An-Najah National University
Faculty of Graduate Studies

In-vitro evaluation of acetylcholinesterase inhibition and antioxidant activity of selected Palestinian medicinal plants: Implications for Alzheimer's disease therapy

By
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This Thesis is submitted in Partial Fulfillment of the Requirements for the Degree of Master of Life Sciences (Biology), Faculty of Graduate Studies, An-Najah National University, Nablus, Palestine.

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This Thesis was defended successfully on 11/9/2014 and approved by

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3. Prof. Waleed Sweileh / Internal Examiner	.....

Dedication

To my mother for her endless support, my father whom I carry his name' may god bless his soul', my dear brothers and sisters for their support and encouragement, with love and respect.

Acknowledgements

I wish to express my deepest gratitude to my supervisor, Dr. Munqez J Y Shtaya, for his encouragement, and support. I would like to express my sincere gratitude to Prof. Mohammed S. Ali-Shtayeh, President of Biodiversity and Environmental Research Center (BERC) and Dr. Rana Jamous, Head of Biodiversity and Biotechnology Research Unit at BERC for their continuous support of my MSc study and research, for their patience, motivation, enthusiasm, and immense knowledge. Their guidance helped me throughout my research and writing of this thesis.

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In conclusion, I recognize that this research would not have been possible without the assistance of BERC. Thank you.

Last but not the least; I am deeply and forever indebted to my mother for her love, support and encouragement throughout my entire life. I am also very grateful to my brothers and sisters for their love and endless support.

الإقرار

أنا الموقعة أدناه مقدمة الرسالة التي تحت عنوان:

In-vitro evaluation of acetylcholinesterase inhibition and antioxidant activity of selected Palestinian medicinal plants: Implications for Alzheimer's disease therapy

التقييم المخبري لفعاليه بعض النباتات الطبيه الفلسطينيه كمثبطات لانزيم الاسيتيل كولين استريز و مضادات للاكسده. كاشاره لعلاج مرض الزهايمر

أقر بأن ما اشتملت عليه هذه الرسالة إنما هو نتاج جهدي الخاص، باستثناء ما تمت الإشارة إليه حيثما ورد، و أن هذه الرسالة ككل، أو أي جزء منها لم يقدم من قبل لنيل أي درجة علمية أو بحث علمي أو بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's name: **اسم الطالبة:**

Signature: **التوقيع:**

Date: **التاريخ:**

List of abbreviations

AD	Alzheimer's disease
Ach	Acetylcholine
AChE	Acetylcholinestrase
AChEI	Acetylcholinestrase inhibitor
NFTs	Neurofibrillary Tangles
ROS	Reactive oxygen species
TAPHM	Traditional Arabic Palestinian herbal medicine
TCM	Traditional Chinese medicine
TRIS-HCL	Tris (hydroxymethyl) aminomethane hydrochloride
BSA	Bovine serum albumin
ATCI	Acetylthiocholiniodide
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
DPPH	2,2-Diphenyl-1-picrylhydrazyl
TNB	5-thio 2- nitrobenzoic acid
NaCl	Sodium chloride
$MgCl_2.H_2O$	Magnesium chloride
mM	Millimolar
M	Molar
µ	Micron
µL	Microliter
BERC	Biodiversity and environmental research center
LE	Leaves
ST	Stem
FR	Fruit
BL	Bulb
FL	Flowers
ST	Stem
SE	Seeds
RFR	Ripen fruits
BHA	Butylated hydroxyanisole
%I	Percentage of inhibition
%	Percent
A_{sample}	Absorbance of the Sample
A_{Blank}	Absorbance of the blank

الإقرار

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Student's name: اسم الطالبة: سلام يوسف جميل أبو زيتون

Signature: التوقيع:

Date: التاريخ: 11.9.2014

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In-vitro evaluation of acetylcholinesterase inhibition and antioxidant activity of selected Palestinian medicinal plants: Implications for Alzheimer's disease therapy

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Abstract

Background: Cholinesterase inhibitory therapy serves as a strategy for the treatment of Alzheimer's disease (AD), several acetylcholineesterase inhibitors are used for the symptomatic treatment of patients with mild to moderate AD. These compounds have been reported to have adverse effects including gastrointestinal disturbances. Numerous medicinal plants have been used in Traditional Arabic Palestinian Herbal Medicine (TAPHM) for the treatment of several diseases, including improvement of memory, Alzheimer's disease (AD) and old age related disease. Ethnopharmacological approach and bioassay guided isolation have provided a lead in identifying potential AChE inhibitors from plant sources.This study was therefore aimed at investigating *in vitro* possible acetylcholinesterase inhibitors (AChEIs) in herbal medicines traditionally used in Palestine for the treatment of memory loss, and to point out the role of these plants as potential sources for development of newly potent and safe natural therapeutic agents of AD.

Methods: The effect on AChE activity of 92 extracts of 47 medicinal plants including ten medicinal plants reported in TAPHM for treatment of age related diseases were evaluated for its anticholinestrase and antioxidant activity.

Results: Thirty eight percent of extracts inhibited AChE by $\geq$ 50%, only 8 extracts showed irreversible inhibition, Antioxidant activity was demonstrated by 73 extracts of which *Majorana syriaca* and *Rosmarinus officinalis* (IC_{50} 0.21 and 0.38 mg/ml) were the most active. Interestingly, differential results have been obtained which indicate the variability of the mode of actions for the selected plants; 27 extracts inhibited the enzyme reversibly while eight extracts showed irreversible inhibition. Additionally, the reversible interaction of *Majorana syriaca, Juglans regia, Rosmarinus officinalis, Menthe spicata*, and *Feoniculum vulgare* against AChE make them effective, new and promising agents for treatment of AD in the future, either as total extracts or their single bioactive constituents.

Conclusions: Palestinian flora have shown to be a rich source for, new and promising agents (AChEIs) for the treatment of AD Further studies are needed to isolate and identify the active compounds responsible for AChEI activities.

Chapter One

General Introduction

Chapter one
General Introduction

1.1 Overview of Alzheimer's disease (AD)

Alzheimer's disease (AD) is the most common form of dementia that affects more than10 million people worldwide (Singhal et al., 2012). It is one of the most widespread neurodegenerative disorders that results in progressive decline of memory and cognition, and mainly affects elderly people (over 65 years of age) as a result of different biochemical pathways (Ferreira et al., 2006; Singhal et al., 2012).

1.2 AD epidemiology and Risk Factors

Most cases of AD are seen in older adults, ages 65 years or above. Between the ages of 65 and 74, approximately 5 % of people have AD. For those over 85, the risk increases to 50 % (Thies & Bleiler, 2011).

In the developed world, AD accounts for 50–60% of all dementia cases. In 2001, an estimated 24.3 million people had dementia (Ferri et al., 2006). The number of people with AD is expected to increase substantially in the coming years as the proportion of the population aged 65 years or more rises sharply (Vinutha et al., 2007). The duration of illness varies between 2 – 10 years (Wolfson et al., 2001) and is reflected in the overall prevalence rates in the 85 years or more, between 10% to 30% (Mayeux, 2003). Deaths from Alzheimer's disease as the underlying cause have increased dramatically since 1991. The changes in the brain caused by AD are not usually the primary cause of death. AD often causes complications,

such as immobility and trouble swallowing. These can lead to malnutrition and increased risk of pneumonia, resulting in death in these patients (Thies & Bleiler, 2011).

Aging is the primary risk factor in AD. However other risk factors include decreased reserve brain capacity, gross brain shrinkage, low mental achievement in early life, and minimal level mentally taxing occupations, followed by latter life reduced mental and physical activity. Head injury has also been cited as a risk factor (Mayeux, 2003).

1.3 Alzheimer's Disease Pathology

The pathology of AD is complex involving many neurotransmitter and pathophysiolgic process, the three hallmarks of the AD including, deposition of extracellular plaques of β-amyloid protein (amyloid plaques), formation of intracellular neurofibrillary tangles (NFTs), and loss of cholinergic neurons are well known and central factors in AD pathology (Figure 1.1) . Plaques and NFTs were first discovered by Alois Alzheimer in 1906 , Although they are defining component of AD, they are not unique to AD; plaques and NFTs occur with normal aging and in some other neurodegenerative disorders (Lei et al., 2010). In AD, plaques and NFTs are localized to areas in the brain that correspond to clinical symptoms.

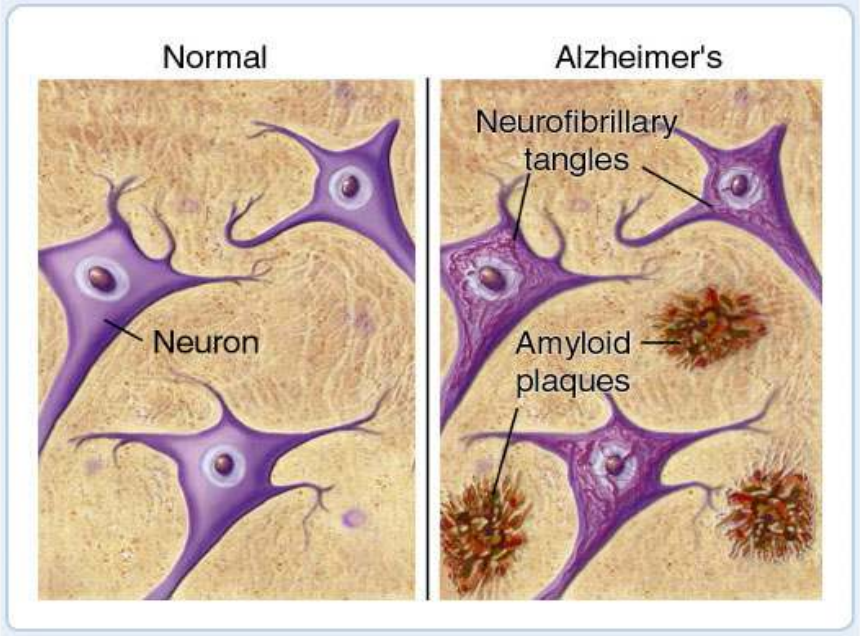

Figure (1.1): A comparison of a normal neuron and Alzheimer ravaged neuron (courtesy American Health Assistance Foundation, 2000-2009)

1.3.1 β-Amyloid Plaques

β-Amyloid plaques are hallmark of Alzheimer's disease . It is cleaved from the amyloid precursor protein with uncertain importance in organism. The precursor protein is degraded by a group of enzymes called secretases resulting in fragments of different length (Morrison & Lyketsos, 2002). Once the precursor protein is degraded by α-secretase, the products are quite harmless.

1.3.2 Intracellular Neurofibrillary Tangles (NFTs)

The formation of NFTs result from destruction of neuronal microtubules caused by the modification of their supporting protein, tau which is involved in stabilization of microtubules in cytoskeleton (Kao et al., 2010). Microtubules are essential components of neuronal cell structure; they act as tracks along which nutrients are delivered and

neuronal transmission is propagated in the neuronal axon. During AD pathogenesis, tau proteins become hyperphosphorylated, disrupting their bonds to microtubules, thus collapsing microtubule structure and destroying the neuron's transport and communication system, neuronal cell death ensures. Although the usual relationship is unclear, hyperphosphorylation of tau is thought to occur after plaque formation (Selkoe, 2002).

1.3.3 Loss of Cholinergic Neurons

Loss of cholinergic neurons is another well established pathology of AD. By late- stage AD, the number of cholinergic neurons is dramatically reduced; in some parts of the brain there is more than 75% loss (Perry et al., 1978).

Acetylcholine is an important neurotransmitter in the brain regions involving memory, and loss of cholinergic activity correlates with some aspects of cognitive impairments. Acetylcholin binds to 2 postsynaptic receptor types: muscarinic and nicotinic. Presynaptic nicotinic receptors influence the release of neurotransmitters important for memory and mood (acetylcholine, glutamate, serotonin, and norepinephrine) all of which have been implicated in AD pathology (Morrison & Lyketsos, 2002)

The brain regions associated with higher mental functions, such as the neocortex and hippocampus are most affected by characteristic pathology of AD (Figure 1.2).

