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Evaluation of Selected Libyan Medicinal Plant Extracts for Their Antioxidant and Anticholinesterase Activities

Thesis submitted for the degree of Doctor of Philosophy

BY

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Declaration

I hereby declare that the contents of this thesis are my own, have not been presented or accepted in any previous application for degree, and are true record of the work carries out my self and all sources of information acknowledged.

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List of Abbreviations

Name	Abbreviation
Acetylcholine	ACh
Acetylcholinesterase	AChE
Acetylthiocholine iodide	ATChI
Adenosine triphosphate	ATP
Alzheimer's disease	AD
Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)	ABTS
Butylated hydroxyanisole	BHA
Butylated hydroxytoluene	BHT
Butyrylcholinesterase	BuChE
Butyrylthiocholine iodide	BuTChI
Cardiovascular disease	CVD
Cardiovascular system	CVS
Central nervous system	CNS
Cholinesterase	ChE
Coronary heart diseases	CHD
Diphenyl-2- Picryl-Hydrazyl	DPPH
Dithiobisnitrobenzoate	DTNB
Ethanol	EOH
Ferric reducing antioxidant power	FRAP
Ferrous Ion Equivalent	FIE
Folin- Ciocalteu method	F-C
Gallic acid equivalent	GAE
Glutathione	GSH
Hydrochloric acid	HCl
Hydrogen atom transfer	HAT
Inhibition percentage	I%
Low density lipoprotein	LDL
Methanol	MeOH
Nicotinamide adenine dinucleotide	NADH

Nicotinamide adenine dinucleotide phosphate	NADPH
Organophosphates	OP
Oxidised low density cholesterol	LDLox
Oxygen radical absorbance capacity	ORAC
Parkinson's disease	PD
Part per million	ppm
Phenolic content	PC
Phosphate buffer saline	PBS
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Single electron tansfer	SET
Standard deviation	SD
Tertiary butylhydroxyquinone	BHQ
Tetrahydroaminoacridine	THA
Thiobarbituric acid reactive substance	TBARS
Total antioxidant capacity	TAC
Total radical- trapping antioxidant parameters	TRAP
Traditional Chinese medicine	TCM
Traditional medicine	TM
Tripyridyltrizine	TPTZ
Trolox Equivalent	TE
Trolox equivalent antioxidant capacity	TEAC

Abstract

In different parts of the world, the use of medicinal plants has always been important in the therapeutic armory of mankind and remains an important source for the discovery of new bio-active compounds. Libya constitutes an apt example where medicinal plants are widely used. While some individual plant species such as *Ginkgo biloba* have been investigated in some detail, there is relatively little information available concerning the antioxidant potential and anticholinesterase activities of plant species in general and Libyan plants in particular.

In this study twenty three Libyan medicinal plants were chosen for the study of antioxidant capacity and phenolic content. Aqueous plant extracts were screened for their antioxidant activity using the FRAP, TEAC and DPPH methods. These methods enable high-throughput screening of potential antioxidant capacity. Results show that of these twenty three plants, hot and cold extracts of *Myrtus communis*, *Quercus robur* and *Syzygium aromaticum* exhibited the strongest antioxidant activity in all tests and this is higher than that of the green tea control. It is suggested that the efficacy of these plants could be explained, at least in part, by their antioxidant activity.

A selection of ten Libyan plants which have various ethnobotanical uses were evaluated for anticholinesterase activity. Most plants screened showed some inhibitory activity with either or both acetyl- and butyrylcholinesterase.

Digestion is an initial step involving changes in pH and activity of proteolytic enzymes. Plant extracts were evaluated for possible changes in antioxidant properties and anticholinesterase activity using an artificial digestion technique. Most extracts showed an increase in antioxidant activity after the final pancreatin step although results varied with the antioxidant assay used. However, almost all anticholinesterase activity was lost at the HCl stage of the *in vitro* digestion procedure. Testing *in vitro* bioavailability of plant extracts is a useful step in evaluating *in vivo* bioavailability.

Chapter 1 General Introduction

1.1 . Definition of Free Radicals:

Free radicals can be defined as any species capable of independent existence that contain one or more unpaired electrons (Halliwell et al., 1995, Halliwell and Gutteridge, 1999, Parsons, 2000, Halliwell, 2000). Oxygen free radicals or, more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are formed in all cells as unwanted by-products of metabolism and as such can be regarded as "toxic agents" with regard to their potential for initiating intracellular damage (Halliwell, 1995, Valko et al., 2007). They are characterized by the presence of unpaired electrons (conventionally denoted by a point suffix $^{\bullet}$). As a result of these unpaired electrons these species tend to attack stable molecules and take one of their electrons (to replace this missing electron), so that the previously stable molecule in turn becomes a free radical seeking to replace its missing electron and attack another molecule. This process is known as oxidation and the free radicals are responsible for the production of a chain of degradation. In biological systems free radicals are typically centred on oxygen, carbon or sulphur atoms. Most investigations have focused on oxygen centred radicals, with the hydroxyl ($OH^{\bullet}$) and superoxide ($O_2^{-\bullet}$) radicals considered as the most physiologically powerful primary tissue damaging free radical species (Table 1.1).

In addition, there are other important free radicals derived from these primary species including the peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) species. The reactive oxygen species (ROS) include both radical and non radical substances. They include $OH^{\bullet}$, $O_2^{-\bullet}$, $RO_2^{\bullet}$and $RO^{\bullet}$, and H_2O_2. Indeed, $OH^{\bullet}$ and its subsequent radicals are the most

Table 1.1 Examples of free radicals (Halliwell and Gutteridge, 1999).

Name	Formula	Comments
Hydrogen atom	$H^{\cdot}$	The simplest free radical.
Trichloromethyl	$CCl_3^{\cdot}$	A carbon- centred radical. These free radicals react rapidly with O_2 to make peroxyl radicals.
Superoxide	$O_2^{\cdot}$	Oxygen- centred radicals.
Hydroxyl	$OH^{\cdot}$	Highly reactive oxygen– centred radicals, attacks all biomoleclues.
Thiyl/ perthiyl	$RS^{\cdot}$ /$RSS^{\cdot}$	A group of radicals that have unpaired electrons residing on sulphur.
Peroxyl, alkoxyl	$RO_2^{\cdot}$, $RO^{\cdot}$	Oxygen-centred radicals formed during the breakdown of organic peroxides and reaction of carbon radicals with O_2 ($RO_2^{\cdot}$).
Oxides of nitrogen	$NO^{\cdot}$, $NO_2^{\cdot}$	Nitric oxide is formed *in vivo* from the amino acid L-aginine; nitrogen dioxide is made when $NO^{\cdot}$ reacts with O_2; both are found in polluted air and smoke from burning organic materials.
Nitrogen-centred radicals-	$C_6H_5N{=}N^{\cdot}$	Formed during oxidation of phenylhydrazine by erythrocytes.

reactive free radical species in biological systems (Erel, 2004, Valko et al., 2007). On the other hand, $O_2^{-\cdot}$ is less reactive than the $OH^{\cdot}$ and its reactivity depends on pH (Morello et al., 2002, Valko et al., 2006). $RO_2^{\cdot}$, $RO^{\cdot}$ are good oxidizing agents as both can abstract hydrogen atoms from other molecules. Moreover, $RO_2^{\cdot}$ can also react with each other to generate singlet oxygen. Although hydrogen peroxide (H_2O_2) is also capable of producing direct damage to the tissues, it is not classed as a free radical since it does not possess unpaired electrons. H_2O_2 is not very reactive but it is considered as a cytotoxic agent. It can inactivate enzymes with essential thiol (- SH) groups at the active site and it is capable of producing cell damage through reacting with metal ions to generate $OH^{\cdot}$, which are more generally reactive (Parsons, 2000, Valko et al., 2006).

1.2 Source of free radicals:

1.2.1 Cellular:

ROS are produced *in vivo* mainly through the mitochondrial respiratory chain brought about by leakage of electrons from the electron carrier directly on to O_2 during the production of adenosine triphosphate (ATP) (Valko et al., 2004). About 1-3% of all electrons in the transport chain tend to generate O_2^{-} instead of contributing to the reduction of oxygen to water (Chance et al., 1979, Valko et al., 2007). In addition, they are also formed from the futile cycling of the various cytochrome P450s in the catalysis of microsomal oxygenation reactions (Basu et al., 1999). ROS are also formed by activation of leukocytes and during phagocytosis. Several enzymes such as xanthine oxidase have been identified that are capable of the production of $O_2^{-\cdot}$. Other ROS generating systems include auto-oxidation reactions; reduction of tissue O_2 by metal redox systems such as Fe^{2+} /Fe^{3+}. The main source of $OH^{\cdot}$, the most dangerous radical, comes from the interaction of hydrogen peroxide with transition ions such as Fe^{2+} or Cu^{2+}. $OH^{\cdot}$ are also formed when $NO^{\cdot}$ and $O_2^{-\cdot}$ react to produce peroxynitrite (Mantle and Preedy, 1999). In general, generation of ROS seems to occur within all aerobic cells (Valko et al., 2006).

2.2.1 Environmental:

Reactive oxygen species can be produced by a set of exogenous processes. Environmental agents including non-genotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells (Valko et al., 2006). Thus, people are exposed to free radicals from the external environment from smoking, chlorine in

drinking water, pollution in the atmosphere such as pesticides, food additives, drugs and antibiotics (Morello et al., 2002, Valko et al., 2007). Local environment and lifestyle play an important role in increasing free radical activity in the body. For example, smoking and poor diets result in increased free radical activity in the body (Scott, 1997, Abou-Seif, 1996). Moreover, it is well established that a number of gaseous products, found to varying degrees in industrial environments as pollutant gases, exert pro-oxidant effects in animal lungs (Scott, 1997, Valko et al., 2006). Epidemiological and experimental studies have indicated that cigarette smoking is considered as a major risk factor for numerous chronic diseases. Smoke contains a complex mixture of toxic agents including ROS and RNS, which mediate cell damage by stimulating lipid peroxidation and oxidation (Proteggente et al., 2006). In addition, cigarette smoke acts as an irritant and induces a localised inflammatory response resulting in generation of ROS by lung macrophages (Kalra et al., 1991, Scott, 1997).

1.3 Mechanisms of ROS Toxicity:

Free radicals have the potential to damage intracellular organelles and components such as nucleic acids, lipids and proteins on which normal cell function depends. Free radical oxidation damages the outer membrane of a cell and then continues into the cell where DNA is affected. Thus damage to the cell will be replicated each time during cell division (Valko et al., 2004, Halliwell and Gutteridge, 1999, Hillestrom et al., 2006). ROS are the mediators of inflammation and are involved in the pathogenesis of infectious disease, including septic shock, and immune diseases such as arthritis and inflammatory bowel disease (Parke et al., 1991). Research in recent years has shown the importance of ROS in degenerative

processes related to aging (Ames et al., 1993) and diseases such as coronary heart disease, chronic renal failure, diabetes mellitus, immune dysfunction, atherosclerosis, cancer and neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Halliwell, 1994, Arts and Hollman, 2005, Ames, 1983, Arredondo et al., 2004, Young and Woodside, 2001, Halliwell, 1996, Halliwell, 2002, Halliwell et al., 1992, Chisolm and Steinberg, 2000, Morello et al., 2002, Basu et al., 1999, Wiseman and O'Reilly, 1997, Vanharanta et al., 2003). The molecular mechanisms of ROS toxicity and ROS- mediated disease include:

i- Oxidation of vital thio compounds to disulphate.

ii- Loss of tissue GSH (glutathione).

iii- Impairment of energy generation (ATP, NADH, and NADPH).

iv- Oxidation of cytoplasm.

v- Inhibition of Ca^{2+} transport and electrolyte homeostasis.

vi- DNA cleavage

vii- The initiation and promotion of mutations and carcinogenesis.

1.4 Pathological Problems Associated with Free Radical Generation:

Under normal physiological conditions there is a balance between both the activities and intracellular level of antioxidants. However, cellular injury resulting from the imbalance between the free radical generating and scavenging system "oxidative stress" has been implicated in the pathogenesis of a variety of disorders. These disorders include:

1.4.1 Cancer

Oxidative damage to DNA, lipids and proteins in the human body is generally considered to be an important factor in carcinogenesis. Cancer initiation and promotion is associated with chromosomal defect and oncogene activation (Borek, 2005, Adlercreutz, 1988, Valko et al., 2004). Exposure of organisms to ionizing radiation has long been known to help development of cancer later in life. Ionizing radiation is a complete carcinogen, being both an initiator and promoter (Kensler and Taffe, 1986, Ames et al., 1993). It is a reasonable possibility that some endogenous free radical reactions will result in tumour formation by serving as a continuous source of tumour initiators and promoters. A tumour may be defined as "an abnormal lump or mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissue, continuing after the stimuli that initiated it have ceased" (Halliwell and Gutteridge, 1999). The formation of tumour results from the breakdown of the normal intercellular collaboration. Some genetic damage by radiation occurs by direct absorption of energy by DNA, but some is mediated by ionization of water resulting in the formation of highly reactive $OH^{\bullet}$, which in turn attacks DNA and generates mutations (Cerutti, 1994, Valko et al., 2006). Defects in DNA repair mechanisms also predispose to the development of cancer (Valko et al., 2007).

1.4.2 Diabetes mellitus

Mellitus is a Latin word meaning ''honey-sweet'' and refers to the taste of diabetic urine. Diabetes mellitus is a chronic disease characterized by elevated blood glucose (hyperglycaemia) and urinary glucose excretion. It is caused by a fault in the insulin production or by a disorder in the response of tissue to it (insulin resistance).

Alloxan, a well known diabetogenic agent, appears to act by producing severe oxidative stress on the β-cell, which is responsible for the production of insulin (Al-Azzawie and Alhamdani, 2006). It is still unclear whether oxidative stress contributes to the origin of diabetes in humans (Tang et al., 2006, Ananthan et al., 2003). Recently, it has been proposed that some of the complications of diabetes, leading to mortality in diabetics, are associated with the increased activity of ROS and oxidative cellular damage (Pratico, 2005, McCune and Johns, 2002, Astaneie et al., 2005, Liu, 2002).

1.4.3 Cardiovascular Diseases (CVD)

The ROS- induced oxidative stress in cardiac and vascular myocytes has been linked with cardiovascular tissue injury. Regardless of the direct evidence for a link between oxidative stress and cardiovascular diseases, ROS- induced oxidative stress plays a major role in various cardiovascular diseases such as atherosclerosis, ischemic heart disease, hypertension, coronary heart disease and congestive heart failure (Adom et al., 2003, Arts and Hollman, 2005, Manach et al., 2005).

1.4.3.1 Atherosclerosis

Atherosclerosis is an arterial disease that is characterized by a local thickening of the vessel wall that develops in the inner coat. Epidemiological evidence suggests that the high concentration of cholesterol in plasma, especially in the form of low-density lipoprotein (LDL), is a high predictor of atherosclerotic risk. LDL is susceptible to modification particularly by oxidation. Besides high levels of cholesterol, uptake of oxidised low density cholesterol (LDLox) seems be a key step

in the development of atherosclerosis. The likely candidate responsible for the oxidative modification of LDL in arterial cells is the oxygen- derived free radical. Oxidised lipoprotein and LDLox have been reported to mediate enhanced superoxide formation, which in turn leads to apoptosis of cells in the umbilical vascular wall. LDLox- mediated formation of ROS also cause plaque formation. In addition to the direct effect of $O_2^{-\cdot}$, oxidation of NO by $O_2^{-\cdot}$ results in the formation of peroxynitrite which is known to initiate lipid peroxidation or lipoprotein oxidation, both important events in the incidence of atherosclerosis (Valko et al., 2007). Furthermore, several reports have discussed the involvement of the free radicals in the development and initiation of atherosclerosis (Darley-Usmar and Halliwell, 1996, Parthasarathy, 1992, Kumpulainen and Salonen, 1996, Pratico, 2005).

1.4.3.2 Coronary heart disease

Coronary heart disease (CHD) is a condition in which the heart muscle receives an inadequate amount of blood because of an interruption of its blood supply. There is increasing evidence to support the hypothesis that free radical-mediated oxidative processes contribute to coronary heart disease (Adlercreutz et al., 2004, Anderson, 2004, Maxwell and Lip, 1997). The development of atherosclerotic lesions in the coronary arteries of heart, if not treated, may lead to CHD, the leading cause of death in affluent communities (Basu et al., 1999).

1.4.4 Inflammatory disease

When cells are damaged by microbes, physical agents, or chemical agents, the injury sets off inflammation (inflammatory response). It is becoming increasingly

apparent that certain types of inflammatory tissue injury are mediated by reactive oxygen metabolites (Aruoma, 2003). The most likely sources of these oxidizing agents are the phagocytic leukocytes (e.g., neutrophils, monocytes, macrophages, and eosinophils) that damage the tissue (Russo et al., 2005, Conner and Grisham, 1996). These reactive radicals and oxidants may injure cells and tissue directly via oxidative degradation of essential cellular components as well as injure cells indirectly by altering the balance that normally exists within the tissue interstitium. It has been demonstrated that nitric oxide ($NO^{\cdot}$) is involved in the pathogenesis of acute and chronic inflammation. During inflammation, nitric oxide is synthesized in significantly large quantities and may be become cytotoxic. The free radical nature of NO and its high reactivity with O_2 to produce peroxynitrite ($ONOO^{-}$), which can cause DNA fragmentation, makes $NO^{\cdot}$ a potent pro-oxidant molecule able to induce oxidative injury and to be potentially harmful toward cellular targets (Vouldoukis et al., 2004, Manach et al., 2005, Tiwari and Tripathi, 2007, Padwad et al., 2006).

1.4.5 Neurodegenerative disorders

The brain is particularly highly susceptible to oxidative damage because of its high oxygen utilisation (Ono et al., 2006, Shen, 2004). It has a high content of oxidizable polyunsaturated fatty acids and possesses redox- active metals such as Fe and Cu. Oxidative stress increases with age and consequently it can be considered as a very important causative factor in several neurodegenerative diseases, typically for older individuals (Auddy et al., 2003, Landsberg, 2005).

1.4.5.1 Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by impairment of memory and cognition, and behavioural problems. It is the most common dementia in Western societies and accounts for two-thirds of all dementia cases (Choudhary et al., 2005b, Inestrosa et al., 2005, Obermayr et al., 2005). Pathologically, AD is characterized by loss of cholinergic neurons from selective brain regions. The first evidence in support of the implication of oxidative stress in pathogenesis of AD was reported by Martins et al. (1986). Cells in the brains of AD patients exhibit an abnormally significant extent of oxidative damage associated with a marked accumulation of amyloid-β peptide (Aβ), the main constituent of senile plaques in the brain, as well as deposition of neurofibrillary tangles and neurophil threads. AD is multifactorial with both genetic and environmental factors implicated in its pathogenesis (Kasa et al., 2000, Gil-Bea et al., 2005). For a number of years, two major hypotheses on the cause of AD have been proposed: the "amyloid cascade hypothesis", which states that the neurodegenerative process is a series of events triggered by abnormal processing of the amyloid precursor protein; and the "neuronal cytoskeletal degeneration hypothesis", which proposes that cytoskeletal changes are the trigger of events. Recently, a new hypothesis seeking to incorporate the multifaceted nature of both the triggering mechanisms and progression of AD has proposed that AD results from a disorder of the mechanisms underlying structural brain self-organization/plasticity (Shen, 2004). Acetylcholinesterase (AChE) is a key component of cholinergic brain synapses and neuromuscular junctions. The major biological role of the enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine (ACh). According to the cholinergic hypothesis, memory impairments

in patients with senile dementia are due to a selective and irreversible deficiency in the cholinergic functions in the brain (Shen, 2004, Choudhary et al., 2005c, Garcia-Alloza et al., 2005).

1.4.5.2 Parkinson's disease

Parkinson's disease (PD) is the second most common degenerative disorder, affecting approximately 10% of the population over 65. It was first described by the English physician James Parkinson as the 'shaking palsy' in 1817. PD is characterised by a rhythmic tremor in foot or hand, especially when the limbs are at rest. As the disease develops, patients begin to have increasing problems in controlling movement including slowing and initiation of movement and rigidity of muscles (Halliwell and Gutteridge, 1999). Pathologically, PD involves a selective loss of dopaminergic neurons in an area of the midbrain called the substantia nigra. These cells use dopamine, a neurotransmitter between brain and nerve cells, to communicate with the cells in another region of the brain called the striatia (Ruberg et al., 1986, Thibaut et al., 1995). The clinical syndrome appears when approximately 80% of striatal dopamine has been lost. Although PD is a multifactorial disorder, cumulative evidence supports an "oxidative stress hypothesis" for initiation of nigral dopamine neuron loss. The foundation for this hypothesis is the pro-oxidative state of the substantia nigra. Essentially, the substantia nigra has a high metabolic rate combined with both a high content of oxidizable species, including dopamine, neuromelanin, poly-unsaturated fatty acids and iron, and a low content of antioxidants (especially glutathione) (Halliwell and Gutteridge, 1999). Thus, oxidative stress can easily overwhelm the natural defences, resulting in production of ROS which serve both to maintain the oxidative stress level and initiate/propagate apoptosis of the dopaminergic neurons. One important feature of this hypothesis is that it allows a transient initiation factor to trigger an active, self-petuating cycle of inflammation and neurodegeneration that ultimately destroys enough of the dopaminergic neurons in the substantia nigra to produce the clinical syndrome of PD (Valko et al., 2007). Occurrence of oxidative stress in PD is supported by both post-mortem studies and by studies demonstrating the capacity of

oxidative stress to induce nigral cell degeneration (Piggott et al., 2003, Valko et al., 2007).

1.4.6 Aging

The process of aging may be defined as a progressive decline in physiological functions of organisms after the reproductive phase of life (Araujo et al., 2005, Ames et al., 1993). Most old animals and humans have obvious or sub clinical diseases such as atherosclerosis and amyloid-β plaques that can influence parameters of oxidative damage. The first direct experimental support of the implication of free radicals in the aging process was in 1957 by Harman (Harman, 1957), who proposed that normal aging results from random deleterious damage to tissue by free radicals (Harman, 1993). Indeed, this report has gradually triggered intense research into the field of the role of free radicals in biological systems (Araujo et al., 2005). Among the many theories describing the process of aging that have been proposed, it is now believed that free radical damage theory seems to be the most considered approach to explain the process of aging (Halliwell and Gutteridge, 1999). It is based on the fact that free radical damage to mitochondria results in mitochondrial DNA mutations, leading to a progressive reduction in cellular energy supply, and consequential physiological decline characterized by the inactivation of key enzymes and accumulation of oxidized proteins (Araujo et al., 2005).

1.5 Biological Defence Systems:

Exposure to free radicals from internal and external sources has led organisms to develop a series of biological defence mechanisms against oxidative damage induced by reactive oxygen species. The biological defence systems involve

preventative mechanisms, repair mechanisms and physical defences. These are dependent upon antioxidants.

