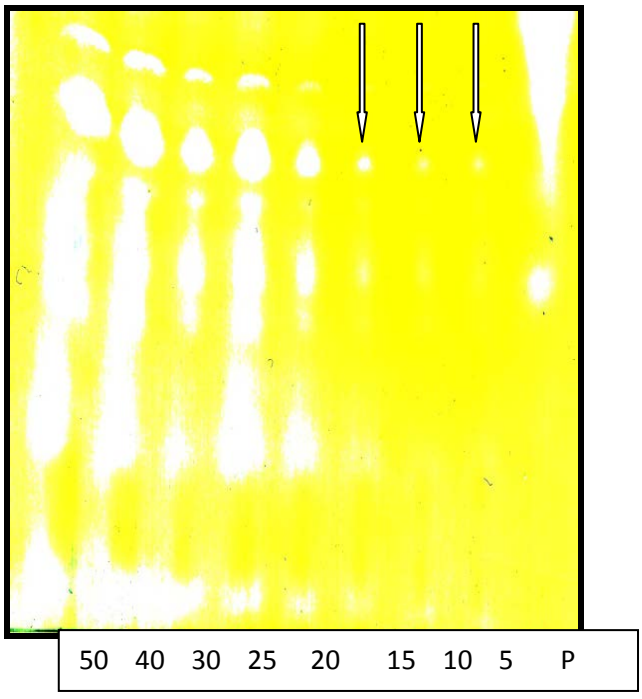

Figure 3-5: Result of bioautographic assay with white area of inhibitory activity of the ethanolic crude extract of the bulbs of *A. coranica* in varying weights (µg) spotted on the TLC plate.

3.4. Conclusions

Ethanolic crude extract of the bulbs of *A. coranica* like other members of the Amaryllidaceae family has acetylcholinesterase inhibitory activity as shown in Figure 3-4. Quantitative determination of acetylcholinesterase inhibitory activity using the microplate assay produced promising results (Table 3-2). There is however, the need to partition this crude extract into various fractions using acid-base fractionation, test them for AChE inhibitory activity and thereafter, isolate active compounds present in the crude extract. This aspect will be discussed in the next chapter.

CHAPTER 4

Isolation of bioactive compounds from ethyl acetate and butanol fractions of the crude extracts of the bulbs of *Ammocharis coranica* (Ker-Gawl.) Herb.

4.1. Introduction

Alkaloids are the largest single class of secondary plant metabolites that have significant biological activity. They are basic substances, with one or more nitrogen atoms connected to at least two carbon atoms, usually as part of a heterocyclic system (Aslanov *et al*., 1987). Alkaloids have provided a wealth of pharmacologically active compounds (Verpoorte *et al*., 1993). These are administered either as pure compounds or as extracts and have often served as model structures for synthetic drugs, example, atropine for tropicamide, quinine for chloroquinine, and cocaine for procaine (Kutchan, l995).

Alkaloids, such as galanthamine and huperzine A of plant origin, form part of the drugs approved for the treatment of Alzheimer's disease (AD); a progressive neurodegenerative disorder, prevalent in elderly people (Houghton *et al*., 2006). Recently, several researchers have examined other secondary plant metabolites such as triterpenoids, pregnanes, and flavonoids from other natural sources for their acetylcholinesterase inhibitory properties (Howes *et al*., 2003; Houghton *et al*., 2006) in the bid to find replacement for AD drugs with side-effects, such as gastrointestinal disorders, liver toxicity and related symptoms (Jann, 2000; Cumming, 2004). Alkaloids from natural and synthetic sources are still the primary targets for new AD drug discoveries due to their unique structure and binding to the acetylcholinesterase enzyme (Sussman *et al*., 1991; Houghton *et al*., 2006). Colorimetric determination based on Ellman's method (Ellman *et al*., 1961), using thin layer chromatographic (TLC) assay (Rhee *et al*., 2001; Marston *et al*., 2002) and the microplate assay (Ingkaninan *et al*., 2000; Rhee *et al*., 2001; Brülhlmann *et al*., 2004) has helped tremendously in the screening of natural products for AChE inhibitory activity. Fractions of ethanolic crude extract of *Ammocharis coranica* investigated by *in vitro* AChE inhibitory assays led to the isolation of two compounds- an alkaloid and a triterpenoid.

4.2. Materials and methods.

4.2.1. Bulk extraction of the bulbs of *A. coranica*.

Powdered dry bulbs of *A. coranica* (200 g) were extracted using (2 x 2000 ml) 96 % ethanol for 48 hours, with shaking on a Labotec® shaker. The supernatant was filtered using Whatman No. 1 filter paper. The filtrate was pooled into a glass container and concentrated, using a Büchi rotavaporator R-

114 (Labotec®). The concentrated extract was poured into a pre-weighed glass jar and left under stream of cold air to dry.

4.2.2. Solvent-solvent fractionation of the ethanolic crude extracts of the bulbs of *A. coranica.*

The residue of the dried ethanolic crude extract of the bulbs of *A. coranica* (16 g) was treated with 6 % aqueous acetic acid (300 ml). The aqueous acid solution was basified with ammonia to pH 9.5, after removal of acidic and neutral material with dichloromethane (200 ml x 3). The basified solution was extracted with ethyl acetate, and butanol respectively (200 ml x 3). The organic phases were evaporated to dryness.

4.2.3. Determination of acetylcholinesterase (AChE) inhibitory activities of the fractions of ethanolic crude extracts of *A. coranica*

Thin layer chromatography (TLC) bioautographic and false positive detection tests; qualitative assays, were used to test the DCM, EtOAc, BuOH and aqueous fractions for acetylcholinesterase (AChE) inhibitory activities (Figure 4-4 A). After which, active fractions were quantitatively assessed using the microplate assay as described in Section 3.4 and 3.5 respectively.

4.3. Isolation of active compound from Ethyl acetate (EtOAc) fraction.

4.3.1. Gradient column chromarography of Ethyl acetate fraction.

The ethyl acetate fraction (2.08 g) was subjected to column chromatography (20 x 5 cm) on 200 g silica gel 60 (Merck), eluted with 100 % dichloromethane enriched gradualy with 10 % methanol up to 100 % methanol. Fractions collected were combined into 9 sub-fractions (A-I) based on UVabsorption, Dragendorff's reagent and AChE bioautographic assays.

4.3.2. Isolation of compound 1

Sub-fraction E (tubes 68-78) was dissolved in dichloromethane: methanol (1:1) and allowed to crystallise at room temperature. White crystals were harvested and rinsed several times with chloroform and ethyl acetate, alternatively, to remove impurities attached to the crystals.

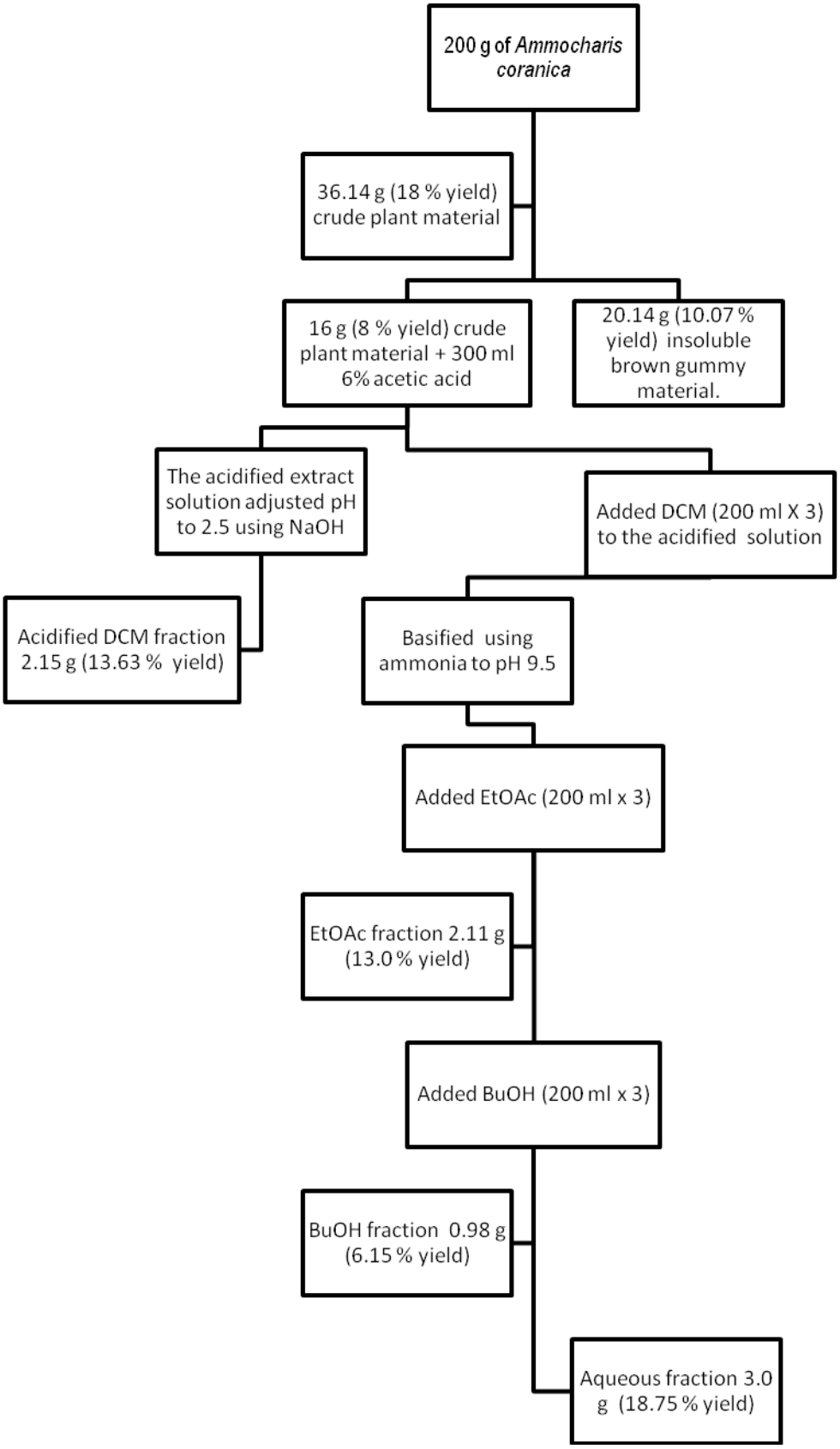

Figure 4-1: Schematic presentation of solvent-solvent fractionation of the ethanolic crude extract of the bulbs of *A. coranica* as adopted from Ghosal *et al*. (1985).

4.3.3. Isolation of compound 2

Sub-fraction C (tubes 19-35) was dissolved in dichloromethane: methanol (1:1). This was then applied on a preparative TLC plate and eluted in hexane:ethyl acetate (2:1). The non-polar target compound was scraped off the plate and the silica gel eluted with dichloromethane.

4.3.4. Isolation of compound 3 from the butanol fraction.

4.3.4.1. Gradient column chromatography of the Butanol (BuOH) fraction.

The butanol fraction was subjected to column chromatography (10 x 5 cm) on 100 g silica gel 60 (Merck) eluted with 100% dichloromethane and then with dichloromethane enriched gradually with 20 % methanol up to 100% methanol. Fractions 7-11 were combined and allowed to precipitate as white crystals to give compound 3

4.4. Results and Discussion

4.4.1. Bulk extraction yield

The total crude extract from the bulbs of *A. coranica* (36.14 g) represented 18% total yield of the plant material extracted (200 g). The percentage yield of the solvent-solvent fractionation of the ethanolic crude extract are presented in figure (4.2). The acidic and the neutral fractions extracted with DCM represented 13.2% (2.11 g), while the basic butanol and ethyl acetate fractions represented 6.1% (0.98 g) and 18.8% (3 g) of the total extract respectively.

4.4.2. TLC fingerprinting ,Dragendorff's test and TLC bioautographc assay of the fractions of the ethanolic crude extract of the bulbs of *A. coranica*.

Several blue fluorescent and dark spots against a green background were observed when the chromatogram was viewed under UV light at 254 nm (Figure 4-3 A). At 365 nm the spots had a light blue fluorescence. The butanol fraction had more moderately polar to very polar compounds, while more non-polar compounds with similar R_f values were seen in the BuOH, DCM, and EtOAc fractions respectively (Figure 4-3 A, B and C). No fluorscence was observed from the aqueous fraction under any of tthe UV wavelengths (Figure 4-3 A,B, and C). Dragendorff's test revealed orange spots in the BuOH, DCM, and EtOAc fractions, but not in the aqueous fraction (Figure 4-3 C). This indicate that the BuOH, DCM, and the ethyl acetate fractions contained alkaloids. TLC bioautographic assay of the various fractions, revealed white spots indicative of acetylcholinesterase inhibitory activity against a yellow background with similar R_f values to the alkaloidal spots. There were no inhibitory spots in the aqueous fraction confirming that the aqueous fraction did not contain compounds with AChE inhibitory

activity, while the inhibitory spots on the DCM fractions were very faint, thus indicating that the acidified DCM did not contain much of AChE inhibitory compounds. The BuOH and EtOAc fractions obtained after basification had strong acetylcholinesterase inhibitory spots indicating a high concentration of AChE compounds. The high acetylcholinesterase inhibitory activity of the BuOH and the EtOAc fractions makes them the obvious choice for the isolation of compounds with AChE inhibitory activity.

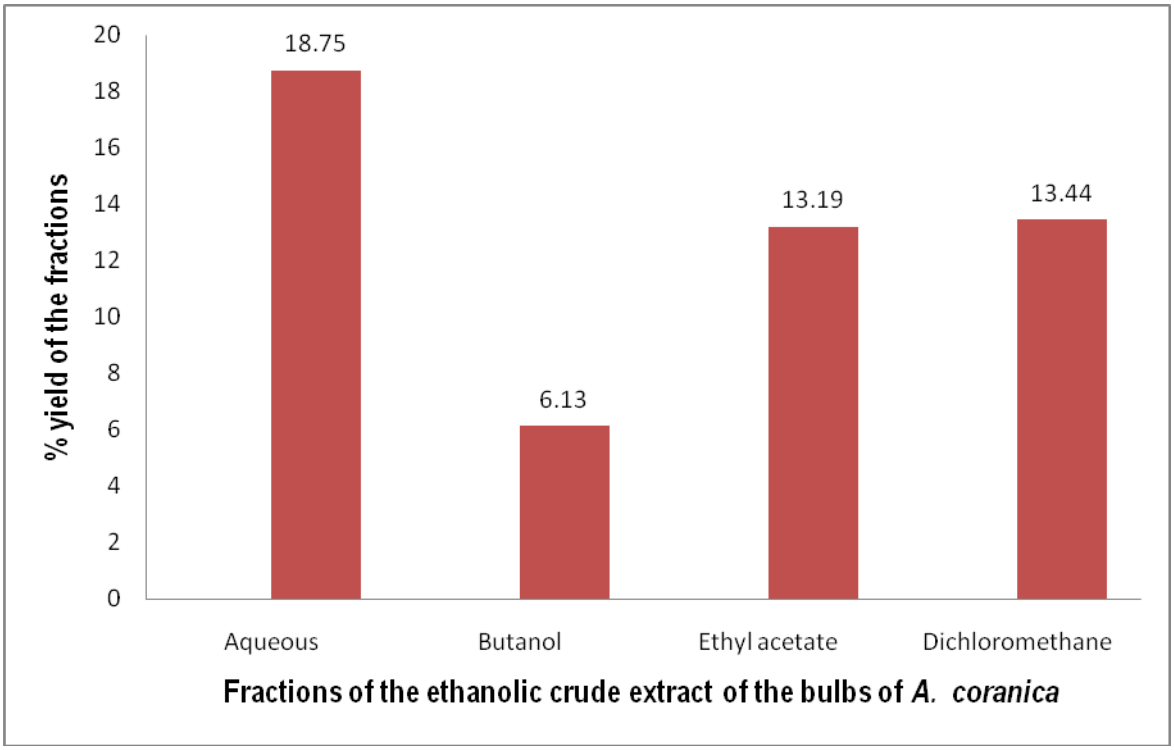

Figure 4-2: Yields of the fraction obtained from the solvent-solvent fractionation of ethanolic crude extract of the bulbs of *A. coranica* .

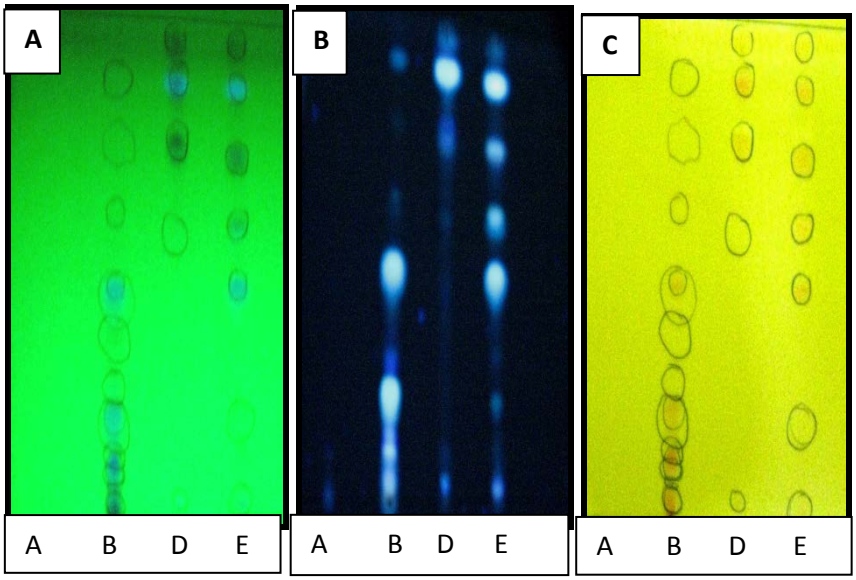

Figure 4-3: Chemical profile of the various fractions of *A. coranica* using (A) UV view of the fractions at 254 nm (B) UV at 365 nm (C) Dragendorff's reagent (A= Aqueous, B= Butanol, D= Dichloromethane and E= Ethyl acetate fractions).