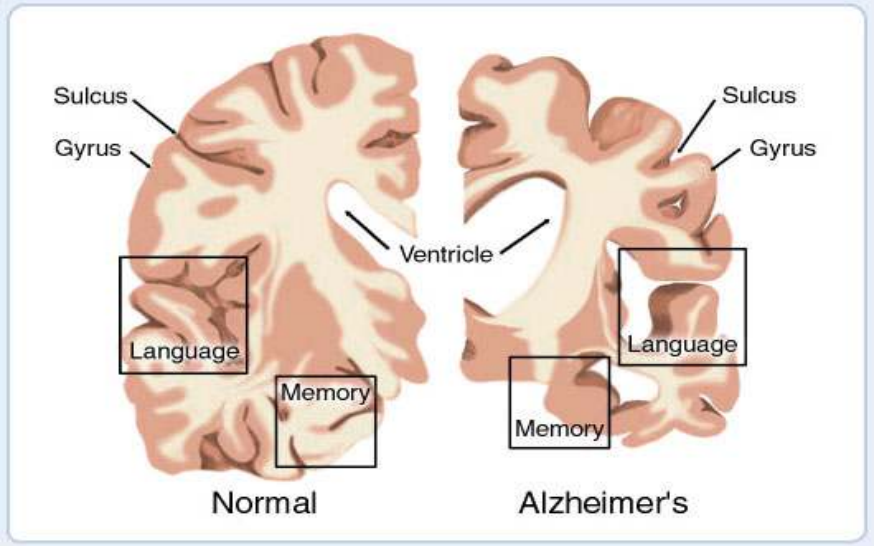

Figure (1.2): Comparison of a normal human brain and Alzheimer ravaged brain (courtesy American Health Assistance Foundation, 2000-2009)

1.4 Acetylcholinesterase (AChE) and its Inhibition

1.4.1. Acetylcholinesterase (AChE)

AChE is a membrane-bound enzyme found in excitable tissues, such as synaptic junctions (figure 1.3). The principle role of AChE is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of the neurotransmitter ACh (Mukherjee et al., 2007). Thus, AChEIs (e.g., the drugs used for the AD therapy) promote an increase in the concentration and duration of action of synaptic ACh (Heinrich & Lee Teoh, 2004; Rollinger et al., 2004).

1.4.2. Inhibitors of AChE (AChEIs)

AChEIs are neurotoxic compounds capable of causing central, peripheral or both central and peripheral cholinergic crises. They are used in two major ways as pharmaceutical and as pesticides (Houghton et al., 2006). There are two basic categories of cholinesterase inhibitors:

reversible and 'irreversible'. In the case of reversible cholinesterase inhibitors, as the name implies, they form transient complexes with the cholinesterases. In essence, they compete for acetylcholine at the enzyme active site. For one group - quaternary alcohols - they act as simple competitive inhibitors, binding and blocking access to the active site. While irreversible inhibitors inactivate the AChE active site by covalent attachment, termed organophosphates and form highly stable (covalent) phospho-intermediates. The organophosphates bind to and undergo initial hydrolysis by AChE, but the acyl intermediate is replaced by a phosphoryl moiety which is cleaved extremely slowly (requiring up to hundreds of hours) and effectively irreversible (Golan et al., 2011)

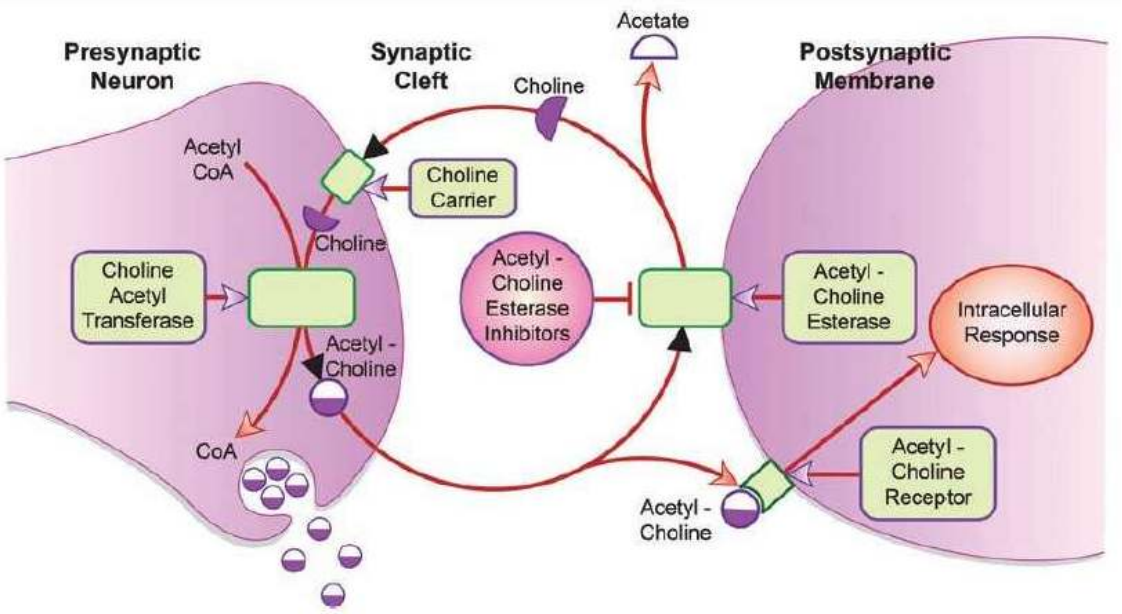

Figure (1.3): Acetylcholine inhibitors (AChEIs) in cholinergic nerve transmission. Acetylcholine is produced in the presynaptic neuron by the enzyme choline acetyltransferase from acetyl-coenzyme A and choline, and later released in the synaptic cleft where it binds to the acetylcholine receptor on the postsynaptic membrane, triggering an intracellular response. The enzyme acetylcholinesterase hydrolyses acetylcholine into acetate and choline in order to terminate synaptic transmission. Choline is transported into the presynaptic neuron by the choline carrier and serves as a substrate for the described production of acetylcholine. AChEIs inhibit the enzyme acetylcholinesterase, which in turn inhibits the breakdown of acetylcholine into acetate and choline and prolongs its duration of action.(Eur Heart J. © 2013 Oxford University Press)

1.4.3 AChEI in the Treatment of AD

There are several strategies to improve cholinergic neurotransmission (Orhan et al., 2004), although the one that has been most successful so far is the "cholinergic hypothesis", i.e., stimulation of cholinergic receptors or increasing the availability of ACh released into the neuronal synaptic cleft by inhibiting ACh hydrolysis by acetylcholinesterase (AChE) through the use of AChE inhibitors (Figure 1.3) (Howes & Houghton, 2003; Lahiri et al., 2002).

Number of AChEI compounds have found application as drugs developed for the treatment of AD and myasthenia gravis (Ofek & Soreq, 2013; Silman & Sussman, 2005). These are based on the premise that increasing the availability of acetylcholine (ACh) at acetylcholine receptors in the brain, resulting in better neuron to neuron transport.

AChEIs as Tacrine, donepezil, galanthamine (an alkaloid from snowdrop) and rivastigmine, have since been developed and are currently in use for the treatment of AD (Shah et al., 2008). However, these drugs are known to have limitations due to their short-half-lives and/or unfavorable side effects (including gastrointestinal disturbances) and problems associated with bioavailability (Knapp et al., 1994; Sung et al., 2002; Wszelaki et al., 2010), which necessitates the interest in finding better AChEIs from natural resources (Adewusi et al., 2011; Ali et al., 2013; Amessis-Ouchemoukh et al., 2014; Benamar et al., 2010; Feitosa et al., 2011; Nicolson, 2003)).

1.4.4 AChEI in Plants

Plants have long been recognized as source of beneficial bioactive such as drugs, cosmetics, food supplements, functional foods and others; a very large proportion of the pharmaceutical compounds on the market are derived, directly or indirectly from plants (Benamar et al., 2010). Many synthetic drugs take their origin from plant based on modern medicine. Since AD, the fourth cause of death worldwide, has become a threat to public health, new treatment strategies based on medicinal plants have become focused. Determination of AChE activity has become an important tool in drug design and discovery as well as in medicine and toxicology. Broad variety of methods has been developed over the past decades for AChE inhibitory activity quantification (Abou-Donia et al., 2014; Miao et al., 2010; Pohanka, 2011).

1.5 Oxidative Stress in the AD

Strong experimental evidences have indicated that reactive oxygen species are associated with the pathogenesis of AD (Figure1.4), as some cellular characteristics of this disease are either causes or effects of oxidative stress theory (refers to the physiological condition at which the capacity of the endogenous antioxidant system fails to cope with the damaging effects of free radicals) of AD pathogenesis (Konrath et al., 2012; Sultana et al., 2006; Zhu et al., 2004). Generally, the physiological role of antioxidant compounds is to attenuate the oxidation chain reactions by removing free-radical intermediates (Liu & Nair, 2010). Since strong

experimental evidences demonstrate that oxidative stress is intimately involved in age-related neurodegenerative diseases, there have been a number of studies which have examined the positive effects of antioxidants in reducing or blocking neuronal death occurring in the pathophysiology of these disorders (Ramassamy, 2006). Consequently, the use of antioxidants has been explored in an attempt to slow AD progression and neuronal degeneration (Howes & Houghton, 2003).

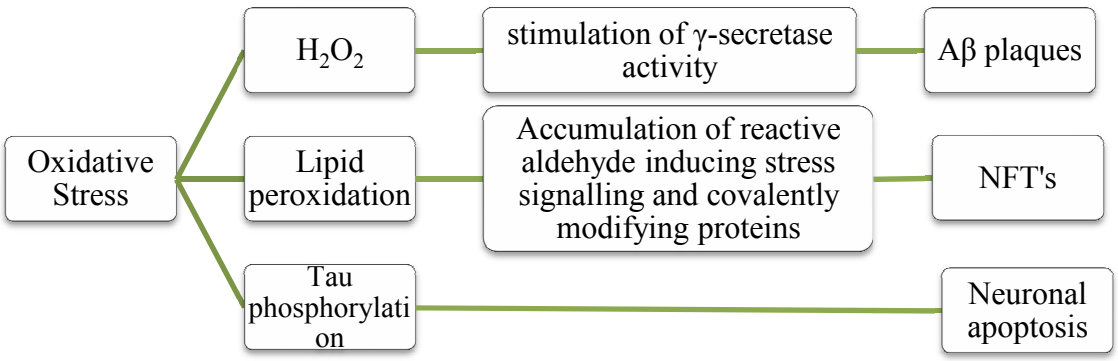

Figure (1.4): Oxidative stress in the pathogenesis of AD. Oxidative stress coincides with elevated levels of ROS, including peroxides such as hydrogen peroxide (H_2O_2), and in the accumulation of oxidation products in neurons. This may result in the stimulation of γ-secretase activity. Further, neuronal lipid peroxidation results in the accumulation of reactive aldehyde inducing stress signalling and covalently modifying proteins. Both pathways, together with phosphorylation of τ protein, eventually lead to the pathognomonic changes associated with AD, that is β-amyloid (Aβ) plaque formation, accumulation of NFT's and neuronal apoptosis

1.6 Traditional Arabic Palestinian Herbal Medicine (TAPHM)

In traditional practices of medicine, including TAPHM, plants have been used to enhance cognitive function and to reduce other symptoms

associated with AD. Plant constituents may not only act synergistically with other constituents from the same plant but may also enhance the activity of compounds, or counteract toxic effects of compounds, from other plant species. This approach has been used in various practices of traditional medicine, including TAPHM and traditional Chinese medicine (TCM) where a combination of plants is frequently prescribed. An ethnopharmacological approach may be useful in providing leads to identify plants and potential new drugs that are relevant for the treatment of cognitive disorders, including AD (Nijs et al., 2002).

Numerous medicinal plants have been used in TAPHM for the treatment of several diseases, including improvement of memory, AD and old age related diseases (Ali-Shtayeh & Jamous, 2006, 2008). However, the use of medicinal plants is mainly based on local tradition and not scientific knowledge.