1.5.1 Definition of Antioxidants:

Antioxidants can be defined as "any substance that when present at low concentrations compared to those of an oxidizable substance significantly delay or prevent a pro-oxidant initiated oxidation of that substrate" (Halliwell, 1995). Antioxidant compounds must be present in biological systems in sufficient concentrations to prevent an accumulation of pro-oxidant molecules, a state known as oxidative stress (Buettner and Schafer, 2000, Wiseman and O'Reilly, 1997). Cells are protected from free radical induced-damage by a complex array of endogenous radical scavenging proteins, enzymes, numerous endogenous antioxidant factors, that include coenzyme Q, glutathione GSH and other tissue thiols, metal ion sequestering proteins such as oxidizing (Fe^{2+}) to (Fe^{3+}), ROS metabolising enzymes such superoxide dismutase, and catalase. In addition, there are other important antioxidant compounds including α-tocopherol (vitamin E), ascorbic acid (vitamin C), uric acid, and the histidine containing dipeptides carnosine and anserine (Hussin et al., 2007). Besides these compounds, there are a variety of plant derived dietary compounds which act as antioxidants such as carotenoids and flavonoids (Prenesti et al., 2007, Montoro et al., 2005, Yu et al., 2005) (Table 1.2).

Table 1.2 Antioxidant defence (adapted from Basu et al, 1999).

Endogenous factors	Endogenous enzymes	Nutritional factors
Glutathione and other thiols	GSH reductase	Vitamin C
Haem proteins	GSH transferase	Vitamin E
Coenenzyme Q	GSH peroxidase	β- Carotene and retinoids
Bilirubin	Superoxide dismutase	Selenium-essential dietary component of glutathione peroxidases
Uric acid	Catalase	Methionine or lipotropes

1.5.2 Antioxidant defence mechanisms:

Antioxidants play the housekeeper's role, "mopping up" free radicals before they get a chance to cause injury. Generally, as shown in Figure 1.1, defence systems against oxidative damage are composed of various antioxidants with different functions including: (a) preventive antioxidants, which suppress the formation of free radicals, (b) radical–scavenging antioxidants, which scavenge radicals to inhibit chain initiation and break chain propagation, (c) repair and *de novo* enzymes, that

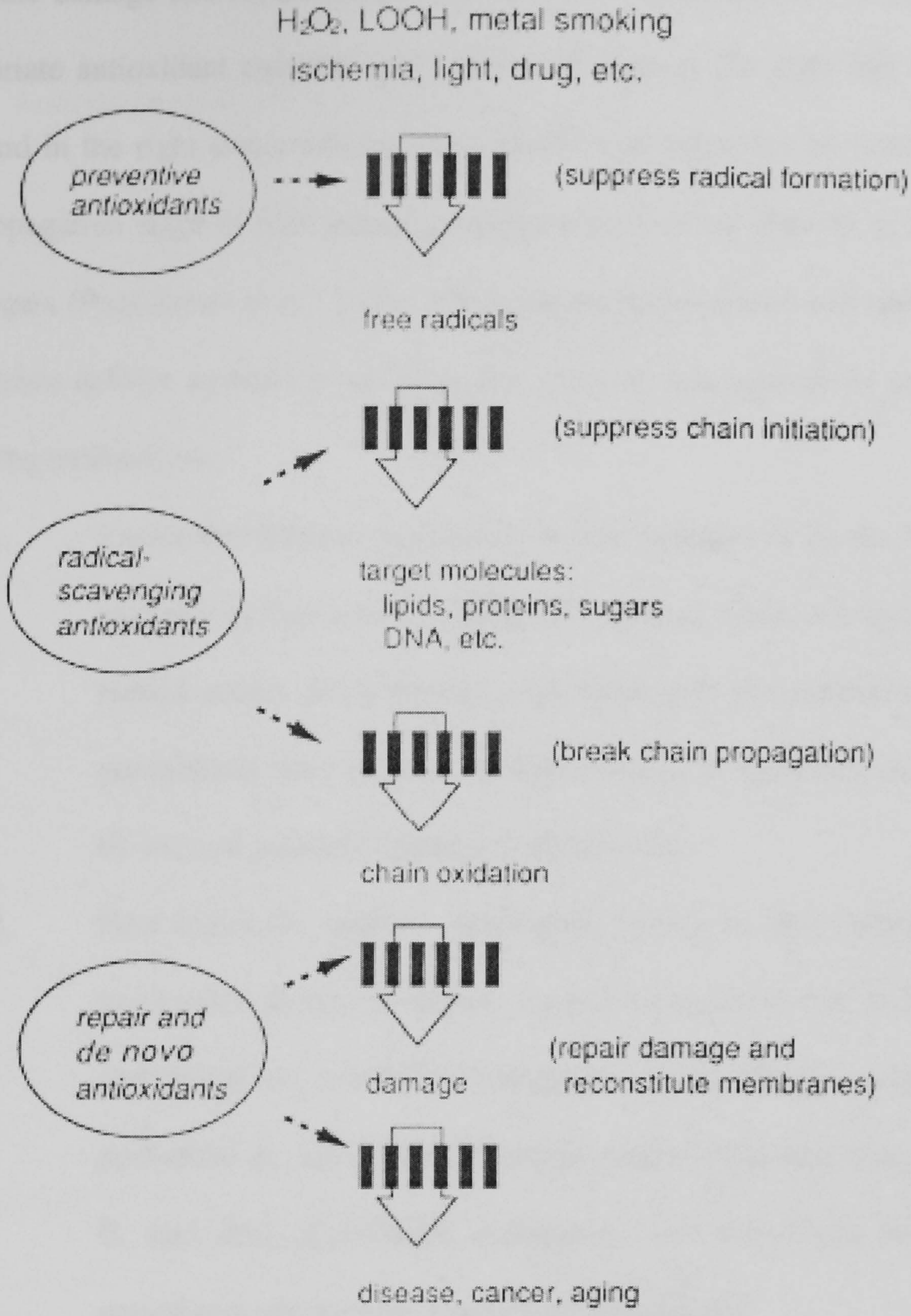


Figure 1.1 Defence system in vivo against oxidative damage (Niki, 1996).

repair the damage and reconstitute membranes and (d) adaptation, by generation of appropriate antioxidant enzymes and transfer of them to the right site at the right time and in the right concentration. Antioxidants can either act by interfering with the propagation stage of free radical generation itself or act directly as free radical scavengers (Pool-Zobel et al., 2000). There are many enzymatic and non enzymatic antioxidant defence systems in the body that remove toxic species by means of the following mechanisms:

1. Enzymatic defence mechanism which is achieved by the endogenous enzymes as mentioned in Table 1.2. These enzymes act against the free radical action, either by enzymatic hydrolysis of ester bonds to remove peroxidized fatty acids or by sequestration of transition metal ions or by enzyme catalysed reduction of peroxides.

2. Non enzymatic defence mechanism, which is also called sacrificial mechanism as the antioxidant compound sacrifices one of its electrons and changes to a relatively unreactive radical. This mechanism can be performed by low molecular weight antioxidants such vitamins C and E, uric acid, glutathione, carotenoids and flavonoids which act as scavengers and prevent free radical propagation.

1.5.3 Analytical methods for measuring antioxidant activity:

Antioxidant activity cannot be measured directly but rather by the effects of antioxidant in controlling the extent of oxidation. A wide range of methods are currently used to assess antioxidant capacity in food, botanicals, nutraceuticals and other dietary supplements. Both *in vivo* and *in vitro* assays are used and all methods have their own advantages and limitations. In the literature, numerous reports have reviewed the advantages and disadvantages of various methods that have been used for the measurement of antioxidants (Frankel and Meyer, 2000, Antolovich et al., 2002, Halliwell and Gutteridge, 1995, Prior and Cao, 1999, Prior et al., 2005, Schlesier et al., 2002). Although there is a great multiplicity of methods used for antioxidant testing, none of these methods provide an ideal individual, approved, standardised method, as within biological systems, there are several sources of antioxidant including enzymes (such as superoxide dimutase, glutathione peroxidise and catalase), large molecules (like albumin and ferritin), small molecules (uric acid and polyphenols), some hormones (melatonin and estrogen) and molecules of dietary origin molecules such as vitamin C, carotenoids, flavonoids, etc. On the other hand, both oxidants and antioxidants may have different chemical and physical features. Furthermore, antioxidants may respond in different manners to different radical or oxidant sources (Prior et al., 2005). Therefore, to assess and describe the total antioxidant activity of any sample, more than one analytical assay must be applied (Frankel and Meyer, 2000). In general, all methods that have been used are based on two major mechanisms by which the antioxidant capability to deactivate the radicals is associated (Prior et al., 2005):

1- Hydrogen Atom Transfer (HAT) based method:

It measures the ability of antioxidant to quench free radicals by hydrogen donation (AH= any H donor).

$$X^{\cdot} + AH \rightarrow XH + A^{\cdot}$$

2- Single Electron Transfer (SET) based method:

It detects the ability of a potential antioxidant to transfer one electron to reduce any compound, including metal, carbonyl and radicals

$$X^{\cdot} + AH \rightarrow X^{-} + AH^{\cdot+} \qquad (1)$$

$$AH^{\cdot+} + H_2O \leftrightarrow A^{\cdot} + H_3O^{+} \qquad (2)$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O \qquad (3)$$

$$M\ (III) + AH \rightarrow AH^{+} + M\ (II) \qquad (4)$$

The most widely and frequently used analytical methods are ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996, Benzie and Strain, 1999) (μmol ferrous ion equivalents); Trolox equivalent antioxidant capacity (TEAC) (Re et al., 1999) (mmol Trolox equivalents) and scavenging activity of 1,1' -diphenyl-2-picryl-hydrazyl (DPPH) radicals (Brand-Williams et al., 1995, Blois, 1958, Koleva et al., 2002) (mmol Trolox equivalents). These methods have gained popularity because they enable high-throughput screening of potential antioxidant capacity.'

1.5.3.1 Ferric reducing antioxidant power (FRAP) assay:

The FRAP assay was first introduced by Benzie and Strain (1996; 1999) for measuring the total antioxidant activity. The assay is based on the reducing power of a compound (antioxidant). A potent antioxidant will reduce the ferric ion (Fe^{3+}) to

the ferrous ion (Fe^{2+}). The ferrous ion forms a blue complex (Fe^{2+}/2, 4, 6-tripyridyl-s-triazine (TPTZ). The resulting blue colour can be measured spectrophotomertically and is linearly related to the total reducing capacity of electron-donating antioxidants. The higher the absorbance the higher the reducing power of the phytochemical, thus higher antioxidant activity (See Methods 2.2.3.1).

1.5.3.2 Trolox equivalent antioxidant capacity (TEAC) assay:

The TEAC assay was originally described by Miller et al. (1993). It has since been improved by modifications regarding free radical generation (Van den Berg et al., 1999, Re et al., 1999) and is based on the relative ability of antioxidants to scavenge the radical cation 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonate ($ABTS^{+}$). The extent of quenching of the $ABTS^{+}$ radical by the test medium is measured spectrophotometrically and compared with that of Trolox, a water-soluble vitamin E analogue (See Methods 2.2.3.2) with proven antioxidant properties.

1.5.3.3 Diphenyl-2-picryl-hydrazyl (DPPH) radical assay:

Radical scavenging activity using the DPPH method was described by Blois (1958). The essence of the DPPH method is that the antioxidant reacts with stable free radicals either by transferring electrons or hydrogen atoms to DPPH, thus neutralising free radical character (Naik *et al.*, 2003). i.e., 1,1′-diphenyl-2-picryl-hydrazyl (deep violet colour) is converted to 1,1′-diphenyl-2-picryl-hydrazine (yellow). Therefore, the colour of the reaction mixture changes from deep violet to yellow and its absorbance decreases. The degree of discolouration indicates the

scavenging potential of the sample antioxidant (Abdille et al., 2005, Tepe et al., 2005a, Tsao and Deng, 2004) (See Methods 2.2.3.3).

1.6 Therapeutic intervention:

Oxidative stress has been confirmed to be involved in all previously mentioned diseases. In some it makes a significant contribution to tissue damage. Such diseases should be amenable to therapeutic intervention with antioxidants. For instance, Tamoxifen is widely used in the treatment of breast cancer and has also reported to have beneficial effects against cardiovascular system (CVS) and particularly CHD by retarding the peroxidation of human LDL (Bingham et al., 1998). In addition, in some acute diseases involving peroxidation, antioxidant therapy is already becoming well established (Scott, 1997, Salonen et al., 2000, Valko et al., 2006, McCune and Johns, 2002). Furthermore, inhibition of NO production in response to inflammatory stimuli is considered as a useful therapeutic strategy in inflammatory disease (Padwad et al., 2006). Although multiple types of intervention by a variety of antioxidants have been shown to have beneficial effects in animals, very few have been developed as therapeutic agents in man (Dunnett and Svendsen, 1993, Obermayr et al., 2005). The reason for this is almost certainly associated with reports regarding their carcinogenic properties (Halliwell and Gutteridge, 1999, Padwad et al., 2006).

1.7 Synthetic antioxidants

Synthetic antioxidants which contain phenolic groups such as gallic acid esters, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and

tertiary butylhydroxyquinone (BHQ), are the most widely used food synthetic antioxidants. In spite of the widespread use of these artificial antioxidants, there is increasing concern over their safety. Currently used synthetic antioxidants have been suspected to cause or promote negative health effects (Amarowicz et al., 2004); thus the replacement of synthetic antioxidants by safe natural antioxidants such as vitamin E and C, flavonoids and other plant phenolics has been increasingly supported (Duan et al., 2006).

On the other hand, there is evidence that some drugs that are already used clinically may exert some part or all of their effects by antioxidant mechanisms. However, these drugs were not designed as antioxidants. Recently, several studies showed that many drugs approved for therapeutic use such as those used in the treatment of diabetes, may have a number of serious adverse effects including hepatotoxicity (Tang et al., 2006, May et al., 2003, Kim and Kim, 2006). However, restrictions have been placed on their application. In general, only a few drugs would appear capable of acting directly as antioxidant *in vivo*. In fact, several drugs that are used in the treatment of various disorders, such as anti-flammatory drugs, might themselves be converted into free radicals *in vivo* and aggravate oxidative damage (Halliwell and Gutteridge, 1999). Therefore, there is a trend to substitute synthetic antioxidant drugs with naturally occurring antioxidants.

1.8 Correlation between antioxidant and neuroprotective effects:

Oxidative stress is involved in acute and chronic neurodegenerative conditions, such as ischaemia–reperfusion injuries, infectious processes (e.g., AIDS) and Alzheimer's and Parkinson's diseases (Sun et al., 2002, Aruoma et al., 2003). A

common feature of these pathological conditions is generation of mitochondrially-derived reactive oxygen species (ROS). In nerve cells, the sources of ROS and NOS are numerous; therefore, cells have to maintain an effective antioxidant system in order to protect themselves against free radical overload and subsequent damage. Antioxidants can scavenge ROS and also can alter inflammation pathways. As a consequence, the use of antioxidants may be useful in the treatment of AD. More recent evidence in the form of antioxidant protection against development of AD lesion suggests vitamins E and C should be taken together to be more beneficial, as vitamin C recycles vitamin E to its active form (El-Demerdash et al., 2005). Epidemiological studies indicate that dietary antioxidants can influence the incidence of neurodegenerative diseases (Arredondo et al., 2004, Cai et al., 2004). Among those, flavonoids have been proposed to be effective cytoprotectors. Flavonoids are polyphenolic compounds and structurally have variations in the carbon ring that characterize the different types. Flavonoids are also considered to be the active ingredients in some medicinal plants. They are increasingly appreciated as chemopreventive agents against pathophysiological conditions, such as cancer, cardiovascular disease and neurodegenerative diseases in which free radicals have been implicated (Aruoma et al., 2003, Eccleston et al., 2002, Wiseman, 1996). The biological functions of these molecules such as anti-mutagenic, anti-carcinogenic and anti-ageing and others could be at least partially due to their antioxidant and free radical-scavenging activity. The primary activity of plant flavonoids is believed to reside in their free radical-scavenging capacity (Sala et al., 2003, Rice-Evans et al., 1996, Ishige et al., 2001). They appear to minimize the number of oxidative DNA mutations and protein modifications by scavenging harmful reactive oxygen species (Djeridane et al., 2006, Sutherland et al., 2006). Moreover, some flavonoids show

other activities such as antiproliferative and pro-apoptotic effects, probably through interactions with key enzymes regulating cell cycle and apoptosis pathways (Torres et al., 2005). The nueroprotective properties of flavonoids, as effective for improving learning and memory, are rapidly becoming established (Sutherland et al., 2006). Consequently, herbs with a high concentration of these compounds such as *Achyrocline satureioides*, *Ginkgo biloba* and *Epilobium parviflorum* are of special interest (Arredondo et al., 2004, Howes and Houghton, 2003).

1.9 Neuroprotective effects of anticholinesterase

Esterases that catalyze the hydrolysis of choline esters at a higher rate than other esterases have been classified as cholinesterases (ChEs). Cholinesterase inhibitors have been used in the treatment of human diseases, the control of insect pests, and more notoriously as chemical warfare agents. Most uses of cholinesterase inhibitors are based on a common mechanism of action initiated by inhibition of acetylcholinesterase (AChE) (Pope et al., 2005). According to substrate specificities and susceptibility to inhibitors, this group of enzymes was divided into two major categories. The first category is plasma butyrylcholinesterase (BuChE) (EC 3.1.1.8), also known as acylcholine acetyl hydrolase, non-specific, pseudo, or type II cholinesterase (Quinn, 1987, Godkar et al., 2006). This enzyme is found principally in plasma and it acts on a variety of choline phenyl, nitro phenyl and other types of esters. The second category is acetylcholinesterase (AChE) (EC 3.1.1.7), which is also called "red cell", true, specific or type I cholinesterase. AchE exists primarily in nerve cells and muscular junctions, but it is also found in other neural and non neural cells. AChE plays an important role in terminating the nerve impulse in the cholinergic synapses by hydrolysis or breakdown of acetylcholine (ACh), the

excitatory neurotransmitter, into acetic acid and choline as shown in the equation below:

$$(CH_3)_3N^+CH_2OCOCH_3 \xrightarrow[H_2O]{AChE} (CH_3)_3N^+CH_2OH + CH_3CO_2H$$

Acetylcholine — choline — acetic acid

Cholinesterase inhibitors thus share a common mechanism of pharmacological or toxicological action, ultimately modifying cholinergic signalling through disruption of acetylcholine degradation. While the use of cholinesterase inhibitors relies on their interaction with AChE, a variety of reports indicate that a number of cholinesterase inhibitors have additional sites of action that may have pharmacological or toxicological relevance (Pope et al., 2005). Several studies suggested that the AChE enzyme has a greater specify for hydrolysing ACh. However, in the absence of the effective AChE enzyme, the reaction which is shown above is inhibited and the liberated ACh accumulates, preventing the smooth transmission of nerve impulses across the synaptic gap at nerve junctions (Quinn, 1987, Pope et al., 2005). This causes loss of coordination, convulsion and ultimately death. AChE is therefore a key component of cholinergic brain synapses and neuromuscular junctions (Pope et al., 2005). The major biological role of the enzyme is the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine. According to the cholinergic hypothesis, memory impairments in patients with senile dementia diseases are due to a selective and irreversible deficiency in the cholinergic functions in the brain. The role of BuChE in normal aging and brain diseases is still indefinable. It has been found that BuChE is present in significantly higher quantities in Alzheimer's plaques than in plaques of

normal age-related non-demented brains (Choudhary et al., 2005b, Orhan et al., 2004). Alzheimer's disease (AD) is neuropathologically associated with a cholinergic deficiency. Therefore, ACh replacement therapy has been used as a potential strategy to improve AD pathology, mainly by inhibiting AChE (Ezoulin et al., 2005, Ferreira et al., 2006, Howes and Houghton, 2003). Encouraging results have been obtained with tacrine, galanthamine, rivastigmine and donepezil, but not without important side effects such as nausea and liver damage (Hu et al., 2005, Orhan et al., 2004). A number of novel natural inhibitors of cholinesterases (AChE and BuChE), isolated from different medicinally important plants, has been reported. (Choudhary et al., 2005c, Godkar et al., 2006, Orhan et al., 2004, Howes and Houghton, 2003). These reports have identified natural compounds that have appreciable inhibitory potential against cholinesterase enzymes.

1.10 Future Therapy

In general, the human body is much better suited to be treated with herbal remedies than synthesized chemical medicines Plants play a major role in the introduction of new therapeutic agents and have received much attention as a source of biologically active substances including antioxidants and anticholinesterase. According to recent epidemiological reports carotenoids and polyphenolic compounds (flavonoids in particular) may exert beneficial effects by reducing cancer risk (Arts and Hollman, 2005, Bravo, 1998, Lopez et al., 2003). Essential nutrients such as vitamins C and E may protect against oxidant-mediated inflammation and tissue damage by virtue of their ability to scavenge free radicals. Thus, maintaining adequate antioxidant status may provide a useful approach in attenuating the cellular injury and dysfunction observed in some inflammatory disorders. The production of

free radicals may stimulate the processes of atherosclerosis, and antioxidant vitamins (including β-carotene), which partly prevent such processes, might favorably influence cardiovascular disease (CVD). Thus, their supplementation might be a useful tool in the prevention of coronary heart disease (CHD). The evidence from case-control studies supports a role of β-carotene in the prevention of CHD (Tavani and La Vecchia, 1999, Visioli et al., 2000, Vanharanta et al., 2003). Recently an intensive search for novel types of antioxidants has been carried out from numerous plant materials.

The synthetic cholinesterase inhibitor tetrahydroaminoacridine (THA) has been approved for patient treatment in several countries, and is effective in reducing cognitive impairment in approximately 30% of AD. However, side effects such as liver damage are recognized as serious problems in the use of such drugs (Mantle et al., 2000a, Orhan et al., 2004, Howes and Houghton, 2003). Therefore, the discovery of new cholinesterase inhibitors has been a challenging area of drug development due to the involvement of cholinesterases in Alzheimer's disease and other related dementias.

1.11 Traditional medicinal plants

In different parts of the world, the uses of medicinal plants have always had an important place in the therapeutic armoury of mankind. Herbal medicine still represents a very important phenomenon in traditional cultures. However, the use of pharmaceutical products of plant origin is a growing area in the treatment of several illnesses and competes with popular medicine. Nevertheless, in some countryside

areas, traditional remedies are still widely used and go side by side with the use of modern pharmaceuticals and often, especially in cases of minor illness, they totally substitute them. Up to 80% of populations in developing counties rely on herbal plants for their primary health care (Ferreira et al., 2006). As a consequence of expensive costs of prescription medicine, the development and reliance on traditional treatment has been encouraged.