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Figure 4-4: TLC bioautographic assay of the fractions showing white areas of inhibition against a yellow background. All the plates were eluted using chloroform: methanol (4:1) as the eluent. (B- Butanol, D-= Dichloromethane, E= Ethylacetate and P= Physostigmine respectively).

4.4.3. Microplate assay of the fractions

The minimum concentration of ethyl acetate and butanol fractions that inhibited 50% (IC_{50}) of acetylcholinesterase enzyme activity were quantitatively determined using microplate reader at 405 nm. The IC_{50} of the butanol fraction was 0.05 ± 0.02 µg/ml, while that of the ethylacetate fraction was 43.1

± 1.22 µg/ml (Table 4-1). The butanol fraction had high activity, which could be attributed to the number of active compounds present, as observed from the TLC bioautographic assay and, assuming that the active compounds are alkaloids, the Dragendorff's test (Figure 4-3 A, B, C and Figure 4-4 A). The ethyl acetate fraction was not as active as the butanol fraction. The microplate assay which is a sensitive quantitative assay confirmed the acetylcholinesterase inhibitory activity qualitatively assessed using the TLC bioautographic assay. Physostigmine used as the positive reference compound in the assay had an IC_{50} of 1.51µg/ml (5.5 ± 0.78 µM). The presence of several active compounds in the butanol fraction might have acted synergistically to inhibit the activity of the enzyme. Thus, the butanol and the ethyl acetate fraction were targets for the isolation of active alkaloids.

Table 4-1: The minimum concentration (µg/ml) inhibited 50 % of AChE (IC_{50}) of the butanol and ethyl acetate fractions of the ethanolic crude extract of the bulbs of *A. coranica*, expressed as mean ± standard error mean (SEM).

Fraction	IC_{50} (µg/ml)
Butanol	0.05 ± 0.02
Ethyl acetate	43.1 ± 1.22
Physostigmine (reference compound)	1.51

4.4.4. Isolation of compound 2 from sub-fraction C of the ethyl acetate fraction of the bulbs of *A. coranica.*

Sub-fraction C (tubes 19-35) of the ethyl acetate fraction (120.9 mg) developed on preparative TLC, yielded 22.6 mg (18.7%) of a white crystalline powder, which appeared as a deep purple compound when stained with the *p*-anisaldehyde spray reagent, and light blue fluorescent compound when viewed under UV light at 365 nm. The compound did not react with the Dragendorff's reagent.

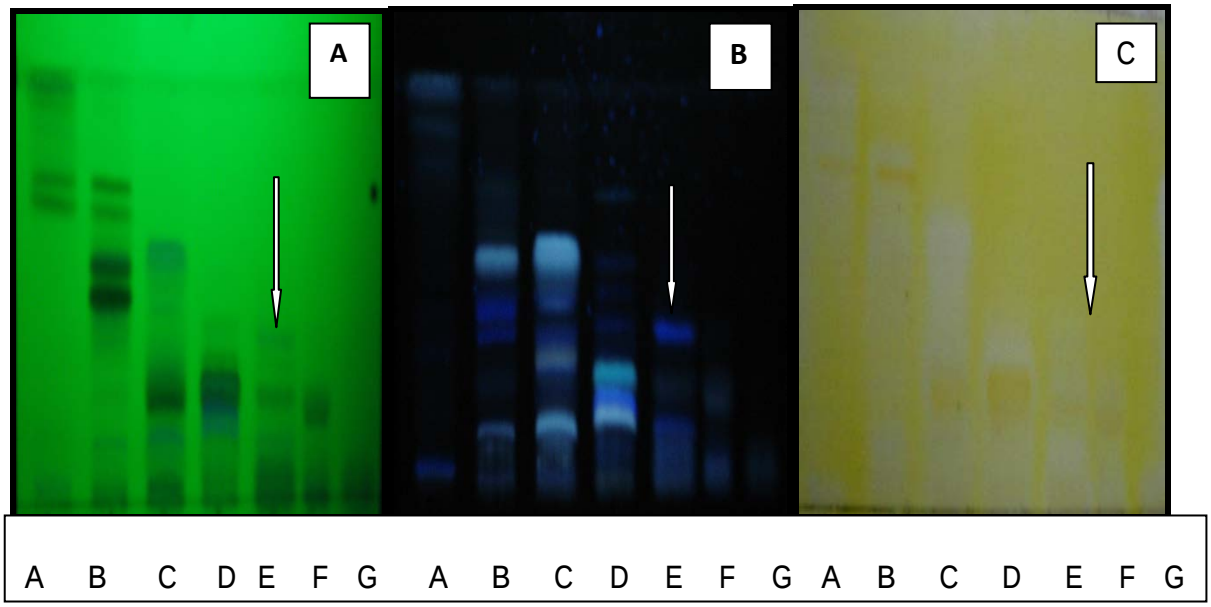

Figure 4-5: Combined sub-fractions of the ethyl acetate fraction of the crude extract of *A. coranica* viewed under UV light at 254 nm (A), 365 nm (B) and TLC bioautographic assay with white areas of inhibition. The arrows indicate the location of compound isolated from both the EtOAc and BuOH fractions.

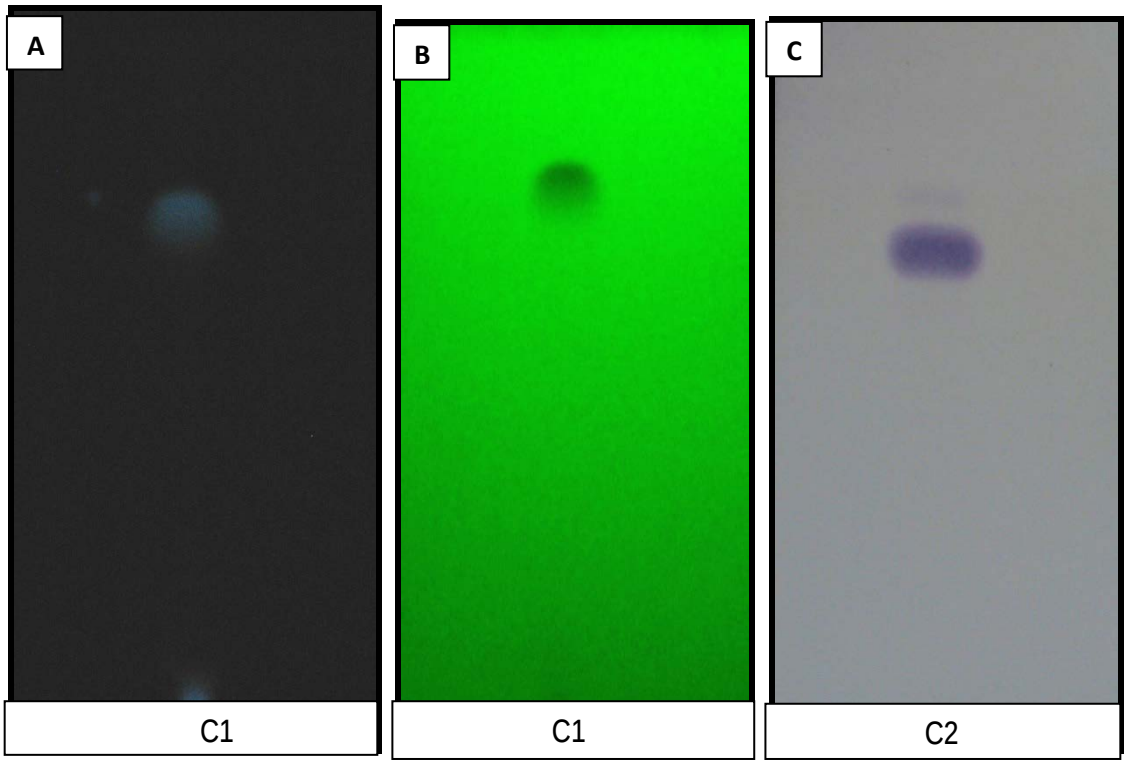

Figure 4-6: A and B- Ultraviolet light response of compound 1 at 365 nm (blue fluorescence) and 254 nm (dark spot). Chromatogram developed in chloroform:methanol (3:1). C- Purple band of compound 2 when stained with *p*-nisaldehyde spray reagent, chromatogram developed in Hexane: Ethyl acetate (2:1).

4.4.5. Gradient column chromatography of butanol fraction of the bulbs of *A. coranica.*

Tubes 1 (A_B), 3-6 (B_B), 7-11 (C_B), 12-14 (D_B), 15-25 (E_B), 33-46 (F_B), were combined based on their similar phytochemical characteristics and acetylcholinesterase inhibitory activities. The crystals formed in sub-fraction C_B were processed using methods described in section 4.5.6., yielding 10 mg white crystals. Preliminary results from nuclear magnetic resonance (NMR) and mass spectroscopy (MS) revealed that the two crystalline compounds isolated from the ethyl acetate and butanol fractions are the same alkaloid. Compound 2, was identified as a triterpenoid.

4.5. Conclusion

The four fractions of the ethanolic crude extracts of the bulbs of *A. coranica* had varying degrees of acetylcholinesterase inhibitory activities. Butanol and ethyl acetate fractions had inhibitory activities against the enzyme. However, the butanol fraction, which contained more alkaloids, exhibited high activity (Figure 4-2 C and Table 4-1). The dichloromethane and aqueous fractions did not have significant inhibitory activity as evident from the TLC bioautographic assay (Figure 4-3 A). Results from the TLC bioautographic test led to quantitative analysis of the BuOH and EtOAc fractions. The microplate assay results confirmed the qualitative investigations; with the BuOH having IC_{50} of 0.05 ± 0.02 µg/ml. Compound 1 was isolated from the EtOAc and the BuOH fractions; as white crystals that appeared as a dark spot and blue fluorescence when viewed at 254 nm and 365 nm respectively (Figure 4-6 A and B). Compound 2 was isolated from the EtOAc fraction; it is a non-polar, white crystalline substance that appeared purple when stained with the *p*-anisaldehyde spray reagent. In the next chapter an attempt will be made to determine the structures of the isolated compounds using NMR spectroscopy.

Chapter 5

Structural elucidation of compounds isolated from ethyl acetate and butanol fractions of *Ammocharis coranica* (Ker. Gawl).

5.1. Introduction

Bioactive natural products are frequently isolated using bioassay guided fractionation; a technique considered rate limiting and capital intensive (Butler, 2004). However, introduction of efficient instrumentation and other automated devices, such as high performance liquid chromatography (HPLC), mass spectroscopy (MS), and column technology has improved the speed of isolation and structural elucidation of natural products (NPs). The advent of new probe technology (Keifer, 2003) and higher magnetic fields has reduced remarkably, the acquisition time for nuclear magnetic resonance (NMR) data, and the structure elucidation of NPs can be achieved routinely on amounts less than 1 mg (Reynolds and Enriquez, 2002), this however is dependent on the frequency of the NMR machine, the purity and solubility of the compound isolated (Holzgrabe *et al*., 1998). In addition to sophisticated apparatus a high level of technical expertise is required that is frequently not available in developing countries.

Developments in NMR techniques during the last two decades have helped in discovering many new natural organic compounds (Topcu and Ulubelen, 2007). NMR is an imaging tool used to identify and/or elucidate detailed structural information of chemical compounds, newly synthesised or isolated from plants and microbes (Holzgrabe *et al*., 1998). NMR is also used quantitatively to analyse the purity profile of a drug or a compound; characterisation of the composition of a drug product and investigating metabolites of a drug in body fluids (Holzgrabe *et al*., 1998). Other uses include, identifying contaminants in food, cosmetics, or medications, and helping researchers determine the site of chemical reaction in a molecule. The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. The nuclei, in a molecule all align in the same direction when a very strong magnetic field is generated using a superconducting electromagnet at very low temperatures (Holzgrabe *et al*., 1998). An NMR spectrum appears as a series of vertical peaks/signals distributed along the x-axis of the spectrum, from the energy transfer which corresponds to radio frequency. Each of these signals corresponds to an atom within the molecule. The position of each signal in the spectrum gives information about the structural environment of the atom producing the signal. Organic compounds are composed mainly of the elements hydrogen, carbon, phosphorus, nitrogen and oxygen. Additionally, there are the halogens fluorine, chlorine, bromine and iodine and sometimes metal atoms. Their isotopic nuclei can be detected using NMR spectroscopy. The most common nuclei observed

using this technique are ^{1}H and ^{13}C, but also ^{31}P, ^{19}F, ^{29}Si and ^{77}Se NMR are available (Holzgrabe et al., 1998; Reynolds and Enriquez, 2002).

5.2. Materials and methods.

The Varian Unit Innova 300 MHz NMR system and Brüker DRX-400 were the NMR instruments used in the ^{13} C and ^{1} H NMR spectroscopy. The compounds isolated from *A. coranica* (Chapter 4) were weighed and dried to yield compound 1 (20 mg) and compound 2 (10 mg). These were dissolved in deuterated dimethylsulphoxide (DMSO-d_6) and deuterated chloroform ($CDCl_3$) respectively. Solutions were loaded into NMR tubes using Pasteur pipettes. After which, NMR spectroscopic analysis were carried out.

5.3. Results and discussion.

5.3.1. Structural elucidation of compound 1 isolated from ethyl acetate and butanol fractions of *A. coranica.*

The compound was isolated from the polar fractions of the plant extract, as colourless crystals and identified based on the spectroscopic data. The ^{1}H- NMR revealed three methylene group signals of C-4, -5, -7 and peaks of H-8, H-11 at 6.79, 6.66, signal of a methylenedioxy group (H-12) at 5.94, signals of H-1, H-2, and H-3 appears at 4.90, 4.26 and 5.35 respectively; in addition to the signals of three methylene groups of H-4, -5 and -7 between 4.00-2.20. The ^{13}C NMR exhibited 16 carbons, four of them were methylene groups at 100.6, 56.7, 53.3 and 28.1 of C-12, -7, -5, -4 and seven methine groups at 118.5, 107.1, 105.1, 71.7, 70.2, 60.8, and 40.2 of C-3, -8, -11, -2, -1, 11c, 11b (Figure 5-1). The data obtained was similar to those published in literature for lycorine (Evidente *et al*., 1983; Schultz *et al*., 1996; Toriizuka *et al*., 2008).

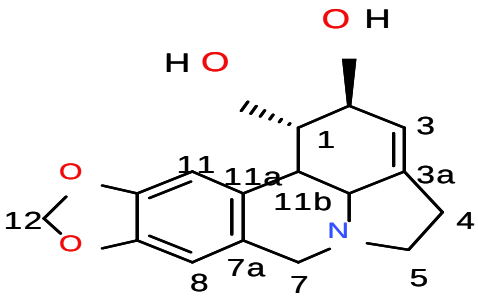

Figure 5-1: The chemical structure of lycorine (1).

5.3.2. Structural elucidation of compound 2 isolated from the ethyl acetate fraction of ethanolic crude extract of the bulbs of *A. coranica*.

The second compound was isolated from the non-polar part of the extract, as colourless crystals and exhibited characteristic purple colour of terpenoids, when sprayed with the vanillin/H_2SO_4 reagent. The chemical structure was established using spectroscopic data, which correlated with the data published and literatures on the same species (Teresa *et al*., 1987; Koornabally *et al.*, 2000; Mesquita *et al*., 2008).

The NMR data revealed in the ^{1}H NMR spectra signals were attributed to H-3 proton at 3.19, two proton singlet of CH_2-28 at 4.69, 4.64, high field signals at 0.13, 0.36 of CH_2-19, in addition to un-resolved seven methyl groups signals around 1.00 ppm. The ^{13}C NMR data confirmed the presence of the terminal double bond at 157.3, 106.3, and the hydroxylated C-3 at 76.5. The remaining data could not be resolved due to the compactness of the spectra. Previous work on the same species by Koorbanally *et al*. (2000) isolated the same compound. This fact and the similarity of the recorded data with the published ones established the structure of the isolated compound as 24-methylenecycloartan-3β-ol.