1.7 Problem Hypothesis

Currently, a few drugs are available in the market as safe and effective for the treatment of AD. Some Palestinian herbal medicines that have been reported in TAPHM literature for treatment of age related diseases, plants with antioxidant and anticholinestrase activities have potential to be used as a novel template for the development of effective drugs for the treatment of AD

1.8 Objectives

- Evaluating in vitro some Palestinian medicinal plants that have been used in Traditional Arabic Palestinian Herbal Medicine (TAPHM) to treat age related diseases and to enhance memory.
- Explore newly potent and safe natural therapeutic agents for the treatment of the disease.

Chapter Two

Materials and Methods

Chapter Two
Materials and Methods

2.1 Reagent and Chemicals

Reagents used in this in vitro study are shown in Table 2.1

Table (2.1): Reagents used in this study

No.	Item	Company	Cat. NO #
1	Acetylcholinesterase (AChE) type VI-S from an electric eel	Sigma	C2888
2	Tris-HCl Tris (hydroxymethyl) aminomethane hydrochloride	invetrogen	15504-020
3	Bovine serum albumin (BSA)	Sigma	A2153
4	Acetylthiocholine iodide (ATCI)	Sigma	01480
5	5,5´-dithiobis [2- nitrobenzoic acid] (DTNB)	Sigma	D8130
6	Galanthamine hydrobromide from lycoris sp.	Sigma	G 1660
7	2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma	D 9132
8	Gallic acid	Sigma	G7384
9	Butylated hydroxyanisole (BHA)	Sigma	B1253
10	Ascorbic acid	Duchefa biochemie	A0602

2.2 Plant Materials

Forty seven plant species were collected in 2014 from Nablus and Tulkarm districts in the Northern part of Palestine (West Bank). Voucher specimens were deposited at the Herbarium of Biodiversity & Environmental Research Center-BERC, Nablus, Palestine (Table 2.2).

Ten of the plant species screened in this study are used in TAPHM for the treatment of age-related diseases, aiming to evaluate their activity as AChEIs and antioxidants (Table 2.2). Remaining thirty seven plant species were screened to Explore newly potent and natural therapeutic containing AChEI and thus can be used for several purposes.

Table (2.2): Palestinian medicinal plants selected for anicholinestrase and antioxidant activity screening

Family Plant species	Voucher No.	Arabic common name	Medicinal uses	References
Amaryllidaceae				
Narcissus tazetta L.	BERC-395	نرجس	skin, hair, and burns, Respiratory system, reproductive system	(Ali-Shtayeh & Jamous, 2008)
Apiaceae				
Smyrnium olusatrum L.	BERC-416		No traditional use reported	
*Foeniculum vulgare Mill.**	BERC-030	شومر	Respiratory system, digestive system, circulatory system, Skin, wounds, hair, eye diseases, ,reproductive system, , nervous system, skeletal and muscular system, kidney and urinary tract system, tooth inflammation, diabetes and cancer	(Ali-Shtayeh & Jamous, 2008)
Asteraceae				
Chrysanthemum coronarium L.	BERC-068	اقحوان ذهبي	Respiratory system, digestive system and circulatory system	(Ali-Shtayeh & Jamous, 2006; Ali-Shtayeh et al., 2000; Khalilia, 2001)
Conyza bonariensis Cronquist.	BERC-259	حشيشة الجبل	constipation and diarrhea	(Bukhari et al., 2013)
Helichrysum sanguineum (L.)\ Kostel.	BERC-091	دم الغزال	diuretic anti-asthmatic , and kidney stones,	(Aslana et al., 2006)
Phagnalon rupestre (L.) DC.	BERC-047	قديح	Weight loss, skin, hair, and burns, skeletal and muscular system, cancer	(Ali-Shtayeh & Jamous, 2006, 2008; Ali-Shtayeh et al., 2003; Ali-Shtayeh et al., 1998; Ali-Shtayeh et al., 2000; Ali Shtayeh & Abu Ghdeib, 1999; Daoud, 2008; Khalilia, 2001)

Family Plant species	Voucher No.	Arabic common name	Medicinal uses	References
Scorzonera papposa L.	BERC-401	لحية التيس الوردية	No traditional use reported	
Cruciferae				
Eruca sativa Miller *	BERC-202	جرجير	Anti-inflammatory and nervous system	(Ali-Shtayeh & Jamous, 2008)
Raphanus rostratus DC.	BERC-368	فجل خنجري	No traditional use reported	
Euphorbiaceae				
Euphorbia hierosolymitana Boiss.	BERC-170	حلبلوب	Skin, wounds and hair	
Fumariaceae				
Fumaria capreolata L.	BERC-367	رز الدجاج المتسلق	anti-hypertensives, diuretics, hepatoprotectants ,gastrointestinal disorders,and treatment of some skin diseases	(Maiza-Benabdesselam et al., 2007; Peris & Stübing, 1996)
Fumaria densiflora DC.	BERC-154	رز الدجاج الكثيف	anti-hypertensives, diuretics, hepatoprotectants ,gastrointestinal disorder and treatment of some skin diseases	(Peris & Stübing, 1996)
Fumaria vaillantii loisel	BERC-396	رز الدجاج	blood purifier , anti-hypertensives, diuretics, hepatoprotectants ,gastrointestinal disorders and treatment of some skin diseases	(Mandal et al., 2012; Peris & Stübing, 1996)
Geraniaceae				
Erodium malacoides (l.) L'Her.	BERC-357	الرقمة الطرية	injuries , legs and hands cracks	(Jaradat, 2005)
Pelargonium odoratissimum (L.) L 'He'r	BERC-049	عطرية	Circulatory system, headache and temperature , kidney and urinary tract system, diabetes	(Ali-Shtayeh & Jamous, 2008)
Juglandaceae				
Juglans regia L. *	BERC-230	جوز	Memory enhancer, antiseptic, for skin diseases antihypoglycaemic and depurative	(Ali-Shtayeh et al., 1997; Ali-Shtayeh & Jamous, 2008)

Family Plant species	Voucher No.	Arabic common name	Medicinal uses	References
Labiatae				
Mentha spicita L. *	BERC-116	نعنع	Memory enhancer, Nerve sedative, headaches	(Ali-Shtayeh & Jamous, 2008)
Rosmarinus officinalis L.*	BERC-018	حصالبان	Epilepsy, Memory enhancer, Sharpens the mind, anti-depressant, anxiety, and rheumatoid arthritis.	(Ali-Shtayeh & Jamous, 2008; Ali et al., 2013)
Salvia fruticosa (L.) Mill. *	BERC-006	مريمية	anti inflammatory gargle, antiseptic, antitussive, antihaemorrhoids pain, antirheumatic; anti stomach disturbances, astringent, carminative, hypotensivea	(Ali-Shtayeh & Jamous, 2008; Yaniv et al., 1987)
Majorana syriaca (L.) Rafin.*	BERC-026	زعتر بري	Circulatory system, eye diseases, weight loss, skin, hair, and burns, reproductive system, respiratory system, nervous system, skeletal and muscular system, digestive system, headache and temperature , kidney and urinary tract system, tooth inflammation , diabetes, cancer	(Akiyama et al., 2000; Ali-Shtayeh & Jamous, 2006, 2008; Ali-Shtayeh et al., 2011, 2012; Daoud, 2008; Khalilia, 2001)
Liliaceae				
Allium neapolitanum Cirillo	BERC-414	الثوم الأبيض	No traditional use reported	
Asphodeline lutea (L.) Rchb.	BERC-371	أبو صوي	Antispasmodic and diuretic	(Ali-Shtayeh et al., 1998)
Asphodelus aestivus Brot. (Asphodelus microcarpus Salzm. & Viv)	BERC-210	بصول	Skin and wounds	(Ali-Shtayeh & Jamous, 2008)
Bellevalia flexuosa Boiss.	BERC-374	بصيلة الفار	No traditional use reported	(Ali-Shtayeh et al., 1998)
Ornithogalum narbonense L.	BERC-464	نجمة بيت لحم	No traditional use reported	
Tulipa sharonensis Dinsm.	BERC-431	زنبق بري	No traditional use reported	

Family Plant species	Voucher No.	Arabic common name	Medicinal uses	References
Myrtaceae				
Myrtus communis L.	BERC-051	ريحان	Epilepsy , treat inflamed eyes and sore throat	(Ali-Shtayeh & Jamous, 2008)
Orchidaceae				
Ophrys dinsmore Schltr	BERC-452	اوركيد الكرمل	No traditional use reported	
*Ophrys lutea (*Gouan*)* Cav	BERC-432	نحله صفراء	No traditional use reported	
Orchis caspia Trautv	BERC-422	سحلب	No traditional use reported	
Orchis collina Banks &Sol.	BERC-448	الاوركيد مروحي الشفة	No traditional use reported	
Oxalidaceae				
Oxalis pes-caprae L.	BERC-265	حمصيص	Skin ,digestive system	(Ali-Shtayeh & Jamous, 2008)
Papilionaceae				
Lupinus pilosus L. (*Lupinus varius* L.)	BERC-019	ترمس بري	No traditional uses reported	(Ali-Shtayeh et al., 2003)
Vicia hybrida L.	BERC-420	قرينه بقره	No traditional use reported	
Ranunculaceae				
Anemone coronaria L.	BERC-355	بنفسج	Skin, wounds and hair	(Ali-Shtayeh & Jamous, 2008)
Nigella sativa L. *	BERC-143	حبة البركة	Memory enhancer, Nerve sedative, headaches, rheumatoid arthritis, Stimulant, improving memory, resolutive, considered as an adaptogen	(Al-Turkimany, 1993; Ali-Shtayeh & Jamous, 2008)
Ranunculus asiaticus L.	BERC-400	برقوق، نموار	No traditional use reported	
Ranunculus millefolius Banks & sol.	BERC-475	ثميره	No traditional use reported	
Rubiaceae				
Galium pisiferum Boiss.	BERC-038	لزيقة	No traditional use reported	
Salvadoraceae				
Retama raetam (Forssk.) Webb	BERC-043	رتم	anti-inflammatory, treat inflamed eyes and sore throat, antirheumatic, treat infertility,treat paralysis, analgesic, treat stomach-ache	

Family Plant species	Voucher No.	Arabic common name	Medicinal uses	References
Sapindaceae				
Dodonaea viscosa L.	BERC-045		Itching, fevers swellings, aches, a antispasmodic agent, toothaches, headaches sprains, bruises, burns and wounds, ulcers and digestive system	(Rani et al., 2009)
Solanaceae				
Mandragora autumnalis Bertol	BERC-286	تفاح المجن	As ointment for external use, pain, insomnia, eye diseases, inflammation, and ulcers, Anesthetic, Relief the pains from the medical operations.	(Hanu et al., 2006; Jaradat, 2005; Karim & Quraan, 1986; Khalilia, 2001)
Urticaceae				
Parietaria judaica L.	BERC-063	عشبة الدم	Cancer, to stop bleeding from fresh skin, wounds, vulnerary, diuretic, cholagogic	(Ali-Shtayeh et al., 2003; Ali-Shtayeh et al., 2011; Ali-Shtayeh et al., 1998; Ali Shtayeh & Abu Ghdeib, 1999; Khalilia, 2001)
Urtica pilulifera L.*	BERC-066	قريص	Memory enhancer, and rheumatoid arthritis	(Ali-Shtayeh & Jamous, 2008)
Zingiberaceae				
Zingiber officinale Rose.*	BERC-	زنجبيل	Memory enhancer andjoints inflammation	(Al-Antaki, 1935; Al-Turkimany, 1993; Ali-Shtayeh & Jamous, 2008; Ibn AlBitar, 1874).
Zygophyllaceae				
Peganum harmala L.	BERC-181	حرمل	Hallucinogenic, epilepsy, mental and nervous illnesses, relieves joints inflammation Cures headaches, strokes, numbness, epilepsy and forget fulness	(Al-Antaki, 1935; Al-Turkimany, 1993)

*** Plant species reported in TAPHM for treatment of age-related diseases**

2.3 Plant Extraction

A total of 92 plant parts (leaves, flowers, stem, seeds, fruits) were collected, separated and ground to fine pieces using an electric mill (Phillips, France) and plant material was exhaustively extracted with 60% Ethanol (2 ml/g), at room temperature for 24 h. In all cases, the solutions were filtered and concentrated to dryness under reduced pressure in a rotary evaporator (45◦C)(Stuart) (Figure 2.1) . Dry extracts were stored at −20 ◦C until used (Figure 2.2).