The preservative effect of many plant spices and herbs suggests the presence of antioxidative and antimicrobial constituents in their tissues (Mansouri et al., 2001, Romani et al., 2004). Polyphenols are a large family of natural compounds widely distributed in plant foods. They can be classified into two categories, phenolic acids, which account for about one third of the total intake of polyphenols, and flavonoids accounting for the remaining two thirds (Table 1.3). Flavonoids and phenolic acids can act as antioxidants by a number of potential pathways. These phytochemicals that possess significant antioxidant capacities are associated with lower occurrence and lower mortality rates of several human diseases. Many medicinal plants contain large amounts of antioxidants such as polyphenols, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Boskou et al., 2006). Therefore, research to identify antioxidative compounds is an important issue. Although it remains unclear which of the compounds of medical plants are the active ones, polyphenols recently have received increasing attention because of some interesting new findings regarding their biological activities. From pharmacological and therapeutic points of view, the antioxidant properties of polyphenols, such as free radical scavenging and inhibition of lipid peroxidation, are the most crucial. Even though a variety of herbs are known to be sources of phenolic compounds, studies isolating polyphenols and evaluating

their antioxidative effects have rarely been carried out. Recently, there has been an upsurge of interest in the therapeutic potential of traditional medicinal plants as antioxidants in reducing such free radicals induced by tissue injury.

Table 1.3 Some dietary sources of polyphenols (adapted from Halliwell and Gutteridge, 1999).

Polyphenols	Source
Phenolic acids	
Caffeic acid	Coffee, grape, olive, plums, cherries
Gallic acid	blueberries
Flavonoids	
Anthocyanins	Bilberry, strawberries, berries
Catechins or Flavanol	Grape seed, tea, apples
flavanones	Citrus fruits
Tannins	Tea, nuts
Procyanidins	Apples, grapes, cocoa
Proanthocyanidins	Grapes
Isoflavones	Soy
Flavonol	Onion, tea, olive, apples

Traditional or folk medicines have been widely employed for centuries, and they remain an important source for the discovery of new bio-active compounds. The scientific evidence that plant-based diets, in particular those rich in vegetable and

fruits, protect against cancer has been found to be strong and consistent by an expert panel (Basu et al., 1999). Recently, several medicinal plants from different countries have been screened for their antioxidant capacity (Table 1.4).

Table 1.4 Summaries of the countries from which native medicinal plants have been screened for antioxidant capacity and their references.

Original country of medicinal plants	References
Brazilian medicinal plants	(Mensor et al., 2001, Silva et al., 2005)
Indian medicinal plants	(Jadhav and Bhutani, 2002)
British medicinal plants	(Mantle et al., 1998, Mantle et al., 2000b, Mantle et al., 2000a)
Portuguese medicinal plants	(Ferreira et al., 2006)
Canadian medicinal plants	(McCune and Johns, 2002, Owen and Johns, 2002)
Bulgarian medicinal plants	(Ivanova et al., 2005)
Lithuanian medicinal plants	(Miliauskas et al., 2004b, Dapkevicius et al., 1998)
Spain medicinal plants	(Navarro et al., 2003)
Thailand medicinal plants	(Chanwitheesuk et al., 2005)
Chinese medicinal plants	(Yingming et al., 2004, Cai et al., 2004)
Croatian medicinal plants	(Katalinic et al., 2006)
Turkish medicinal plants	(Tepe et al., 2006, Tepe et al., 2005a)
Algerian medicinal plants	(Djeridane et al., 2006)
Iranian medicinal plants	(Souri et al., 2004)

Medicinal plant extracts could be therapeutically useful for several apparently unrelated syndromes by virtue of synergistic effects of two or more components that complement each other *in vivo* through restorative / activation functions. Several studies have reported that many medicinal plants, such as *Bauhinia monandra, Olea europaea, Gymnema montanum, Rhizoma coptidis* and *Eugenia jambolana* which have proven successful in treating diabetes, also possess high antioxidant capacity. Therefore, this activity might be correlated with the presence of antioxidant components (Argolo et al., 2004, Al-Azzawie and Alhamdani, 2006, Ananthan et al., 2003). Over the last thirty years, there have been numerous broad-base screening programmes in which large numbers of plant species world wide have been evaluated for their therapeutic activities such as antioxidant and anticholinesterase activities. The screening results showed that some plants have a variety of chemical constituents such as flavonoids, tannins, alkaloids and monoterpenes, which have the ability to inhibit AChE and act as free radical scavengers (Perry et al., 2003, Howes and Houghton, 2003, Vasudevan and Parle, 2006, Ferreira et al., 2006, Ravi et al., 2004, Tang et al., 2006).

In recent times, the focus on plant research has increased globally and the large body of evidence collected shows the immense potential of medicinal plants. Numerous herbal extracts, containing several active constituents and often more than one plant species, have been used to treat CNS-related disorders. Several studies have now been undertaken to scientifically investigate the traditional (CNS-related) use of *Salvia lavandulaefolia* (a sage species that contains only trace amounts of the

convulsant thujone) relevant to the treatment of Alzheimer's disease. These have centred on the activity of the essential (volatile) oil (Perry et al., 2003). In a study of the traditional Chinese herbs, Hu et al. (2005) found that aqueous extracts of the traditional Chinese herb *Rhizoma anemarrhenae* improved memory and raised the brain Muscarinic receptor density in aged animals by inhibition of acetylcholinesterase. Recently, extracts of *Thespesia populnea*, a large tree found in the tropical regions and coastal forests of India, have been shown to elevate acetylcholine levels in the brain and ultimately improve memory of both young and aged mice. In the light of the above, it may be worthwhile to explore the potential of this plant in the management of Alzheimer patients (Vasudevan and Parle, 2006). In addition, *Celastrus paniculatus* (CP), a medicinal plant from India, reputed to be useful as a pharmaceutical aid for learning and memory, has been investigated (Gattu et al., 1997). The inhibitory activities of selected Turkish medicinal plants, with various ethnobotanical uses, have been screened with the aim of discovering new candidates for anticholinesterase compounds (Orhan et al., 2004). Other studies have reviewed the pharmacological basis of some plants and their active constituents that have been used in traditional Ayurvedic medicine and Traditional Chinese medicine (TCM) for their reputed cognitive-enhancing effects (Howes and Houghton, 2003). The reputed effects of some traditional herbal drugs may not only be relevant in managing the cognitive decline that can be associated with general ageing but may also be relevant in the treatment of specific cognitive disorders such as AD (Perry et al., 2003). In Amazonian communities the use of traditional remedies prepared from *Ptychopetalum olacoides* (Olacaceae) roots for treating various central nervous system conditions, including those associated with aging, was found to facilitate memory recovery (Siqueira et al., 2003). Monoterpenes and their derivatives were

found to be excellent inhibitors of AChE. Anticholinesterase activity of essential oils of *Citrus paradisi* (Miyazawa et al., 2001), *Mentha* species (Miyazawa et al., 1998) , and *Salvia lavandulaefolia* (Spanish sage) (Perry et al., 2002, Perry et al., 2003, Savelev et al., 2003) have been well established. Mechanisms of AChE inhibition by monoterpenoids have not been fully understood. However, several studies have considered them as reversible competitive inhibitors especially for AChE from *Tribolium confusum* and *Rhizopertha domicia*, but at relatively high concentrations of the terpenes (Greenberg-Levy et al., 1993). In contrast, Perry et al. (2002) found that these compounds are non-competitive reversible inhibitors for erythrocyte AChE.

1.11.1 Libyan Medicinal plants

In 2005 the World Health Organization (WHO, 2005) defined traditional medicine (TM) as `` traditional medicine is a comprehensive term used to refer to both traditional medicine such as traditional Chinese medicine, Indian ayurvola and Arabic unani medicine, and to various forms of indigenous medicine``. In Asia and Latin America the populations continue to use TM as a result of their historical circumstances and cultural beliefs. In China, TM accounts for around 40% of all health care needs, while in Africa, nearly 80% of the population uses TM to help to meet their health care requests (WHO, 2005). In Arabic countries in general, and particularly in Libya, there is a long history of people using herbs or plants to treat various diseases successfully (El Gadi, 1992, Kotb, 1983). Libya constitutes an apt example where medicinal plants are widely used in everyday life as a part of folk medicinal remedies. Here the traditional use of medicinal plants as spice, food,

flavours, etc., is becoming more and more popular. There are many traditional Libyan plants such as marjoram, sage, nigella, myrtle, thyme, absinthe, clove, oak, karkade, rosemary. These are the most popular plants, and account for approximately 80% of the Libyan medicinal plants being used. Indeed, the extensive use of medicinal herbs has proven to be the most efficient traditional way of maintaining good health (Kotb, 1983).

However, while some individual plant species such as *Ginkgo biloba* have been investigated in some detail (Sener et al., 2005, Stefanovits-Banyai et al., 2006, Das et al., 2002), there is relatively little knowledge and information available concerning the antioxidant potential and anticholinesterase activities of plant species in general and Libyan plants in particular. Considering the importance of these areas, selected medicinal plants which are being used traditionally in Libya for various disorders where free radicals are thought to be involved, were evaluated for their antioxidant and anticholinesterase activities. According to the traditional medicine uses the plants were extracted with water. The information on the plants that are used in this study is summarized in Table 1.5. Figure 1.2 illustrates the city names of Libya.

Table 1.5 A list of Libyan medicinal plants as used in this study (adapted from El Gadi, 1992 and Kotb, 1983).

Local name	English name	Scientific name	Family name	Locations	part used	Uses
شاي أخضر	Green tea	*Camellia sinensis*	Theaceae	-	Leaves	Stimulant to the CNS, diuretic
الدفلة, دفلي, ورد الحمار	Oleander, Rose bay	*Nerium oleander* (LINN.)	Apocyanaceae	Many places all over Libya.	The whole plant.	Treatments of heart diseases, dried leaves are rubbed upon afflicted parts for headache or neuralgia.
زعتر, دوس, تومس	Thyme, common thyme	*Thymus vulgaris* (LINN.)	Labiatae	Tripoli, Garian and Benghazi	Leaves and flowering tops	Digestive, stimulant, carminative intestinal antiseptic, antifungal, used for whooping cough and bronchitis.
بردقوش, ريحان داوود	Sweet marjoram	*Origanum majorana* (LINN.)	Labiatae	Derna	the flowering plant without the root	Carminative, condiment, antispasmodic, very mild laxative.
مرسين, جدره	Myrtle, common myrtle	*Myrtus communis* (LINN.)	Myrtaceae	common in Tripoli and Benghazi areas	Leaves, berries and the volatile oil	Antidiabetic, astringent, in eczema epilepsy, wound and ulcers.
عاقول, شوك الجمال	Manna tree, camel thorn, prickly alhagi	*Alhagi maurorum* (MEDIK)	Leguminosae	Sebha, Wadi el Ajial, Chat and Ghadames	Leaves and the exudates of branches	Diuretic and expectorant, treatment of rheumatism, mild laxative.

جعدة, حشيشة الريح	Hulwort, cat thyme	*Teucrium polium* (LINN.)	Labiatae	Tripoli , Benghazi and Fezzan	Leaves	Antidiabetic, antiintestinal inflammation and antimalarial, bitter tonic.
حريق, شعر العجوز	Stinging nettle, small nettle	*Urtica urens* (LINN.)	Urticaceae	different areas in Libya	The whole plant.	Antianemic, haemostatic, antidiabetic, diuretic.
عشبة الارنب, طعام الارنب	African fleabane	*Phagnalon rupestre* (LINN.)	Compositae	Tripoli , Benghazi, Derna,Trhuna and Tobruk	The whole herb	Effective in cases of urinary calculi, reduce the renal colic pain.
عنصل, بصل فرعون	Squill	*Urginea maritima* (LINN.)	Liliaceae	along the north coast of Libya	The bulbs	Heart diseases (very low doses)
ورق الزيتون	Common olive	*Olea europaea* (LINN.)	Oleaceae	everywhere in Libya	Leaves	show hypoglycemic activity, increases blood circulation and urine secretion and hypotensive
اكليل الجبل, حصالبان	Common rosemary	*Rosmarinus officinalis* (LINN.)	Labiatae	Many places all over Libya	Leaves and flowering tops	Antirhematic, antiseptic, antispasmodic, carminative, cholgogue, respiratory antiseptic.

فجل بري, عيش وجبن	Wild radish, runch	*Raphanus raphanistrum* (LINN)	Cruciferae	cultivated areas	seeds	Rubfacient, stimulant, emetic, antihaemorrhagic.
دمسيسة, افسنتين	Absinthe, worm-wood	*Artemisia absinthium*(LINN)	Compositae	grows wildly in waste areas	Dried herbs especially leaves and flowering tops	An excellent bitter tonic, antiseptic and diuretic, increase the hepatic secretion, affect the CNS.
اقحوان, صفيرة	Marygold flower, garden marygold	*Calendula officinalis* (LINN.)	Compositae	common in the gardens	Flowers and leaves	Diuretic, diaphoretic, assist antiemetic, antianemic, healing of ulcers, oxytocic
مرمية, شاي درنه, تفاح, تيه	Sage	*Salvia officinalis* (LINN.)	Labiatae	Many places all over Libya	The volatile oil and shade-dried leaves	In cases of nervous disorders, dizziness and trembling
روبيه, فرسيون ابيض	White horehound, horehound	*Marrubium vulgare* (LINN.)	Labiatae	Tripoli, Benghazi, Shahat and many places in Libya.	The flowering plant without the root	To cure cough, sore throat and cold. Hypoglycaemic, cholgogue.

فيجل, سذب	Herb of grace, rue	*Ruta graveolens* (LINN.)	Rutaceae	Many places all over Libya	Leaves	Relieve teeth and ear pains, to ease delivery, emmenagogue and ecbolic
قميلة, فلية, بابونج	Chamomile, German chamomile	*Matricaria chamomilla* (LINN.)	Compositae	Many places all over Libya	The flower heads and their volatile oil	Tonic, mild laxative, diuretic, antispasmodic, diaphoretic, carminative, urinary and respiratory antiseptic.
كركديه	Roselle, karkade	*Hibiscus sabdariffa* (LINN.)	Malvaceae	Many places in Libya	Calyx, leaves and seeds	As a source of vit. C, laxative, diuretic, reduce blood pressure, mild laxative and intestinal antiseptic.
بلوط	Chestnut- oak	*Quercus robur* (LINN.)	Fagaceae	Between Garian and Yefern	Fruits	Very astringent, used to treat haemorrhoids
القزاح		*Pituranthos tortousus*	Apiaceae	Many places in Libya	The whole plant	Reduce blood pressure
قرنفل	Clove	*Syzygium aromaticum*	Myrtaceae	-	Dried flower buds	Headaches, respiratory disorders
زنجبيل	Ginger root	*Zingiber officinale*	Zingiberaceae	-	Rhizomes	Cardiotonic, pain relief

Figure 1.2 Libya map illustrate the city names.

http://atlas.freegk.com/world/africa/libya/libya.jpg

1.12 Aims and Objectives

Plant extracts may have multiple pharmacological effects via a variety of mechanisms; the choice of plants investigated in this study was based on two criteria: first, in this domain there is no published study in Libya that deals with these plants, and the second criterion was that these plants have an ethnopharmacologically good reputation indicating their traditional utilization in the treatment of some abdominal diseases, and they have been proved to be efficient in the treatment of various disorders of the stomach, colon, rectum, oesophagus and liver. Furthermore, they have been described as treating hypertension and oedema, and as detoxicant, diuretic, or anti-inflammatory agents. Due to their traditional utilization and active components, these plants are also considered to be efficient for the treatment of free radical-related disorders. Therefore, the objectives of the current investigation were fourfold:

Firstly, to screen plant extracts (cold water and boiled water extracts) for their antioxidant activity by three methods (FRAP, TEAC and DPPH assays) and to evaluate the relative level of antioxidant activity of these selected plants which are being used traditionally in Libya for various disorders where free radicals are thought to be involved.

Plant phenolics comprise one of the major groups of primary antioxidants or free radical terminators. There are no publications on phenolic content and related antioxidant properties of the medicinal plants traditionally used in Libya. Therefore, it was reasonable to determine the total phenolic content in the selected plant extracts. Consquently the second goal of this study was to determine the phenolic contents of these selected plant extracts by the Folin-Ciocalteu method and to correlate or otherwise these contents with antioxidant activity.

Thirdly, to determine the *in vitro* inhibitory potential of the selected plant extracts against AChE and BuChE by a modified Ellman's method, aiming to discover novel candidates for anticholinesterase compounds from natural resources.

Fourthly, to investigate the *in vitro* enzymatic digestion of the selected plant extracts in order to predict bioavailability of their effusions after ingestion.

Xerophytes could reasonably be thought of as possible sources of known and novel antioxidants, sources conceivably richer than might be found in plants native to more mesic habitats. These plants are used in traditional medicine but may have other uses yet to be described in this comparatively little-known area of ethnobotany. Consequently, there has been an increased interest to discover and search for "new" compounds with an antioxidant potential from xerophytes. Therefore, a range of xerophytic species were assayed for their antioxidant and anticholinesterase activities. It is of interest to compare medicinal usage and chemical activity of this unrelated group of plants with those of Libyan origin.

Finally, in the long term the data obtained here could be useful to develop an extended database regarding bioactivity of traditional herbal medicine plants of Libya and their correlations with various diseases and disorders.

Chapter 2 Screening of Antioxidant Activity of Selected Traditionally Used Libyan Medicinal Plant Extracts

2.1 Introduction

Reactive Oxygen Species (ROS) are highly reactive and potentially damaging transient chemical species created in all cells through various physiological and biochemical process (such as activation of phagocytes, mitochondrial respiration, and biosynthesis of endoperoxide) as undesirable metabolic by-products of normal aerobic metabolism (Rice-Evans and Miller, 1994, Parke et al., 1991, Rice-Evans, 2000). Most ROS such as superoxide radical ($O^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), and nitric oxide radical ($NO^{\cdot}$), attack biological molecules such as proteins, lipids, DNA and RNA leading to cell or tissue damage and injury associated with many diseases, from malignancy to cardiovascular disease and dementia (Rice-Evans and Parker 1998; Hashim et al., 2005). ROS are responsible in part for the ageing process finally leading to death. Parke (1999) has summarised the molecular mechanisms of ROS toxicity as: oxidation of vital thio-compounds to disulphate; loss of tissue GSH (glutathione); impairment of energy generation (ATP, NADH, and NADPH); oxidation of cytoplasm; inhibition of Ca^{2+} transport and electrolyte homeostasis; DNA cleavage and the initiation and promotion of mutations and carcinogenesis.

There are, however, many naturally occurring substances which function to protect against the potentially harmful effects of pro-oxidants. These substances,

termed antioxidants, are simply defined as "chemical compounds or substances that inhibit oxidation"(Balcerczyk and Bartosz, 2003). Antioxidant compounds must be present in biological systems in sufficient concentrations to prevent an accumulation of pro-oxidant molecules, a state known as oxidative stress (Buettner and Schafer, 2000). Antioxidants can interfere with the production of free radicals and/or inactivate them once they are formed. In other words, these antioxidants can act by either interfering with the propagation stage of free radical generation itself or act directly as free radical scavengers. For example, vitamins E and C act as free radical scvengers which can quench free radicals as well as singlet oxygen (Rice-Evans and Miller, 1996).

Currently used synthetic antioxidants have been suspected to cause or promote negative health effects (Amarowicz et al., 2004); hence stronger restrictions have been placed on their application. Therefore, there is a trend to substitute synthetic antioxidants with naturally occurring antioxidants. Some natural antioxidants such as rosemary, sage (Ollanketo et al., 2002) and *Ginkgo biloba* (Arredondo et al., 2004) are exploited commercially either as antioxidant additives or as nutritional supplements. The widespread uses of traditional herbs and medicinal plants have been traced to the occurrence of natural products with medicinal properties. Moreover, the role of herbal teas in disease prevention and cure has been attributed in part to antioxidant properties of their constituent lipo-soluble vitamins A and E, the water soluble vitamin C, and a wide range of amphipathic molecules, broadly termed phenolic compounds (Firuzi et al., 2005, Mello et al., 2005, Duan et al., 2006).

Phenolic compounds are commonly found in both edible and nonedible plants. They have been reported to have multiple biological effects including antioxidant activity (Ivanova et al., 2005, Kaur and Kapoor, 2002). Besides the well known and traditionally used natural antioxidants from tea (Mello et al., 2005), fruits (Prior, 2003, Velioglu et al., 1998, leong and Shui, 2002), vegetables (Gazzani et al., 1998, Kaur and Kapoor, 2002) and spices (Owen and Johns, 2002) many other plants species (which are rich in phenolic content) have been investigated in search of novel antioxidants (Rosa et al., 2003, El-Ghorab et al., 2004, Hayder et al., 2004, Tepe et al., 2005b, Tepe et al., 2005a, Tepe et al., 2006, Tepe et al., 2004, Montoro et al., 2005, Sun and Ho, 2005, Katalinic et al., 2006).

During the last 20 years many publications have appeared on the measurement of antioxidants and a large number of different methods and strategies have been proposed and developed for the evaluation of the total antioxidant capacity in diverse samples such as biological samples, plant tissue and foodstuffs (Prior and Cao, 1999). Although there is a great multiplicity of methods used for antioxidant testing, none of them provides an ideal individual, approved, standardised reference method. This is simply because within biological systems, there are various sources of antioxidants including enzymes (such as superoxide dimutase, glutathione peroxidase and catalase), large molecules (like albumin and ferritin), small molecules (uric acid and polyphenols), some hormones (melatonin and estrogen) and dietary origin molecules such as vitamin C, carotenoids and flavonoids. On the other hand, both oxidants and antioxidants may have different chemical and physical features. Furthermore, antioxidants may respond in different manners to different radical or oxidant sources (Prior et al., 2005).

Consequently, in order to give an overall picture to describe the total antioxidant activity in any sample, more than one analytical assay must be applied (Frankel and Meyer, 2000). In general, most of the methods are based on the generation of the radical in the assay, and the antioxidant response in the sample against the radical is measured. However, the most widely and frequently used assays among these are ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996, Benzie and Strain, 1999) (μmol ferrous ion equivalents); Trolox equivalent antioxidant capacity, (TEAC) (Re et al., 1999) (mmol Trolox equivalents) and scavenging activity of 1,1′-diphenyl-2-picryl-hydrazyl (DPPH) radicals (Brand-Williams et al., 1995, Blois, 1958, Koleva et al., 2002) (mmol Trolox equivalents).

The FRAP assay was first introduced by Benzie and Strain (1996, 1999) for measuring the total antioxidant activity. The assay is based on the reducing power of a compound (antioxidant). A potent antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}). The ferrous ion forms a blue complex (Fe^{2+}/2, 4, 6-tripyridyl-s-triazine (TPTZ), which absorbs at 593 nm. A high absorbance at this wavelength indicates high reducing power of the phytochemical, thus high antioxidant activity.

The TEAC assay (Re et al., 1999) is based on the relative ability of antioxidants to scavenge the radical cation 2,2′-azinobis3-ethylbenzothiazoline-6-sulphonate ($ABTS^{+}$). The extent of quenching of the ABTS radical is measured spectrophotometrically at 734 nm and compared with Trolox, a water-soluble vitamin E analogue.