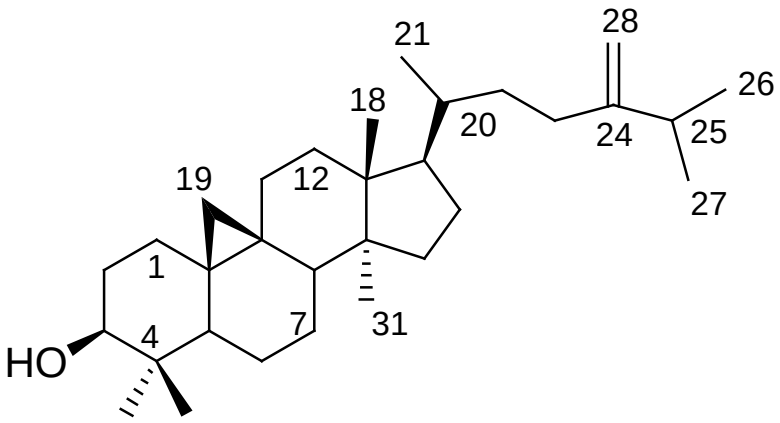

Figure 5-2: The chemical structure of 24-methylenecycloartan-3β-ol (2).

5.4. Conclusions.

The structural elucidation of the isolated compounds was limited to the NMR spectroscopy and comparison of NMR structures to previous work of other researchers. Lycorine (alkaloid) and 24-methylenecycloartan-3β-ol (triterpenoid) were the two compounds elucidated. See Appendix 1 and 2 for NMR spectra of the isolated compounds. The biological activities of these compounds are discussed in the following chapter.

CHAPTER 6

Acetylcholinesterase (AChE) inhibitory activities of lycorine and 24-methylenecycloartan-**3β**-ol, isolated from ethylacetate and butanol fractions of the bulbs of *Ammocharis coranica* (Ker-Gawl.) Herb.

6.1. Introduction

Lycorine (2,4,5,7,12b,12c-hexahydro-1H-[1,3]dioxolo- [4,5-j]pyrolo[3,2,1-de]phenathridine-1,2-diol) is a toxic natural alkaloid present in various Amaryllidaceae plants (Ghosal *et al*., 1985; Lin *et al*., 1995). It is well documented that lycorine inhibits growth and cell division in higher plants, algae, and yeasts, by interfering with the peptidyltransferase centre on ribosomes (De Leo *et al.*, 1973 A; De Leo *et al.*, 1973 B; Arrigoni *et al.*, 1975; Jimenez *et al*., 1976; Kukhanova *et al*., 1983 Arrigoni *et al.*, 1994) and has antimalarial, antitumour , anti-inflammatory and AChE inhibitory activities (Ghosal *et al*., 1985; Likhitwitayawuid *et al*., 1993; Elgorashi *et al.*, 2004; Houghton *et al* ., 2004; Liu *et al*., 2004). Lycorine and haemanthamine isolated from *Hymenocallis littoralis* (Amaryllidaceae) have potent cytotoxic effect against eleven different cell lines (Lin *et al.*, 1995).

Triterpenoids are secondary metabolites isolated in the non-polar fractions of plant extracts (Mukherjee *et al*., 1990; Singla and Pathak, 1990). Apart from the isolation of 24-methylenecycloartan-3β-ol, also known as 24-methylenecycloartanol (Mitova *et al*., 2003) from the bulbs of *Ammocharis coranica*, it has been identified in the bulbs of *Crinum augusturn* (Tram *et al.,* 2002), an Amaryllidaceae. There is however no report on the inhibitory effect of 24-methylenecycloartan-3β-ol on AChE. The compound has been reported to have the following biological activities: anti-inflammatory (Akihisa *et al*., 1996; Yasukuwa *et al*., 1998), inhibition of tumour promotion (Yasukuwa *et al*., 1991; Yasukuwa *et al*., 1995) and anti-hypercholesterolaemia (Kiribuchi *et al*., 1983). When tested for their *in vitro* inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumour promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells; 24-methylenecycloartanol, along with forty-seven other cycloartane type and related triterpenoids, caused high viability (70%) of Raji cells even at 32 *nM* (Mol ratio of compound to TPA= 1000:1), indicating their low cytotoxicity at high concentration (Kikuchi *et al*., 2007).

Lycorine and 24-methylenecycloartan-3β-ol isolated from the bulbs of *A. coranica* in this project were not subjected to cytotoxicity tests due to evidence of their cytotoxic effect from other plants (Lin *et al.*, 1995; Kikuchi *et al*., 2007).

6.2. Materials and method

6.2.1. Determination of AChE inhibitory activity of Lycorine and 24-Methylenecycloartan-**3β**-ol using the Microtitre plate assay.

The IC_{50} of AChE by lycorine and 24-methylenecycloartan-3β-ol were determined using the microplate assay as described in sections 3.2.4.13 and 3.2.4.14 respectively.

6.3. Results and discussion.

6.3.1. IC_{50} of acetylcholinesterase of the isolated compounds.

In our experiment the IC_{50} of lycorine was 29.4 ± 3.15 µg/ml (102 ± 7.8 *µM*). This is the first report of IC_{50} of lycorine isolated from *A. coranica*. Lycorine isolated from other Amaryllidaceae plants have also been tested for AChE inhibitory activity. Elgorashi *et al*. (2004) found that the IC_{50} of Lycorine isolated from *Crinum macowanii* was 213 µ*M*, while Houghton *et al*. (2004) established an IC_{50} of 450 µ*M*, from lycorine isolated *Crinum jagus* and *C. glaucum* from south-western part of Nigeria. The discrepancies in the IC_{50} of lycorine are obvious, even from plants of the same genus. The differences in the method and conditions adopted in the independent experiments and the solubility and purity of lycorine in methanol could be attributable. Although DMSO is a better solvent for lycorine, its use is avoided because it has been found to inhibit acetylcholinesterase (Giovanni *et al*., 2008). Although the calculated values of the three independent IC_{50} of lycorine varied, the most consistent deduction from the three experiments is that lycorine has appreciable inhibitory activity against AChE. In the experiment carried out at the Phytomedicine laboratory, physostigmine (positive control) is about 68 times a stronger inhibitor of AChE than lycorine. The fact that lycorine is toxic (Ghosal *et al*., 1985; Lin et al., 1995) and has weak inhibitory effect on AChE (Elgorashi *et al*., 2004; Houghton *et al*., 2004) makes it a less suitable candidate for the development of an AD drug. Physostigmine (eserine) was first isolated from the seeds of *Physostigma venenosum* (Fabaceae) in the nineteenth century and has long been known as an AChE inhibitor (Shu, 1998). It is also reported to have significant cognitive benefits in both normal and Alzheimer's patients (Shu, 1998). Clinical use has been limited by the very short *in vivo* half-life of the compound. Adverse effects of physostigmine are mainly gastrointestinal complaints, with high rates of withdrawal from treatments (Coelho and Birks, 2001). The chemical structure of physostigmine provided a template for the development of rivastigmine, a long acting reversible and competitive inhibitor that is indicated as an oral treatment for patients with mild to moderately severe Alzheimer's disease (Polinsky, 1998). Lycorine and the galanthamine alkaloids have been isolated from quite a number of plants from the Amaryllidaceae family. In a similar study, Lopez *et al*.(2002 screened 26 extracts prepared from various *Narcissus* species along with 23 pure Amaryllidaceae-type alkaloids

against acetylcholinesterase and reported that the alkaloids having galanthamine and lycorine skeletons possess inhibitory activity (Lopez *et al*., 2002).

The second compound, 24-methylenecycloartan-3β-ol, did not exhibit acetylcholinesterase inhibitory activity. The negative values indicate that the compound had no inhibitory activity against AChE at the concentration used in the microplate assay, although it showed zone of inhibition when screened using the thin layer chromatography bioautographic assay. This is however, the first report of the test of this compound for AChE inhibitory activity.

6.4. Conclusion

Lycorine isolated from the bulbs of *A. coranica* possesses AChE inhibitory activity. Quantitative assay using the microplate assay showed an IC_{50} of 29.4 ± 3.15 µg/ml (102 ± 7.8 *µM*). The second compound 24-methylenecycloartan-3β-ol, however, did not show AChE inhibitory activity using the quantitative method. This is the first report of the assessment of the activity of this compound against AChE, based on intensive literature search. The cytotoxicity test of these compounds and active butanol fraction was not carried out in this research, however available literature information indicate that lycorine has cytotoxic effect on several cell lines, while 24-methylenecycloartan-3β-ol, has very low effect on cells even at high concentrations. There might also be some level of cytotoxicity in the other fractions which contain lycorine. Re-establishment of the AChE inhibitory activity of lycorine confirms the works of Elgorashi *et al*. 2004 and Houghton *et al*. 2004 respectively. However, there were differences in the IC_{50} values of the independent experiments due to likely differences in the methods, conditions and solubility of the isolated compound.

Chapter 7

General Conclusions

The aim of this project was to screen the bulbs of *Ammocharis coranica* (Ac) for acetylcholinesterase (AChE) inhibitory activity, isolate and elucidate structures of active compounds and investigate their biological properties. Preliminary screening and subsequent tests were conducted using TLC bioautographic (TLCBA) and microplate assays (MPA) based on Ellman's method.
Ethanolic crude extract of the bulbs of *A. coranica* like other members of the Amaryllidaceae family showed AChE inhibitory activity. The preliminary thin layer chromatography bioautographic assay investigation of the ethanolic crude extract of the bulbs of *A. coranica* showed several white spots of inhibition, confirmed not to be false positives. Quantitative determination of AChE inhibitory activity of the ethanolic crude extracts of the bulbs of *A. coranica* using the microplate assay produced promising results, the crude extracts had IC_{50} 14.3 ± 0.50 µg/ml.

The residue of the dried ethanolic crude extracts of the bulbs of *A. coranica* (36.14 g) subjected to acid-base fractionation represented 18% total yield of the plant material extracted (200 g). The acidic and the neutral fractions extracted with dichloromethane (DCM) represented 13.2% (2.11 g), while the basic butanol, ethyl acetate and aqueous fractions represented 6.1% (0.98 g), 18.8% (3 g) and18.75% of the total extract respectively.

The fractions tested for AChE inhibitory activity using thin layer chromatography bioautographic and microplate assays had varying degrees of activities. Butanol and ethyl acetate fractions were active against the enzyme. However, the butanol fraction which had more bands of alkaloids on the TLC plate when sprayed with Dragendorff's reagent exhibited high activity. Dichloromethane and aqueous fractions showed very week inhibitory activity as evident in the thin layer chromatography bioautographic assay. The microplate assay results showed BuOH, EtOAc fractions having IC_{50} 0.05 ± 0.02 and 43.1 ± 1.22 µg/ml respectively. The butanol fraction was about 30 times more active than the positive reference compound-Physostigmine, which had IC_{50} 1.51 µg/ml, while the ethyl acetate fraction was about 28 times less active than physostigmine. The promising results obtained from butanol and ethylacetate fractions made them the primary targets for the isolation of active compounds.

Compound 1 was isolated from the EtOAc and the BuOH fractions; as a polar white crystal, while Compound 2 was isolated from the EtOAc fraction as a non-polar, colourless crystalline substance that

appeared purple when stained with the *p*-anisaldehyde reagent. The structural elucidation of the isolated compounds was limited to the NMR spectroscopy and comparison of NMR structures to previous work of other researchers. Compound 1 was identified as Lycorine (alkaloid) and compound 2 was elucidated as 24-methylenecycloartan-3β-ol (triterpenoid).

The two isolated compounds, Lycorine (1) and 24-methylenecycloartan-3β-ol (2), when tested for AChE activity using the microplate assay. Lycorine showed an IC_{50} of 29.4 ± 3.15 µg/ml (102 ± 7.8 μM), while 24-methylenecycloartan-3β-ol did not show AChE inhibitory activity. This is the first report of the assessment of the AChE inhibitory activity of 24-methylenecycloartan-3β-ol.

The cytotoxicity test of these compounds and active butanol fractions was not performed in this research. However, available literature information revealed that lycorine is cytotoxic on several cell lines, while 24-methylenecycloartan-3β-ol has very low effect on cells even at high concentrations. It is possible that the butanol fraction may have therapeutic uses if it is not toxic and the results imply that substantial synergism may have existed between different compounds in the butanol fraction. The 24-methylenecycloartan-3β-ol (2) was not active when tested using the microtitre plate assay although it did inhibit colour development in the bioautography assay.

The re-establishment of the AChE inhibitory activity of lycorine confirmed the works of Elgorashi *et al*. 2004 and Houghton *et al*. 2004 respectively. However, the differences in the IC_{50} values of the independent experiments are likely due to difficulty of lycorine to dissolve in most organic solvents.

References

Abramov, A.Y., Canevari, L., Duchen, M.R., 2004. "Calcium signals induced by amyloid beta peptide and their consequences in neurons and astrocytes in culture". Biochim Biophys Acta 1742 (1-3): 81–87.

Adams, M., Gmünder, F., Hamburger, M., 2007. Plants traditionally used in age related brain disorders—a survey of ethnobotanical literature. Journal of Ethnopharmacology 113: 363–381.

Akihisa, T., Yasukawa, K., Oinuma, H., Kasahara, Y., Yamanouchi, S., Takido, M., Kumaki, K., Tamura, T., 1996. Triterpene alcohols from the flowers of Compositae and their anti-inflammatory effects. Phytochemistry, 43 (6): 1255-1260.

Arrigoni, O., 1994. Ascorbate system in plant development. Journal of Bioenergetics and Biomembranes 26: 407–419.

Arrigoni, O., Arrigoni, R., Calabrese, G., 1975. Lycorine as an inhibitor of ascorbic acid biosynthesis. Nature 256: 513–514.

Aslanov, K.A., Kushmuradov, Y.K., Sadykov, A.S., 1987. Lupine alkaloids. In: The alkaloids, ed. Brossi, A., Vol. 31, pp. 117-192, Academic Press, New York.

Bai, D.L., Tang, X.C., He, X.C., 2000. Huperzine A: a potential therapeutic agent for treatment of Alzheimer's disease. Current Medicinal Chemistry 7 (3): 355-374.

Barghorn, S., Nimmrich, V., Striebinger, A., 2005. "Globular amyloid beta-peptide oligomer - a homogenous and stable neuropathological protein in Alzheimer's disease". Journal of Neurochemistry 95 (3): 834–847.

Blanchard, B.J., Hiniker, A.E., Lu, C.C., Margolin, Y., Yu, A.S., Ingram, V.M., 2000. "Elimination of Amyloid beta Neurotoxicity". Journal of Alzheimer's Disease 2 (2): 137–149.

Bourne, Y., Taylor, P., Marchot, P., 1995. Acetylcholinesterase inhibition by fasciculin: crystal structure of the complex. Cell 83: 503–512.

Brain with Alzheimer's disease.www.ahaf.org.Accessed November 2009.

Brown, A.G., 1991. The nerve cells and the nervous system: An introduction to neuroscience. Springer-Verlag: 1-252.

Brühlmann, C., Marston, A., Hostettman, K., Carrupt, P.A., Testa, B., 2004. Screening of non-alkaloidal natural compounds as acetylcholinesterase inhibitors. Chemical Biodiversity 1: 819-829.

Burns, A., Iliffe, S., 2009. Alzheimer's disease. British Medical Journal 338 (b158): 467-471.

Butler, M.S., 2004 .The role of natural product chemistry in drug discovery. Journal of Natural Product 67: 2141-2153.

Chandra, V., Ganguli, M., Ratcliff, G., 1994. Studies of the epidemiology of dementia: Comparisons between developed and developing countries. Aging Clinical Experimental Research 6: 307–321.

Chemical synapse- Wikipedia, the free encyclopedia. Cholinergic synapse. Accessed August 2009 http://en.wikipedia.org/wiki/File:chemical synapse schema.

Coelho, F., Birks, J., 2001. Physostigmine for Alzheimer's disease. Cochrane Database Systematic Review (2): CD001499.

Cordell, G.A., Colvard, M.D., 2005. Some thoughts on the future of Ethnopharmacology. Journal of Ethnopharmacology 10 (1-2): 5-14.

Corey-Bloom, J., Anand, R., Veach, J., 1998. A randomised trial evaluating the efficacy and safety of ENA 713 (rivastigmine tartrate), a new acetylcholinesterase inhibitor, in patients with mild to moderately severe Alzheimer's disease. International Journal of Geriatric Psychopharmacology 1: 55-65.

Court, J.A., Perry, E.K., 1991. Dementia: the neurochemical basis of putative transmitter orientated therapy. Pharmacology and Therapeutics 52: 423-443.

Cummings, J.L., 2004. Alzheimer's disease. The New England Journal of Medicine 351: 56-67.

De Leo, P., Dalessandro, G., De Santis, A., Arrigoni, O., 1973 A. Inhibitory effect of lycorine on cell division and cell elongation. Plant and Cell Physiology 14: 481–486.

De Leo, P., Dalessandro, G., De Santis, A., Arrigoni, O., 1973 B. Lycorine as an inhibitor of ascorbic acid biosynthesis. Plant and Cell Physiology 14: 487–496.

De Villiers, C., Louw, S.J., 1996. Determining the prevalence of Alzheimer's disease in elderly South Africans. South African Medical Journal 86: 135–136.

Eichler, J., Anselmet, A., Sussman, J.L., Massoulie, J., Silman, I., 1994. Differential effects of 'peripheral' site ligands on Torpedo and chicken acetylcholinesterase. Molecular Pharmacology 45: 335–340.

Elgorashi, E.E., Malan, S.F, Stafford, G.I., Van Staden, J., 2006. Quantitative structure- activity relationship studies on acetylcholinesterase enzyme inhibitory effects of amaryllidaceae alkaloids. South African Journal of Botany 72: 224-231.