Figure (2.1) Rotary evaporator used for drying plant extracts under reduced pressure.

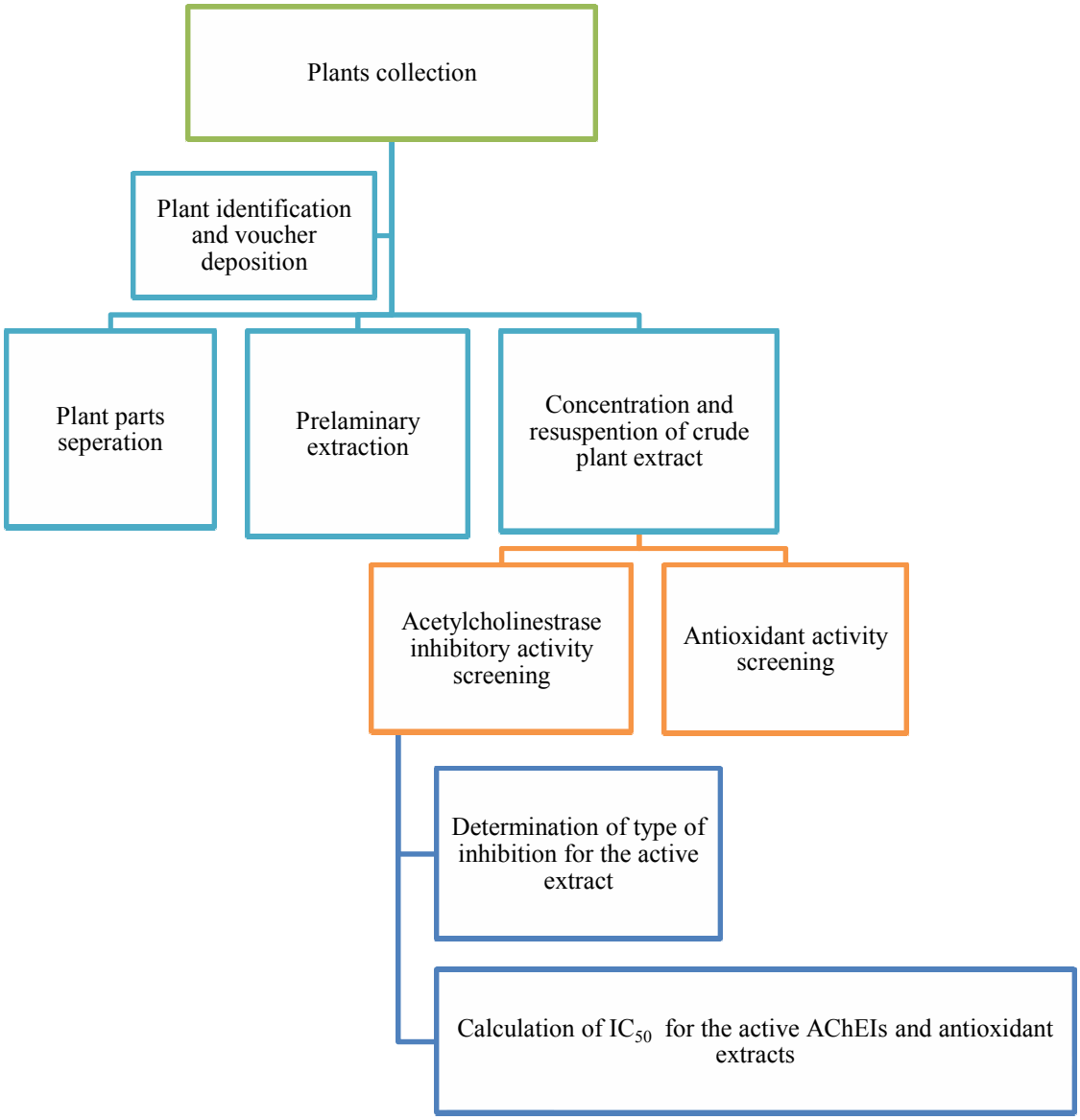

Figure (2.2): Flow diagram showing the layout of plant extracts preparation, anticholinestrase and antioxidant activity evaluation

2. 4 Evaluation of AChE Inhibitory Activity

AChE inhibitory activity was determined spectrophotometrically using a 96-well microplate reader (Biotek USA) (Figure 2.3) based on previously published method (Ellman, Courtney, & Featherstone, 1961; Lee, Lee, Yang, Baek, & Kim, 2004).

Figure (2.3) samples absorbences measurment using spectrophotometr

2. 4.1 Principle of the Reaction

The chemical principle of the reaction is depicted in Figure 2.2. The enzyme hydrolyzes the substrate Acetylthiocholine iodide (ATCI) to thiocholine and acetic acid. Thiocholine is allowed to react with 5,5'-dithiobis-nitrobenzoic acid (DTNB) and this reaction results in the development of a yellow color. The color intensity of the product is measured at 405 nm, and it is proportional to the enzyme activity.

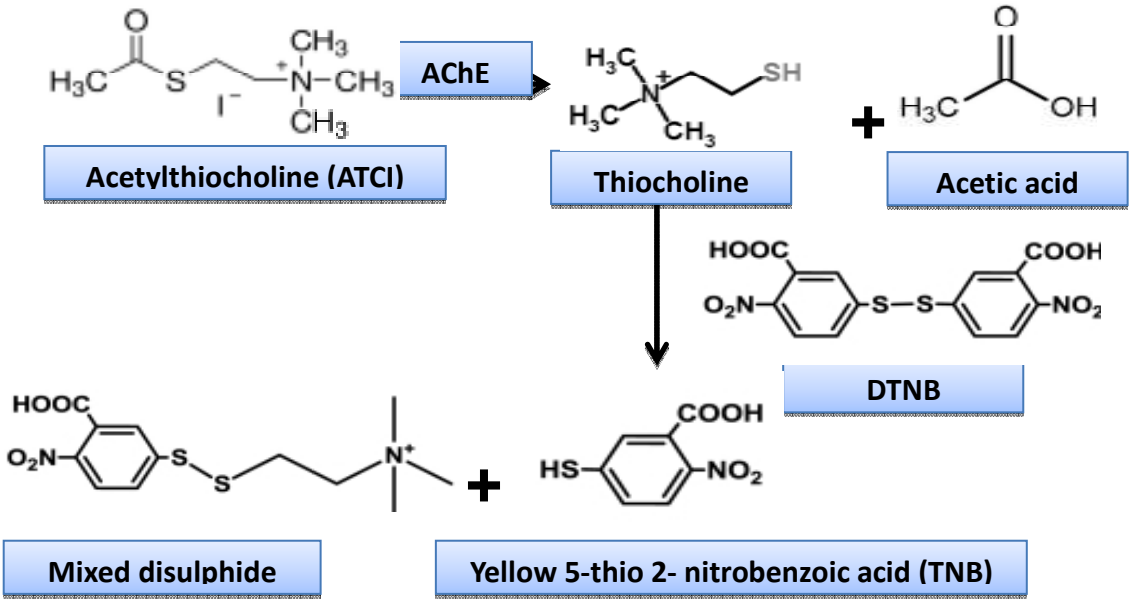

Figure (2.4): Chemical Mechanism of Ellman's method.

2.4.2 Preparation of the Enzyme and Solutions

The lyophilized enzyme was dissolved in buffer A (50 mM Tris – HCl, pH 8) to make al 1000 units/ml stock solution. It was further diluted with buffer B (50 mM Tris –HCl, pH 8 containing 0.1% bovine serum albumin) to get 0.22 unit /ml enzyme solution for the microplate assay. The enzyme stock solution was kept in -20 freezer , ATCI (15 mM) was prepared by dissolving in distilled water , DTNB (0.3 mM) was prepared in buffer C (50 mM Tris –HCL, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$); galanthamin (1 mg/ml) was prepared in distilled water and used as a reference standard . The evaporated ethanolic plant extracts were dissolved in distilled water so obtain the required concentrations**.**

2.4.3 The Multi-well Plate AChE Inhibition Assay

Acetylcholinestrase inhibition was determined spectrophotometrically as described in (Lee et al., 2004). In the 96-well plates, a reaction mixture of 25 µL of 15 mM ATCI, 125 µL of 3 mM DTNB and 25 µL of the plant extract were added and the absorbance was measured at 405 nm. Thereafter, 25 µL of AChE solution (0.22 U/mL) was added to the wells and the microplate was read again at the same wavelength 10 times with 1 min intervals. Galanthamine used as standard drug at 1 mg/ml concentrations; a blank of water in 50 mM Tris-HCl, (pH 8) was used.

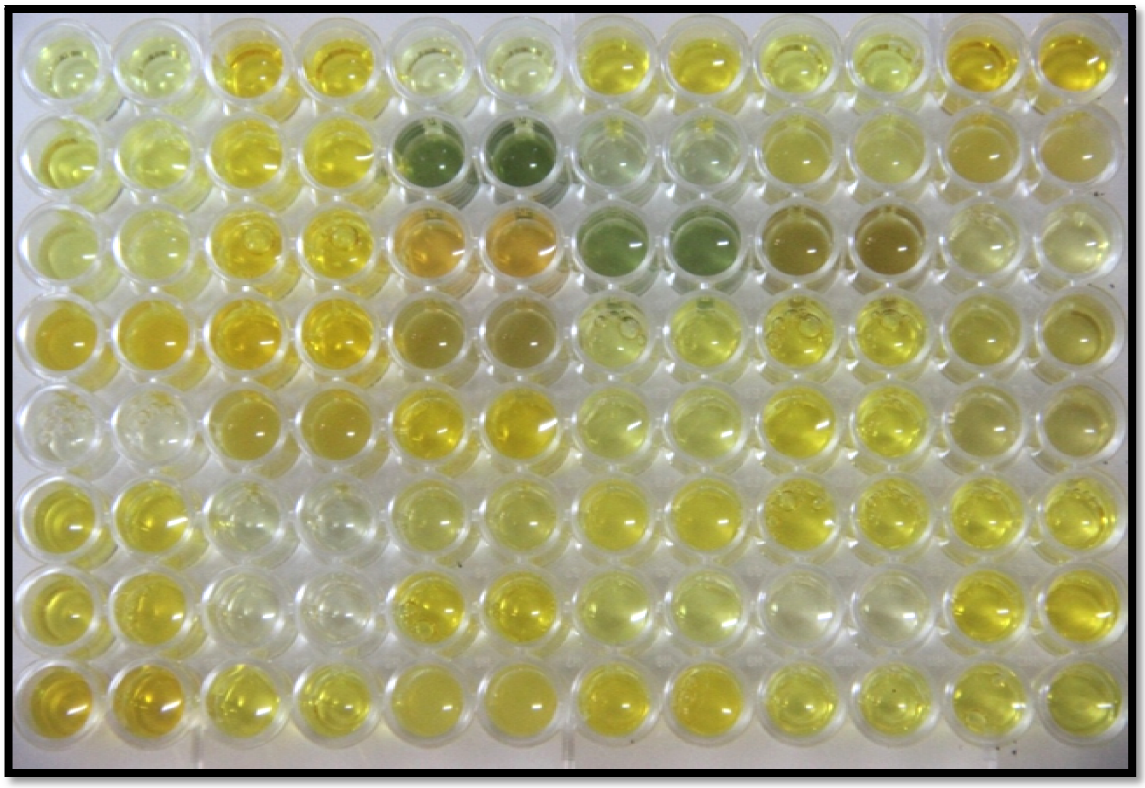

Figure (2.5): The multi-well plate AChE inhibition Assay. 96 micro-well plate containing a reaction mixture of 25 µL of 15 mM ATCI, 125 µL of 3 mM DTNB and 25 µL of the plant extract and 25 µL of AChE solution (0.22 U/mL).Galanthamine used as standard drug at 1 mg/ml concentrations placed in first two wells; a blank of water in 50 mM Tris-HCl, (pH 8) placed in the second two wells.