Radical scavenging activity using the DPPH method was described by Blois (1958). The essence of the DPPH method is that the antioxidant reacts with stable free radicals either by transferring electrons or hydrogen atoms to DPPH, thus neutralising free radical character (Naik et al., 2003). i.e., 1,1′-diphenyl-2-picryl-hydrazyl (deep violet colour) is converted to 1,1′-diphenyl-2-picryl-hydrazine (yellow). Therefore, the colour of the reaction mixture changes from deep violet to yellow and its absorbance at a wavelength of 517 nm decreases. The degree of discolouration indicates the scavenging potential of the sample antioxidant (Tsao and Deng, 2004, Abdille et al., 2005, Tepe et al., 2005a). These three methods are representative of the methods employing radicals in the evaluation of radical scavengers; such methods have gained high popularity over the last decade because of their rapidity and sensitivity (Katalinic et al., 2006).

Recently, there has been an upsurge of interest in the therapeutic potential of traditional medicinal plants as antioxidants in reducing such free radicals induced by tissue injury. However, Mantle et al. (2000b) have determined the relative levels of endogenous antioxidant activity in a range of British medicinal plants selected on the basis of their widespread use in traditional herbal medicine including rosemary, sage, and mint. In Iran Souri et al. (2004) have screened sixty Iranian plants for their antioxidant activity. In addition, a large number of plants which have been used as food and medicinal herbs in Thailand (Chanwitheesuk et al., 2005), Bulgaria (Ivanova et al., 2005), Croatia (Katalinic et al., 2006), Tunisia (Bouzouta et al., 2003), Spain (Navarro et al., 2003), China (Yingming et al., 2004), Turkey (Tepe et al., 2005a, Tepe et al., 2006), India (Jadhav and Bhutani, 2002) and Lithuania

(Dapkevicius et al., 1998, Miliauskas et al., 2004b) have been investigated for their antioxidant potential.

Traditional medicine is widely practiced in Arabic countries in general, and particularly in Libya. Libya constitutes an apt example where medicinal plants are widely used in everyday life as part of folk medicinal remedies. Ethnopharmacological surveys conducted among herbal practitioners of traditional Arab medicine in these countries revealed a large number of indigenous plants are used as sources of their herbal therapies (Ali-Shtayeh et al., 2000). Some of these herbal therapies are used to treat diabetes, heart disease, high blood pressure, and liver disease, conditions in which oxidative stress is prominent (Kotb, 1983). At present, no laboratory data on the bioactivity of herbal medicines used to treat these diseases in traditional Arab medicine in Libya exist.

Moreover, there is relatively little knowledge and information available concerning the antioxidant potential of plant species in general and in Libyan plants in particular. We hypothesized that the beneficial effects of these plants might be due to their antioxidant properties. Considering the importance of this area, the objective of this study is, therefore, to evaluate the relative level of antioxidant activity of selected medicinal plants which are being used traditionally in Libya for various disorders where free radicals are thought to be involved using the three different assays (FRAP, TEAC and DPPH). In the long term plants identified as having high levels of antioxidant activity *in vitro* may prove of value in the design of clinical trials of novel treatment in which free radical induced tissue damage has been implicated.

2.2 Materials and methods

2.2.1 Plant materials:

2.2.1.1 Plant collection and identification:

The medicinal plant species used in this study (Table 1.5) were collected fresh from different areas of Libya, during the period from 12th April to 5th May 2005. The green tea, clove and ginger roots were purchased from a local market for herbs in Benghazi. The botanical identification of the plant species was determined with the aid of descriptions given by Kotb (1983) and confirmed by Dr Osama Rahoma. The plant parts used were allowed to dry in air and then ground into a powder state using a commercial miller and finally used for the preparation of extracts within approximately one month of collection.

2.2.1.2 Plant extract:

Plant extracts with either hot or cold water (hot means freshly boiled water and cold means room temperature water) were prepared in triplicate in ways that mimic the traditionally used methods in folk medicine as follows.

2.2.1.2.1 Hot water extract:

One gram of each dried powdered plant was soaked with boiling de-ionized water (1:20 w/v) in a conical flask and shaken for 30 minutes at 160 strokes per minute (IKA- VIBRAX-VXR, The Scientific Instrument Centre Ltd, Liverpool, UK). The extracts were filtered through glass wool then the filtrates were centrifuged (3000×g, 10 min) (Hettich Universal Centrifuge, Germany) to remove insoluble

matter, and the supernatants retained for antioxidant analysis (Koleva et al., 2002; Mantle et al., 2000b).

2.2.1.2.2 Cold water extract:

One gram of each dried powdered plant was soaked with de-ionized water (1:20 w/v) in a conical flask and shaken for 24 hours at 160 strokes per minute (IKA-VIBRAX-VXR, The Scientific Instrument Centre Ltd, Liverpool, UK). The extracts were filtered through glass wool then the filtrates were centrifuged (3000×g, 10 min) (Hettich Universal Centrifuge, Germany) to remove insoluble matter, and the supernatants retained for antioxidant analysis (Koleva et al., 2002, Mantle et al., 2000b).

2.2.2 Chemicals:

The chemicals used in these experiments, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) powder ($ABTS^{+}$), potassium persulphate, (+/-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), ferrous sulphate, ferric chloride, 2,4,6 tripyridyl-s-triazine (TPTZ), Folin-Ciocalteu reagent, gallic acid and 1,1′-diphenyl-2-picryl-hydrazyl (DPPH) were supplied by Sigma Chemical Company, UK. All other solvents, salts and reagents were obtained from VWR International, Country Durham, UK.

2.2.3 Antioxidant analysis:

2.2.3.1 FRAP assay:

The FRAP assay was originally developed by Benzie and Strain (1996) to measure reducing power in plasma, but the assay subsequently has also been adapted and used for the assay of antioxidants in botanical tissues. The reaction measures reduction of Fe^{3+} 2,4,6-tripyridyl-s-triazine (TPTZ) to Fe^{2+} 2,4,6-tripyridyl-s-triazine (TPTZ), a coloured product. The assay solution was prepared fresh daily using 25 ml of 300 mM sodium acetate buffer pH 3.6 plus 2.5 ml of 10 mM TPTZ solution in 40 mM HCl plus 2.5 ml of 20 mM ferric chloride ($FeCl_3$ $6H_2O$). A range of ferrous standard solutions (0 to 2 mM) were prepared from a stock 2 mM ferrous sulphate solution in distilled water. Absorbances were measured using a COBAS Mira (Roche Diagnostics, Welwyn Garden City, Herts.). 10 µl of plant extract or standard solution was mixed with 30 µl de-ionized water as a diluter and 300 µl of the reactive solution and incubated for 250 s at 37°C. The change in absorbance at 600 nm over the incubation period was measured and the ferrous ion equivalent (FIE) unit of activity was calculated by comparison with a standard calibration curve of ferrous ions (figure 2.1).

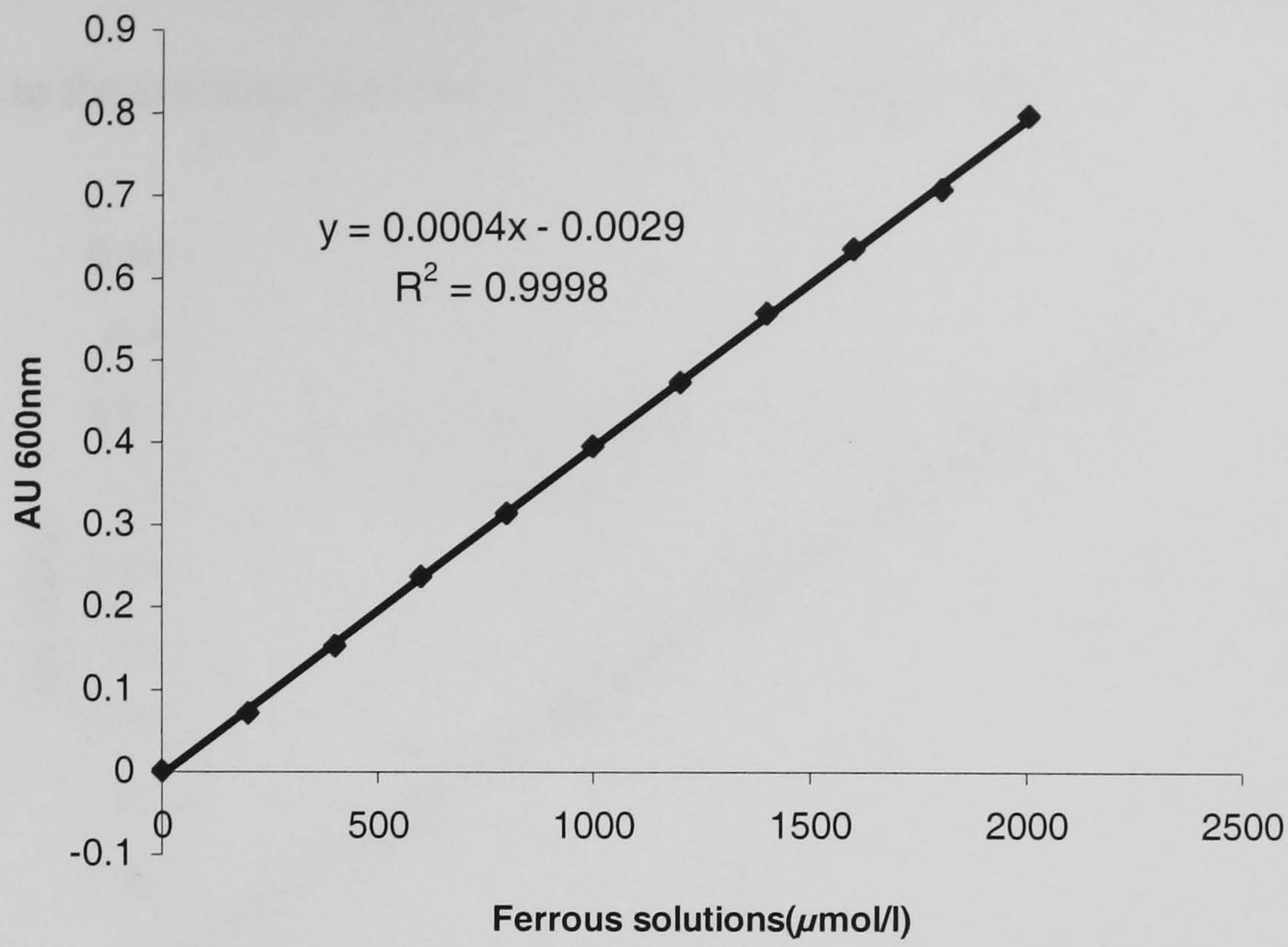


Figure 2.1 FRAP Standard Calibration Curve.

2.2.3.2 TEAC assay:

The standard TEAC assay described by Van den Berg et al. (1999) and Re et al. (1999) was used with minor modifications for the determination of TEAC values. This assay assesses the total radical scavenging capacity based on the ability of compounds to scavenge the stable ABTS radical (ABTS$^{\cdot}$). ABTS$^{\cdot}$ (blue green) solution was produced by the reaction between one part of ABTS solution (7mM) and nine parts of potassium persulphate solution (2.45 mM) (both solutions were prepared in distilled water) in a total volume of 100ml. This radical solution was stored in the dark for 12-16 hours before use. The concentrated solution was diluted with phosphate buffered saline (PBS), pH 7.4 to give a final absorbance of 0.70 ± 0.02 at 420 nm. The assay for the COBAS Mira was as follows: 5 µl of plant extract or Trolox standard and 10µl water were mixed with 500µl of ABTS$^{\cdot}$ solution and incubated for 250s at 37°C. The change in absorbance at 405nm over the incubation period was measured. A range of Trolox standards (0-2.5 mmol) was prepared from a

stock Trolox solution in ethanol. The TEAC equivalent antioxidant was calculated by reference to the standard solution on a molar basis (figure 2.2).

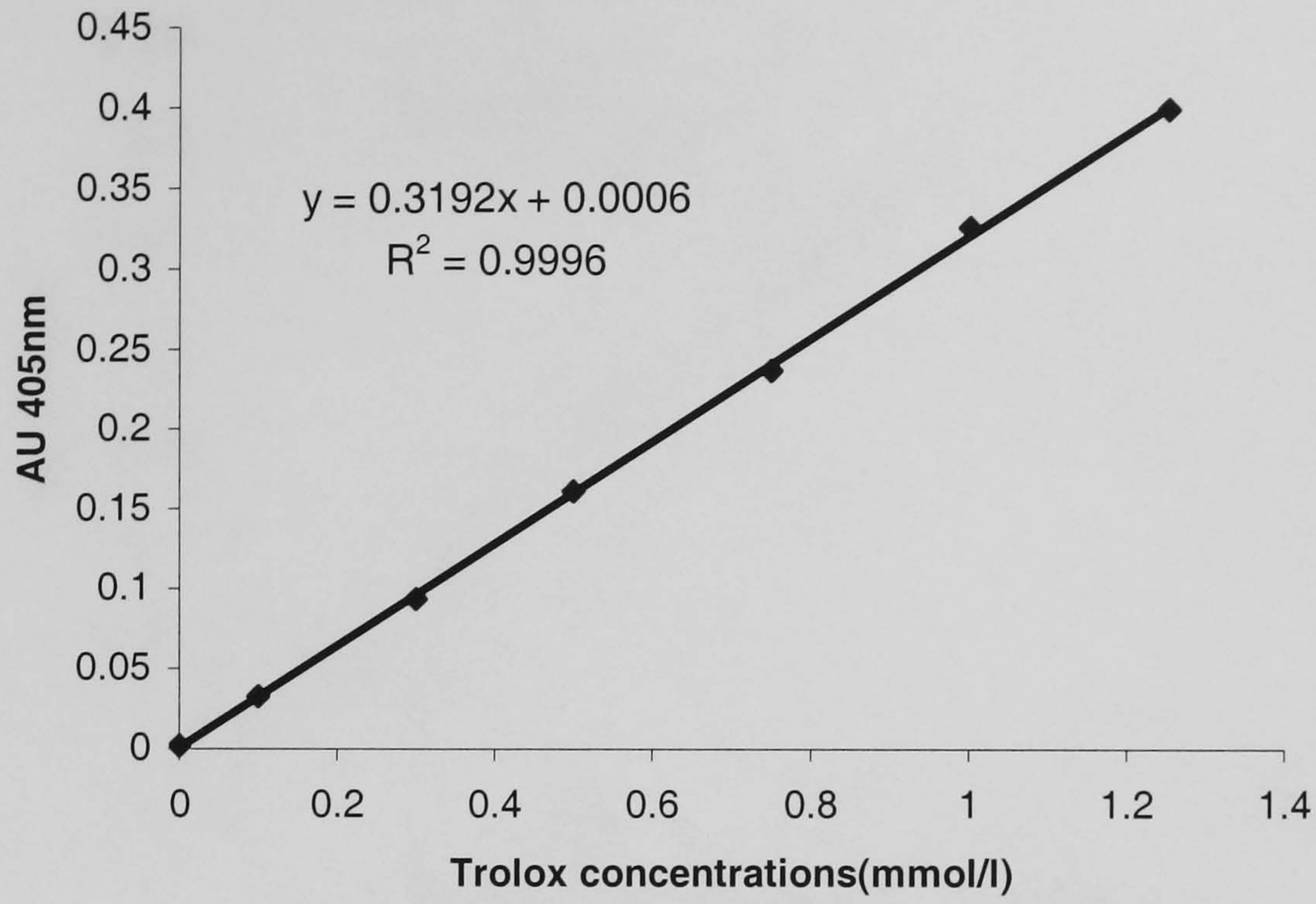


Figure 2.2 TEAC Standard Calibration Curve.

2.2.3.3 DPPH· assay:

The original DPPH· (1,1'-diphenyl-2-picrylhydrazyl) method of Brand-William et al. (1995) was modified by Fukumoto and Mazza (2000). This method of Fukumoto and Mazza was further modified as follows: DPPH· (150 μM), a stable free radical, was dissolved in 80% (v/v) methanol. Using 80% (v/v) methanol had the advantage of a faster reaction rate for some compounds such BHA and lower evaporation losses. The assay was adapted to be carried out in the COBAS Mira. 10 μl of plant extract or standard solution and 30 μl de-ionized water as a diluter were mixed with 300 μl of DPPH· solution and incubated for 25 min at 30°C. The change in absorbance at 500 nm over the incubation period was measured and compared to a standard calibration curve of Trolox standards (0 to 2.5 mmol in methanol; figure.

2.3). The TEAC of the antioxidant was calculated from standard Trolox solutions on a molar basis as shown in figure 2.3.

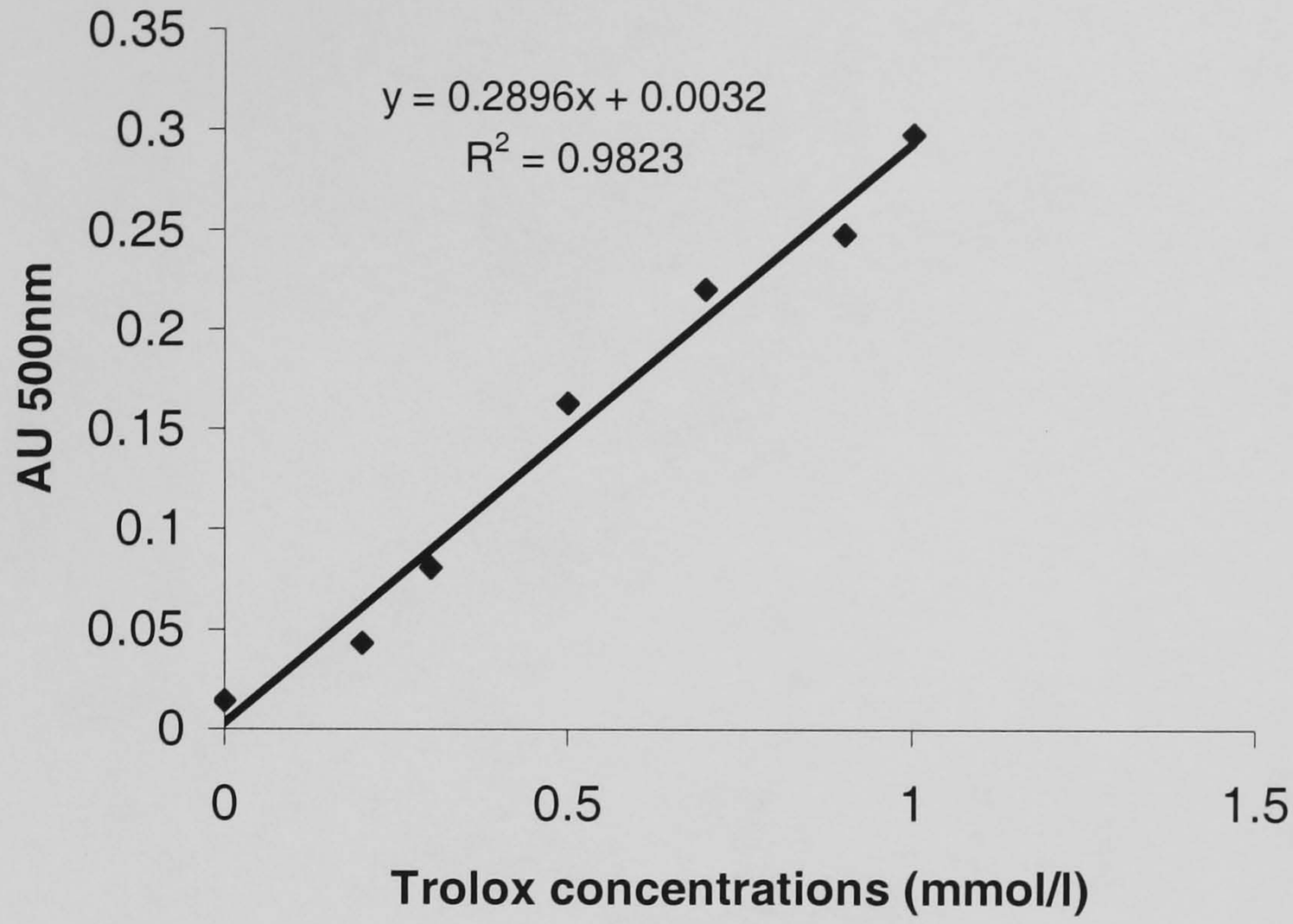


Figure 2.3 DPPH Standard Calibration Curve.

2.2.4 Determination of total phenolic content:

The total phenolic content of the plant extracts was determined according to the Folin- Ciocalteu method (Duan et al., 2006) with a slight modification. Instead of reading samples spectrophotometrically the assay was performed in a Multiskan Ascent micro plate reader (Thermo Labsystem, Helsinki, Finland). In each well of a 96-well flat-bottom polystyrene micro plate a 10 µl aliquot of plant extract or calibration standard was added to 130 µl of Folin-Ciocalteu reagent (the concentrated commercial 2 N reagent was diluted 1:10 (v/v) with de-ionized water). After 5 min 100 µl of 7.5% (w/v) sodium carbonate solution was added. The plates were shaken in the automated micro plate reader for 1minute and incubated for 30minutes at 37°C. The absorbance was measured at 750 nm, and then compared to a gallic acid

calibration standard curve (0 to 1500 mg/L in de-ionized water) (figure 2.4). The total phenolic content was expressed as gallic acid equivalents (GAE) (figure 2.4).