Elgorashi, E.E., Stafford, G.I., Van Staden, J., 2004. Acetylcholinesterase enzyme inhibitory effects of amaryllidaceae alkaloids. Planta Medica 70: 258-260.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemistry and Pharmacology 7: 88-95.

Esch, F.S., Keim, P.S., Beattie, E.C., 1990. Cleavage of amyloid beta peptide during constitutive processing of its precursor. Science 248: 1122-1124.

Esiri, M.M., 1996. The basis for behavioural disturbances in dementia. Journal of Neurology, Neurosurgery and Psychiatry 61: 127-130.

Evidente, A., Cicala, M.R., Giudicianni, I., Randazzo, G., Riccio, R., 1983. ^{1}H and ^{13}C NMR analysis of lycorine and a-dihydrolycorine. Phytochemistry 22: 581–584.

Exelon® (rivastigmine tartrate), 2000. Capsules prescribing information. East Hanover, NJ: Novartis Pharmaceuticals Corporation.

Fennell, C.W., Lindsey, K.I., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, M.O., Van Staden, J., 2004. Assessing African medicinal plants for efficacy and safety: Pharmacological screening and toxicology. Journal of Ethnopharmacology 94: 205-217.

Fennell, C.W., Van Staden, J., 2001. *Crinum species* in traditional and modern medicine. Journal of Ethnopharmacology 78: 15-26.

Francis, P.T., Palmer, A.M., Snape, M., Wilcock, G.K., 1999. The cholinergic hypothesis of Alzheimer's disease: a review of progress. Journal of Neurology Neurosurgery and Psychiatry 66: 137-147.

Gerstner, J., 1941. A preliminary check list of Zulu names of plants, with short notes. Bantu Studies 15 (4): 369-383.

Geula, C., Mesulam, M.M., 1994. Cholinergic systems and related neuropathological predilection patterns in Alzheimer disease. In: Terry RD, Katzman R, Bick KL, eds. Alzheimer Disease. New York, NY: Raven Press: 263-291.

Geula, C., Wu, C.K., Saroff, D., 1998. Aging renders the brain vulnerable to amyloid ß-protein neurotoxicity. Nature Medicine 4: 827-831.

Ghosal, S., Saini, K.S., Razdan, S., 1985. Crinum alkaloids: their chemistry and biology. Phytochemistry 24: 2141–2156.

Giess, W., Snyman, J.W., 1972. The naming and utilization of plant life by the Zul'hoasi Bushmen of the Kau-Kauveld, Verlag, Hamburg.

Giovanni, S.D., Borloz, A., Urbain, A., Marston, A., Hostettmann, K., Carrupt, P., Reist, M., 2008. *In vitro* screening assays to identify natural or synthetic acetylcholinesterase inhibitors: Thin layer chromatography versus microplate methods. European Journal of Pharmaceutical Sciences 33: 109-119.

Gray, E.G., Paula-Barbosa, M., Roher, A., 1987. "Alzheimer's disease: paired helical filaments and cytomembranes". Neuropathology and Applied Neurobiology 13 (2): 91–110.

Greenblatt, H.M., Dvir, H., Silman, I., Sussman, J.L., 2003. Acetylcholinesterase: a multifaceted target for structure-based drug design of anticholinesterase agents for the treatment of Alzheimer's disease. Journal of Molecular Neuroscience 20: 369–383.

Gregory, G.C., Halliday, G.M., 2005. "What is the dominant Abeta species in human brain tissue? A review". Neurotoxicity Research 7 (1-2): 29–41.

Guillou, C., Mary, A., Zafiarisoa, D., Reno, D., Gras, E., Thai, C., 2000. Potent acetylcholinesterase inhibitors: design, synthesis and structure–activity relationships of alkylene linked bis-galanthamine and galanthamine–galanthaminium salts. Bioorganic Medicinal and Chemical Letters 10: 637–639.

Guillozet, A.L., Smiley, J.F., Mash, D.C., 1997. Butyrylcholinesterase in the life cycle of amyloid plaques. Annals of Neurology 42: 909-918.

Guyton, A.C., Hall, J.E., 1996. Textbook of Medical Physiology, 9th Edition: 563-582.

Harborne J.B., 1980. Phytochemical Methods -A Guide to Modern Techniques of Plant Analysis, Chapman and Hall, London, 278 pp.

Harel, M., Kleywegt, G.J., Ravelli, R.B.G., Silman, I., Sussman, J.L., 1995. Crystal structure of an Acetylcholinesterase-fasciculin complex: Interaction of a three-fingered toxin from snake venom with its target. Structure 3: 1355-1366.

Hauth, H., Stauffacher, D., 1962. Die alkaloide von *Ammocharis coranica* (Ker-Gawl.) Herb. Emend. M-Redh. Helvetica Chimica Acta 44: 491-502.

Heinrich, M., Teoh, H.L., 2004. Galanthamine from snowdrop: The development of a modern drug against Alzheimer's disease from local Caucasian knowledge. Journal of Ethnopharmacology 92: 147-162.

Holzgrabe, U., Diehl, B.W.K., Wawer, I., 1998. NMR spectroscopy in pharmacy. Journal of Pharmaceutics and Biomedical Analysis 17(4-5): 557-616.

Hostettmann, K., Borloz, A., Urbain, A., Marston, A., 2006. Natural product inhibitors of acetylcholinesterase. Current Organic Chemistry 10: 825-847.

Houghton, P.J., Agbedahunsi, J.M., Adegbulugbe, A., 2004. Cholinesterase inhibitory properties of alkaloids from two Nigerian Crinum species. Phytochemistry 65: 2893-2896.

Houghton, P.J., Ren, Y., Howes, M.J., 2006. Acetylcholinesterase inhibitors of plant and fungi. Natural Product Report 23: 181-199.

Howes, M.J.R., Perry, N.S.L., Houghton, P.J., 2003. Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders. Phytotherapy Research 17: 1–18.

Hulme, M., 1954. Wild flowers of Natal. Shuter and Shooter, Pietermaritzburg.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A.B., 1996. Zulu medicinal plants: An inventory. University of Natal press, Pietermaritzburg.

Iijima, K., Liu, H.P., Chiang, A.S., Hearn, S.A., Konsolaki, M., Zhong, Y., 2004. "Dissecting the pathological effects of human Abeta40 and Abeta42 in Drosophila: a potential model for Alzheimer's disease". Proceedings of National Academic of Science U.S.A. 101 (17): 6623–6628.

Ineichen, B., 2000. The epidemiology of dementia in Africa: a review. Social Science and Medicine 50: 1673–1677.

Ingkaninan, K., Best, D., Heijden, V.D., Hofte, A.J.P., Karabatak, B., Irth, H., Tjaden, U.R., Greef, V.D., Verpoorte, R., 2000. High-performance liquid chromatography with on-line coupled UV, mass spectrometric and biochemical detection for identification of acetylcholinesterase inhibitors from natural products. Journal of Chromatography A 872: 61–73.

Jacot-Guillarmod, A., 1971. Flora of Lesotho (Basutoland). Verlag von Cramer, Lehre.

Jann, M.W., 2000. Rivastigmine, a new-generation cholinesterase inhibitor for the treatment of Alzheimer's disease. Pharmacotherapy 20: 1-12.

Jann, M.W., Grossberg, G., 2000. Cholinesterase inhibitors in the treatment of Alzheimer's disease. Consultant Pharmacist 15 (Supplementary B): 7-15.

Jimenez, A., Santos, A., Alonso, G., Vazquez, D., 1976. Inhibitors of protein synthesis in eukaryotic cells. Comparative effects of some amaryllidaceae alkaloids. Biochim Biophys Acta 425: 342–348.

Kandel, E.R., Schwartz, J.H., Jessel, T.M., 2000. Principles of Neural Science. McGraw-Hill. Professional.

Keefover, R.W., 1996. The clinical epidemiology of Alzheimer's disease. Neurological Clinics 14: 337-351.

Keifer, P. A., 2003. Flow NMR applications in combinatorial chemistry. Current Opinion in Chemistry and Biology 7: 388-394.

Kikuchi, T., Akihisa, T., Tokuda, H., Ukiya, M., Watanabe, K., Nishino, H., 2007. Cancer chemopreventive effects of cycloartane-type and related triterpenoids in *in vitro* and *in vivo* models. Journal of Natural products 70: 918-922.

Kiribuchi, M., Miura, K., Tokuda, S., Kaneda T., 1983. Hypocholesterolemic effect of triterpene alcohols with soysterol on plasma cholesterol in rats. Journal of Nutrition and Scientific Vitaminology 29(1): 35-43.

Knapp, M.J., Knopman, D.S., Solomon, P.R., 1994. The Tacrine Study Group. A 30- week randomized controlled trial of highdose tacrine in patients with Alzheimer's disease. Journal of American Medical Association 271: 985-991.

Koorbanally, N., Mulholland, D.A., Crouch, N., 2000. Alkaloids and triterpenoids from *Ammocharis coranica* (amaryllidaceae). Phytochemistry 54 (1): 93-97.

Kua, J., Zhang, Y., Eslami, A.C., Butler, J.R., McCammon, J.A., 2003. Studying the roles of W86, E202, and Y337 in binding of acetylcholine to acetylcholinesterase using a combined molecular dynamics and multiple docking approaches. Protein Science 12 (12): 2675-2684.

Kukhanova, M., Victorova, L., Krayevsky, A., 1983. Peptidyltransferase center of ribosomes: on the mechanism of action of alkaloid lycorine. FEBS Letters 160: 129–133.

Kutchan, T.M., 1995. Alkaloid biosynthesis-the basis for metabolic engineering of medicinal plants. The Cell 7:1059-1070.

Levkoff, S.E., Macarthur, I.W., Bucknall, J., 1995. Elderly mental health in the developing world. Social Science Medicine 41: 983–1003.

Likhitwitayawuid, L., Angerhofer, C. K., Chai, H., Pezzuto, J. M., Cordell, G. A., Ruangrungsi, N., 1993. Cytotoxic and antimalarial alkaloids from the bulbs of *Crinum amabile*. Journal of Natural Products 56: 1331–1338.

Lin, L., Hu, S., Chai, H., Pengsuparp, T., Pezzuto, J.M., Cordell, G.A., Ruangrungsi, N., 1995. Lycorine alkaloids from *Hymenocallis littoralis*. Phytochemistry 40: 1295–1298.

Liu, j., Hu, W., He, L., Ye, M., Li, Y., (2004). Effects of lycorine on HL-60 cells via arresting cell cycle and inducing apoptosis. FEBS Letters 578: 245–250.

Lopez, S., Bastida, J., Viladomat, F., Codina, C., 2002. Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and *Narcissus* extracts. Life Science 71: 2521–2529.

Louw, C.A.M., Regnier, T.T.C., Korsten, L., 2002. Medicinal bulbous plants of South Africa and their traditional relevance in the control of infectious diseases. Journal of Ethnopharmacology 82: 147-154.

Madden, S., Spaldin, V., Park, B.K., 1995. Clinical pharmacokinetics of tacrine. Clinical Pharmacokinetics 28: 449-457.

Marston, A., Kissling, J., Hostettmann, K., 2002. A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants. Phytochemical Analysis 13: 51-54.

Maslow, K., 2008. Alzheimer's disease: Facts and Figures. Alzheimer's and Dementia 4: 110-133.

Mason, L.H., Puschett, E.R., Wildman, W.C., 1955. Alkaloids of the amaryllidaceae. Part IV: Crystalline alkaloids of *Ammocharis coranica* (Ker-Gawl.) Herb., *Brunsvigia rosea* (Lam.) Hannibal and Two *Crinum Species*. Journal of the American Chemical Society 77: 1253-1256.

Masuda, Y., 2004. Cardiac effect of cholinesterase inhibitors used in Alzheimer's disease—from basic research to bedside. Current Alzheimer's Research 1: 315–321.

McGaw, L.J., Eloff, J.N., 2008. Ethnoveterinary use of southern African plants and scientific evaluation of their medicinal properties. Journal of Ethnopharmacology 119: 559-574.

Mesquita, S.G., Martinez, M.F., Romoff, P., Fávero, O.A., Lieber, S.R., Enrique, J., Lago, G., 2008 Constituintes químicos das folhas de *Murraya paniculata* (Rutaceae). Brazilian Journal of Pharmacognosy 18(4): 563-568.

Mitova, M., Tashova, R., Popov, S., Berger, R. G., Krings, U., Handjieva, N., 2003. GC/MS analysis of some bioactive constituents from *Carthamus lanatus* L. Zeitschift Naturforschung 58C: 697-703.

Mudher, A., Lovestone, S., 2002. "Alzheimer's disease-do tauists and baptists finally shake hands?" Trends in Neuroscience 25 (1): 22–26.

Mukherjee, K.S., Bhattacharjee, P., Mehrotra, A., 1990. Triterpenoids of Euphorbiaceae family: 1980–1989. Journal of Scientific Indian Research 49: 449–456.

Neuwinger, H.D., 2000. African Traditional Medicine. A Dictionary of Plant Use and Applications. Medpharm GmbH Scientific Publishers, Stuttgart, Germany.

Nistor, M., Don, M., Parekh, M., 2007. "Alpha- and beta-secretase activity as a function of age and beta-amyloid in Down syndrome and normal brain". Neurobiology of Aging 28 (10): 1493–1506.

Noback, C.R., Strominger, N.L., Demarest, R.J., Ruggiero, D.A., 2005. The human nervous system: structure and function, 6th Edition. Humana Press, Totowa, New Jersey: 1-76.

Nordberg, A., Svensson, A.L., 1998. Cholinesterase inhibitors in the treatment of Alzheimer's disease: a comparison of tolerability and pharmacology. Drug Safety 19: 465-480.

Normal neuron and Alzheimer ravaged neuron.www.ahaf.org. Accessed August 2009.

Orhan, I.; Sener, B., Atici, T., 2003. Antioxidant flavonoids from *Tamus communis ssp. Cretica*. Chemistry of Natural Compounds 39 (4): 383-386.

Page, B.A., 1998. In: Extractives from three Southern African medicinal plants, M.Sc Dissertation, University of Natal, Durban: 36-40.

Pelvig, P., Pakkenberg, H., Stark, K., Pakkenberg, B., 2008. Neocortical glial cell numbers in human brains". Neurobiology of aging 29 (11): 1754–1762.

Pole-Evans, I.B., 1938. *Ammocharis coranica*. Flowering Plants of South Africa 18: 712.

Polinsky, R.J., 1998. Clinical pharmacology of rivastigmine: a new-generation acetylcholinesterase inhibitor for the treatment of Alzheimer's disease. Clinical Therapeutics 20: 634-647.

Quinn, D.M., 1987. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. Chemical Review 87: 955-979.

Raskin, I., Ribnicky, D.M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno D.A., Ripoll C., Yakoby, N., O'Neal, J.M., Cornwell, T., Pastor, I., Fridlender, B., 2002. Plants and human health in the twenty-first century. Trends in Biotechnology 20(12): 522-531.

Reynolds, W. F.; Enriquez, R. G., 2002. NMR methods for analyzing natural substances. Journal of Natural Products 65: 221-244.

Rhee, I.K., Meent, M.V., Ingkaninan, K., Verpoorte, R., 2001. Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. Journal of Chromatography A 915: 217–223.

Rhee, I.K., Van Rijn, R.M., Verpoorte, R., 2003. Quantitative determination of false-positive effects in the acetylcholinesterase assay using thin layer chromatography. Phytochemical Analysis.14: 127-131.

Rhee, I.K.; Appels, N.; Hofte, B.; Karabatak, B.; Erkelens, C.; Stark, L.M.; Flippin, L.A.; Verpoorte, R., 2004. Isolation of the acetylcholinesterase inhibitor ungeremine from *Nerine bowdenii* by preparative HPLC coupled on-line to a flow assay system. Biological and Pharmaceutical Bulletin 27: 1804-1809.

Risa, A., Risa, J., Adsersen, A., Stafford, G.I., Van Staden, J., Jäger, A.K., 2004. Acetylcholinesterase inhibitory activity of plants used as memory enhancers in traditional South African medicine. South African Journal of Botany 70: 664-666.

Robertson, M.R., Harrell, L.E., 1997. Cholinergic activity and amyloid precursor protein metabolism. Brain Research Reviews 25 (1): 50-69.

Rogers, S.L., Farlow, M.R., Doody, R.S., 1998. Donepezil Study Group. A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Neurology 50: 136-145.

Rollinger, J.M., Hornick, A., Langer, T., Stuppner, H., Prast, H., 2004. Acetylcholinesterase inhibitory activity of scopolin and scopoletin discovered by virtual screening of natural products. Journal of Medicinal Chemistry 47: 6248–6254

Schmitz, C., Rutten, B.P., Pielen, A., 2004. "Hippocampal neurone loss exceeds amyloid plaque load in a transgenic mouse model of Alzheimer's disease". American Journal of Pathology 164 (4): 1495-1502.

Schultz, A.G., Holoboski, M. A., Smyth, M. S., 1996. The First Asymmetric Total Syntheses of (+)-Lycorine and (+)-1-Deoxylycorine. Journal of American Chemical Society 118: 6210-6219.

Shadlen, M.F., Larson, E.B., 1999. What's new in Alzheimer's disease treatment? Reasons for optimism about future pharmacologic options. Postgraduate Medicine 105: 109-118.