2.4.4 Calculation of Enzyme Activity

The rate of color change per min was calculated for each reading. Any increase in the absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the absorbance before addition of the enzyme from the absorbance after addition of the enzyme. The percentage inhibition of the enzyme activity for each test solution was calculated using the following equation:

$$\text{Inhibition (\%)} = 1 - \left(\frac{A\ sample}{A\ Blank}\right) \times 10$$

Where A_{sample} and A_{Blank} represent the change in the absorbencies of sample and blank.

2.4.5 Estimation of IC_{50} Values

The IC_{50} values (concentration of test compounds that inhibits the hydrolysis of substrates by 50 %) were determined by spectrophotometric measurement of the effect of increasing concentrations of test compounds (plant extracts and positive controls) on AChE activity. To calculate the IC_{50} values (Figure 2.3), each sample was assayed at eight concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 mg/ml). IC_{50} values were obtained from dose-effect curves by linear regression.

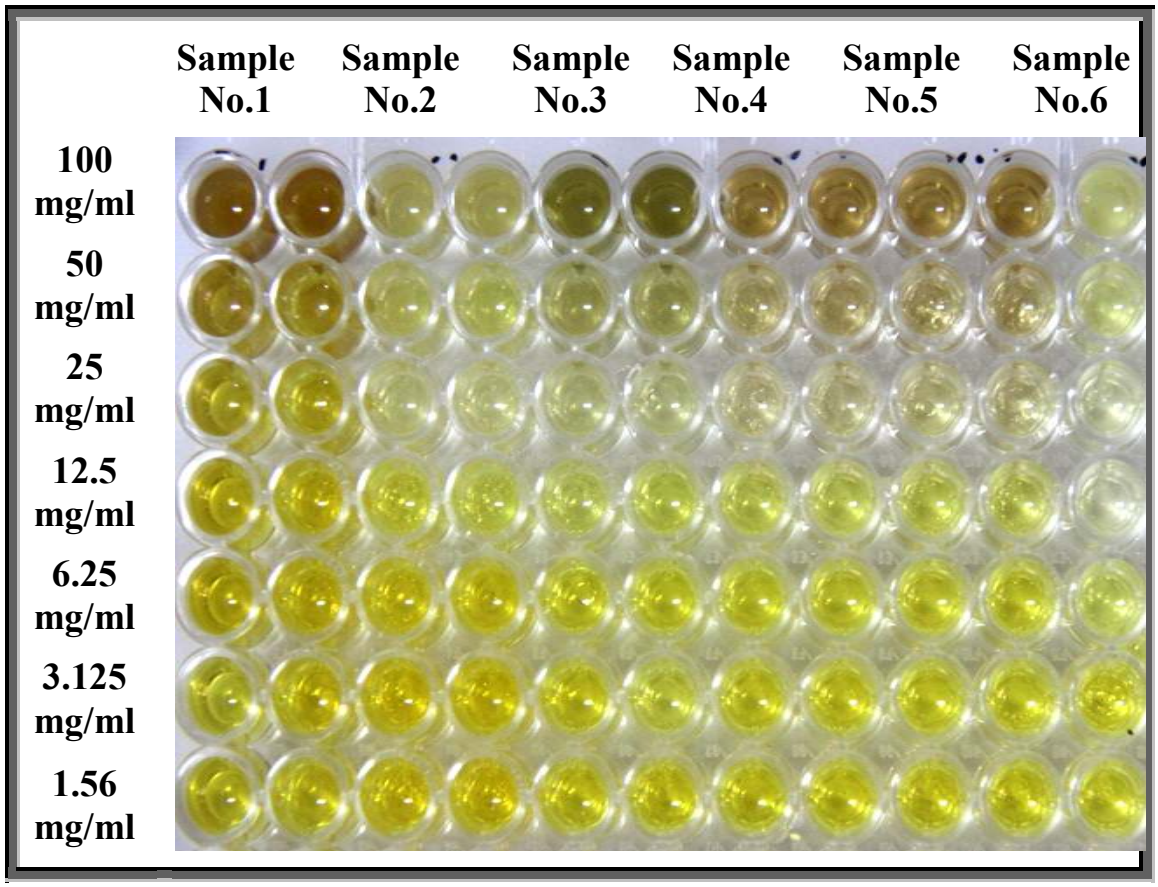

Figure (2.6): The Microtiter plate for estimation of IC_{50} values, plate made up of 12 columns (1-12) and 7 rows (A-G), with the different concentrations (mg/ml) of plant extracts

2.5 Determination of the Inhibition Type of Plant Extracts on AChE

The type of inhibition of AChE by extracts (reversible or irreversible inhibition) was determined by measuring the restored AChE activity by 10 time dilution of plant extract concentration after mixing and incubation of AChE and plant extract. In reversible inhibition, AChE activity can be restored by dilution of plant extract, while there is no change in AChE activity with dilution of plant extract in irreversible inhibition (Ali et al., 2013).

2.6 Determination of Antioxidant Activity Using Scavenging Activity of DPPH Radical

Free radical scavenging activity of plant extracts was determined using the free radical 1,1-diphenly-2-picrylhydrazyl-hydrate (DPPH), which is a molecule containing a stable free radical (Sharma & Bhat, 2009). In the presence of an antioxidant which can donate an electron to DPPH, the purple color which is typical for free DPPH radical decays and the change in absorbance at 517 nm is followed spectrophotometerically. The effect of the plant extracts on DPPH radical was estimated using the method of (Liyana-Pathirana & Shahidi, 2005) with minor modification. Twenty five micro liter of plant extract were added to 175µl of 0.004% DPPH methanolic solution, in a 96-well plate. Appropriate blanks were prepared using the solvent only in addition to the same amount of DPPH reagent to overcome any inherent solvent activity. The reaction mixture was shaken well and allowed to stand at room temperature in the dark for

30 min, and then the decrease in absorbance at 517 nm was measured against a control (methanol solution) by using UV-vis spectrophotometer. The radical-scavenging activity of samples, expressed as percentage inhibition of DPPH (I %), and it was calculated according to the formula:

$$\%\, I \;=\; [((Acontrol - Asample)/)Acontrol]x100$$

Where $A_{control}$ is the absorbance of DPPH radical

IC_{50} of extracts and standard was determined using non-linear regression analysis of the dose-%I relationship.

2.7 Data Analysis

Tests were carried out where possible at least in duplicate and on two different occasions. Results are reported as mean ± standard deviation (S.D.). Standard curves were generated and calculation of the 50% inhibitory concentration (IC_{50}) values was done using Excel.

Chapter Three

Results

Chapter Three
Results

Forty seven plant species were selected based on their uses as remedies for the central nervous system diseases, as antidotes for human and animal poisoning or to improve memory and cognitive function (Table 2.1).The plants were mainly collected from their natural habitats or rarely from "Attarin" shops. A total of 92 plant extracts were tested *in-vitro* for its anticholinestrtase and antioxidant activities.

3.1 Screening of Plant Extracts Acetylcholinestrase Inhibitory Activity

The inhibition effect of plant extracts from 92 different extracts on AChE activity was screened using the Ellman's method. The results obtained by Ellman's method of all plant extracts are shown in Table 3.1

Table (3.1): Anticholinestrase and antioxidant activity % inhibition) of selected Palestinian plants.

Scientific name	Plant Part*	Ellman's	DPPH
Allium neapolitanum	LE	6.21± 0.30	42.12±1.24
	FL	58.04±1.19	53.26±1.78
	ST	28.75±2.33	58.36±2.32
	BU	48.46±3.05	73.4±0.85
Anemone coronaria	FL	35.67±2.02	68.94±1.33
	LE	33.33±.33	76.73±1.03
Asphodeline lutea	LE	6.08±0.11	70.16±0.23
	BU	41.34±3.76	22.1±1.27
Asphodelus microcarpus	BU	4.64±0.91	53.55±2.05
	FL	1.73±0.38	25.3±0.99
Bellevalia flexuosa	ST	2.47±0.75	14±1.41
	LE	11.38±0.54	52.3±0.99
	BU	22.37±0.52	65.34±0.93
	FR	10.96±1.36	82.69±1.85
	FL	19.75±1.06	25.3±1.5
Chrysanthemum coronarium	LE	38.91±1.29	22.4±0.2

Scientific name	Plant Part*	Ellman's	DPPH
Conyza bonariensis	LE	81.23±2.50	65.25±1.06
	FL	83.12±2.66	83.94±0.08
Dodonaea viscosa L.	LE	40.27±0.38	138.83±0.24
Erodium malacoides	LE	33.69±0.98	70.5±0.71
	FL	1.11±1.26	74.25±1.06
Eruca sativa	LE	79.41±1.08	75.8±0.54
Euphorbia hierosolymitana	LE	19.75±1.06	93.07±0.10
	FL	29.81±1.15	99.33±1.88
Foeniculum vulgare	LE	52.68±0	72.12±0.76
Fumaria capreolata	FR	97.37±0.52	37.08±1.30
	LF	90.27±0.38	69±1.41
	FL	98.14±1.61	76.41±0.83
Fumaria densiflora	LE	83.12±0.17	56±1.41
	FL	82.31±0.44	73.66±1.90
Fumaria vaillantii	LE	95.98±1.39	67.25±0.35
Galium pisiferum	FL	13.38±0.54	60.5±0.71
	LE	18.04 ± 0.06	81.58±0.59
Helichrysum sanguineum	LE	28.46 ±0.65	100±1.41
	FL	16.5±0.71	100±1.41
Juglans regia	FR	11.36±1.50	78.69±0.20
Lupinus pilosus	FL	53.98±1.39	44.23±1.09
	LE	70.52±0.74	88.16±1.19
	FR	44.93±1.5	71.26±0.27
Majorana syriaca	LE	88.1±1.98	100±1.3
Mandragora autumnalis	FR	98.12±0.17	27.21±0.30
	RT	97.46±0.65	48.08±1.30
	LE	94.4±0.57	68.91±0.13
	FR(ripen)	72.0±1.94	62.11±1.45
Mentha spicata	LF	74.17±0.176	93.52±0.33
Myrtus communis	LE	85.59±0.75	94.23±0.35
Narcissus tazetta	BU	95.34±0.93	25.21±1.54
Nigella sativa	SE	92.49±0.38	88.08±0.35
Ophrys dinsmore	BU	20.96±1.36	17.6±0.57
	ST	32.88±1.24	70.67±0.95
	FL	39.79±1.12	85.19±1.15
	LE	25.58±0.82	143.33±2.36
Ophrys lutea	FL	51.75±1.06	64.32±0.96
	ST	31.33±0.47	88.3±0.99
	LE	54.5±0.71	94.23±1.09

Scientific name	Plant Part*	Ellman's	DPPH
Orchis caspia	ST	27.75±1.06	79.32±0.96
	LE	97.61±0.86	81.75±0.35
	FL	43.79±1.12	87.21±1.12
Orchis collina	BU	26.5±0.71	10.19±1.15
	ST	23.04±0.06	55.61±0.55
	LE	38.33±0.95	85.38±2.29
	FL	33.25±0.35	91.41±0.83
Ornithogalum narbonense	BU	- 5.67 ± 0 .95	54.71±0.41
	FL	- 0.33 ± 0 .95	46.25±1.06
	ST	17.21 ± 1.12	67±1.41
	LE	-3.71 ± 1.00	80.86±1.22
Oxalis pes-caprae	FL	100.22±0.31	110.75±1.06
	LE	88.26±0.37	147.43±0.61
Parietaria judaica	LE	31.91±1.29	71.66±0.93
Peganum harmala	SE	81.06±0.08	80.12±0.15
Pelargonium odoratissimum	LE	- 1.58±1.24	88.91±0.13
Phagnalon rupestre	FL	93.24±0.34	57±0.00
	LE	95.61±0.86	150.2±.036
Ranunculus asiaticus	FL	88.47±0.66	45.75±0.35
Ranunculus millefolius	LE	76.75±1.06	57.58±0.82
	FL	17.33 ± 0 .47	64.61±0.55
Raphanus rostratus	LE	24.1±1.27	81.75±0.35
Retama raetam	FL	29.59±0.83	51.5±0.71
	LE	16.62±3.37	59.58±0.59
Rosmarinus officinalis	LE	95.32±0.45	99.16±1.19
Salvia fruticosa	LE	46.81±2.26	73.1±0.97
Scorzonera papposa	FL	17.33 ± 0 .47	44.8±1.13
	LE	15.83 ± 0.24	49.03±1.37
Smyrnium olusatrum	LE	5.01±1.98	57.83±1.17
	FL	3.98±1.39	82.41±0.83
Tulipa sharonensis	LE	56.5±0.71	38.08±1.30
	ST	50.79±0.30	61.9±0.14
	FL	37.42±0.82	64.41±0.58
	BU	- 18.63±0.89	71.82±0.25
Urtica pilulifera	LE	2.73±0.77	86.66±0.96
Vicia hybrida	LE	49.96±1.36	41.05±1.34
Zingiber officinale	BU	48.04±0.06	99±1.2
Galanthamin	NA	93.44±2.21	