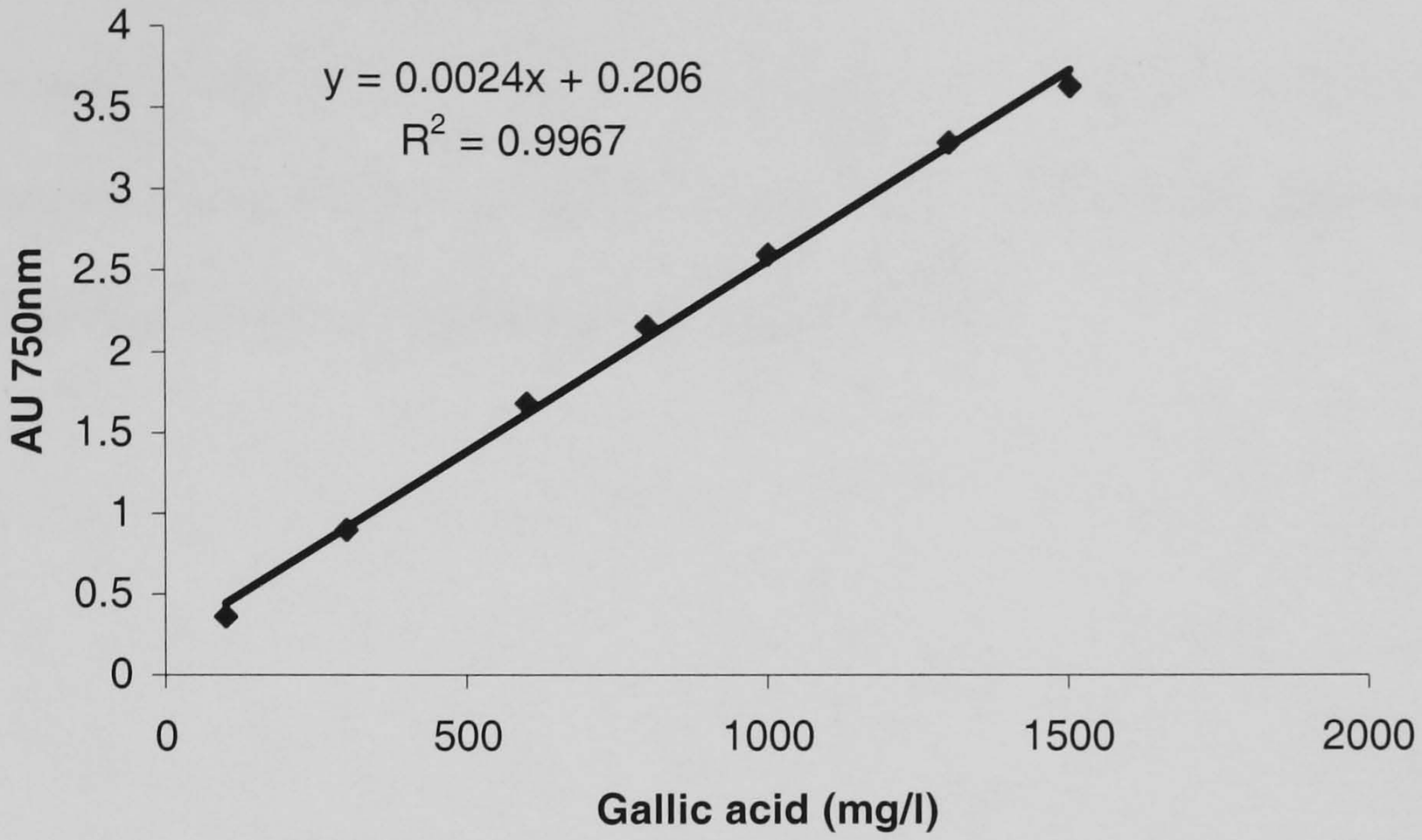


Figure 2.4 Phenolic Content Standard Calibration Curve.

The absorbances of the standard curves were plotted versus reference concentrations (ferrous sulphate conc. for FRAP and Trolox conc. for TEAC and DPPH). The absorbances of the test samples were read from the standard curve to give antioxidant activity as ferrous ion equivalent (FIE) for FRAP assay and as mmol Trolox equivalent for TEAC and DPPH assays (the data were corrected for any initial dilution of samples if required). Samples with high antioxidant activity need to be diluted for example, typically by 1:5, 1:10, or 1:20 before pipetting the sample into the assay tube.

2.2.5 Statistical analysis:

The experiments were carried out in triplicate extracts. The results are given as mean ± standard deviation (SD). The data for antioxidant activity for each assay

were analysed by one-way analysis of variance (ANOVA), and for comparison with green tea extract (standard antioxidant reference), the Dunnett's post–test was used which is designed to compare several treatments with one control treatment (Ljubuncic et al., 2005, Arredondo et al., 2004). A difference was considered statistically significant when $p < 0.05$. All statistical tests were completed using Minitab version 14.0 and Microsoft Excel. Linear regressions between the content of phenolics and data for the antioxidant assays were assessed.

2.3 Results

2.3.1 Antioxidant analysis:

2.3.1.1 FRAP assay:

In this study, the water phase antioxidant activity of plant extracts (hot and cold) produced from twenty three different Libyan plant species in comparison with the well established antioxidant properties of green tea have been investigated. As shown in Table 2.1 and Table 2.2 there were big differences in total antioxidant capacity for Ferric Reducing Antioxidant Power between the studied plants. The FRAP values varied from 748 to 40263 and 864 to 71010 (μmol Fe^{2+} E/g dried weight) for hot and cold water extracts, respectively. According to the comparison of reducing ability/antioxidant power of the green tea (standard antioxidant reference) with FRAP of the twenty three selected plant extracts, these plant extracts were divided into three groups (Table 2.3). Group (a) represented the plant extracts that shown high FRAP values as compared with the green tea (highly significant, p values ranged from less than 0.0001 to 0.05), group (b) moderate FRAP values (lower than the green tea), and group (c) low FRAP (lower than 1mmol Fe^{2+} E/g). Out of the twenty three selected plant extracts which were assayed for FRAP antioxidant activity, nine of the hot extracts and three of the cold extracts were found in the group of high antioxidant (group a) (Table 2.3). The group with moderate activity was represented by *Teucrium polium*> *Hibiscus sabdariffa*> *Matricaria chamomilla*> *Marrubium vulgare*> *Artemisia absinthium*> *Phagnalon rupestre*> *Pituranthos tortousus*> *Ruta graveolens*> *Urtica urens*> *Zingiber officinale*> *Raphanus raphanistrum* for the hot extracts and *Hibiscus sabdariffa*> *Calendula*

officinalis> Salvia officinalis>Rosmarinus officinalis> Teucrium polium> Thymus vulgaris> Olea europaea> Origanum majorana> Marrubium vulgare> Phagnalon rupestre> Nerium oleander> Ruta graveolens> Artemisia absinthium> Pituranthos tortousus> Raphanus raphanistrum> Matricaria chamomilla> Zingiber officiale> Urtica urens for the cold extracts. *Urginea maritima* and *Alhagi maurorum* represented the group with low antioxidant activity of both hot and cold water extracts (Table 2.3). Figure 2.5 and Table 2.4 illustrate the comparison of antioxidant activity for the hot and the cold water extracts assayed by the FRAP method.

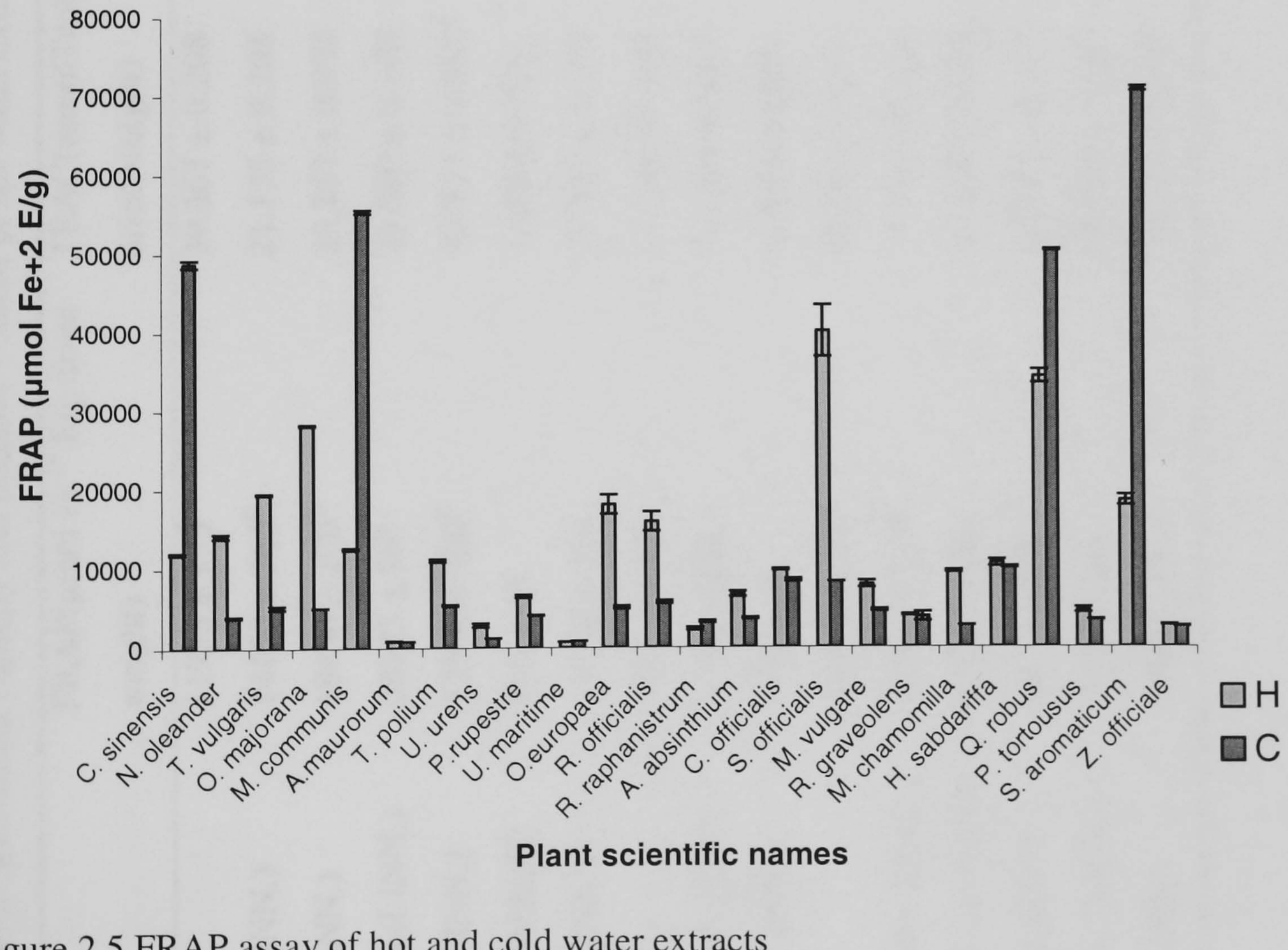


Figure 2.5 FRAP assay of hot and cold water extracts

Table 2.1. Water soluble antioxidant capacity and phenolic content of hot water extracts.

Scientific name	FRAP(μmol Fe^{2+} E/g dried weight)	TEAC(mmolTroloxE/g dried weight)	DPPH(mmolTrolox E/g dried weight)	Phenolic content(mgGAE/g dried weight)
Camellia sinensis	11933 ± 153	39.467 ± 0.208	19.433 ± 0.321	1385.9 ± 21.0
Nerium oleander (LINN.)	14150 ± 265[b]	21.133 ± 0.208	7.767 ± 0.208	1022.3 ± 30.5
Thymus vulgaris (LINN.)	19450 ± 100[a]	23.267 ± 0.058	10.267 ± 0.153	1101.5 ± 52.9
Origanum majorana (LINN.)	28183 ± 76[a]	21.500 ± 0.100	0.515 ± 0.005	1125.8 ± 3.8
Myrtus communis (LINN.)	12500 ± 100[d]	43.333 ± 0.252[a]	20.433 ± 0.058[d]	4456.0 ± 51.5[a]
Alhagi maurorum (MEDIK)	899 ± 35	1.100 ± 0.006	0.504 ± 0.007	303.2 ± 21.5
Teucrium polium (LINN.)	11050 ± 218	21.367 ± 0.252	5.867 ± 0.153	895.9 ± 30.7
Urtica urens (LINN.)	2867 ± 232	11.767 ± 0.058	0.399 ± 0.017	382.3 ± 14.9
Phagnalon rupestre (LINN.)	6500 ± 200	21.700 ± 0.004	0.510 ± 0.009	760.9 ± 41.8
Urginea maritima (LINN.)	748 ± 20	0.900 ± 0.002	0.464 ± 0.005	267.0 ± 10.4
Olea europaea	18117 ± 206[a]	22.200 ± 0.100	13.267 ± 0.115	1252.1 ± 8.9
Rosmarinus officinalis (LINN.)	15967 ± 126[a]	20.767 ± 0.153	12.900 ± 0.173	1123.9 ± 31.5
Raphanus raphanistrum (LINN.)	2350 ± 200	25.467 ± 0.058	0.360 ± 0.009	332.3 ± 17.6
Artemisia absinthium(LINN.)	6750 ± 304	23.333 ± 0.115	5.267 ± 0.115	711.8 ± 42.7
Calendula officinalis (LINN.)	9847 ± 104	18.300 ± 0.100	4.867 ± 0.058	996.6 ± 13.8
Salvia officinalis (LINN.)	40263 ± 3287[a]	19.833 ± 0.115	19.533 ± 0.153	1327.9 ± 10.9

Marrubium vulgare (LINN.)	7947 ± 419	22.167 ± 0.115	3.833 ± 0.115	682.5 ± 14.2
Ruta graveolens (LINN.)	4073 ± 76	11.067 ± 0.058	0.704 ± 0.007	527.5 ± 28.8
Matricaria chamomilla (LINN.)	9563 ± 144	20.967 ± 0.115	4.967 ± 0.115	833.2 ± 21.8
Hibiscus sabdariffa (LINN.)	10630 ± 436	21.800 ± 0.100	5.200 ± 0.100	856.0 ± 26.2
Quercus robur	34447 ± 852[a]	22.167± .351	23.600 ± 0.520[c]	2472.7 ± 17.0[a]
Pituranthos tortousus	4640± 331	11.400 ± 0.006	0.902 ± 0.021	439.3 ± 17.7
Syzygium aromaticum	18560 ± 656[a]	25.167 ± 0.850	29.867 ± 0.153[a]	6937.5 ± 75.4[a]
Zingiber officinale	2773 ± 76	11.933 ± 0.058	0.367 ± 0.058	271.4 ± 2.9

Data expressed as mean ± SD, (n = 3)

[a] Extremely statistically Significantly higher than the green tea (the standard antioxidant), P<0.0001

[b] P<0.001

[c] P<0.005

[d] P<0.01

Table 2.2 Water soluble antioxidant capacity and phenolic content of cold water extracts.

Scientific name	FRAP(μmol Fe^{2+}E/g dried weight)	TEAC(mmolTroloxE/g dried weight)	DPPH(mmolTrolx E/g dried weight)	Phenolic content(mgGAE/g dried weight)
Camellia sinensis	48783 ± 439	35.852 ± 0.104	17.044 ± 0.069	3039.8 ± 8.9
Nerium oleander (LINN.)	3964 ± 126	10.1167 ± 0.058	3.156 ± 0.026	593.3 ± 32.1
Thymus vulgaris (LINN.)	5106 ± 238	10.833 ± 0.153	0.949 ± 0.021	704.2 ± 8.8
Origanum majorana (LINN.)	5078 ± 25	9.215 ± 0.072	0.859 ± 0.0199	960.5 ± 28.1
Myrtus communis (LINN.)	55433 ± 270 [a]	36.443 ± 0.060 [b]	19.047 ± 0.0398 [a]	3829.2 ± 38.2 [a]
Alhagi maurorum (MEDIK)	864 ± 8	1.085 ± 0.007	0.472 ± 0.007	312.4 ± 7.7
Teucrium polium (LINN.)	5389 ± 126	10.533 ± 0.058	0.227 ± 0.005	620.0 ± 5.1
Urtica urens (LINN.)	1186 ± 1	1.130 ± 0.004	0.365 ± 0.017	262.6 ± 29.1
Phagnalon rupestre (LINN.)	4086 ± 63	5.657 ± 0. 125	3.881 ± 0.117	678.3 ± 18.9
Urginea maritima (LINN.)	866 ± 14	1.000 ± 0.002	0.724 ± 0.004	270.9 ± 3.7
Olea europaea	5101 ± 176	6.133 ± 0.208	4.088 ± 0.052	765.7 ± 42.1
Rosmarinus officinalis (LINN.)	5751 ± 176	5.800 ± 0.200	0.517 ± 0.003	673.3 ± 21.4
Raphanus raphanistrum (LINN.)	3226 ± 126	2.100 ± 0.100	0.472 ± 0.007	353.1 ± 6.4
Artemisia absinthium(LINN.)	3659 ± 104	3.567 ± 0.058	0.897 ± 0.017	426.3 ± 26.0
Calendula officinalis (LINN.)	8502 ± 153	7.833 ± 2.08	0.573 ± 0.007	916.3 ± 11.9

Salvia officinalis (LINN.)	8286 ± 29	7.060 ± 0.053	0.811 ± 0.025	771.5 ± 29.6
Marrubium vulgare (LINN.)	4651 ± 144	3.467 ± 0.115	0.434 ± 0.009	494.1 ± 10.2
Ruta graveolens (LINN.)	3893 ± 588	4.500 ± 0.700	0.600 ± 0.009	519.9 ± 58.3
Matricaria chamomilla (LINN.)	2719 ± 14	3.397 ± 0.060	2.155 ± 0.052	610.3 ± 37.5
Hibiscus sabdariffa (LINN.)	10072 ± 76	8.486 ± 0.419	5.622 ± 0.150	806.3 ± 10.9
Quercus robur	50558 ± 75 [c]	36.443 ± 0.060 [b]	20.635 ± 0.242 [a]	3018.0 ± 38.4
Pituranthos tortousus	3448± 14	3.667 ± 0.231	0.462 ± 0.009	355.8 ± 3.4
Syzygium aromaticum	71010 ± 265 [a]	36.165 ± 0.104 [d]	20.497 ± 0.210 [a]	6361.1 ± 20.5 [a]
Zingiber officinale	2561 ± 52	2.053 ± 0.040	1.080 ± 0.007	248.0 ± 6.0

Data expressed as mean ± SD, (n = 3)

[a] Extremely statistically Significantly higher than the green tea (the standard antioxidant), P<0.0001

[b] P<0.001

[c] P<0.005

[d] P<0.05

Table 2.3 Comparative Antioxidant Activities by various methods of selected Libyan medicinal plants.

Plant Scientific Name	Hot Water Extracts				Clod Water Extracts			
	FRAP	TEAC	DPPH	PC	FRAP	TEAC	DPPH	PC
Nerium oleander (LINN.)	+++	++	++	++	++	++	++	++
Thymus vulgaris (LINN.)	+++	++	++	++	++	++	+	++
Origanum majorana (LINN.)	+++	++	+	++	++	++	+	++
Myrtus communis (LINN.)	+++	+++	+++	+++	+++	+++	+++	+++
Alhagi maurorum (MEDIK)	+	+	+	+	+	+	+	+
Teucrium polium (LINN.)	++	++	++	++	++	++	+	++
Urtica urens (LINN.)	++	++	+	+	++	+	+	+
Phagnalon rupestre (LINN.)	++	++	+	++	++	++	++	++
Urginea maritima (LINN.)	+	+	+	+	+	+	+	+
Olea europaea	+++	++	++	++	++	++	++	++
Rosmarinus officinalis (LINN.)	+++	++	++	++	++	++	+	++
Raphanus raphanistrum (LINN.)	++	++	+	+	++	++	+	+
Artemisia absinthium(LINN.)	++	++	++	++	++	++	+	++
Calendula officinalis (LINN.)	++	++	++	++	++	++	+	++
Salvia officinalis (LINN.)	+++	++	+++	++	++	++	+	++
Marrubium vulgare (LINN.)	++	++	++	++	++	++	+	++
Ruta graveolens (LINN.)	++	++	+	++	++	++	+	++

Matricaria chamomilla (LINN.)	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Hibiscus sabdariffa (LINN.)	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Quercus robur	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +
Pituranthos tortousus	+ +	+ +	+	+ +	+ +	+ +	+	+
Syzygium aromaticum	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Zingiber officinale	+ +	+ +	+	+	+ +	+ +	+	+

P C = Phenolic content.
+ + + = group a (significantly higher than the green tea)
+ + = group b (moderate group, lower than the green tea)
+ = group c (low antioxidant activity or phenolic content)

Table 2.4 Antioxidant activities shown by hot and cold water extracts selected Libyan medicinal plants.

Plant Scientific Name	FRAP	TEAC	DPPH	Phenolic Content
Camellia sinensis	C	H/C	H/C	C
Nerium oleander (LINN.)	H	H	H	H
Thymus vulgaris (LINN.)	H	H	H	H
Origanum majorana (LINN.)	H	H	C	H/C
Myrtus communis (LINN.)	C	H	H/C	H/C
Alhagi maurorum (MEDIK)	H/C	H/C	H/C	H/C
Teucrium polium (LINN.)	H	H	H	H
Urtica urens (LINN.)	H	H	H/C	H
Phagnalon rupestre (LINN.)	H	H	C	H/C
Urginea maritima (LINN.)	H/C	H/C	C	H/C
Olea europaea	H	H	H	H
Rosmarinus officinalis (LINN.)	H	H	H	H
Raphanus raphanistrum (LINN.)	C	H	C	H/C
Artemisia absinthium(LINN.)	H	H	H	H
Calendula officinalis (LINN.)	H	H	H	H/C
Salvia officinalis (LINN.)	H	H	H	H
Marrubium vulgare (LINN.)	H	H	H	H
Ruta graveolens (LINN.)	H/C	H	H/C	H/C

Matricaria chamomilla (LINN.)	H	H	H	H
Hibiscus sabdariffa (LINN.)	H/C	H	H/C	H/C
Quercus robur	C	C	H/C	C
Pituranthos tortousus	H	H	H	H
Syzygium aromaticum	C	C	H/C	H/C
Zingiber officinale	H/C	H	C	H/C

H = Hot water extract value is higher than the cold extract value.
C = Cold water extract value is higher than the hot extract value.
H/C = No differences between hot extract values and cold extract values.

2.3.1.2 TEAC assay:

The water phase antioxidant activities of hot and cold extracts produced from 23 selected Libyan plants were studied in comparison with green tea, the established antioxidant reference. Measurement of antioxidant activity of these plant extracts by the TEAC assay showed a significant variation between studied species (Tables 2.1, 2.2 and 2.3). The low antioxidant activity included *Urginea maritima* and *Alhagi maurorum* of both hot and cold plant extracts and *Urtica urens* of cold plant extract. Interestingly, *Myrtus communis* (hot and cold extracts) and *Quercus robur* and *Syzygium aromaticum* (cold plant extracts) exhibited high antioxidant potential compared to that of green tea (p value ranged between < 0.001 and < 0.05). Out of twenty three studied plant extracts a group of twenty (hot extracts) and seventeen (cold extracts) species were identified, with considerable (above 2 mmol) TEAC values ranging between 25.467 and 11.067 mmol T E, and between 10.833 and 2.053 mmol T E of hot and cold extracts respectively. In general, the TEAC values of cold plant extracts were lower than those of hot extracts (figure 2.6 and Table 2.4).