Shen, Z.X., 2004. "Brain cholinesterases: II. The molecular and cellular basis of Alzheimer's disease". Medical Hypotheses 63 (2): 308–321.

Shu, Y.Z., 1998. Recent natural products based drug development: a pharmaceutical industry perspective. Journal of Natural Product 61:1053–1071.

Siek, G.C., Katz, L.S., Fishman, E.B., 1990. Molecular forms of acetylcholinesterase in subcortical areas of normal and Alzheimer disease brain. Biological Psychiatry 27: 573-580.

Singla, A.K., Pathak, K., 1990. Phytoconstituents of Euphorbia species. Fitoterapia 61: 483–516.

Skolnick, A.A., 1997. Old Chinese herbal medicine used for fever yields possible new Alzheimer disease therapy. Journal of the American Medical Association 277 (10): 776.

Small, G.W., 1998. Treatment of Alzheimer's disease: current approaches and promising developments. American Journal of Medicine 104: 32-38.

Snijman, D.A., Linder, H.P., 1996. Phylogenetic relationships, seed characters, and dispersal system evolution In Amaryllideae (Amaryllidaceae). Annals of the Missouri Botanical Gardens 83: 362-386.

Sramek, J.J., Frackiewics, E.J., Cutler, N.R., 2000. Review of the acetylcholinesterase inhibitor galanthamine. Expert Opinion on Investigational Drugs 9: 2393-2402.

Stafford, G.I., Pedersen, M.E., Van Staden, J., Jäger, A.K., 2008. Review on plants with CNS effects used in traditional South African medicine against mental diseases. Journal of Ethnopharmacology 119: 513-537.
Sussman, I.L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., Silman, I., 1991. Atomic structure of acetylcholinesterase from *Torpedo californica*; a prototype acetylcholine-binding protein. Science 253: 872-879.

Tallarida, R.J., Jacob, L.S., 1979. A dose-response relationship in pharmacology. New York: Springer-Verlag.

Teresa, J.P., Urones, J.G., Marcos, S., Basabe, P., Cuadrado, J.S., Oro, R.F.M., 1987. Tritepenes from *Euphorbia broteri.* Phytochemistry 26: 1767-1776.

Terry, R.D., Masliah, E., Hansen, L.A., 1994. Structural basis of cognitive alterations in Alzheimer's disease. In: Terry R.D., Katzman R, Bick K.L, eds. Alzheimer Disease. New York, NY: Raven Press: 179-196.

Thibodeau, G.A., Patton, K.I., 2003. Anatomy and Physiology, 5th Edition, Mosby, 362-413.

Topcu, G., Ulubelen, A., 2007. Structure elucidation of organic compounds from natural sources using 1D and 2D NMR technique. Journal of Molecular Structure 834-836: 57-73.

Toriizuka, Y., Kinoshita, E., Kogure, N., Kitajima, M., Ishiyama, A., Otoguro, K., Yamada, H., Omura, S., Takayama, H., 2008. New lycorine-type alkaloid from *Lycoris traubii* and evaluation of antitrypanosomal and antimalarial activities of lycorine derivatives. Bioorganic and Medicinal Chemistry 16: 10182–10189.

Toro, R., Perron, M., Pike, B., Richer, L., Veillette, S., Pausova, Z., Paus, T., 2008. Brain size and folding of the human cerebral cortex. Cerebral Cortex 18 (10): 2352–2357.

Tram, N.T.N., Titorenkova, T.V., Bamkova, V., Handjieva, N.V., 2002. *Crinum L*. (Amaryllidaceae). Fitoterapia 73: 183-208.

United Nations, 2003. Long-Range Population Projections Report. http://www.un.org/esa/population/publications/longrange/long-range working-paper final. PDF.

Verpoorte, R., Van der Heijden, R., Schripsema, J., Hoge, J.H., Ten Hoopen, H.J., 1993. Plant cell biotechnology for the production of alkaloids: Present status and prospects. Journal of Natural Products 56: 186-207.

Weinstock, M., 1999. Selectivity of cholinesterase inhibition. CNS Drugs 12: 307-323.

Wenk, G.L., 2003. "Neuropathologic changes in Alzheimer's disease". The Journal of clinical Psychiatry 64 (Supplementary 9): 7–10.

Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T., Delon, M.R., 1982. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215: 1237–1239.

Williams, D.R., 2006. "Tauopathies: classification and clinical update on neurodegenerative diseases associated with microtubule-associated protein tau". Internal Medicine Journal 36 (10): 652–660.

Woodruff-Pak, D.S., Vogel III, R.W., Wenk, G.L., 2001. Galantamine: Effect on nicotinic receptor binding, acetylcholinesterase inhibition, and learning. Proceedings of the National Academy of Sciences of the United States of America 98 (4): 2089-2094.

World Health Report, Executive Summary, 1998. http://www.who.int/whr/ 1998/exsum98e.htm.

Yankner, B.A., Duffy, L.K., Kirschner, D.A., 1990. "Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides". Science 250 (4978): 279-282.
Yasukawa K., Takido M., Matsumoto T., Takeuchi M., Nakagawa, S., 1991. Sterol and triterpene derivatives from plants inhibit the effects of tumour promoter and sitosterol and betulinic acid inhibits tumour formation in mouse skin two-stage carcinogenesis. Oncology 41: 72-76.

Yasukawa, K., Akihisa, T., Kimura, Y., Tamura, T., Takido, M., 1998. Inhibitory effect of cycloartenol ferulate, a component of rice bran, on tumour promotion in two-stage carcinogenesis in mouse skin. Biological and Pharmaceutical Bulletin 21: 1072-1076.

Yasukawa, K., Yu, S.Y., Yamanouchi, S., Takido, M., Akihisa, T., and Tamura, T. (1995) Some Lupane-Type Triterpenes Inhibit Tumor Promotion by 12-*O*-Tetradecanoylphorbol-13-acetate in Two-Stage Carcinogenesis in Mouse Skin, Phytomedicine 1: 309–313.

Zhang, Y., Kua, J., McCammon, J.A., 2002. Role of the catalytic triad and oxyanion hole in acetylcholinesterase catalysis: An ab initio QM/MM study. Journal of the American Chemical Society 124 (35): 10572-10577.

APPENDIX 1 NMR SIGNALS FOR COMPOUND 1

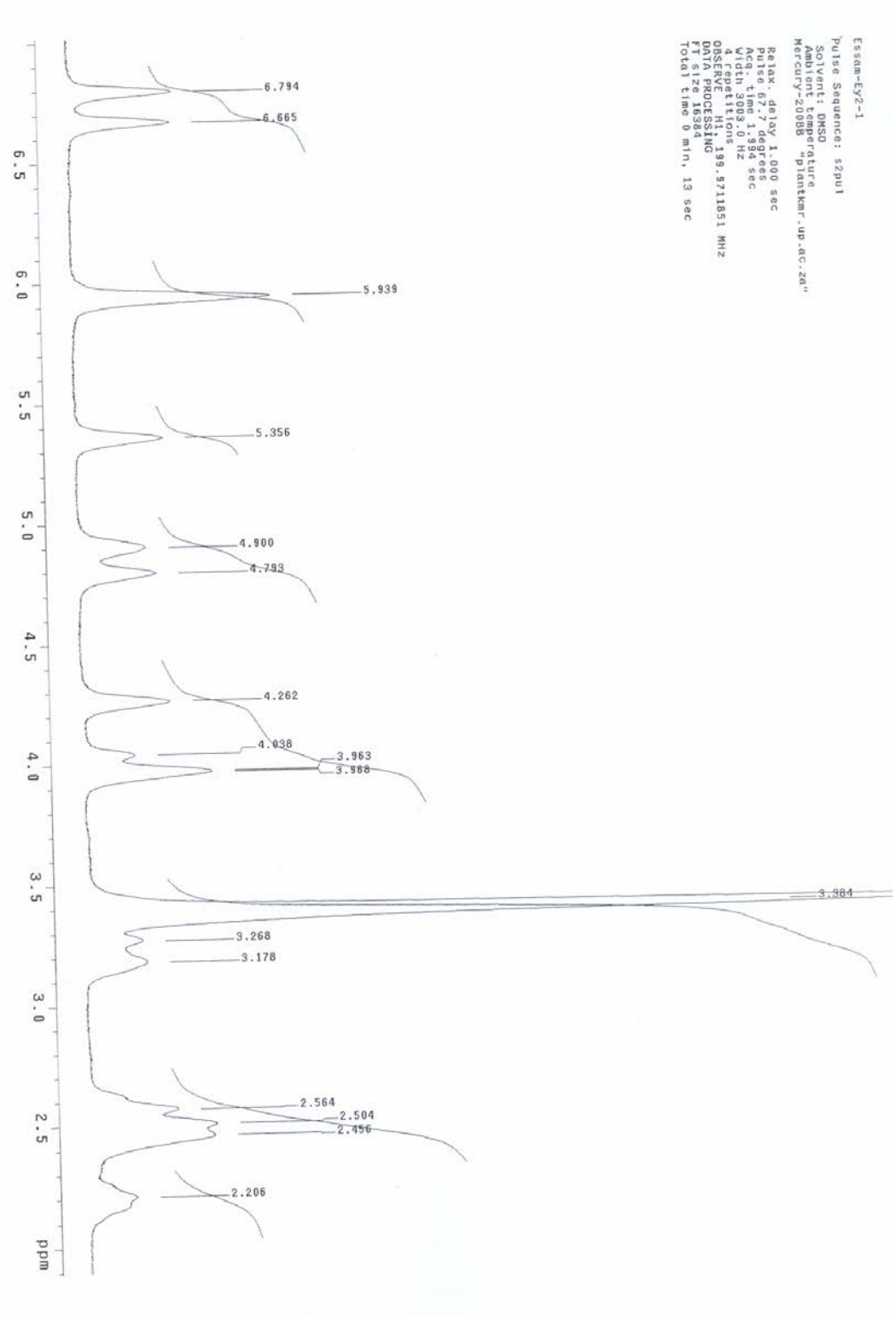

Figure A.1: ^{1}H NMR spectrum of Lycorine isolated from the bulbs of *Ammocharis coranica.*

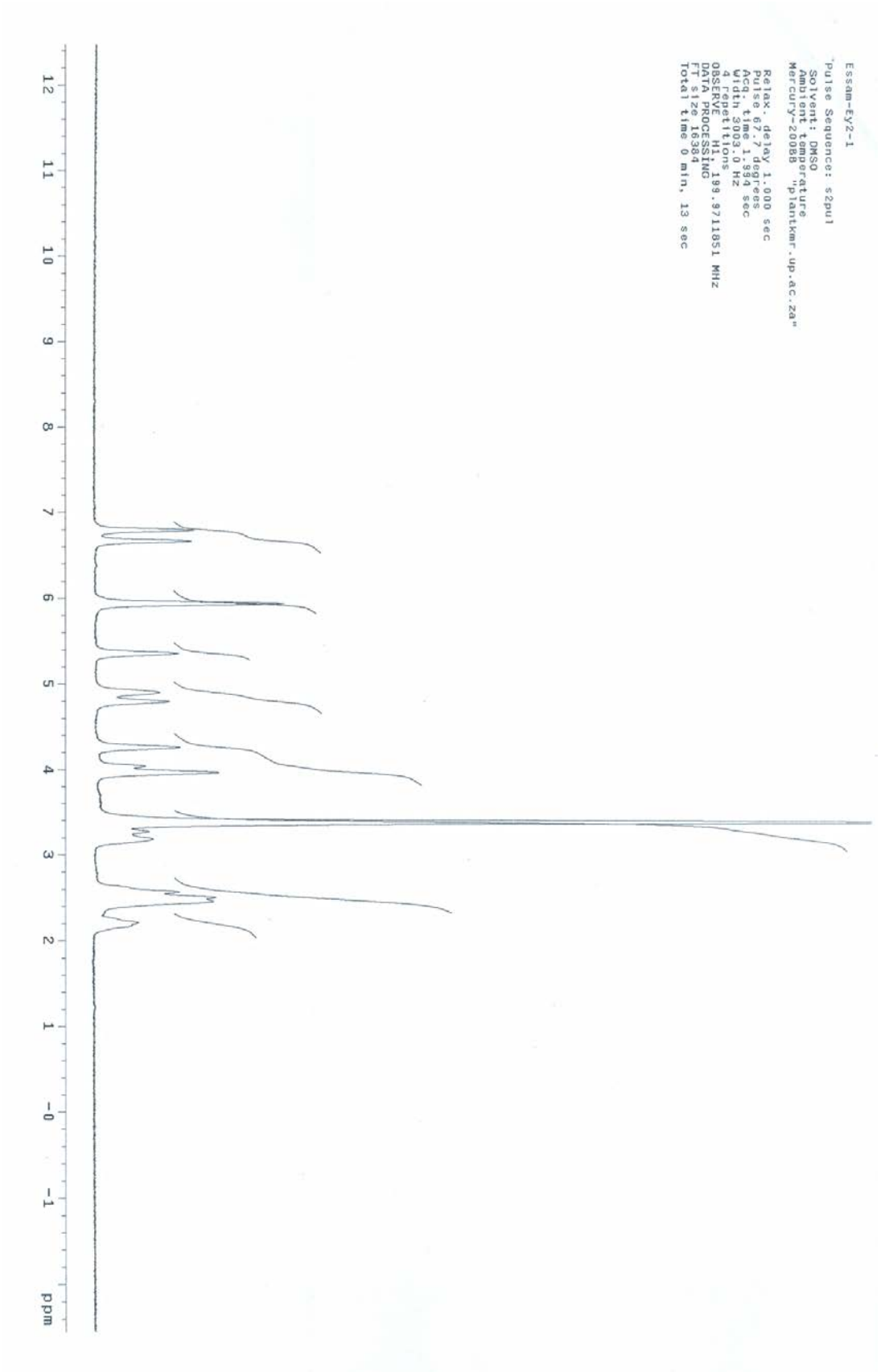

Figure A.2: ^{1}H NMR spectrum of Lycorine isolated from the bulbs of *Ammocharis coranica*.

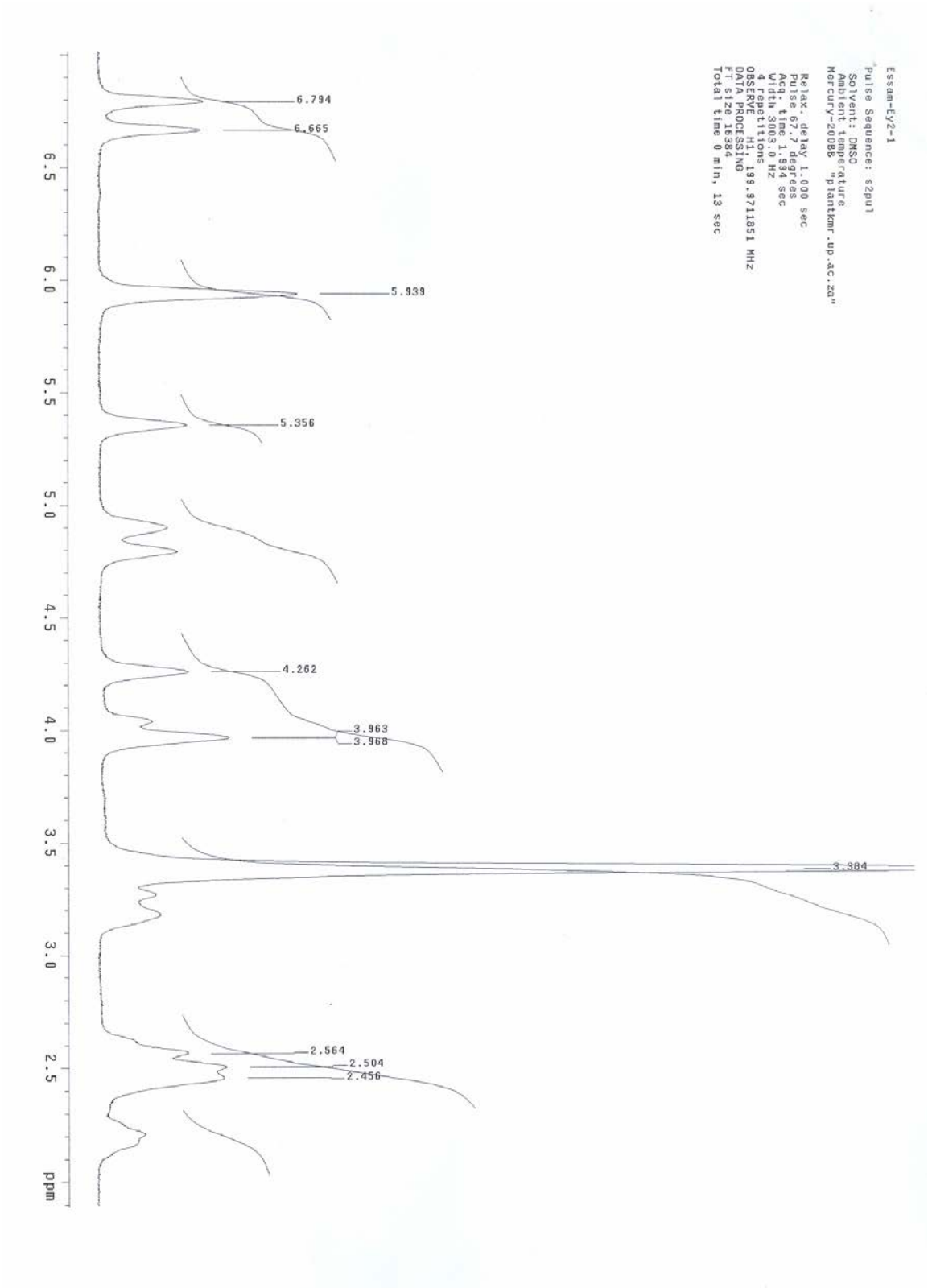

Figure A.3: ^{1}H NMR spectrum of Lycorine isolated from the bulbs of *Ammocharis coranica*.