*** BU, Bulb, FL, Flower; FR, Fruit; LE, Leaves; SE, Seeds; ST, Stem; RT, Roots.**

Values were expressed as mean of percentage of inhibition ± standard deviation

The screenings were performed at a concentration of 100 mg/ml and the extracts were considered as active if they only inhibited the enzyme more than 50%. Active extracts divided into two categories according to their anticholinestrase activity (Table 3-2); high activity with percentage of inhibition more than 75 % , moderate activity with percentage of inhibition between 50-75 %, while extracts with percentage of inhibition less than 50% were divided into three categories: no, very low, and low (Table 3.2)

Table (3.2): Categories of AChE inhibitory activities based on Ellman's inhibition assays

Categories of AChE inhibitory activities (percentage of inhibition)	Number of extracts (%)
Activated AChE (<0%)	5 (5.4)
Very low activity (0%-25%)	26 (28.3)
Low activity (25%-50%)	26 (28.3)
Moderate activity (50%-75%)	10 (10.9)
High activity (75%-100%)	25 (27.1)
Total	**92 (100)**

Thirty five 35 (38%) extracts inhibited AChE by > 50%, of these extracts 25 (27.1%) have high AChEI activity and 10 (10.9%) have moderate activity. Fifty two extracts inhibited AChE by ≤ 50 %, 26 (28.3 %) have low activity and 26 (28.3%) have very low activity, the remaining 5 extracts activated the enzyme (Table 3.2)

The most potent plant extracts which inhibited AChE more than 90 % were the Leaves, fruits and flowers of *Fumaria capreolata*, leaves, roots and fruits of *Mandragora autumnalis,* leaves of *Orchis caspia*, flowers of

Oxalis pes-caprae, leaves and flowers *Phagnalon rupestre*, seeds of *Nigella sativa, leaves of Rosmarinus officinalis,* Bulbs of *Narcissus tazetta,* and Leaves of *Fumaria vaillantii* (Table 3.1)

3.1.1 Distribution of Active AChEI Extracts on Different Plant Families

The screened plant species belong to 41 genera and 22 families (Table 3.3). All extracts belonging to the Fumariacea family inhibited AChE more than 80 %. While extracts belonging to Liliaceae and Orchidaceae did not reduce the enzyme activity, the percentage of inhibition for plant extracts belonging to these two families ranged from 1.73 to 58.4. Moreover, the enzyme activity was increased by leaves, flowers and stem of *Ornithogalum narbonense* and bulbs of *Tulipa* increased the enzyme activity.

Table (3.3): distribution of active AChEI and antioxidant extracts on different plant families.

Family name	No.of plant species	No. of extracts tested	No. of active extracts (AChEIs) (%)	No. of active extracts (Antioxidant) (%)
Amaryllidaceae	1	1	1(100)	0(0)
Apiaceae	2	3	1(33)	3(100)
Asteraceae	5	9	4(44)	6(67)
Brassicaceae	2	3	1(33)	3(100)
Euphorbiaceae	1	2	0(0)	2(100)
Fumariaceae	3	6	6(100)	5(83)
Geraniaceae	2	3	0(0)	3(100)
Juglandaceae	1	1	0(0)	1(100)
Labiatae	4	4	3(75)	4(100)
Liliaceae	6	21	3(14)	14(67)
Myrtaceae	1	1	1(100)	1(100)
Orchidaceae	4	14	3(21)	12(85)
Oxalidaceae	1	2	2(100)	2(100)
Papilionaceae	2	4	2(50)	2(50)
Ranunculaceae	3	6	3(50)	5(83)
Rubiaceae	1	2	0(0)	2(100)
Salvadoraceae	1	2	0(0)	2(100)
Sapindaceae	1	1	0(0)	1(100)
Solanaceae	1	4	4(100)	2(50)
Urticaceae	2	2	0(0)	2(100)
Zingiberaceae	1	1	0(0)	1(100)
Zygophyllaceae	1	1	1(100)	1(100)
Total	**47**	**92**	**35(38)**	**73(79)**

3.2. Determination of IC_{50} of the active extracts AChEI

The dose-dependent AChE inhibitory activity of the active herbs was further studied and the IC_{50} values of inhibition were determined (Figure 3.1). Ethanol extracts of the tested plants were found to have high AChE inhibitory activities in a dose-dependent manner. Further testing and analyses of the inhibition of AChE by fruits, leaves and flowers of *Fumaria capreolata*, flowers of *Conyza bonariensis* and *Fumaria densiflora*, leaves

of *Mandragora autumnalis and Lupinus pilosus* revealed IC_{50} values less than 3.5 mg/ml

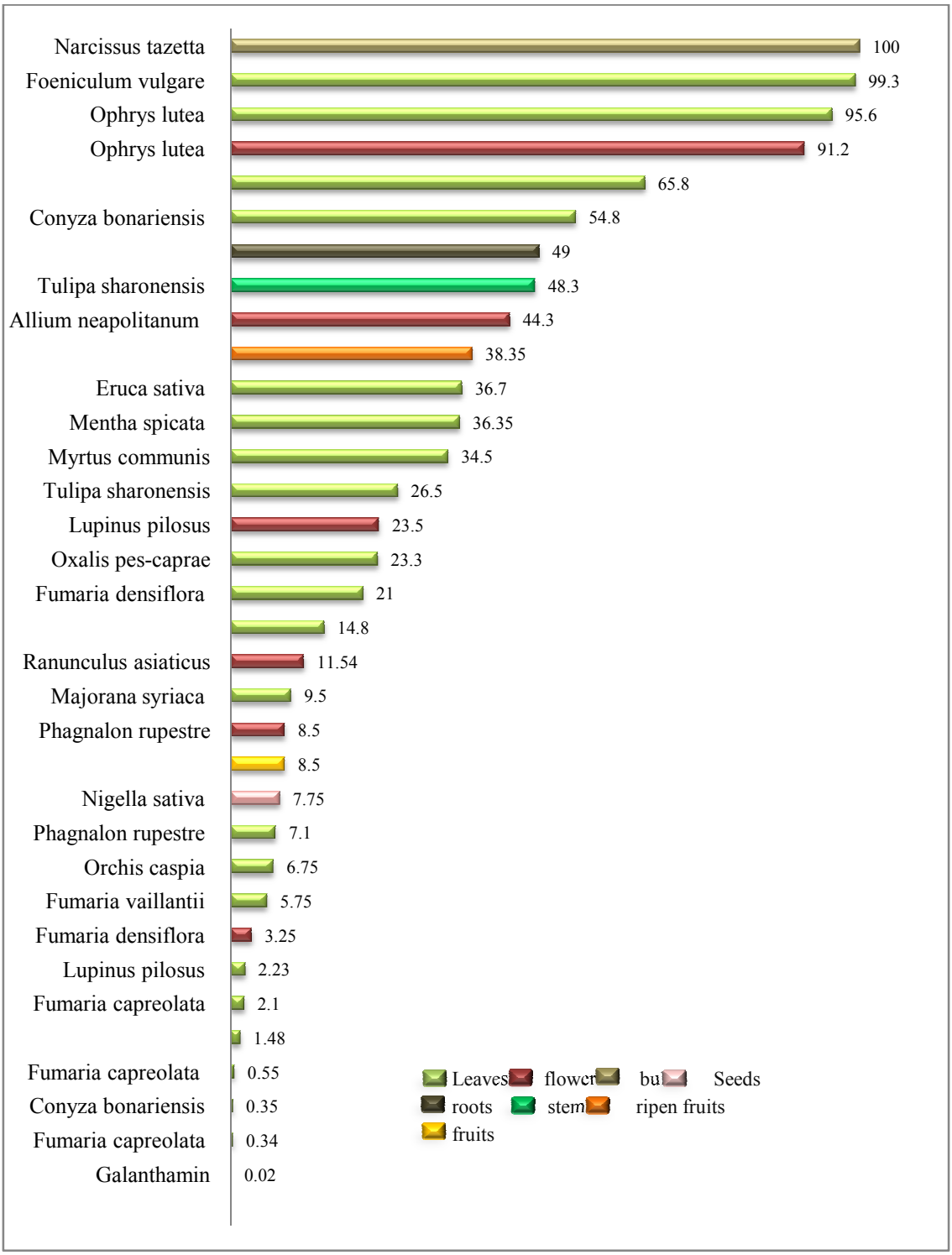

Figure (3.1): IC_{50} values for acetylcholinestrase inhibition by plant extracts.

3.3. Determination of AChEIsType

The inhibition type of plant extracts which showed ≥ 50% inhibition activity was determined by assaying the change in the remaining AChE activity of the mixture of AChE and the plant extract before and after the dilution of the plant extract in the same mixture. While AChE activity was restored more than 11 and 25 fold by 10 times dilution of seeds of *Nigella sativa* and leaves of *Rosmarinus officinalis,* respectively. However, the same dilution of flowers, and fruits of *Fumaria capreolata,* the leaves and flowers of *Fumaria densiflora,* the seeds of *Peganum harmala,* and the leaves of *Lupinus pilosus* and *Mandragora autumnalis,* did not show any effect on the remaining activity of AChE after dilution. These results indicate that AChE is inhibited reversibly by *Nigella sativa and Rosmarinus officinalis* and irreversibly *Fumaria capreolata, Fumaria densiflora Lupinus pilosus*, *Peganum harmala* and *Mandragora autumnalis*

Table (3.4): Acetylcholinestrase inhibitors type of inhibition

Type of inhibition	No of extracts (%)
Reversible	27 (77.1)
Irreversible	8 (22.9)
Total	**35(100)**

3.4 Screening of Antioxidant Activity

Table 3.1 shows the antioxidant activity results of the tested plant extracts. Seventy three extracts showed ≥ 50% antioxidant activity, of which *Phagnalon rupestre, Oxalis pes-caprae, Ophrys dinsmor, Dodonaea viscosa, Helichrysum sanguineum,* and *Majorana syriaca* were the most active.

3.5 IC_{50} of the antioxidant activity

The IC_{50} of the antioxidant activity for the plants extracts which showed $\geq$ 50 % AChE inhibition activity was determined (Figure 3-3). Of theseleaves of *M. syriaca* (IC_{50} 0.212mg/ml), leaves of *Rosmarinus officinalis* (0.377 mg/ml), leaves of *Fumaria densiflora* (0.514 mg/ml), leaves of O*rchis caspia* (0.514 mg/ml), leaves of *Mentha apicata* (0.56 mg/ml), flowers of *Fumaria densiflora* (0.678 mg/ml), flowers of *Fumaria capreolata* (0.69 mg/ml), and flowers of *Phagnalon rupestre* (0.928 mg/ml) were particularly strong antioxidants when compared to the reference radical scavengers (BHA, gallic acid, and ascorbic acid) recording IC_{50}'s < 1 mg/ml.