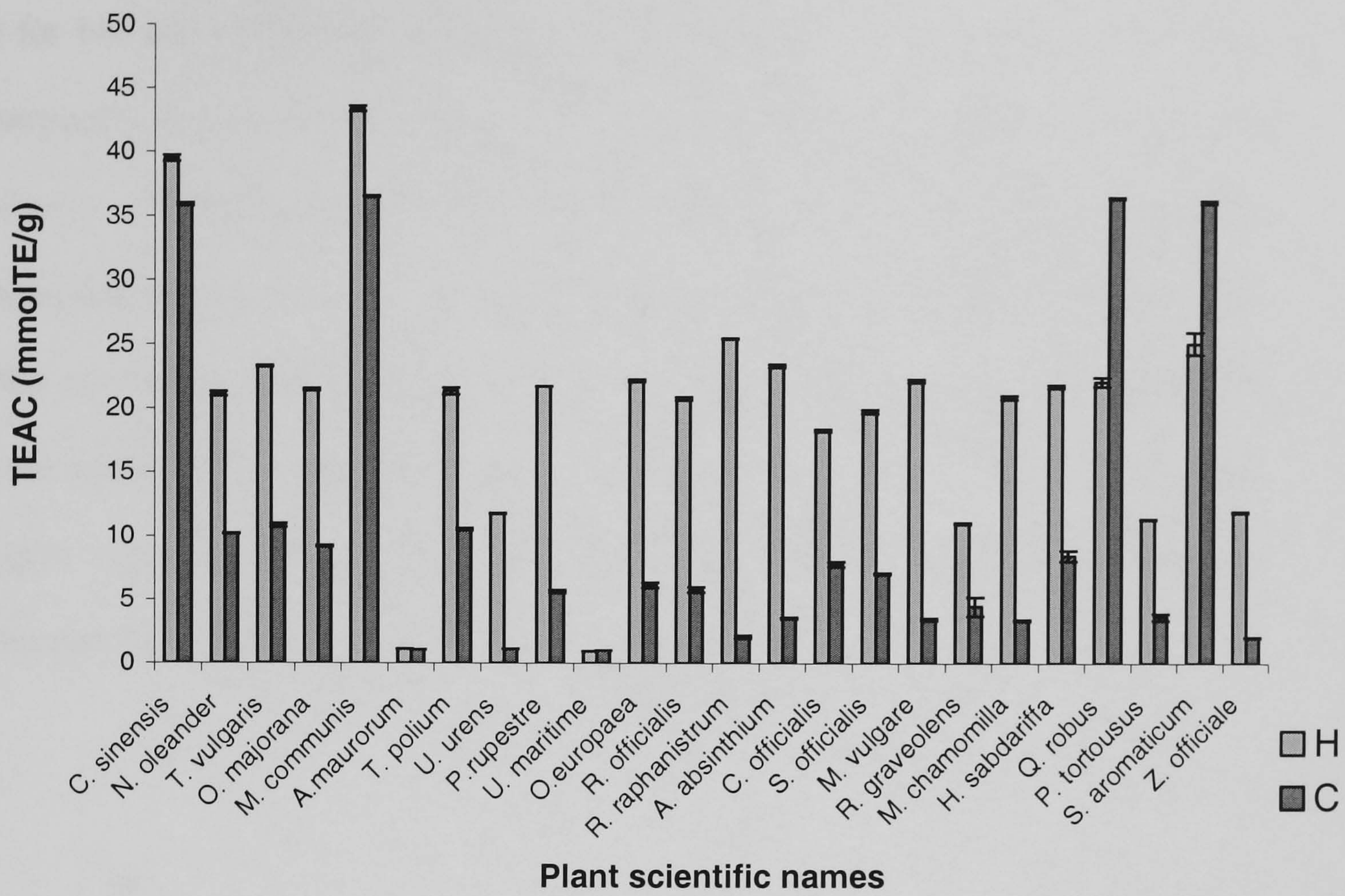


Figure 2.6 TEAC assay of hot and cold water extracts.

2.3.1.3 DPPH˙ assay:

Results of DPPH reduction by hot and cold plant extracts are summarized in Tables 2.1, 2.2 and Table 2.3. The results obtained show that the hot and cold extracts of *Myrtus communis*, *Quercus robur* and *Syzygium aromaticum* had a significant higher capacity of scavenging activity against the DPPH free radical (p <0.01, 0.005, and 0.0001 of hot extracts respectively, and < 0.0001 of cold extracts) than the green tea extract used as a standard (positive control). The hot extract of *Salvia officinalis* also showed activity against DPPH (19.533 mmol TE/ g) which was almost the same as those obtained from the green tea extract (19.433 mmol TE/ g). Nine hot and fifteen cold extracts exhibited low DPPH values below 2 mmol T E

which is considered as the group with a slight scavenging activity against the DPPH free radical. The remaining plant extracts showed a moderate scavenging activity with values ranging from 13.267 to 3.833 mmol T E /g and 5.156 to 2.155 mmol T E /g for hot and cold extracts respectively, in the following decreasing order: *Olea europaea*> *Rosmarinus officinalis*> *Thymus vulgaris*> *Nerium oleander*> *Teucrium polium*> *Artemisia absinthium*> *Hibiscus sabdariffa*> *Matricaria chamomilla*> *Calendula officinalis*> *Marrubium vulgareh* (hot extracts) and *Hibiscus sabdariffa*> *Olea europaea*> *Phagnalon rupestre*> *Nerium oleander*> *Matricaria chamomilla* (cold extracts). Generally, in this assay, the antioxidant values of hot extracts exhibit higher capacity of scavenging activity against the DPPH free radical as compared with the cold extract capacity (figure 2.7 and Table 2.4).

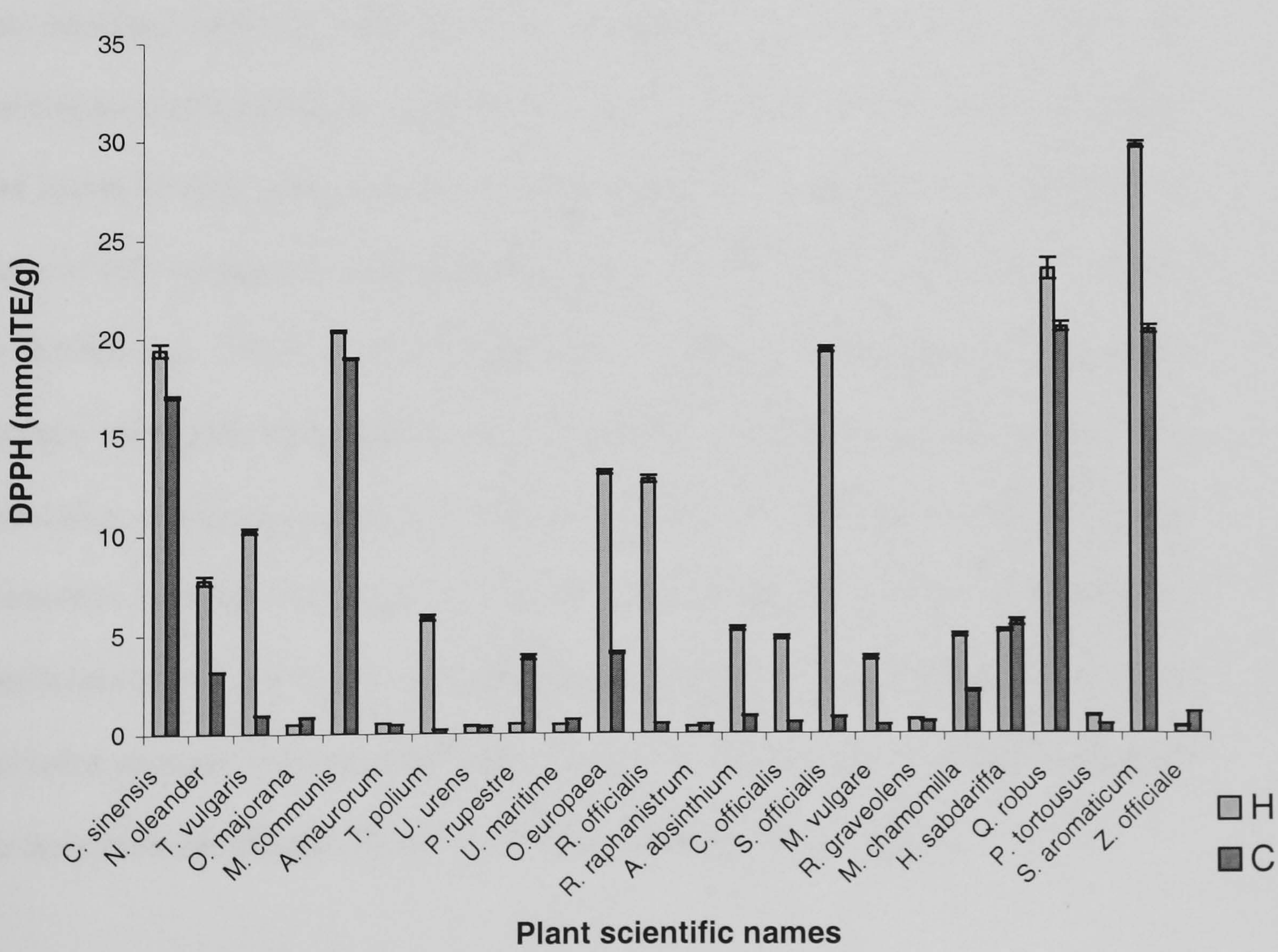


Figure 2.7 DPPH assay of hot and cold water extracts.

2.3.2 Phenolic content:

The total phenolic content of the hot and cold plant extracts investigated in this study varied widely from 6937.5 to 267.0 mg GAE/ g of dry material and 6361.1 to 248.0 mg GAE/ g of dry material for hot and cold extracts respectively (Table 2.1, 2.2, and 2.3). *Myrtus communis* and *Syzygium aromaticum* of hot and cold extracts and *Quercus robur* hot extract presented the highest total phenol content as compared with the green tea ($p < 0.0001$; figure 2.8 and Table 2.4). Correlation between the content of phenolic compounds and antioxidant activity was examined. Strong correlation between the total phenolic content and antioxidant capacity by FRAP assay was found in cold water extracts with correlation coefficient equal to 0.972 ($r^2 = 0.95$) (figure 2.9). As shown in figure 2.10 a positive relationship was also observed between total phenolic content and the TEAC assay result with correlation coefficient equal to 0.901 ($r^2 = 0.837$) for cold extracts. Weak correlation was found between total phenolic content and the FRAP and TEAC assays for hot extracts with correlation coefficient equal to 0.376 and 0.505 ($r^2 = 0.143$, $r^2 = 0.255$) for FRAP and TEAC assays respectively. A positive correlation was obtained between the DPPH assay results and the total phenolic content in hot extracts with correlation coefficient equal to 0.813 ($r^2 = 0.661$). Cold extracts showed a strong correlation between the content of phenolic compounds and DPPH with correlation coefficient equal to 0.915 ($r^2 = 0.837$) (figure 2.11). In general, cold water extracts showed a stronger correlation between the phenolic content and total antioxidants for the three methods (FRAP, TEAC and DPPH) than hot water extracts.

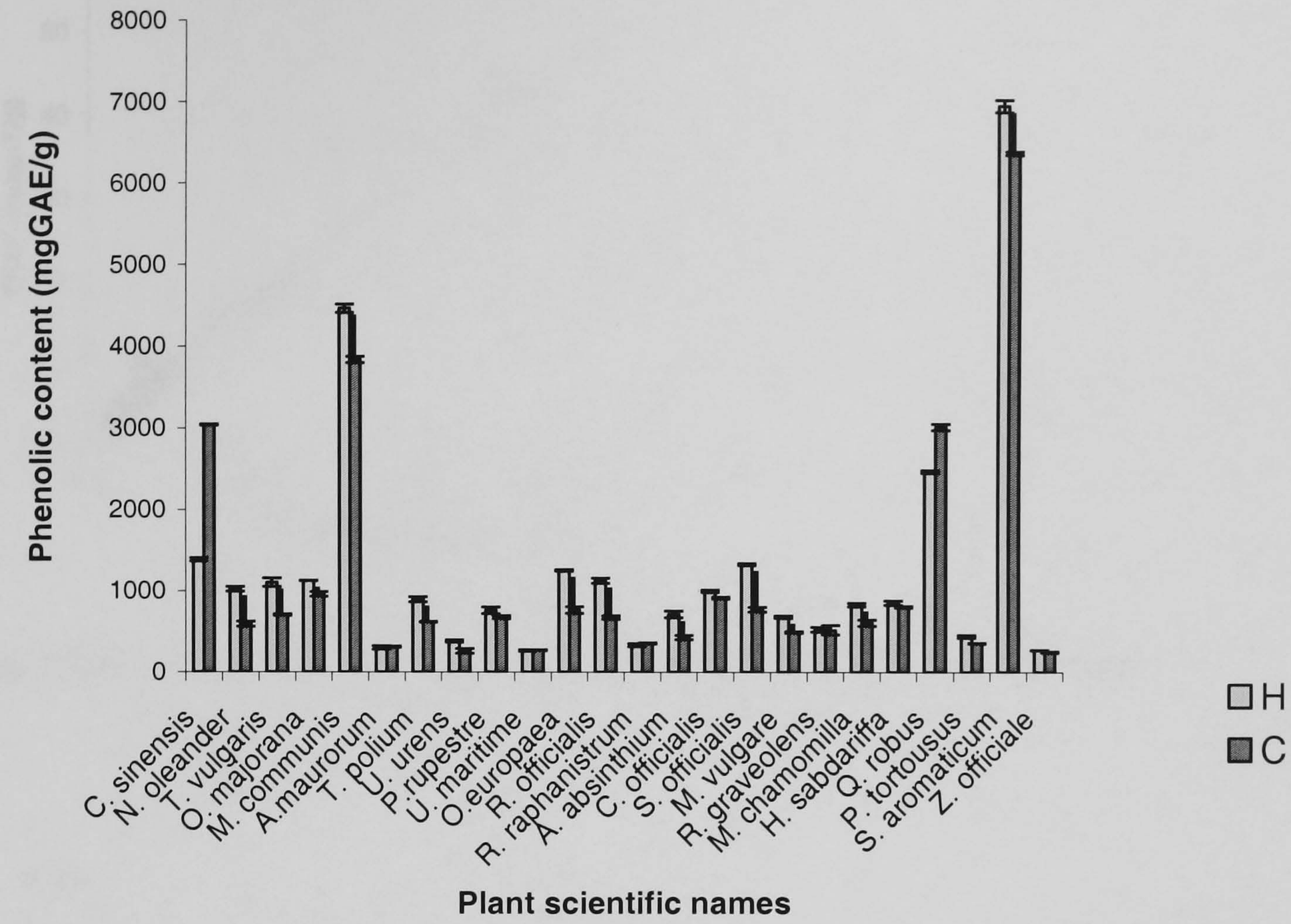


Figure 2.8 Phenolic content of hot and cold water extracts

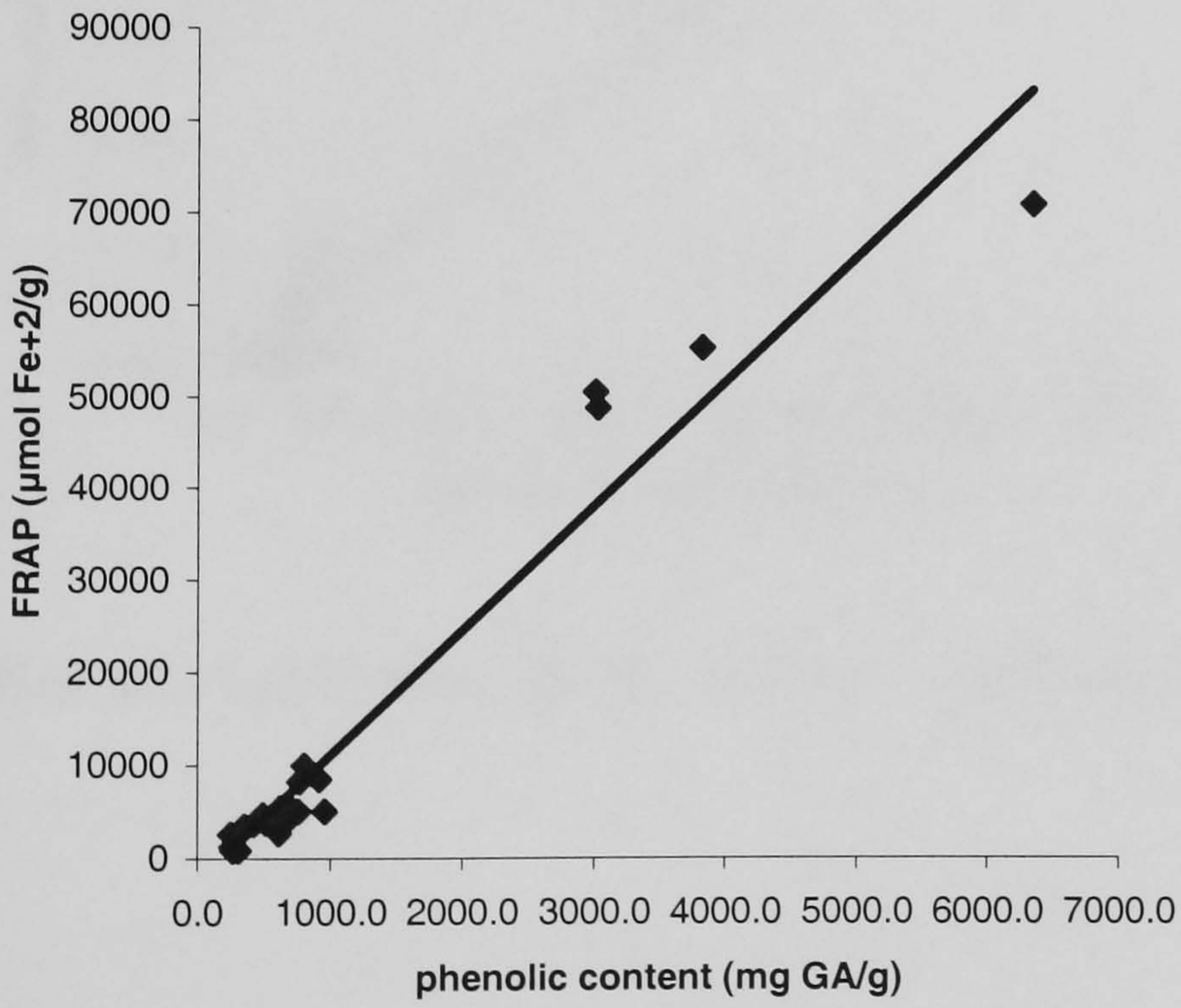


Figure 2.9 Correlation between phenolic content and FRAP for cold extracts.

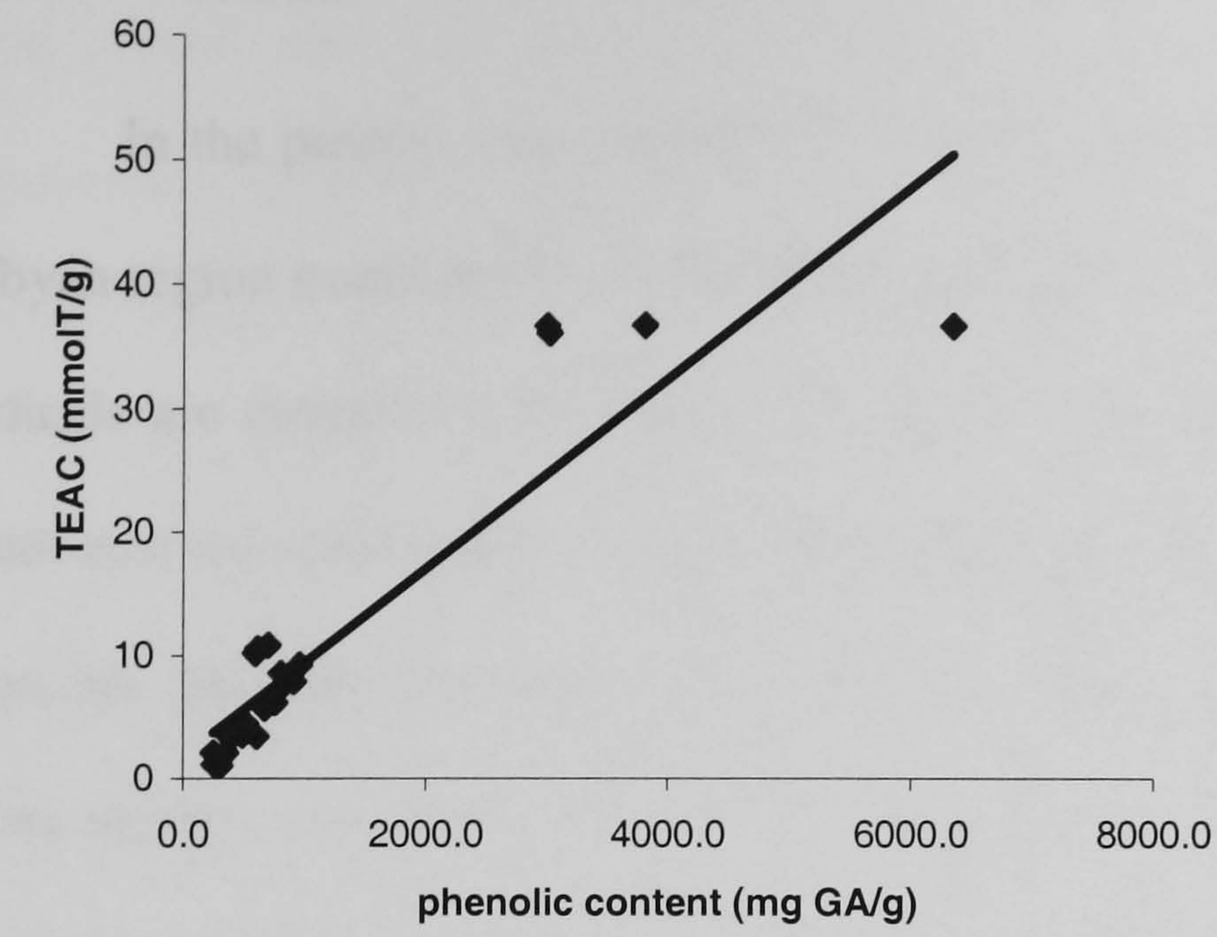


Figure 2.10 Correlation between phenolic content and TEAC for cold extracts

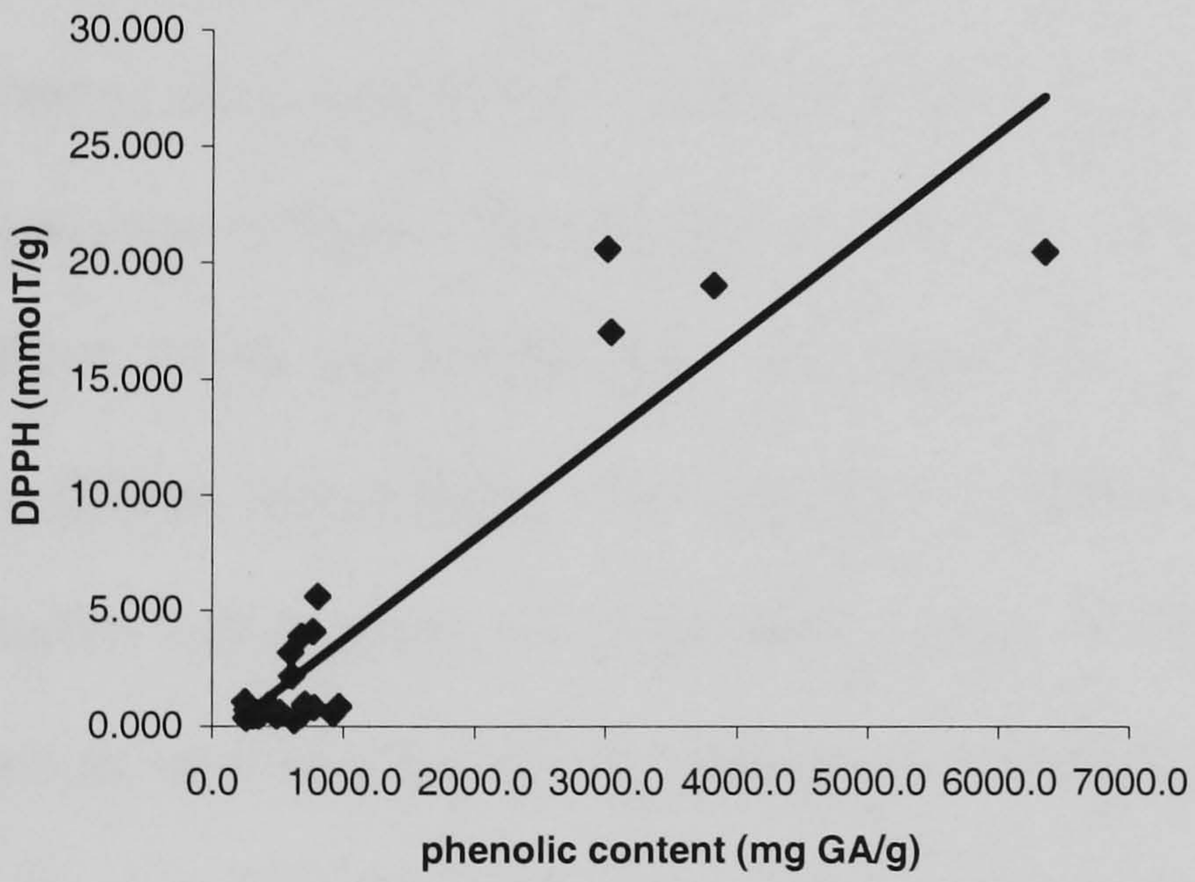


Figure 2.11 Correlation between phenolic content and DPPH for cold extracts.