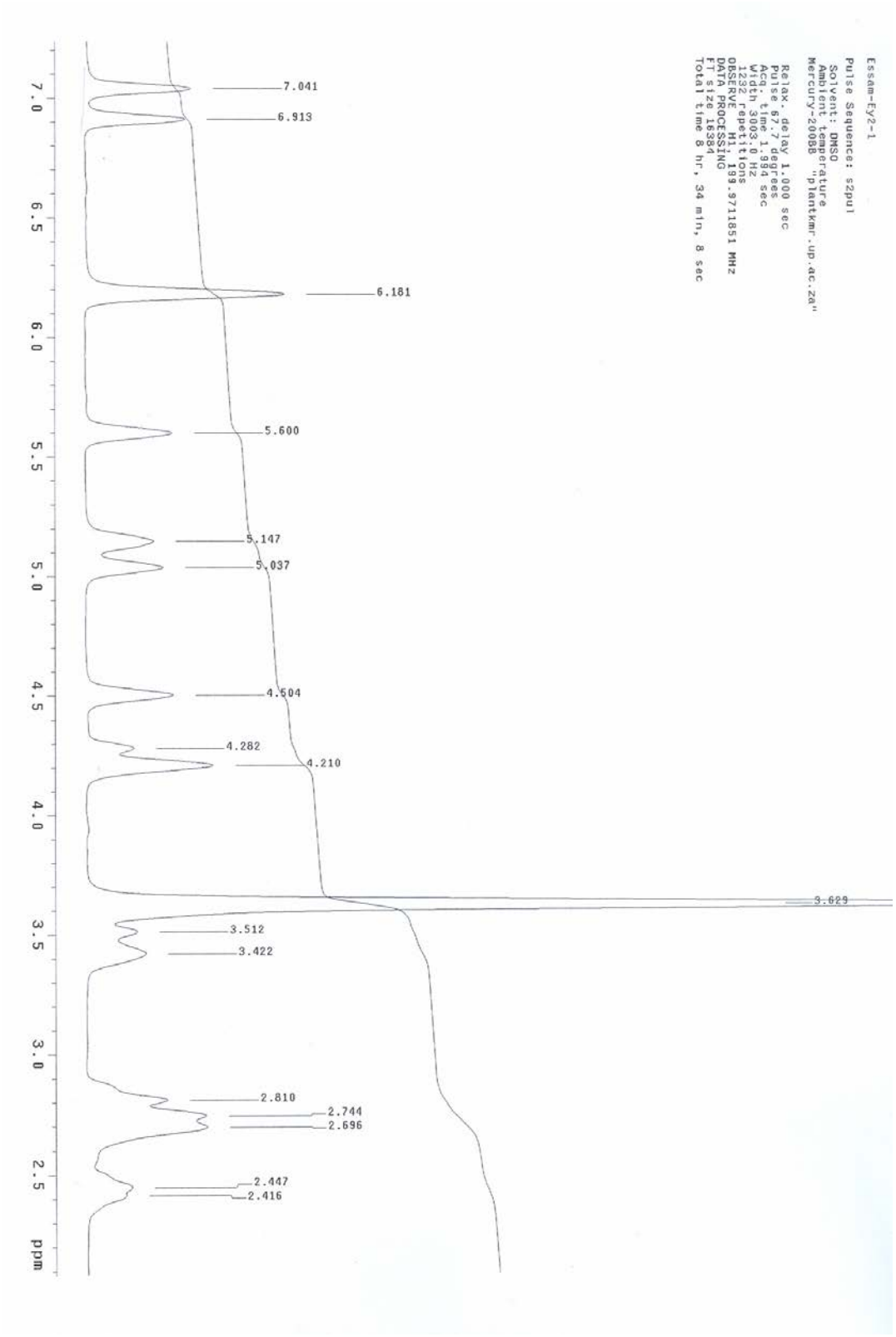

Figure A.4: ^{1}H NMR spectrum of Lycorine isolated from the bulbs of *Ammocharis coranica*.

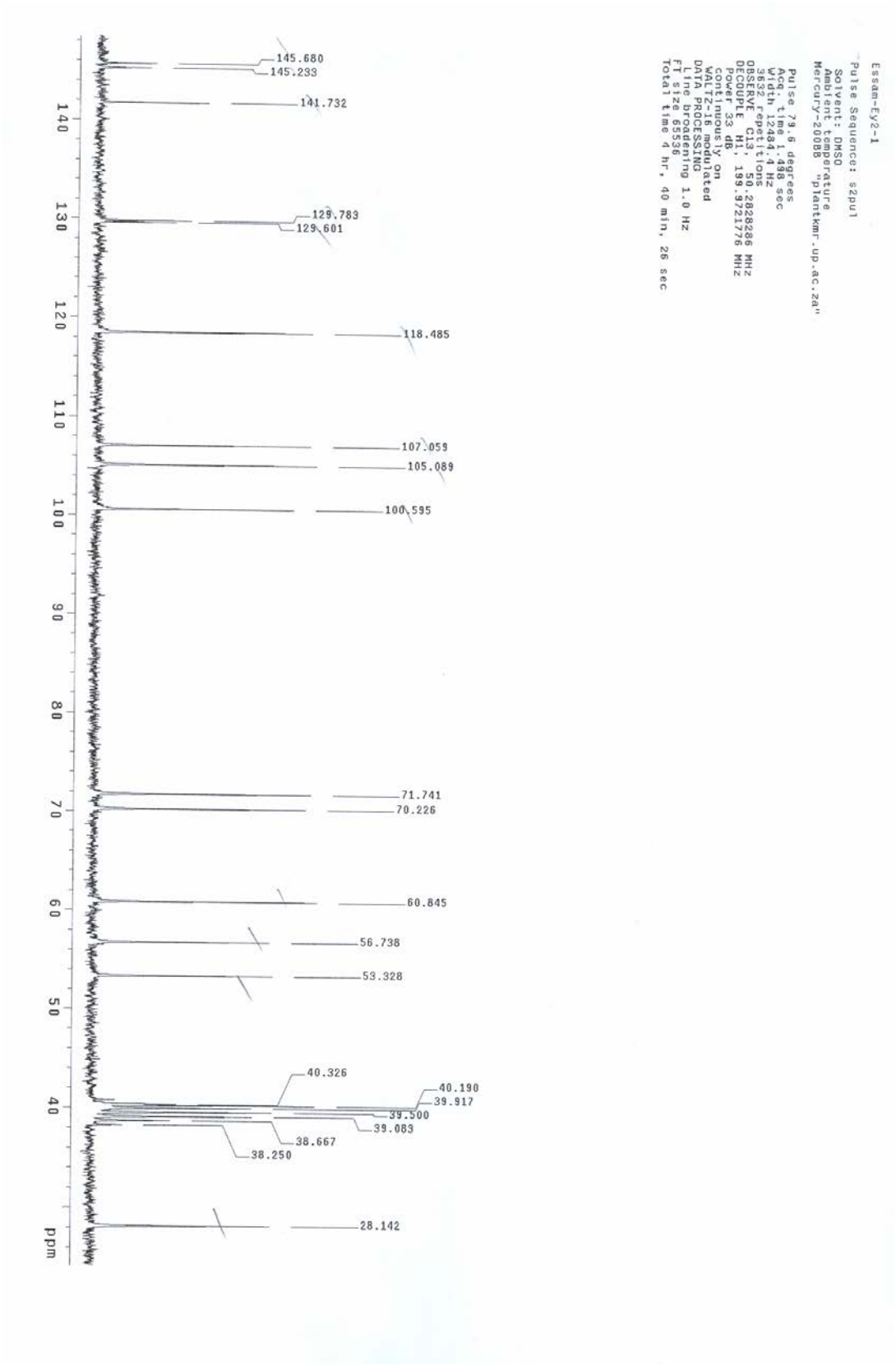

Figure A.5: ^{13}C NMR spectrum of lycorine isolated from the bulbs of *Ammocharis coranica*.

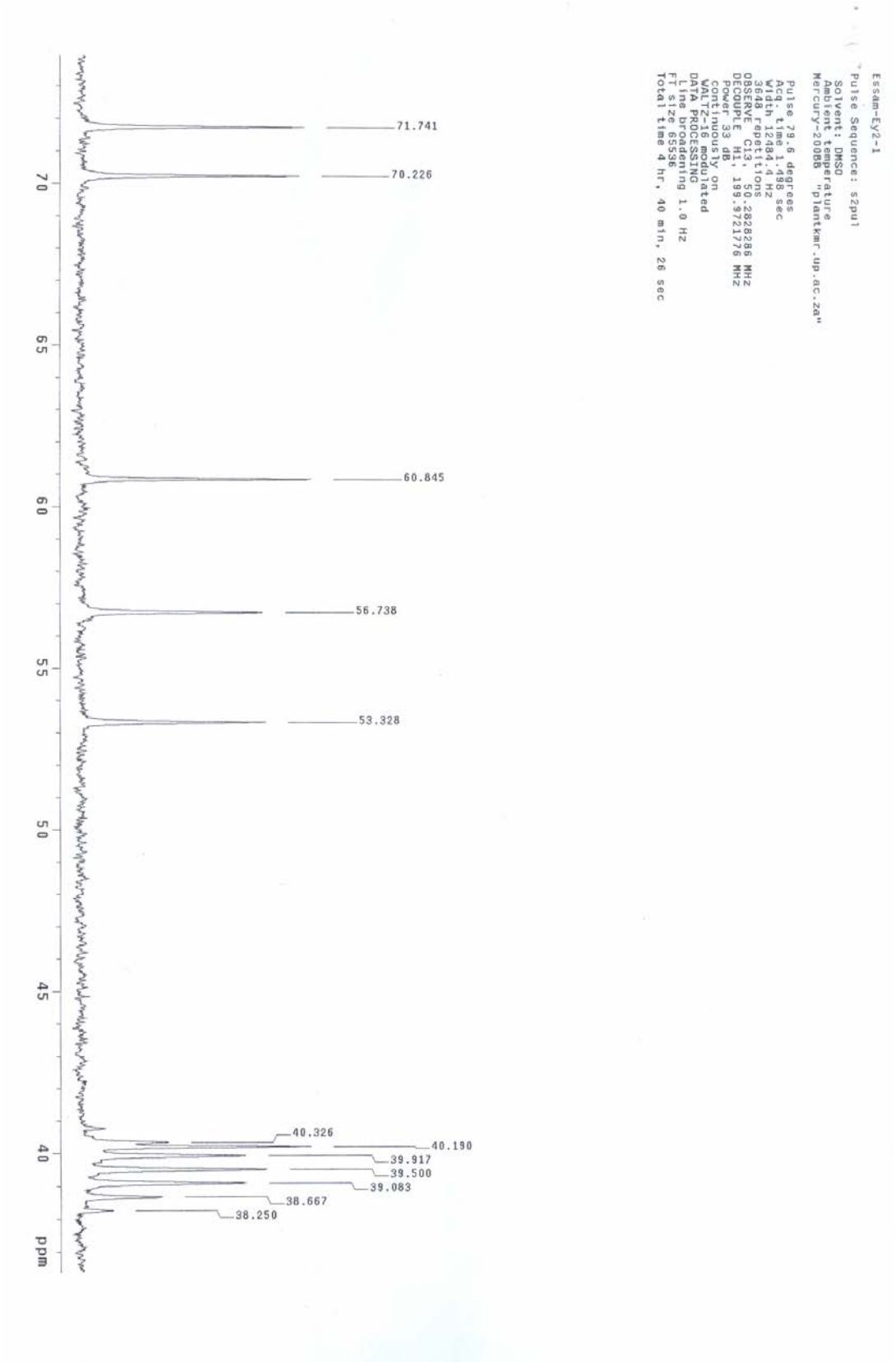

Figure A.6: ^{13}C NMR spectrum of lycorine isolated from the bulbs of *Ammocharis coranica*.

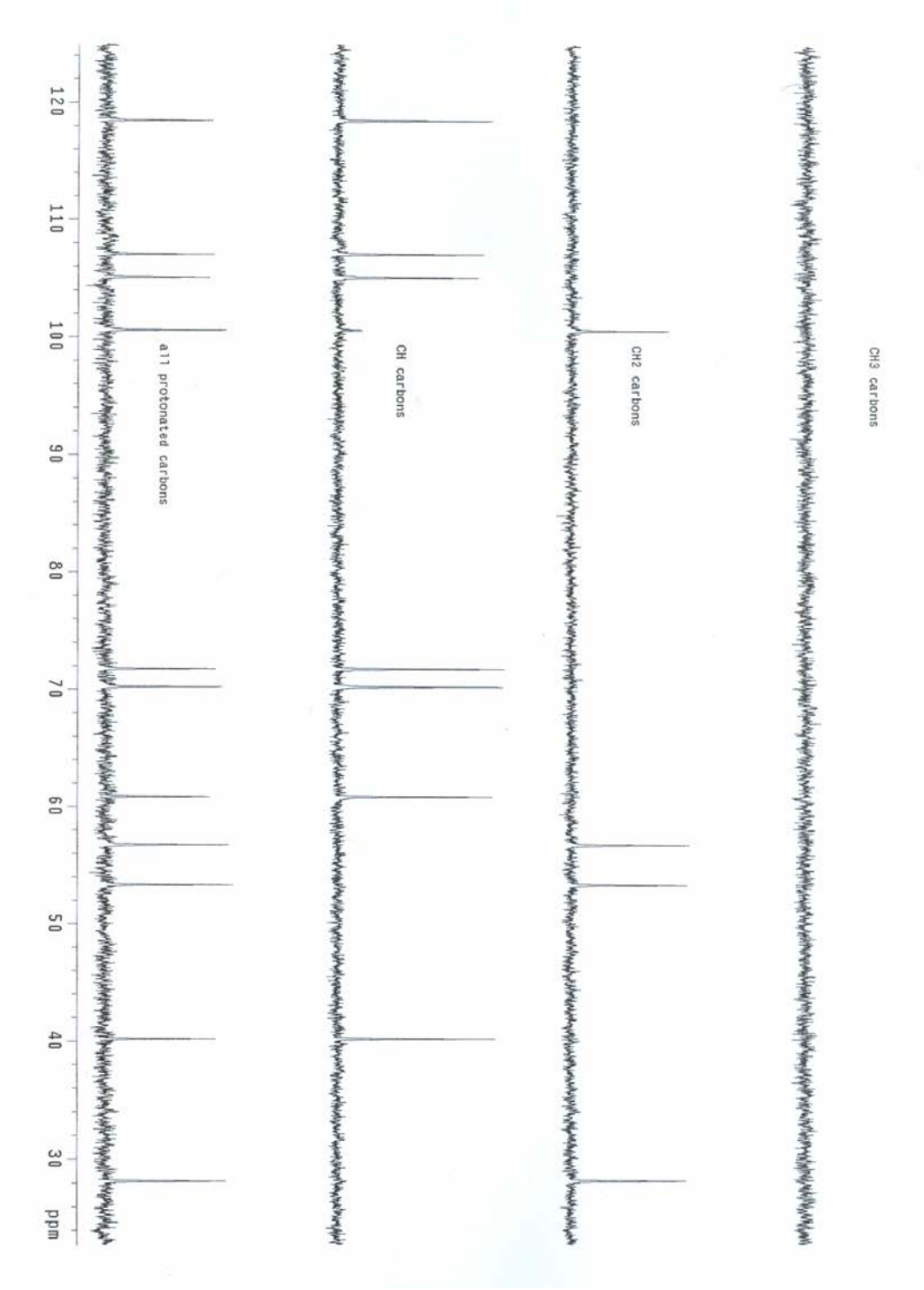

Figure A.7: DEPT experiment data of Lycorine isolated from the bulbs of *Ammocharis coranica*