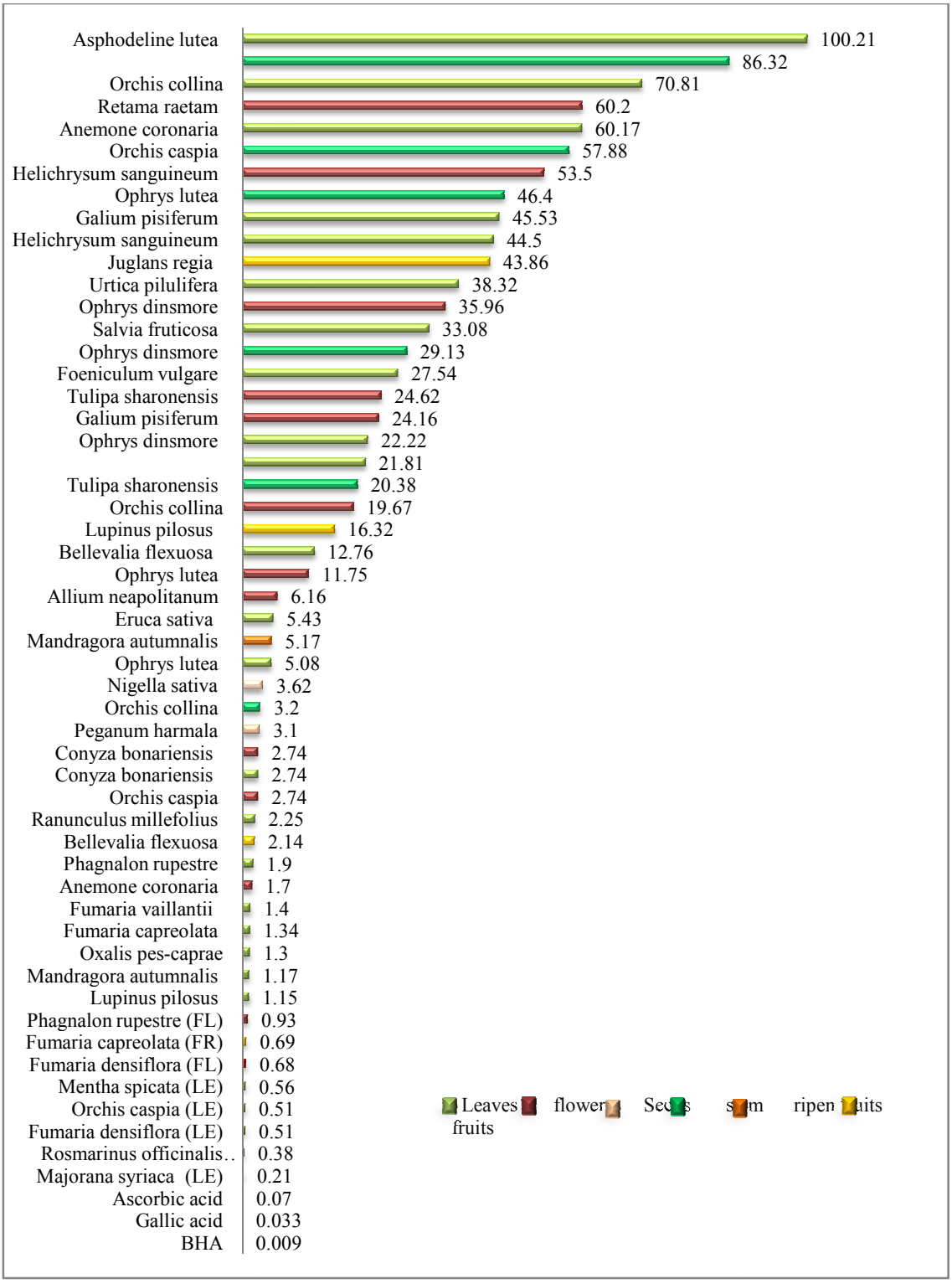

Figure (3.2): IC_{50} values for antioxidant activity of plant extracts

3.6 Acetylcholinestrase inhibitory activity of plants used in TAPHM

Ten of the plant species screened in this study were reported in TAPHM for the treatment of age-related diseases (Figure 3.3), of which

Urtica pilufera and Juglans regia revealed very low reversible inhibitory activity, *Salvia fruticosa* and *Zingiber officinale* exhibited low reversible inhibitory activity, *Foeniculum vulgare, Mentha spicata* and *Eruca sativa* reversibly reduced the enzyme activity to 47.37%, 25.83% and 20.59% with IC_{50} 99.3mg/ml , 36.35mg/ml and 36.7 mg/ml, respectively (Figure 3.1). *Majorana syriaca, Nigella* sativa and *Rosmarinus officinalis* exhibited the highest reversible inhibitory activity more than 85 % and IC_{50} less than 15mg/ml.

All of these extracts revealed relatively high antioxidant activity ranging between 72.1 % for *Foeniculum vulgare* and 100 % *for Majorana syriaca* , which has the lowest IC_{50} value of 0.21 mg/ml (Figure 3.2).

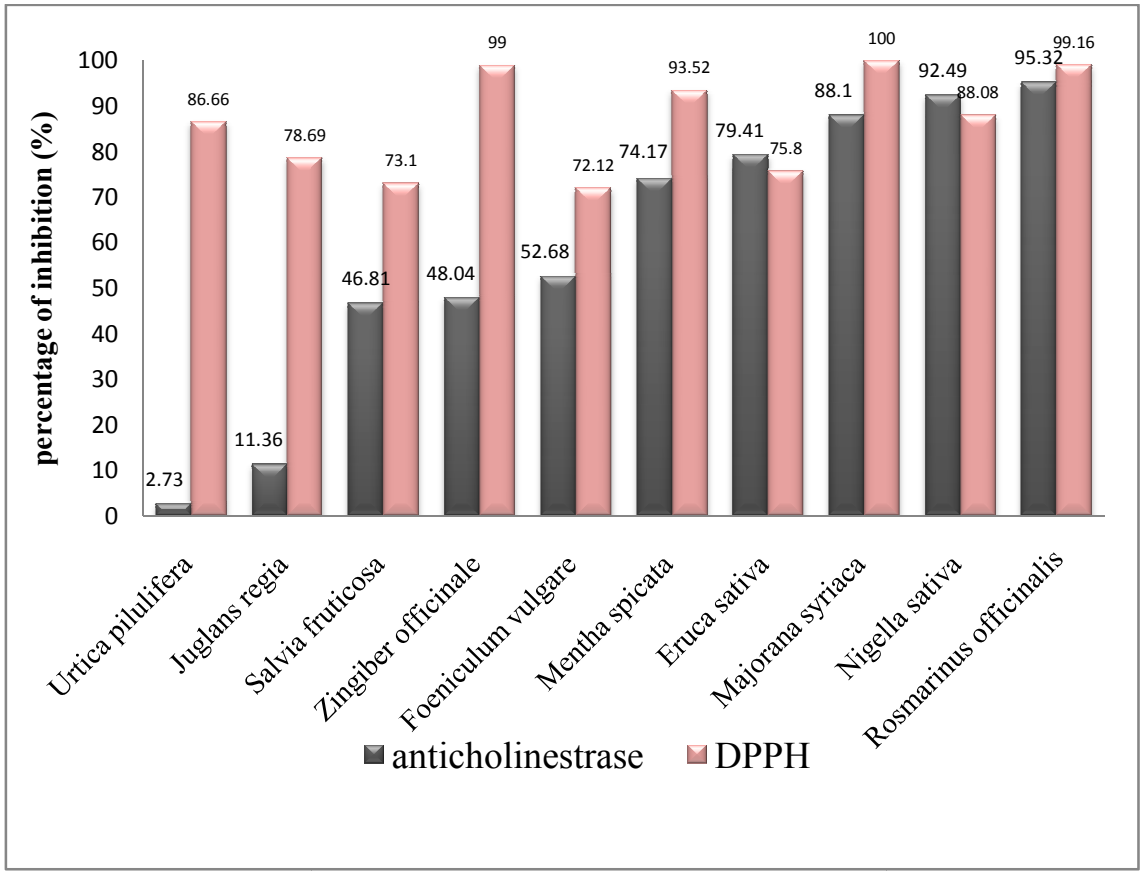

Figure (3.3): Antioxidant and acetylcholinestrase inhibitory activities of plants used in TAPHM for the treatment of age-related diseases.

Chapter Four

Discussion

Chapter Four
Discussion

Palestine is enriched with great plant diversity, and 368 of these plants have been reported to be used in TAPHM for the treatment of several diseases (Ali-Shtayeh et al., 2014). However, the use of medicinal plants is mainly based on local tradition and not scientific knowledge (Ali-Shtayeh et al., 2012; Ali-Shtayeh et al., 2000). The chemical constituents of most of these plants are unknown and may have dangerous effect on human health. On the other hand, some plants which are not reported to be used in herbal medicine might possess potential activity.

The deficiency of ACh is one of characteristics of AD and responsible for most of its symptoms such as decline of memory and cognition of the patients. AChE inhibitors such as tacrine, donepezil, rivastigmine, and galantamine are currently used as anti-AD drugs (Mehta et al., 2012). The side effects of these anti-AChE drugs such as toxicity, tolerability, and loss of efficiency have stimulated researchers to search for alternative natural anti-AD drugs for medication switch (Gauthier et al., 2003).

Thirty five extracts belonging to 23 plant species (Table 3-2) have been identified to be effective in inhibition of AChE enzyme which is considered to be related to the mechanism of memory dysfunction in this study. In the light of these findings, we can conclude that most of the plant extracts screened herein showed inhibitory activity against AChE and they could be considered for further studies in the treatment of AD. In particular,

the species belonging to Oxalidaceae, Lamiaceae, Fumariaceae, Myrtaceae, Ranunculaceae, Solanaceae, Asteraceae and Amaryllidaceae families had highest inhibitory activity ranging between 100.22 and 85.59% at 100 mg/ml concentration against AChE. Since most of the AChE inhibitors are known to contain nitrogen, the higher activity of these extracts may be due to their rich alkaloidal content (Orhan et al., 2004).

Species belonging to Liliaceae and Orchidaceae families showed the lowest inhibitory activity ranging between -18.63 to 58.4, Although members of Orchidaceae family have already shown to be therapeutically successful in all cases of nervous irritability, hysteria, spasm, fits and all derangements of the function of the brain such as madness and delirium. There are several orchid species, which are valued, as febrifuge in treating malaria, and in clearing tapeworms and other intestinal parasites. They are also used in treating skin diseases such as boils, pimple, rashes, eruptions and skin lesions, either in the form of ointment or poultice. Its roots, seeds, leaves, flowers and stems are used in various ways for their curative powers (Gutierrez, 2010)

The inhibition type of AChE varied among plant extracts, while 27 extracts showed reversible inhibition, 8 showed irreversible inhibitions. Although IC_{50} values of *Fumaria capreolata*, *Fumaria densiflora*, are lower than that of *Rosmarinus officinalis* and *Nigella sativa*, the inhibition type in this study showed that *Rosmarinus officinalis* and *Nigella sativa* reversibly inhibit AChE and can be used for AD's medication rather than

Fumaria capreolata, *F. densiflora* which inhibit irreversibly AChE. This recommendation is supported by the toxicity reports in literature which indicated higher safety margin of *R. officinalis* and *N. sativa*as compared to *Fumaria* species (Ali & Blunden, 2003; Ca, 1999; Noureddine et al., 2013)

Fumaria species have been used in traditional medicine as antihypertensives, diuretics, hepatoprotectants and laxatives (to treat gastrointestinal disorders), as well as in the treatment of rachis and conjunctivitis (Stübing & Peris, 1988). The plant has also been evaluated pharmacologically and shown to possess antihelmintic, antipyretic and hypoglycemic properties (Akhtar et al., 1984; Hordgen et al., 2003; Khattak et al., 1985). The biological activities of *Fumaria species* have been mainly associated with the presence of isoquinoline alkaloids (Noureddine et al., 2013). The toxicity of the species has been evaluated, and *Fumaria capreolata* has shown to be non-toxic (Noureddine et al., 2013), while *F. densiflora* was reported to be toxic (Erdoğan, 2009). The AChEI activity of *Fumaria* species has been reported by several researchers; the plant was reported to have strong AChEI activity (Orhan et al., 2004). In this study, *Fumaria* spp were among the most active plant extracts against AChE. However, the reaction was shown to be irreversible, and thus the plant cannot be used for the treatment of AD as the activity of the enzyme cannot be restored.