2.4 Discussion

In the present study twenty three medicinal plants were chosen native to the Libyan region traditionally used for the treatment of various disorders, where the free radicals are thought to be implicated. Based on the traditional way of ingestion of plant derived antioxidants it was appropriate to study the water-soluble antioxidant capacity and phenolic content of herbal teas prepared from Libyan medicinal plants. Plant extracts with either hot (freshly boiled water) or cold (room temperature water) were prepared simply in ways that mimic the traditionally used methods in folk medicine (Triantaphyllou et al., 2001). The extracts from these plants were screened for their antioxidant activity using the three most popular methods (FRAP, TEAC and DPPH). The results (Table 2.1, 2.2, 2.3, 2.4) show that out of these twenty three extracts, the crude extracts (hot and cold) of *M. communis, Q. robur* and *S. aromaticum* exhibited the strongest antioxidant activity, in all tests used in this study (FRAP, TEAC and DPPH), and were higher than that of the positive control, green tea. Indeed, this suggests that for these extracts any of these methods will offer a reliable measurement of antioxidant status. Similar activities of *M. communis* extracts have been previously assessed by the linoleic acid assay (Rosa et al., 2003, Souri et al., 2004), the β-carotene/ linoleic acid system and the DPPH free radical scavenging assay (Yadegarinia et al., 2006, Hayder et al., 2004). The results presented here were consistent with these reports. To the best of the knowledge of the author, no previous reports have dealt with *M. communis* antioxidant activity by FRAP and TEAC assays. The results obtained from *S. aromaticum* extracts in this study exhibited a high antioxidant activity which confirmed the existing literature using different antioxidant assays. *S. aromaticum* demonstrated a good degree of antioxidant capacity tested using the thiobarbituric acid reactive substance (TBARS)

and the β-carotene agar assays (Lean and Mohamed, 1999, Dorman et al., 2000), the lipid / MA and aldehyde/ carboxylic assays (Lee, 2001); reductive potential, superoxide anion radical scavenging (Gulcin et al., 2004), TEAC (Juliani et al., 2004) and DPPH free radical scavenging (Owen and Johns, 2002, Gulcin et al., 2004, Nassar, 2006). No data have been found in the literature for the FRAP assay. Despite few reports in the literature regarding antioxidant activity of *Q. robur* bark (Andrensek et al., 2004), only one recent report has dealt with *Q. robur* fruit as a convenient nutritional source with antioxidant effects (Rakic et al., 2006).

The results show that, in the experimental conditions described, most of the extracts possess moderate antioxidant capacity (especially the cold extracts) (figures 2.5, 2.6, and 2.7). Their behaviour differed according to the type of substrate in the assay and the temperature used for extraction. Accordingly, the differences in the activity of various extracts (the cold and the hot) can be explained by the loss of certain phytoconstituents (mainly polyphenols), probably because of the difference in temperature of the extraction procedures (Velioglu et al., 1998, Gazzani et al., 1998, Sun and Ho, 2005). In contrast, Zhou et al. (2000) and Katalinic et al. (2006) have suggested that a higher temperature promotes extraction of polyphenols and consequently free radical scavenging ability. On the other hand, at a higher temperature certain amounts of polyphenols may react with other components to produce an insoluble complex or may be oxidized (Vijayakumari et al., 1995). Preparation of plant inffusions with hot (100°C) and cold (25°C) water revealed that although antioxidants were liberated from leaves into the water at both the temperatures studied, infusions of hot water had higher antioxidant capacities as determined with FRAP. This notion was proposed when *Melissae folium* inffusions

were tested for their antioxidant capacity at different effusion temperatures by FRAP, TEAC and DPPH free radical scavenging (Katalinic et al., 2006).

In this study, there was good agreement between the antioxidant activity (especially for FRAP assay) and the extraction method used in folklore for most plant species. For instance, *N. oleander, M. chamomilla, T. vulgaris, S. officinalis* and *O. europaea* were suggested to be used as a hot infusion only in the traditional uses (El Gadi, 1992). Our results showed a higher antioxidant activity for those plants as hot extracts. Similarly, for those suggested to be used as cold infusions, such as *Q. robur* and *S. aromaticum*, the highest activities have been found in the cold extracts.

Plant phenolics comprise one of the major groups of compounds of primary antioxidant or free radical terminators (Gao et al., 2000). In addition, numerous studies have suggested that the potent antioxidant activities of green tea (Rechner et al., 2002, Campanella et al., 2003) are due largely to its polyphenolic content. There are no publications on phenolic content and related antioxidant properties of the medicinal plants traditionally used in Libya; therefore, it was reasonable to determine their total phenolic content in the selected plant extracts. Phenolic content for these plant extracts were assayed by the Folin- Ciocalteu (F-C) method (Duan et al., 2006). The F-C method is actually not an antioxidant test but instead an assay for the quantity of oxidizable substances, i.e. phenolic compounds (Wangensteen et al., 2004). Again, *M. communis, Q. robur* and *S. aromaticum* showed the highest phenolic content among the examined extracts (figure 2.8). The report by Romani et al. (2004) has offered quantitative and qualitative details of *M. communis* polyphenol

composition. Interestingly, they found that *M. communis* had the same main polyphenols that exist in green tea, such as galloyl derivatives, and this explained the high antioxidant capacity of *M. communis*. In addition, Abdel-Wahhab and Aly (2005) have demonstrated that the antioxidant capacity of *S. aromaticum* is due in part to the contribution of aromatic chemicals such as eugenol and eugenol acetate.

The values of FRAP, TEAC and DPPH in the crude extracts (the cold) were highly correlated with the content of total phenolics (figures 2.9, 2.10, 2.11). In the results shown here the higher radical scavenging activity of the polar extracts confirms that the phenolic compounds are likely to contribute to the radical scavenging activity of these plants. According to Tepe et al. (2004) the presence of polar phenolics in the extracts is considered as an important factor in the free radical scavenging activity. These results from this experiment are in agreement with the literature (Gao et al., 2000, Arredondo et al., 2004, Tsai et al., 2002, Tepe et al., 2004, Katalinic et al., 2006, Duan et al., 2006, Sun and Ho, 2005), which found a good correlation between antioxidant capacity (by FRAP, TEAC and DPPH) and phenolic contents. Furthermore, the correlation between total phenolic and ORAC values was previously reported by Velioglu et al (1998). In contrast, the previous report by Kahkonen et al. (1999) and Velioglu et al. (1998) did not show any correlation between total phenolic content and oxidation with the Mello assay. The difference between the results shown in this study and reports in the literatures could be explained by the difference in the procedure of the assays used. There are many investigations of antioxidant activity of medicinal plant extracts (Mantle et al., 1998, Mantle et al., 2000b, Souri et al., 2004, Chanwitheesuk et al., 2005, Ivanova et al., 2005, Katalinic et al., 2006, Bouzouta et al., 2003, Navarro et al., 2003, Yingming et

al., 2004, Tepe et al., 2005b, Tepe et al., 2005a, Tepe et al., 2006, Kaur and Kapoor, 2002, Dapkevicius et al., 1998, Miliauskas et al., 2004a, Miliauskas et al., 2004b). However, it is difficult to compare the results of these studies unless they are based on the same assay and extraction procedures.

Antioxidant capacity estimation is assay dependent. The specificity and sensitivity of one method does not lead to complete examination of all phenolic compounds and antioxidants in the extracts. Therefore, a combination of several methods *in vitro* could provide a more reliable assessment of antioxidant activity. The FRAP assay is a method of antioxidant activity evaluation based on redox-reactions. It is quick and simple to perform and it is a reasonable screen for the ability to maintain redox status in cells or tissues. Reducing power appears to be related to the degree of hydroxylation and extent of conjugation in polyphenols (Zaporozhets et al., 2004). The FRAP mechanism measures electron transfer. However, it cannot detect compounds that act by radical quenching (hydrogen transfer), such as thiols and proteins (Prior et al., 2005). Thus it is helpful to use FRAP in combination with other methods. The TEAC assay gained popularity because it enables high-throughput screening on potential antioxidant capacity (Van den Berg et al., 1999, Re et al., 1999). This assay assesses the total radical scavenging capacity, based on the ability of a compound to scavenge the stable ABTS radical (Arts et al., 2004a, Arts et al., 2003, Arts et al., 2004b). The extracts that showed high TEAC values could contain substances that have a redox potential lower than that of $ABTS^{\cdot}$. Indeed, many phenolic compounds have low redox potential and can thus react with $ABTS^{\cdot}$ (Frankel et al 2000; Prior et al 2005). The $DPPH^{\cdot}$ system is a stable radical generating procedure. It can be used to assay a large

number of samples in a short period of time, and is sensitive enough to detect active compounds even at low concentrations and was used in the present study. In the DPPH· assay hydrogen –donating ability is an index of the primary chain-breaking antioxidant. These antioxidants donate hydrogen to free radicals which are then converted to non-radical species and thus inhibit the propagation phase of lipid peroxidation (Koleva et al., 2002, Apati et al., 2003). Consequently, the extracts that showed strong or remarkable activity against DPPH· free radical scavenging could have substances rich in available hydroxyl groups such as flavonoids.

The results of the present work suggested that the efficacy of these plants could be explained, at least in part, by their antioxidant activity. These plants, rich in polar phenols, could be a good source of compounds that would help to increase overall antioxidant capacity of an organism and protect it against lipid peroxidation induced by oxidative stress.

Chapter 3 Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Libyan medicinal plants.

3.1 Introduction

Cholinesterases (ChE) are members of the serine hydrolase family, so named because they utilize a serine at the active site. Acetylcholinesterase (AChE) is distinguished from butyrylcholinesterase (BuChE) by its greater specificity for hydrolyzing acetylcholine (ACh), the cationic neurotransmitter (Quinn, 1987, Giacobini, 2004). ACh is involved in the signal transfer in the synapses. The ACh molecules are released from the presynaptic nerve in response to an action potential. They diffuse across the synapse and bind to the ACh receptor, which among other functions serves an ion gate for the entry of K^+ into the postsynapse (Pope et al., 2005). Under normal conditions, AChE rapidly and efficiently hydrolyzes ACh to acetic acid and choline. Extensive inhibition of this enzyme leads to accumulation of the neurotransmitter ACh and enhanced stimulation of postsynaptic cholinergic receptors. When the liberated ACh accumulates, they prevent the smooth transmission of nerve impulses across the synaptic gap at nerve junctions (Quinn, 1987, Pope et al., 2005), and this causes loss of coordination, convulsion and ultimately death. Therefore, AChE is a key component of cholinergic brain synapses and neuromuscular junctions (Pope et al., 2005). Because of the essential role that AChE plays in the nervous system, this enzyme has long been an attractive target for the rational design of mechanism-based inhibitors (Giacobini, 2004).

Most uses of ChE inhibitors are based on a common mechanism of action initiated by inhibition of AChE. This action is beneficial in cases where a reduction in cholinergic transmission contributes to clinical symptoms, for instance, low muscle tone in the autoimmune disorder myasthenia gravis due to loss of nicotinic receptors (Pope et al., 2005, Taylor, 2001). Alzheimer's disease (AD) is the most common cause of dementia in the elderly population; it is a chronic, slowly progressive neurodegenerative disorder. The gradual loss of memory, decline in other cognitive functions and decrease in functional capacity results in death approximately 10 years after the onset of symptoms. In addition, AD is neuropathologically associated with a cholinergic deficiency. Therefore, ACh replacement therapy has been used as a potential strategy to improve AD pathology, mainly by inhibiting AChE (Ezoulin et al., 2005, Ferreira et al., 2006, Howes and Houghton, 2003). ChE inhibitors are the only class of compounds to date that have consistently proven to be efficacious in treating the cognitive and functional symptoms of AD (Fawcett et al., 2002, Schliebs et al., 1997, McQueen, 1995, Nagabukuro et al., 2004). It has been suggested that the inhibition of AChE and BuChE enzymes should be one of the objectives in the treatment of cognitive dysfunctions associated with AD (Giacobini, 2004, Okello et al., 2004). On this basis, the *cholinergic hypothesis* became the leading strategy for the development of drugs for the treatment of AD, even if today other approaches are also being followed in the search for agents able to treat and/or prevent the disease (Pope et al., 2005, Giacobini, 2004, Furey et al., 2000). The medications that have been approved for the symptomatic treatment of mild to moderate AD are tacrine (an aminoacridine), donepezil (a benzylpiperidine), rivastigmine (a carbamate) and galantamine (a tertiary alkaloid). Tacrine was the first AChE inhibitor to be launched

as a treatment for AD but, although there was evidence of beneficial effects on AD symptoms, the drug also caused showed several adverse side effects such as nausea and liver damage which in some cases made the usage of these drugs a serious problem (Orhan et al., 2004, Rampa et al., 2000, Knapp et al., 1994, Hu et al., 2005). Several plant species contain alkaloid cholinesterase inhibitors. For example, galantamine, an alkaloid from snowdrop (*Galanthus nivalis*) was recently approved for use in AD therapy (Howes and Houghton, 2003, Svedberg et al., 2004).

Although plant alkaloids are best known for inhibiting cholinesterase enzymes, recent reports indicate new classes of cholinesterase-inhibiting phytochemicals such as terpenoids, especially monoterpenes (Perry et al., 2000, Perry et al., 2003, Greenberg-Levy et al., 1993) and coumarins (Howes and Houghton, 2003). The monoterpenes are amongst the most widespread and chemically interesting groups of natural products found in plants. They have been known for hundreds of years as components of the fragrant oils which can be obtained from leaves, flowers and fruits of many plants (Newman, 1972). The major commercially-obtained monoterpenes are able to inhibit human, bovine and electric eel AChEs (Savelev et al., 2003, Miyazawa et al., 2001, Miyazawa et al., 1998, Perry et al., 2000, Perry et al., 2002).

In the last few years, much research has been carried out to evaluate the effectiveness and safety of the use of plants or their metabolites for the prevention or treatment of diseases. Consequently, screening of plant extracts represents a continuous effort to find new bioactive molecules or extracts and approximately 20% of the plants in the world or their extracts have been submitted to pharmacological or

biological tests. Several reports have been published on the biological effects of plants traditionally used either in infusion or in traditional remedies as cholinesterase inhibitors *in vitro* (Nagabukuro et al., 2004, Choudhary et al., 2005b, Perry et al., 2000, Perry et al., 2002, Perry et al., 2003, Choudhary et al., 2005c, Zaheer ul et al., 2003, Choudhary et al., 2005a, Okello et al., 2004). However, new naturally occurring anticholinesterases continue to be identified in a wide variety of medicinal plants from different countries, such as Turkey (Orhan et al., 2004, Tasdemir et al., 2004); Portugal (Ferreira et al., 2006), New Zealand (Kellam et al., 1992), South Africa (Eldeen et al., 2005), Korea (Oh et al., 2004); China and India (Howes and Houghton, 2003). A recent study has reported that the extracts of *Hypericum undulatum*, *Melissa officinalis*, *Laurus nobilis* and *Lavandula pedunculata* showed high values for antioxidant and anticholinesterase activities (Ferreira et al., 2006).

The main goal of the current study is to screen some recorded and unrecorded Libyan medicinal plants, particularly those which have different antioxidant activities (high to moderate or low), for possible anticholinesterase activity. The aim is also to introduce new plant sources whose biological activities as potential anticholinesteases have not been previously documented. Thus in the study reported in this chapter, the plant species *Camellia sinensis; Myrtus communis; Alhagi maurorum; Urginea maritima; Olea europaea; Matricaria chamomilla; Hibiscus sabdariffa; Quercus robur; Syzygium aromaticum;* and *Zingiber officinale*, which have various ethnobotanical uses, were evaluated for their anticholinesterase activities.

3.2 Materials and methods

3.2.1 Plant materials:

3.2.1.1 Selection of the Plants

The medicinal plant species used in this experiment were selected according to the results of the previous experiments reported in Chapter 2. According to antioxidant activity of the plants, they were divided into three categories: group (a), high antioxidant activity; group (b), moderate antioxidant activity and group (c), low antioxidant activity. Four plant species from group (a) *Camellia sinensis; Myrtus communis*; *Quercus robur* and *Syzygium aromaticum*, three from group (b) *Olea europaea; Matricaria chamomilla* and *Hibiscus sabdariffa* and three from group (c) *Alhagi maurorum; Urginea maritima; Zingiber officinale* were selected for this experiment. Details of these plants are presented in Table 1.5 (see Chapter 1)

3.2.1.2 Plant extract:

Plant extracts prepared with either hot or cold water (hot means freshly boiled water and cold means room temperature water) was prepared in ways that mimic the traditionally used methods in folk Libyan medicine as follows:

3.2.1.2.1 Hot water extract:

Four grams of each dried powdered plant were soaked in freshly boiled de-ionized water (1:5 w/v) as described in section (2.2.1.2.1).

3.2.1.2.2 Cold water extract:

Four grams of each dried powdered plant was soaked with de-ionized water (1:5 w/v) as described in section (2.2.1.2.2).

3.2.2 Chemicals:

Acetylthiocholine iodide (ATChI), butyrylthiocholine iodide (BuTChI), 5,5′-dithiobisnitrobenzoic acid (DTNB), butyrylcholinesterase (BuChE, E. C.3.1.18, from human serum), acetylcholinesterase (AChE, E. C.3.1.1.7, from human erythrocytes) and sodium bicarbonate were purchased from Sigma Chemical Company, UK. Buffers and other chemicals were all of analytical grade.

3.2.3 Anticholinesterase Assay:

Anticholinesterase inhibition was determined spectrophotometrically using the method described by Ellman (Ellman et al., 1961). The principle of this method is the measurement of the rate of production of the yellow coloured anion of 5-thio-2-thionitrobenzoic acid (TNB). The assay involves two linked reactions which produce the coloured compound (TNB):

1- ATChI is hydrolysed enzymatically by acetylcholinesterase to give acetic acid and thiocholine.

2- Thiocholine reacts with DTNB to produce TNB (yellow colour).

The production of TNB is monitored by measuring the absorbance of light by the reaction mixture (maximum at 412nm) over the time. Any inhibition of AchE would result in reduction of hydrolysis by the enzyme and that would reduce the formation of the yellow anion.

The percentage of inhibition can be calculated as:

$$\text{Inhibition (I \%)} = \frac{(a - b) - (c - d)}{(a - b)} \times 100$$

Where:

a = Buffer + substrate + enzyme + DTNB + de-ionised water (control)

b = Buffer + substrate + DTNB + de-ionised water (blank)

c = Buffer + substrate + enzyme + DTNB + sample (inhibitor)

d = Buffer + substrate + DTNB + sample (colour control).

In this experiment, a typical assay consisted of 5µl of AchE solution at a final assay concentration of 0.03 U/mL or 5 µl of BuchE solution at a final assay concentration of 0.02 U/mL; 200 µl of 0.1 M sodium phosphate buffer (PB) pH 8.0; 5 µl of DTNB at a final concentration of 0.3 mM prepared in 0.1 M PB pH 7.0 with 0.12 M sodium bicarbonate and 5 µl of sample solution. The reactants were pre-incubated at 30°C for 15 minutes (for AChE) and 10 minutes (for BuChE) in a 96-well, flat bottom polystyrene microlitre plate. The reaction was initiated by adding 5µl of ATChI or BuTChI at a final assay concentration of 0.5mM. A blank for each assay was prepared by replacing the 5µl of enzyme with PB (pH 8) and 5 µl of the sample with de-ionised water. As a control 5 µl of the sample was replaced with de-ionised water. A colour control was assayed by replacing the 5µl of enzyme with PB (pH 8). Each sample was assayed in triplicate. For the assay absorbance was measured at 405nm in a Titertek Multiskan MCC/340 microplate reader for a period

of 6 minutes at 30°C with Genesis-Lite Windows microplate software (Labsystem International).

3.2.4 Dose-response curves and equations:

Each plant extract was tested over a range of concentrations. Each concentration was tested with triplicate extracts then by using Microsoft Excel software, straight line dose-response curves were fitted to the data points. The concentration of the plant extracts which gave 50% inhibition of the enzymes was expressed as an IC_{50} value. The activity of extracts which did not achieve 50% inhibition was expressed as the measured percent inhibition.

3.3 Results

Results for the hot and the cold water extracts and their activities against AChE enzyme are presented in Table 3.1. An example of a dose response curve for the hot and the cold *Camellia sinensis* extracts against the AChE is shown in Figure 3.1. There was a good agreement between the results obtained from the hot and the cold extracts against the AChE enzyme. Table 3.2 shows the results of hot and cold water extracts and their inhibitory activity toward BuChE. For the majority of plants, the hot water extracts exhibited higher activity against this enzyme than cold water extracts. However, the cold water extracts of *M. chamomilla* and *Q. robur* showed higher anti- BuChE activity.

Most the plant extracts analyzed exhibited various inhibitory activities toward both enzymes. According to their activities, plant extracts were divided into four categories (Table 3.3). The first category includes the plants that did not show any inhibitory activities. The second category includes the plants that showed low activity (1- 25% inhibition); the third category contains the plants that exhibited moderate activity (25-50% inhibition) and the last category includes the plants which possessed high activity (50-100% inhibition).