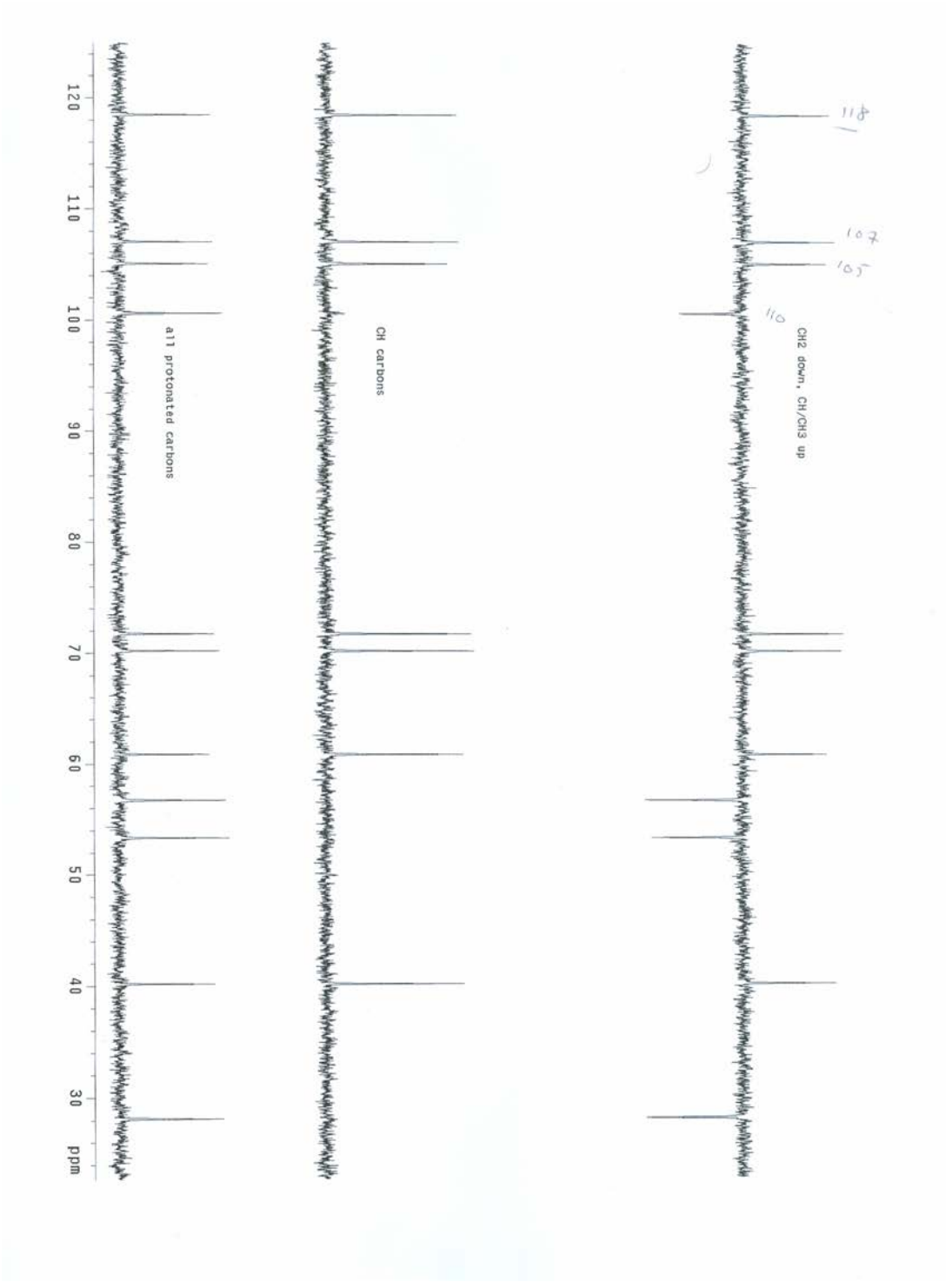

Figure A.8: DEPT experiment data of Lycorine isolated from the bulbs of *Ammocharis coranica.*

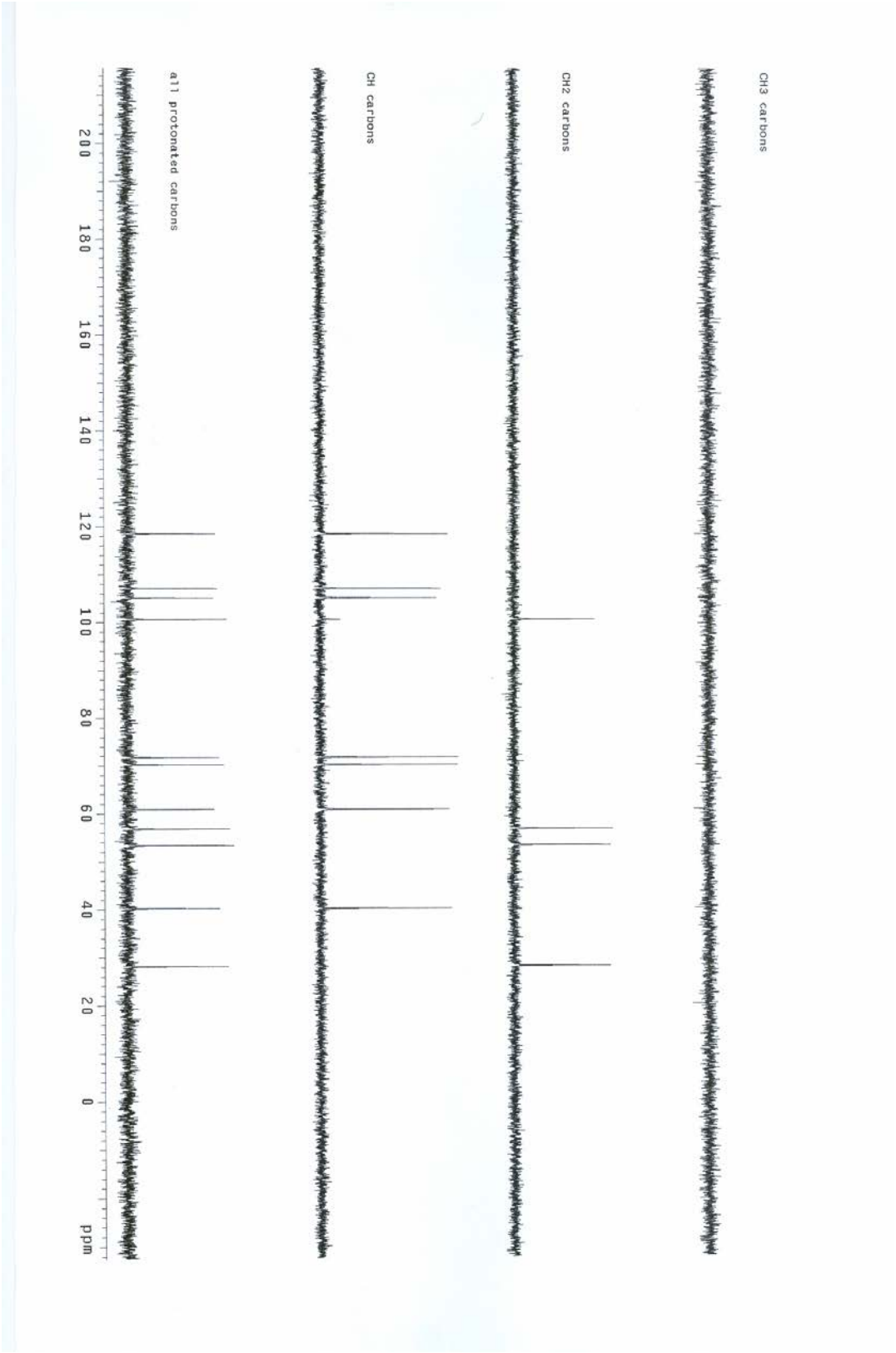

Figure A.9: DEPT experiment data of Lycorine isolated from the bulbs of *Ammocharis coranica.*

APPENDIX 2 NMR SIGNAL OF COMPOUND 2

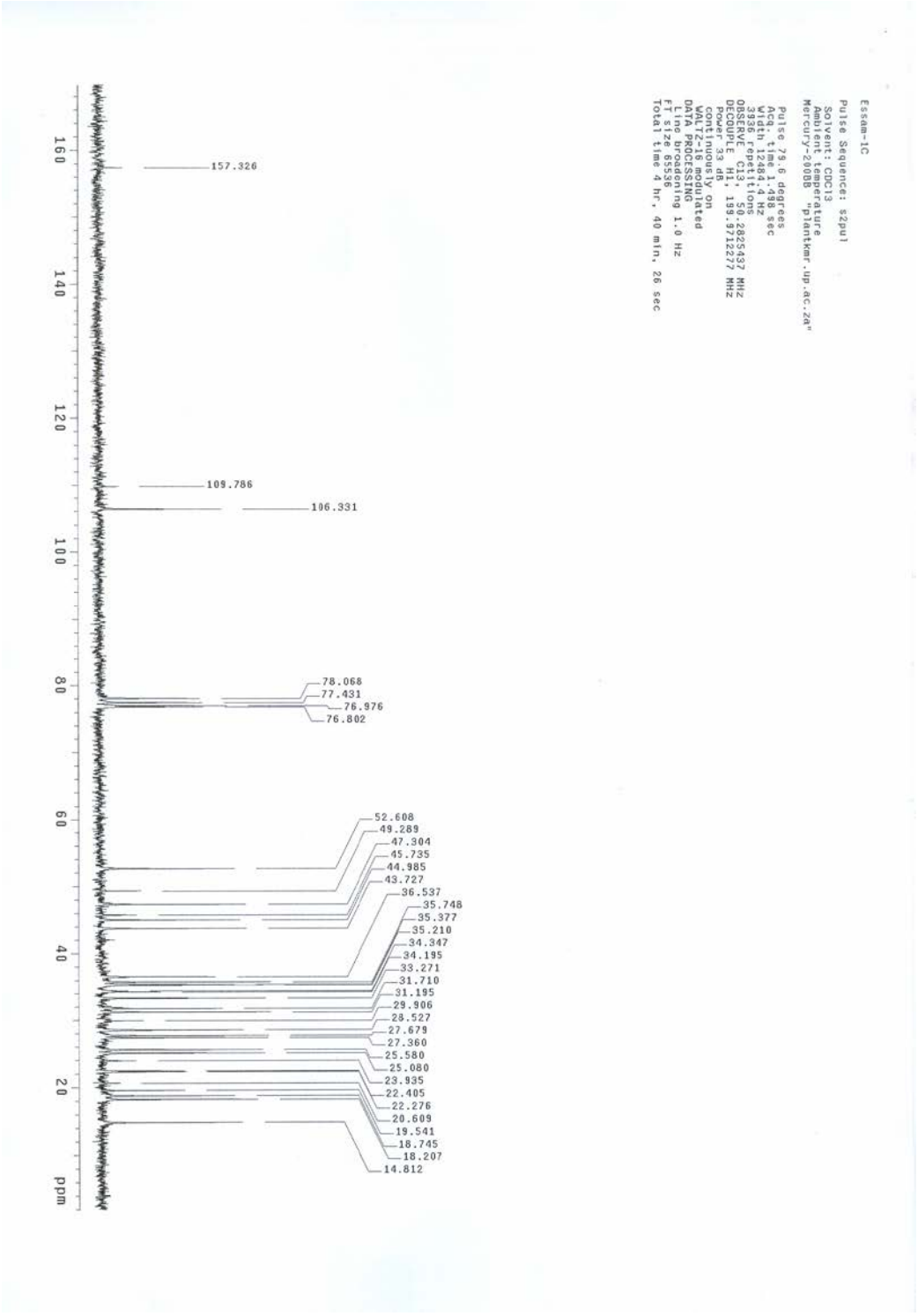

Figure B.1: ^{13}C NMR spectrum of 24-methylenecycloartan-3β-ol isolated from the bulbs of *A. coranica.*

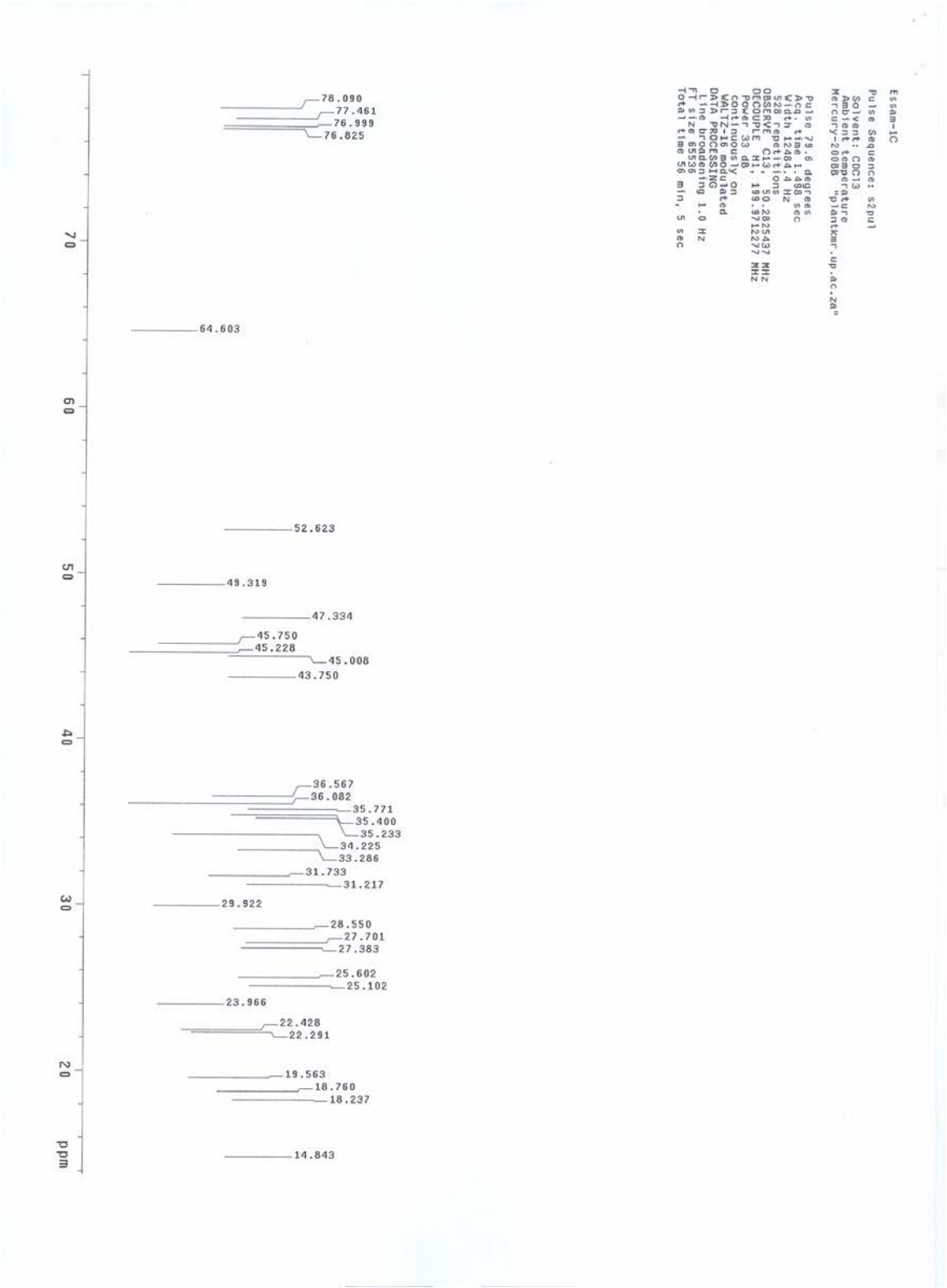

Figure B.2: ^{13}C NMR spectrum of 24-methylenecycloartan-3β-ol isolated from the bulbs of *A. coranica.*

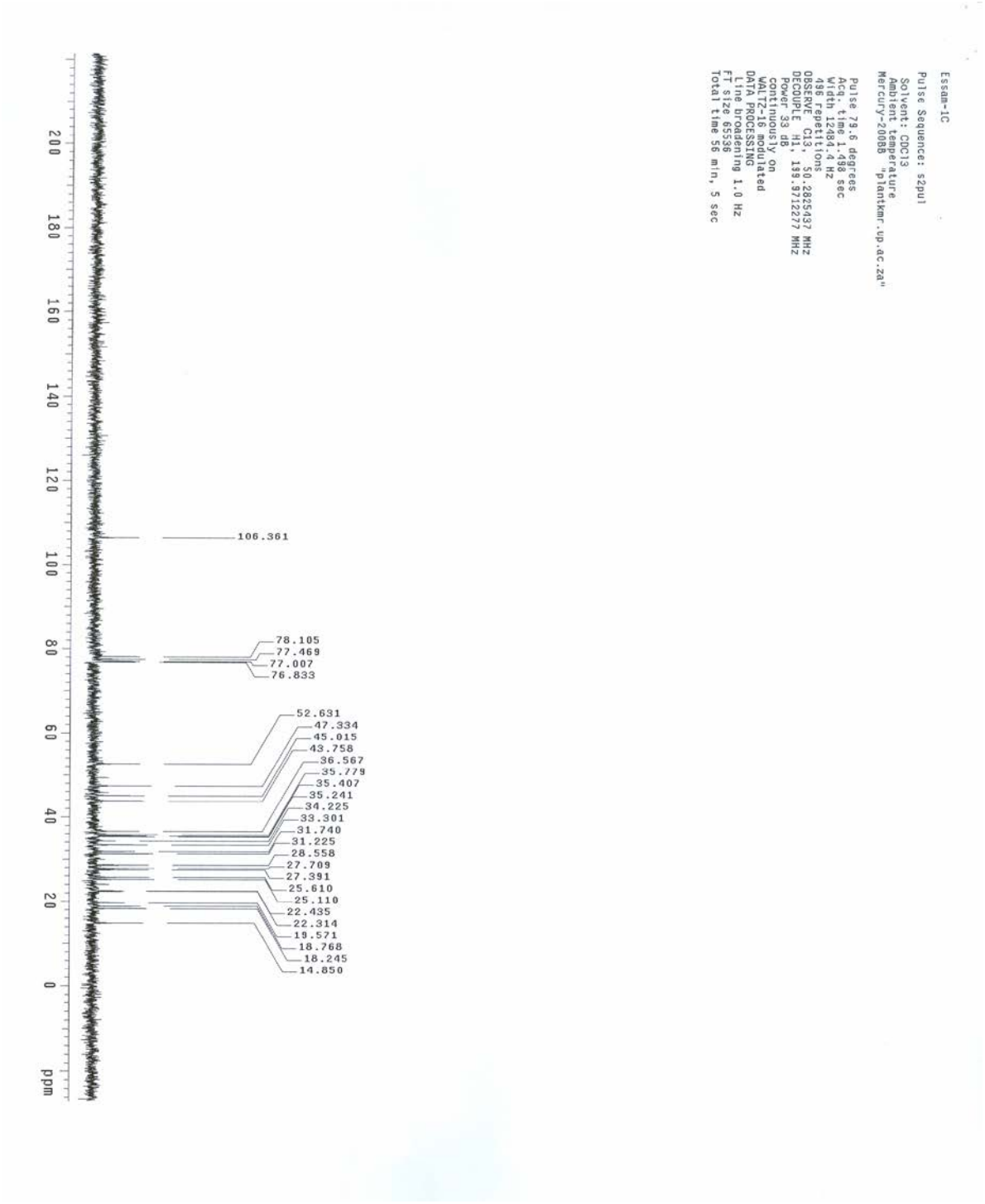

Figure B.3: ^{13}C NMR spectrum of 24-methylenecycloartan-3β-ol isolated from the bulbs of *A. coranica.*