AChEI activity of the methanolic extract of *Peganum harmala,* has previously been reported by Ali et al. (2013). The plant contains β-

carboline alkaloids, which demonstrated potent activity against AChE (Cao et al., 2007). Harmaline, the major active constituent of *P. harmala* is a common dihydro β-carboline type. It possesses interesting pharmacological activities and can interact with several enzymes and neurotransmitters including topoisomerase I, and monoamine oxidase-A (Herraiz et al., 2010; Sobhani et al., 2002). Although, *P. harmala* has been used in traditional medicine, there are reports of severe intoxication in cattle, donkeys, sheep and horses (Bailey, 1979). Digestive and nervous syndromes have been reported in animals that consume a sub-lethal amount of the plant. Harmaline and harmine are toxic alkaloids characterized in the seeds of *P. harmala*. Harmaline is almost twice as toxic as harmine and in moderate doses cause tremors and clonic convulsions, but with no increase in spinal reflex excitability (Budavari & Neil, 1996). The seeds of *P. harmala* were among the potent plant extracts against AChE activity, however, the reaction has been shown to be irreversible, thus the plant cannot be used for the treatment of AD.

Mandragora autumnalis is a member of the Solanaceae plant family. Since ancient times, this plant was believed to be to have magic properties, because of the "human body" shape of its root and its narcotic and poisonous effect, and it is still known as witch's or devil's herb (Piccillo et al., 2006; Piccillo et al., 2002). The plant contains tropane belladonna anticholinergic alkaloids that have different actions and clinical effects. The alkaloids of the tropane group, principally atropine, scopolamine, and hyoscyamine, act on the peripheral and central nervous systems.

Scopolamine is the principal alkaloid in Mandragora. These substances have parasympatholytic properties, producing similar peripheral effects (mucosal dryness due to inhibition of sweat, salivary and bronchial gland activity, urinary retention, reduced gastrointestinal motility) while having different central actions: hyoscyamine stimulates the cerebral cortex, whereas scopolamine is a depressant and produces sedative and hypnotic effects . Different parts of the plant showed different degrees of acetylcholinestrase inhibition this may be due to variability of the alkaloid concentration in different parts of the plant

Some insecticides including organophosphate and carbamates cause AChE inhibition which leads to the accumulation of ACh at neuromuscular junctions causing rapid twitching of voluntary muscles and eventually paralysis of the insects. However, in this study, leaves and flowers of *Fumaria* species, seeds of *P. harmala,* and the leaves of *Lupinus pilosus* and *Mandragora autumnalis,* which have shown high irreversible AChEI activity, can be considered potent natural insecticides.

Plant species reported in TAPHM for the treatment of age related diseases exhibited different anticholinesterase activity, as extracts of *Urtica pilulifera* and *Juglans regia* have very low inhibitory activity, *Salvia fruticosa* and *Zingiber officinale* exhibited low inhibitory activity*, Foeniculum vulgare, Mentha spicata* and *Eruca sativa* reduced the enzyme activity to 47.37%, 25.83% and 20.59 with IC_{50} 99.3mg/ml, 36.35mg/ml and 36.7 mg/ml respectively*, Majorana syriaca, Nigella* sativa and

Rosmarinus officinalis exhibited the highest inhibitory activity more than 85 % and IC_{50} less than 15mg/ml

Rosmarinus officinalis (rosemary) contains the natural COX‑2 inhibitors (e.g. apigenin, carvacrol, eugenol, oleanolic acid, thymol, and ursolic acid, which can prevent Alzheimer's disease (Duke et al., 2007) . In addition, rosemary contains antioxidants and anti‑inflammatory compounds. Some of the strongest antioxidant substances in the plant are carnosic acid and ferulic acid, which have been reported to posses antioxidant activity much higher than the widely common synthetic antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Singhal et al., 2012).

Majorana syriaca an important food-flavouring ingredient in the Middle Eastern culture, known commonly as Za'atar. It reduced the enzymatic activity of AChE to 11.9 %, The plant is used traditionally for the treatment of several ailments and is associated to be used as memory enhancer. The main components of the plant extract according to GS-MS analysis were thymol, and carvacrol. The remaining compounds comprise flavonoids and phenolic acids that provide the antiradical and antioxidant activity (Ghada Al-Bandak et al., 2009). The plant has shown to have strong activity against AChE. Ursolic acid which was isolated from the plant, has shown to be a potent AChE inhibitor in Alzheimer's Disease (Chung et al., 2001).

Nigella sativa (black seeds) is considered as an adaprogenic herb and is widely used in Palestine and other Arabic countries; in *vitro* it reduced AChE activity to 8.51 at 100 mg/ml with IC_{50} 7.75 mg/ml. The fixed oil has demonstrated noticeable spatial cognitive preservation in rats challenged with chronic cerebral hypoperfusion which indicates a promising prospective neuroprotective effect (Azzubaidi et al., 2011)

Juglans regia L. (Juglandaceae), known as 'walnut', is an important food plant containing many nutritive elements. It is an important medicinal plant used in TAPHM. , It has been believed that walnut is good for the brain due to its similar shape to human brain. Our findings indicated that the fruit extracts had usually better antioxidant activity in the assays performed. However, the traditional belief in the beneficial effects of walnut for brain 'due to its similar shape to human brain' can be explained by its antioxidant activity, rather than its cholinesterase inhibitory properties.

Alzheimer's appears to be caused to a large degree by oxidative damage (Pratico & Delanty, 2000). Therefore, antioxidants, in general, should have positive effects in both the prevention and treatment of AD. A study found that antioxidants such as vitamin A, vitamin D, lycopene, and beta carotene were all significantly lower in AD patients compared to controls (Foy et al., 1999). Another study of 633 patients aged $\geq$ 65 years found that high dose supplementation with vitamin C decreased the risk of developing AD (Morris et al., 1998). Therefore, the plant extracts which demonstrated potent free radical scavenging properties are expected to play

a vital role in reducing the oxidative stress and this may explain their use in traditional medicine for improvement of AD and/or ageing related diseases. It is worth mentioning that some of the plant extracts which have showed high antioxidant activity including *Mentha spicata* (93.52), *Zingiber officinale* (99), *R. officinalis* (99.16), *M. syriaca* (100), and the leaves of *Oxalis pes-caprae* (147.43), are wild edible plants widely consumed among the Palestinian population (Ali-Shtayeh et al., 2008). Some of these plants have been reported to be used traditionally for memory enhancement (Ali-Shtayeh & Jamous, 2008; Ali-Shtayeh et al., 2008; Ali et al., 2013; Singhal et al., 2012).

Limitation

In the present work, the selected extracts were screened for AChE inhibition using the Ellman's method. The Ellman's method is the most widely used AChE inhibitory assay (Miao et al., 2010). This method has some advantages and disadvantages. Its main advantages are simplicity, rapid processing of large numbers of samples, fast conversion of ACTI comparing to other artificial substrates such as naphthyle acetate and relatively low cost (Pohanka et al., 2012; Rakonczay & Brimijoin, 1986). On the other hand, Ellman's method has some disadvantages, including the interference of some compounds. The -SH groups in the plant extract may react with DTNB and ATCh, thus the natural substrates are not identical from a kinetic point of view. False positive reaction of enzyme activity can be provided by samples containing a lot of thiol-bearing molecules.

Recommendations

Palestinian flora have shown to be a rich source for AChEIs, especially those plants with strong reversible AChEI and strong antioxidant activities. Further studies are needed to isolate and identify the active compounds responsible for AChE inhibitory activities.

It is also highly recommended to develop an economic, accurate, reproducible, and convenient colorimetric micro-well plate assay for qualitative as well as quantitative spectrophotometric analysis of phytochemical ingredients with activity against AChE to make the evaluation of the enzyme activity easier and more accurate.

Conclusion

Palestinian flora have shown to be a rich source for AChEIs, especially those plants with strong reversible AChEI and strong antioxidant activities. The extracts of *Rosmarinus officinalis*, *Mentha spicata*, *Majorana syriaca*, and *Nigilla sativa* were proved to have a great potential as AChEIs.

Overall such plants provide promising sources for alternatives to current therapies for AD and other neurodegenerative disorders. It is also hoped that these plants will aid in earlier intervention at a stage of AD when some disease-modifying therapies may be most efficacious. Further studies are needed to isolate and identify the active compounds responsible for AChE inhibitory activities.

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جامعة النجاح الوطنية
كليه الدراسات العليا

التقييم المخبري لفعالية بعض النباتات الطبية الفلسطينية كمثبطات لإنزيم الاسيتيل كولين استريز ومضادات للأكسدة. كإشارة لعلاج مرض الزهايمر

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قدمت هذه الأطروحة استكمالا لمتطلبات الحصول على درجه الماجستير في العلوم الحياتية بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين.

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الملخص

خلفيه: تستخدم مثبطات انزيم الاسيتيل كولين استريز كاستراتيجيه لعلاج مرض الزهايمر والخرف الشيخوخي والوهن العضلي والشلل الرعاشي ومن هذه المثبطات ال galanthamin و donepezilو rivastigmine لكن هذه المركبات تؤثر بشكل سلبي على الجهاز الهضمي. هناك العديد من النباتات تستخدم في الطب العربي التقليدي الفلسطيني لعلاج الأمراض المرتبطة بالجهاز العصبي ولقد تم فصل بعض مثبطات انزيم الاسيتيل كولين استريز من مصادر نباتيه والتي تستخدم لعلاج الأمراض المرتبطة بنقصان الذاكرة.

الأهداف: تهدف هذه الدراسة إلى التحقق من وجود مثبطات أنزيم الأسيتيل كولين إستريز في النباتات المستخدمة تقليديا في فلسطين لعلاج الاضطرابات المعرفية والإشارة إلى دور هذه النباتات كمصادر محتمله لتطوير امكانيات حديثه وآمنه وطبعيه لعلاج مثل هذه الاضطرابات.

الطرق: تم اختبار قدرة 92 مستخلص نباتي مستخلصة من 47 نبات منها عشرة نباتات مستخدمة في الطب العربي التقليدي الفلسطيني لعلاج الأمراض المرتبطة بتقدم السن وذلك باستخدام طريقه أيلمان و بالإضافة إلى ذلك تم اختبار فعالية هذه النباتات كمضادات للأكسده باستخدام طريقه الDPPH .

النتائج: ثمانيه وثلاثون بالمئه من المستخلصات ثبطت نشاط أنزيم الأسيتيل كولين إستريز بنسبه تزيد عن 50% فقط ثمانية مستخلصات منها كان لها نشاط تثبيط غير مسترجع. وكانت النباتات التي ثبطت الأنزيم بنسبه تزيد عن 90 %هي أرز الدجاج المتسلق وتفاح المجن

والسحلب والحمصيص والقديح و حبه البركه وحصالبان والنرجس وأرز الدجاج .وكان هنـــاك 73 مستخلص لها خواص مضادة للأكسدة منها الزعتر وحصالبان والتي كانت قيمه ال IC50 لها 0.21 و0.38 مغ/مل وكانت الأفضل فعالية. ومن المثير للاهتمام وجود تباين واضح في آلية عمل هذه المثبطات فقد تفاعل27 مستخلص بشكل مسترجع في حين تفاعلت 8 مستخلصات بشكل غير مسترجع مما جعل الزعتر وحبه البركه وحصالبان والنعنع والشومر اكثـــر فعاليـــه والتي يمكن استخدامها لعلاج مرض الزهايمر وتم الكشف عن مثبطات للانزيم تثبيطا لا رجعه فيه من مستخلصات أرز الدجاج المتسلق وأوراق وأزهار أزر الدجاج الكثيف وأوراق كل مـــن الترمس البري تفاح المجن بذور الحرمل ومثل هـــذه النبـــات يمكـــن اســـتخدامها كمضـــادات للحشرات.

الاستنتاجات: النباتات الفلسطينية مصدر غني لمثبطات الأسيتيل كولين إستريز خاصةً النباتات التي كان لها نشاط تثبيط عكسي ولها خواص مضادة للأكسدة وتوفر مثل هذه النباتات مصـــادر واعده لتستخدم كبدائل للعلاجات الحالية المستخدمة في علاج مرض الزهايمر وهنـــاك حاجـــه لمزيد من الدراسات لعزل وتحديد المركبات النشطة المسؤولة عن تثبيط الأنزيم.