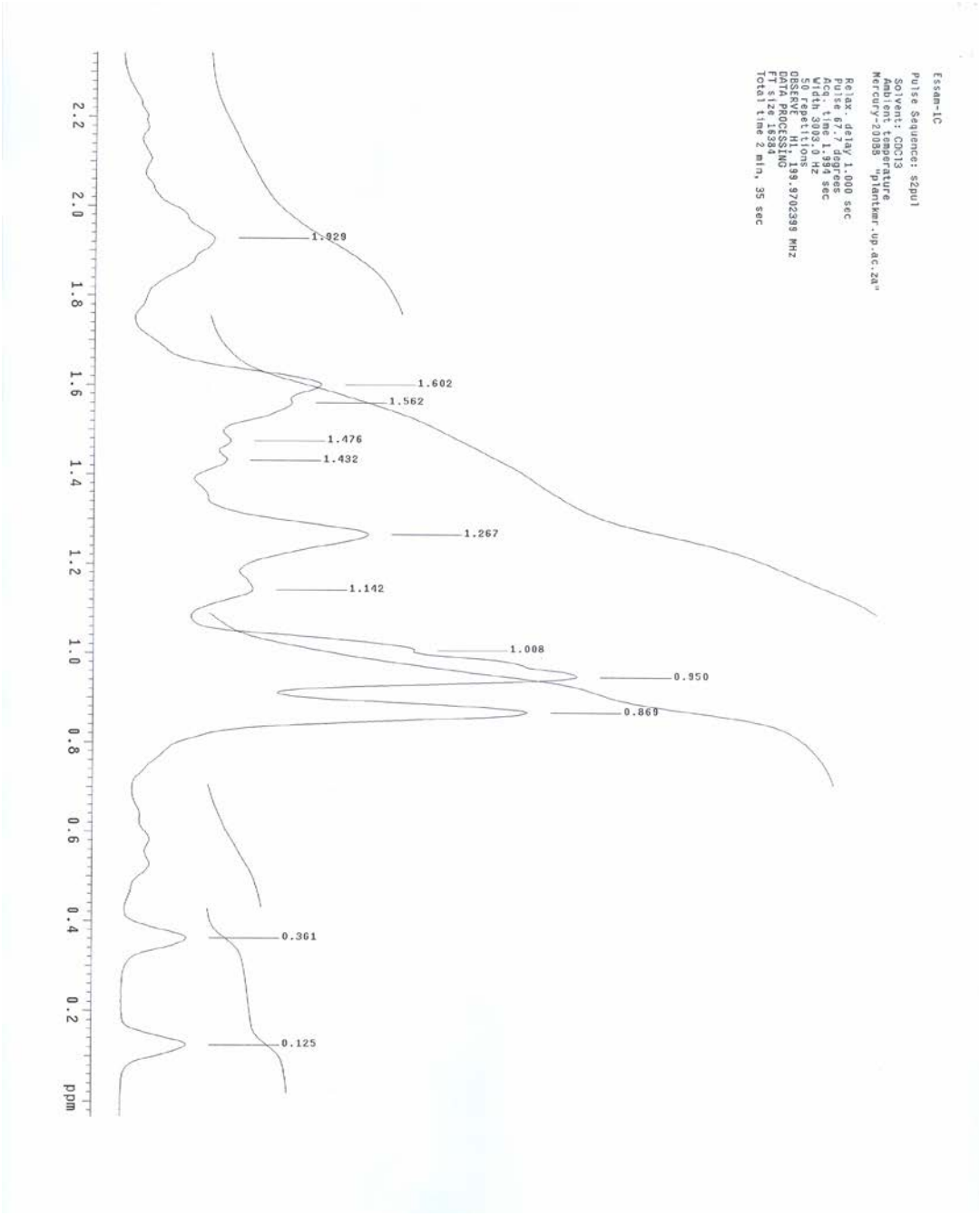

Figure B.4: ^{1}H NMR spectrum of 24-methylenecycloartan-3β-ol isolated from the bulbs of *A. coranica.*

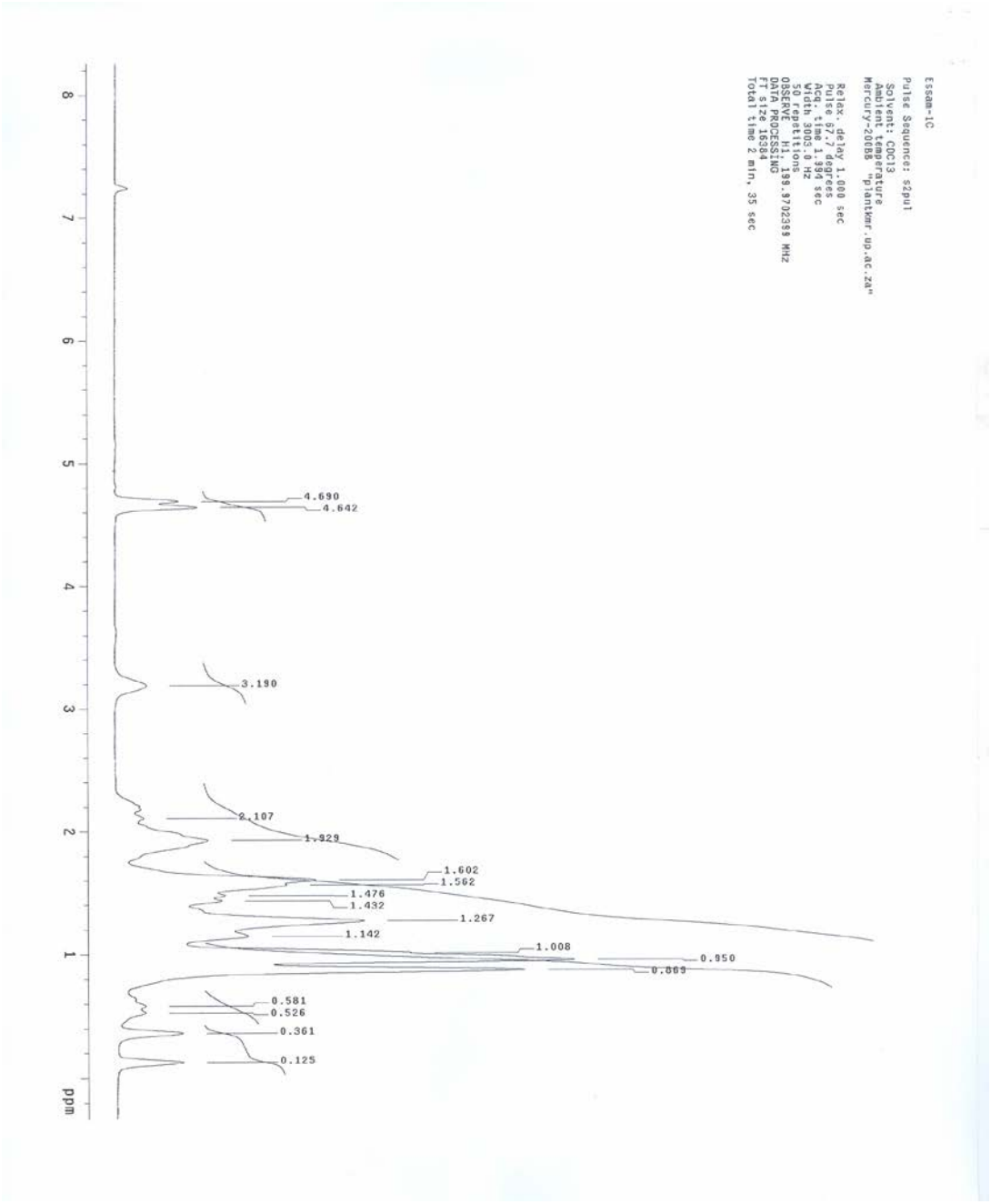

Figure B.5: ^{1}H NMR spectrum of 24-methylenecycloartan-3β-ol isolated from the bulbs of *A. coranica.*

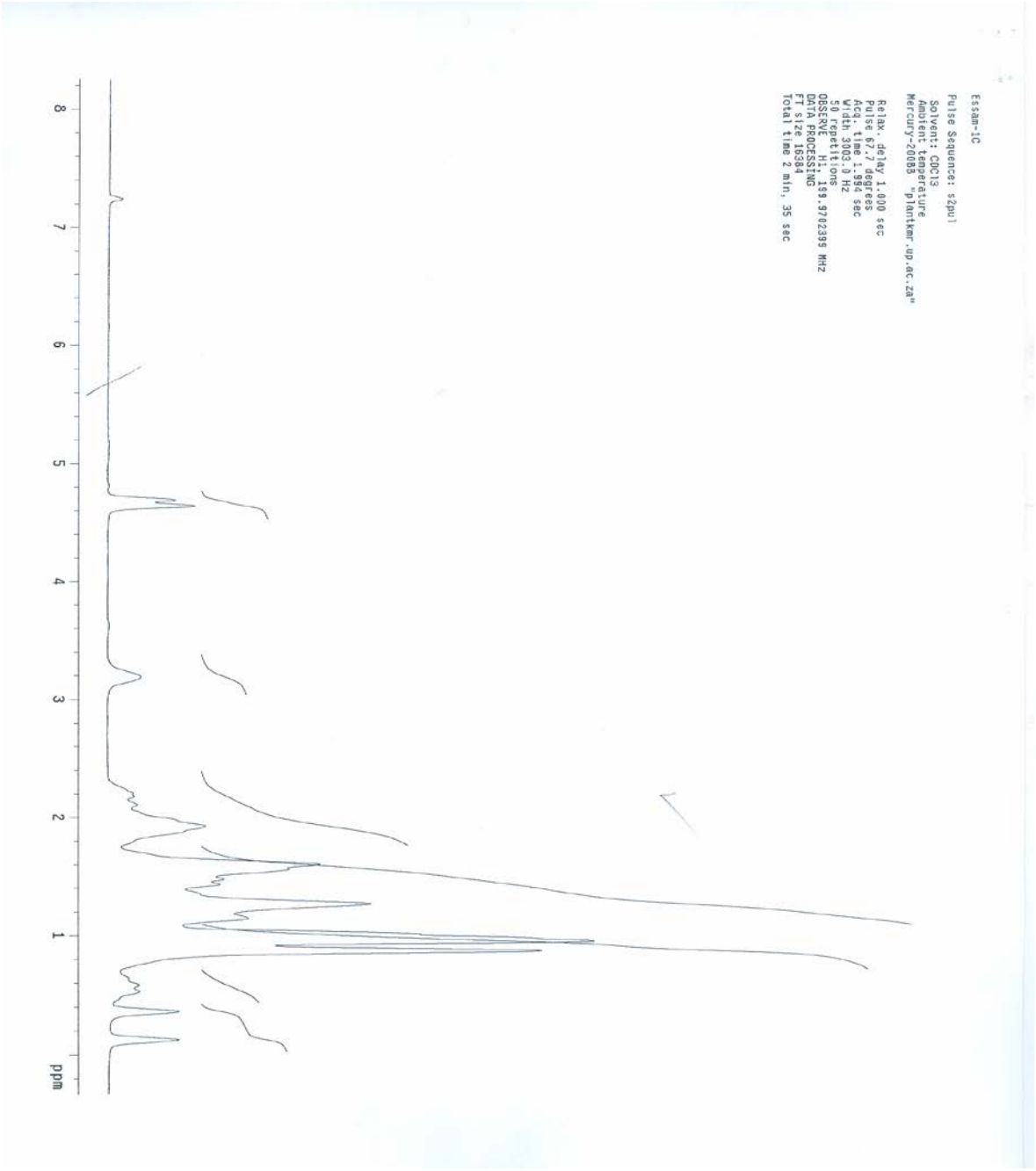

Figure B.6: ^{1}H NMR spectrum of 24-methylenecycloartan-3β-ol isolated from the bulbs of *A. coranica.*

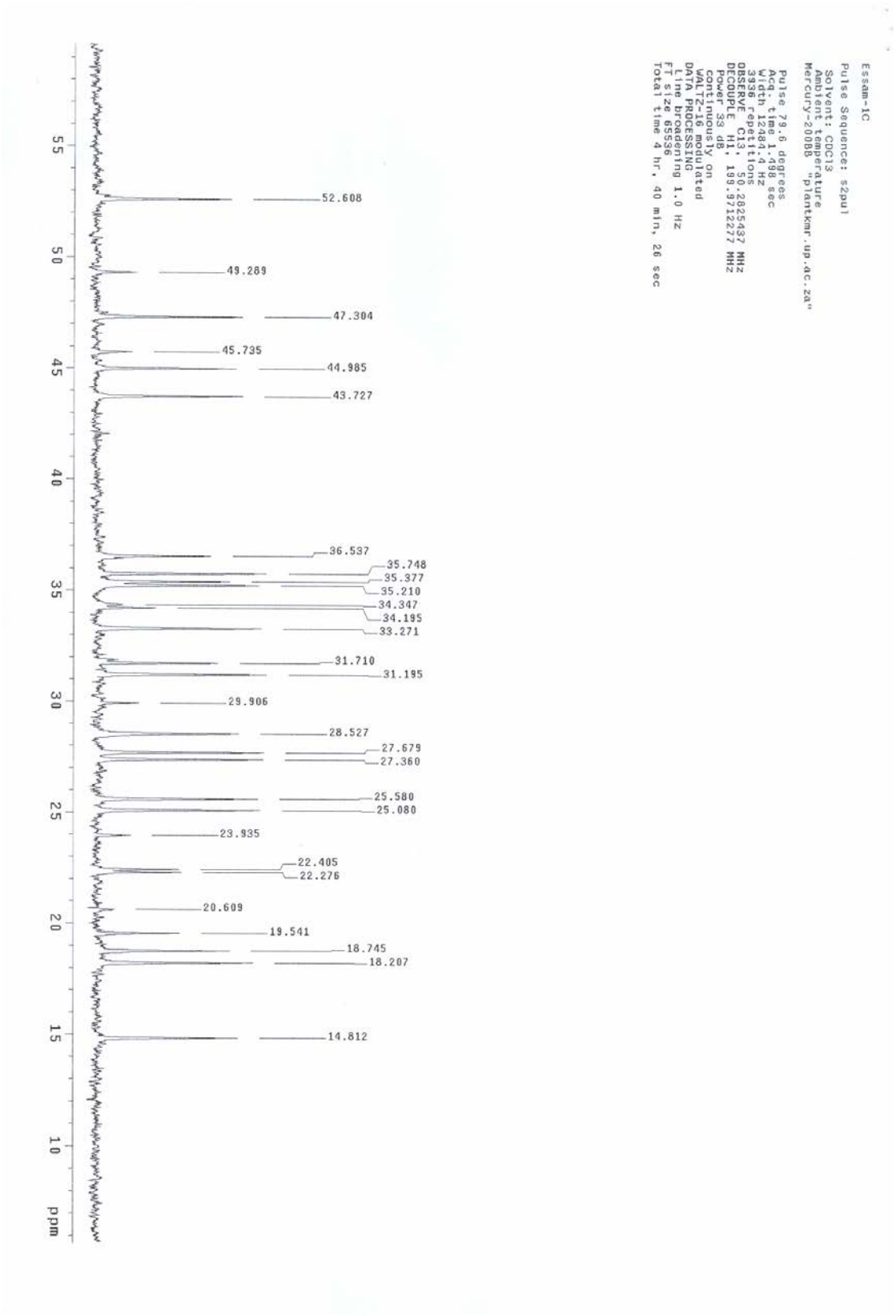

Figure B.4: ^{13}C NMR spectrum of 24-methylenecycloartan-3β-ol isolated from the bulbs of *A. coranica.*