Some of the plant extracts such as *Olea europaea* which had no inhibitory activity against AChE (Table 3.1), exhibited low (22.63 ± 1.2 % at 0.12mg/ml in cold water extracts) and moderate (34.79 ± 0.38 % at 0.15mg/ml in hot water extracts) activity against BuChE (Table 3.2). Similarly, the extracts of *Quercus robur* which had lower activity against AChE showed much higher activity against BuChE

for both hot and cold water extracts. In contrast, the extracts of *Urginea maritima* (both in hot and cold water extracts) showed high activity (74.1 ± 0.77 %; 79.8 ± 0.50 %) toward AChE. Both extracts had no noticeable activity against BuChE. On the other hand, while *Matricaria chamomilla* extracts appeared to possess no activity against AChE enzyme (Table 3.1), only the cold water extract displayed a noticeable inhibition with IC_{50} value of 0.109 ± 0.001 mg/ml against BuChE (Table 3.2). The lowest levels of activity were obtained from *Zingiber officinale, Hibiscus sabdariffa* and *Alhagi maurorum* extracts which showed negligible or no inhibitory activity against either enzyme (Table 3.3). The cold extract of *Myrtus communis* showed moderate activity against both enzymes, whereas the hot extracts exhibited good anti- BuChE activity with IC50 value of 0.148 ± 0.003mg/ml, and moderate (44.63 ± 2 %) anti- AChE activity at a final concentration of 0.15mg/ml. Among the ten Libyan plants analysed, the green tea (*Camellia sinensis*) and clove (*Syzygium aromaticum*) were the most potent inhibitors against both AChE and BuChE enzymes.

Table 3.1. Inhibition of Human AChE enzyme by selected Libyan medicinal plants.

Plant Scientific Name	Hot Water Extracts	Cold Water Extracts
Camellia sinensis	0.068 ± 0.001 [a]	0.070 ± 0.003 [a]
Myrtus communis	0.15 (44.63 ± 2) [b]	1.31 (40.13 ± 0.90)[b]
Alhagi maurorum	0.172 [c]	0.173 [c]
Urginea maritima	0.200 ± 0.005 [a]	0.114 ± 0.002 [a]
Olea europaea	0.148 [c]	0.118 [c]
Matricaria chamomilla	0.165 [c]	0.178 [c]
Hibiscus sabdariffa	0.279 [c]	0.295 [c]
Quercus robur	0.12 (15.80 ±0.97)[b]	0.11 (34.37 ± 0.92)[b]
Syzygium aromaticum	0.075 ± 0.003 [a]	0.088 ± 0.001 [a]
Zingiber officinale	0.207 [c]	0.110 [c]

Data expressed as mean ± SD (n = 3)

a = IC_{50} value (mg/ml) (final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations).

b = Concentration (mg/ml) providing this percentage inhibition (Inhibition %, inhibitory activity of extracts which did not reach 50% enzyme inhibition).

c = No Inhibition at this concentration (mg/ml).

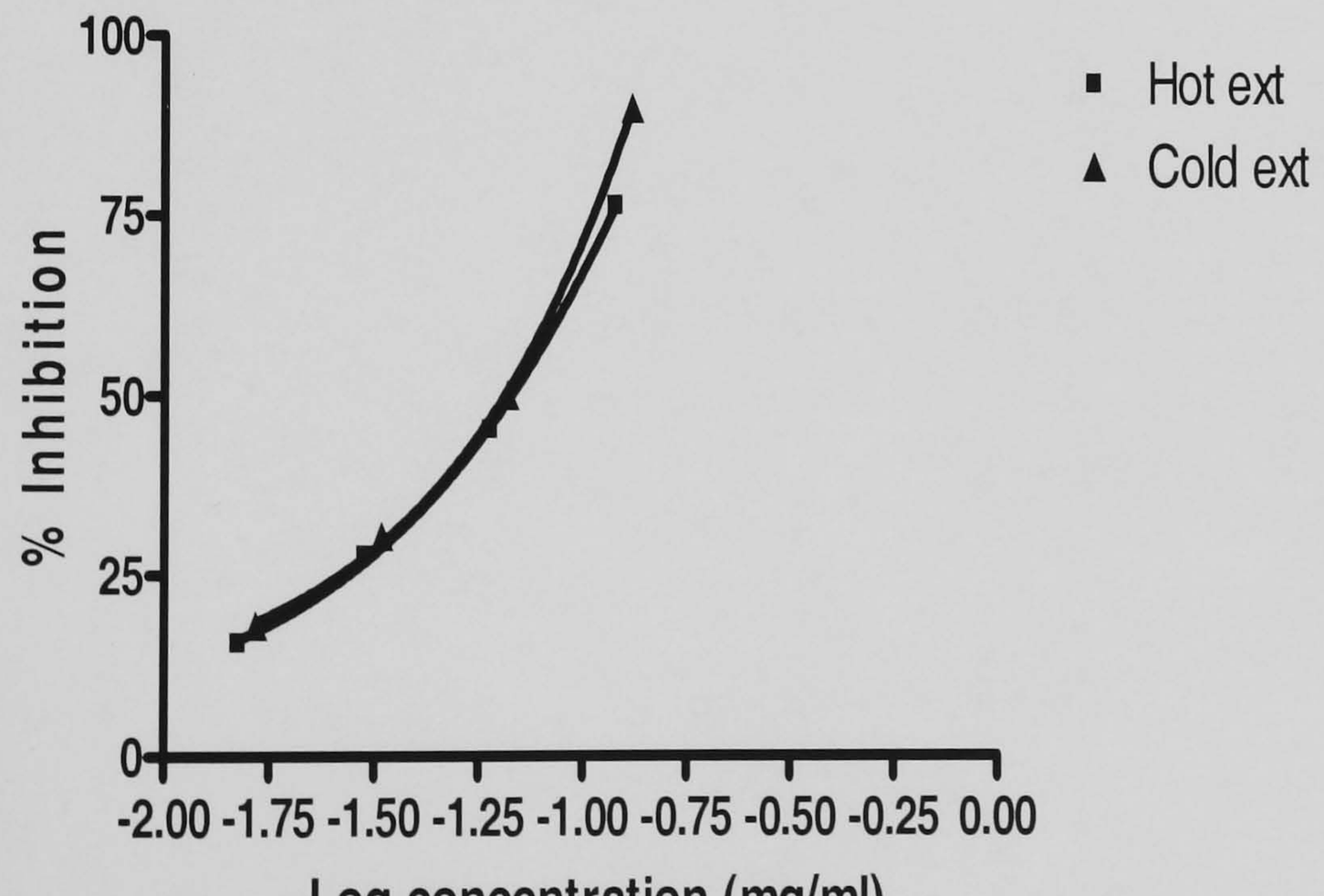


Figure 3.1 Dose response curve for the hot and the cold *Camellia sinensis* extracts against AChE.
The graph was produced using Graphpad Prism to show the sigmoid pattern where concentrations are plotted on a logarithmic scale.

Table 3.2. Inhibition of Human BuChE enzyme by selected Libyan medicinal plants.

Plant Scientific Name	Hot Water Extracts	Cold Water Extracts
Camellia sinensis	0.066 ± 0.004^{a}	$0.12\ (7.42 \pm 0.46)^{b}$
Myrtus communis	0.148 ± 0.003^{a}	$0.13\ (38.74 \pm 1.12)^{b}$
Alhagi maurorum	0.172^{c}	$0.17\ (2.89 \pm 0.21)^{b}$
Urginea maritima	$0.33\ (4.99 \pm 0.68)^{b}$	0.199^{c}
Olea europaea	$0.15\ (34.79 \pm 0.38)^{b}$	$0.12\ (22.63 \pm 1.2)^{b}$
Matricaria chamomilla	0.165^{c}	0.109 ± 0.001^{a}
Hibiscus sabdariffa	0.279^{c}	$0.29\ (21.68 \pm 0.8)^{b}$
Quercus robur	0.066 ± 0.002^{a}	0.055 ± 0.002^{a}
Syzygium aromaticum	0.116 ± 0.007^{a}	$0.15\ (44.7 \pm 0.72)^{b}$
Zingiber officinale	0.207^{c}	0.110^{c}

Data expressed as mean ± SD, (n = 3)

a = IC_{50} value (mg/ml) (final concentration of inhibitors required for 50% enzyme inhibition, as calculated from the dose response-curve equations).

b = Concentration (mg/ml) providing this percentage inhibition (Inhibition %, inhibitory activity of extracts which did not reach 50% enzyme inhibition).

c = No Inhibition at this concentration (mg/ml).

Table 3.3 Plant extracts categories.

Plant Scientific Name	Hot Water Extracts		Cold Water Extracts	
	AChE	BuChE	AChE	BuChE
Camellia sinensis	+++	+++	+++	+
Myrtus communis	++	+++	++	++
Alhagi maurorum	-	-	-	+
Urginea maritima	+++	+	+++	-
Olea europaea	-	++	-	+
Matricaria chamomilla	-	-	-	+++
Hibiscus sabdariffa	-	-	-	+
Quercus robur	++	+++	+	+++
Syzygium aromaticum	+++	+++	+++	++
Zingiber officinale	-	-	-	-

- = no inhibition.

+ = low activity (1-25% inhibition).

++ = moderate activity (25-50% inhibition).

+++ = good activity (50-100% inhibition).

3.4 Discussion

Several studies have suggested that inhibition of cholinesterase is a promising approach for the treatment of Alzheimer's disease (AD) and for possible therapeutic applications in the treatment of Parkinson's disease, ageing, and myasthenia gravis (Atta-ur-Rahman et al., 2001). In all cases, the therapeutic strategy is to increase the persistence of synaptic ACh by blocking its degradation, such that there is a net increase in cholinergic receptor activation. This overall strategy is based on a clinical condition wherein activation of cholinergic receptors is deficient. Thus, increasing the residence of acetylcholine molecules within synapses by inhibiting AChE can at least partially counteract a deficiency in either the release of neurotransmitter or a reduction in cholinergic receptors/signalling. Traditional medicines have been widely used for a long time, and are still a vital source for the discovery of novel bio-active compounds. There is some evidence suggesting that butyrylcholinesterase activity may be involved in the pathogenesis of Alzheimer's disease. This has led to the hypothesis that the use of non-selective cholinesterase inhibitors that inhibit both butyrylcholinesterase and acetylcholinesterase may be more beneficial to patients with Alzheimer's disease than the use of selective cholinesterase inhibitors that inhibit acetylcholinesterase alone (Liston et al., 2004, Giacobini, 2004). In these experiments, the plant species of *Camellia sinensis; Myrtus communis; Alhagi maurorum; Urginea maritima; Olea europaea; Matricaria chamomilla; Hibiscus sabdariffa; Quercus robur; Syzygium aromaticum;* and *Zingiber officinale*, which have various ethnobotanical uses, were examied for their anticholinesterase (AChE and BuChE) activities. The plant extracts used in this study, either hot (freshly boiled water) or cold (room temperature water), were prepared simply in ways that were

similar to the traditionally used methods in folk medicine (Triantaphyllou, et. al., 2001).

Tea belongs to the Theaceae family and is the most widely consumed beverage in the world next to water alone. Recent studies have shown that green tea possesses pharmacologically protective and biologically active properties such as anticarcinogenic (Borek, 2005), antioxidative and neuroprotective (Katalinic et al., 2006, Beecher et al., 1999, Benzie and Szeto, 1999) activities. In this study, the IC_{50} values for hot extracts of green tea (*Camellia sinensis*) (Table3.1) were 0.068 ± 0.001mg/ml and 0.066 ± 0.004mg/ml. These results were higher than those of Okello *et al* (2004), who also found that green tea possesses potent inhibitory activities against AChE and BuChE enzymes with IC50 values of 0.05 ± 0.005 mg/ml and 0.05 ± 0.007 mg/ml, respectively. One chemical constituent which may cause this anticholinesterase activity in green tea has been suggested to be thymol (Duke, 1992). Similar anticholinesterase activities were obtained from the hot extracts of *S. aromaticum* with IC_{50} of 0.075 ± 0.003 mg/ml and 0.116 ± 0.007 mg/ml toward the AChE and BuChE enzymes respectively. Although it was reported that *S. aromaticum*, among the clove species, has been successfully used to treat asthma as an antimicrobial against oral bacteria and various allergic disorders by oral administration (Gulcin et al., 2004), to the best of the knowledge of the author, no previous reports have dealt with *S. aromaticum* anticholinesterase activity. The low activity of the cold water extracts of green tea and clove against the BuChE enzyme could be due to the nature of the component which is responsible for this activity and the difference in temperature during the extraction procedures where the active component is not soluble until higher temperatures. The high and moderate activities

of extracts of *M. communis* could be explained by the presence of 1, 8-cineole (Duke, 1992), a monoterpene, that previously has been proven to be a cholinesterase inhibitor (Perry et al., 2000, Savelev et al., 2003, Greenberg-Levy et al., 1993, Perry et al., 2003, Miyazawa et al., 2001, Miyazawa et al., 1998). In this experiment, some extracts such as *M. chamomilla* and *Q. robu,r* which had lower activity against AChE, exhibited much higher activity against BuChE. In contrast, other extracts such as *U. maritime* possess high anti AChE activity and low anti- BuChE activity. These results are consistent with the recent report by Orhan et al. (2004), which also show differential activities for extracts from Turkish plants against the two enzymes. This might be due in part to the difference between the two enzymes genetically, structurally and in their kinetics (Giacobini, 2004) or could be because these plant extracts interact with the enzymes by different mechanisms (Orhan et al., 2004).

Focusing on the possible correlations between antioxidant and anticholinesterase activities, a recent report has pointed out that the extracts of *Hypericum undulatum*, *Melissa officinalis*, *Laurus nobilis* and *Lavandula pedunculata* which showed high values for antioxidant activity also exhibited anticholinesterase activities (Ferreira et al., 2006). Excluding the *U. maritime* extracts, all the plant extracts that have been identified here to possess a noticeable inhibitory activity against either AChE or BuChE or both (excluding the *U. maritime* extracts) were found to have a potent antioxidant activity and presented in group (a) (Table 2.4, chapter 2). All the plant extracts screened herein showing high inhibitory activity (except the green tea) is reported here for the first time as cholinesterase inhibitors. In the light of these findings these plants could be considered for further studies in the treatment of AD.

Chapter 4 *In vitro* enzymatic digestion to predict apparent antioxidant and anticholinesterase release from selected Libyan medicinal plants.

4.1 Introduction

Plants play a major role in the introduction of new therapeutic agents and have received much attention as a source of biologically active substances such as antioxidant and anticholinesterase activities. Recently, there has been an upsurge of interest in the therapeutic potential of traditional medicinal plants as antioxidants in reducing free radicals induced by tissue injury. In addition, several classes of antioxidant dietary compounds have been suggested to present health benefits, and there is much evidence that consumption of these products leads to a reduction of the expression of various biomarkers of pro-inflammatory and /or oxidative stress (Silva et al., 2005). The active agents in these vegetative extracts are principally water-soluble or lipophilic antioxidant molecules. Indeed, most of these plant extracts contain various amounts of vitamin E, vitamin C, β-carotene, and polyphenols including flavonoids (Zin et al., 2006, Chen et al., 2006, Anagnostopoulou et al., 2006). Most phenolic compounds present in vegetables and fruits show beneficial physiological properties, e.g. cardioprotective, anticarcinogenic and anti-inflammatory activities, and, based on their ideal chemical structure for free radical-scavenging activities, they are reported to protect human health against oxidative damage. Phenolic compounds may also act as cardioprotective agents or at least they possess *in vitro* vasorelaxing effects and inhibit platelet aggregation (Amin et al., 2006). In nature these compounds are normally present in plants as polymeric

complexes, which require to be degraded adequately before being absorbed (Serrano et al., 2005, Bourne et al., 1999, Capecka et al., 2005).

Some of these antioxidant compounds can be inactivated and digested as they pass along the gastro-intestinal tract thus destroying the antioxidant and pharmacological properties of these molecules. These compounds may be degraded in the gastrointestinal tract and lose their activity even before absorption (Serrano et al., 2005). Nowadays, there is increasing information concerning absorption of phenolics and some of them have been detected in human plasma and other biological fluids (Bourne et al., 1999). Studies performed on rats and humans fed flavonols and catechins have confirmed that they are partly absorbed by the intestine (Martinez-Ortega et al., 2001). Furthermore, catechin was detected in human plasma after the intake of a diet containing fruits and vegetables. In the past, it was assumed that glycosylated forms of flavonoids were not absorbable from the digestive tract. In a human study with ileostomy subjects, however, it was found that the absorption of orally-administered quercetin was 24%, while the absorption of quercetin glycosides from onions was 52% (Hollman et al., 1995). In addition, rutin and other glycosides, such as phloretin, have been measured in the plasma of non-supplemented healthy volunteers (Paganga and Rice-Evans, 1997).

Excessive or unregulated enzyme activities are implicated in a wide range of pathological conditions and control of these activities by administration of specific enzyme inhibitors has proven to be a viable means of their control. It has been suggested that the inhibition of AChE and BuChE enzymes should be one of the

objectives in the treatment of cognitive dysfunctions associated with Alzheimer's disease (AD) (Giacobini, 2004, Okello et al., 2004). On this basis, the *cholinergic hypothesis* became the leading strategy for the development of drugs to treat AD, even if today other approaches are also being followed in the search for agents able to treat and/or prevent the disease (Pope et al., 2005, Giacobini, 2004, Furey et al., 2000). Although plant alkaloids are best known for inhibiting cholinesterase enzymes, recent reports indicate new classes of cholinesterase-inhibiting phytochemicals such as terpenoids, especially monoterpenes (Perry et al., 2000, Perry et al., 2003, Greenberg-Levy et al., 1993) and coumarins (Howes and Houghton, 2003).

There is little information concerning the extent to which the antioxidant and the anticholinesterase compounds are absorbed in humans, their metabolism, pharmaco-kinetics and bioavailability. Although a small number of reports have provided evidence for uptake of specific flavonoids in rats (Ames et al., 1993, Verma et al., 1988), few studies have established flavonoid uptake in humans *in vivo*, and no publications regarding the digestion of antioxidant and anticholinesterase compounds from natural resources were found in the literature. Consequently, there is a need to elucidate the extent to which these compounds are absorbed and whether they are degraded or not in the intestinal tract (Serrano et al., 2007). Previously, such *in vitro* assays have been applied to compare the bioavailability of minerals from rich dietary sources such as tea (Powell et al., 1998).

Digestion is an initial step involving changes in pH and activity of proteolytic enzymes (Serrano et al., 2007). It would be interesting to evaluate possible changes in antioxidant properties and anticholinesterase activity of these plant extracts during digestion. According to their antioxidant activity, plants were divided into three categories: group (a), high antioxidant; group (b), moderate antioxidant and group (c), low antioxidant (see chapter 2). Four plant species from group (a) *Camellia sinensis; Myrtus communis*; *Quercus robur* and *Syzygium aromaticum*; three from group (b) *Olea europaea; Matricaria chamomilla* and *Hibiscus sabdariffa*, and three from group (c) *Alhagi maurorum; Urginea maritima; Zingiber officinale* were selected for experiments on antioxidant property changes during *in vitro* digestion. Only the plant extracts that showed acceptably high anticholinesterase activities were chosen for *in vitro* anticholinesterase release experiments (see chapter 3). The purpose of the present work was to ascertain if gastrointestinal conditions could affect the antioxidant properties of original plant extracts and evaluate their stability or possible transformation to other compounds during the *in vitro* digestion steps. The objective was to investigate whether *in vitro* bioavailability of these plant extracts could be used as a step prior to the evaluation of plant extracts *in vivo*. The aim was to use gastrointestinal fluids to mimic digestion and to qualify changes in apparent availability of antioxidant components and anticholinesterase active components from water-soluble extracts.

4.2 Materials and methods.

4.2.1 Plant materials:

4.2.1.1 Selection of the Plants

The medicinal plant species used in this experiment were selected according to the results of previous experiments. According to antioxidant activity, plants were divided into three categories: group (a), high antioxidant; group (b), moderate antioxidant and group (c), low antioxidant. Four plant species from group (a) *Camellia sinensis; Myrtus communis*; *Quercus robur* and *Syzygium aromaticum* and three from (b) *Olea europaea; Matricaria chamomilla* and *Hibiscus sabdariffa* and (c) *Alhagi maurorum; Urginea maritima; Zingiber officinale* were selected for the experiments to measure antioxidant properties during *in vitro* digestion. Only the plant extracts that showed acceptable high anticholinesterase activity were chosen for *in vitro* anticholinesterase release experiments. Details of these plants are presented in Table 1.5.

4.2.1.2 Plant extract:

Plant extracts in either hot or cold water (hot means freshly boiled water and cold means room temperature water) were prepared in ways that mimic the traditionally used methods in Folk medicine as follows:

4.2.1.2.1 Hot water extract:

Four grams of each dried powdered plant was soaked in freshly boiled de-ionized water (1:5 w/v) as described in the previous chapter (section 2.2.1.2.1).

4.2.1.2.2 Cold water extract:

Four grams of each dried powdered plant was soaked with de-ionized water (1:5 w/v) as described in the previous chapter (section 2.2.1.2.2).

4.2.2 Chemicals:

Acetylthiocholine iodide (ATChI), butyrylthiocholine iodide (BuTChI), 5,5′-dithiobisnitrobenzoic acid (DTNB), butyrylcholinesterase (BuChE. E.C.3.1.18, from human serum), acetylcholinesterase (AChE, E. C.3.1.1.7, from human erythrocytes), sodium bicarbonate, 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) powder $ABTS^{+}$, potassium persulphate, (+/-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), ferrous sulphate, ferric chloride, 2,4,6 tripyridyl-s-triazine (TPTZ), 1,1′-diphenyl-2-picryl-hydrazyl (DPPH), pancreatin, and pepsin were purchased from Sigma Chemical Company, UK. Buffers and other chemicals were of analytical grade. All other solvents, salts and reagents were obtained from VWR International, Country Durham, UK.

4.2.3 *In vitro* enzymatic procedure

The *in vitro* digestion using sequential enzymatic steps is based on the method described by Aura *et al.* (1999) and recently modified by Nagah and Seal (2005). Briefly, one millilitre (for antioxidant experiments) and three millilitres (for anticholinesterase experiments) of the plant extract were added into a 50 millilitre plastic screw-topped tube containing five glass marbles. 19 ml of water was added only to the tubes used for antioxidant analysis. The tubes were incubated in a shaking water bath set at 37°C and at 120 strokes/ min. After 10 min, 10 ml of 0.05 M HCl

was added to each tube (simulating pH conditions in the stomach). After 20 minutes incubation, one mg pepsin dissolved in 0.5 ml of 0.05 M HCl was added. Then after further 20 min incubation, 1ml of 0.5M NaOH was added (simulating pH conditions in the small bowel). Finally, after 20 min, 5 ml of pancreatin (3 gram of pancreatin dissolved in 20ml distilled water, centrifuged for 10minutes at 1500 × g and then 15 ml of supernatant sufficient for three tubes, removed) was added and incubated for 20 min. Each plant extract was run in triplicate. Aliquots of 1ml were taken from each tube immediately before each addition and stored at -20°C for antioxidant and anticholinesterase analysis. The digestion protocol is shown in figure 4.1. A blank was run in triplicate for each experiment to detect if the reagents themselves have any antioxidant or anticholinesterase activity.

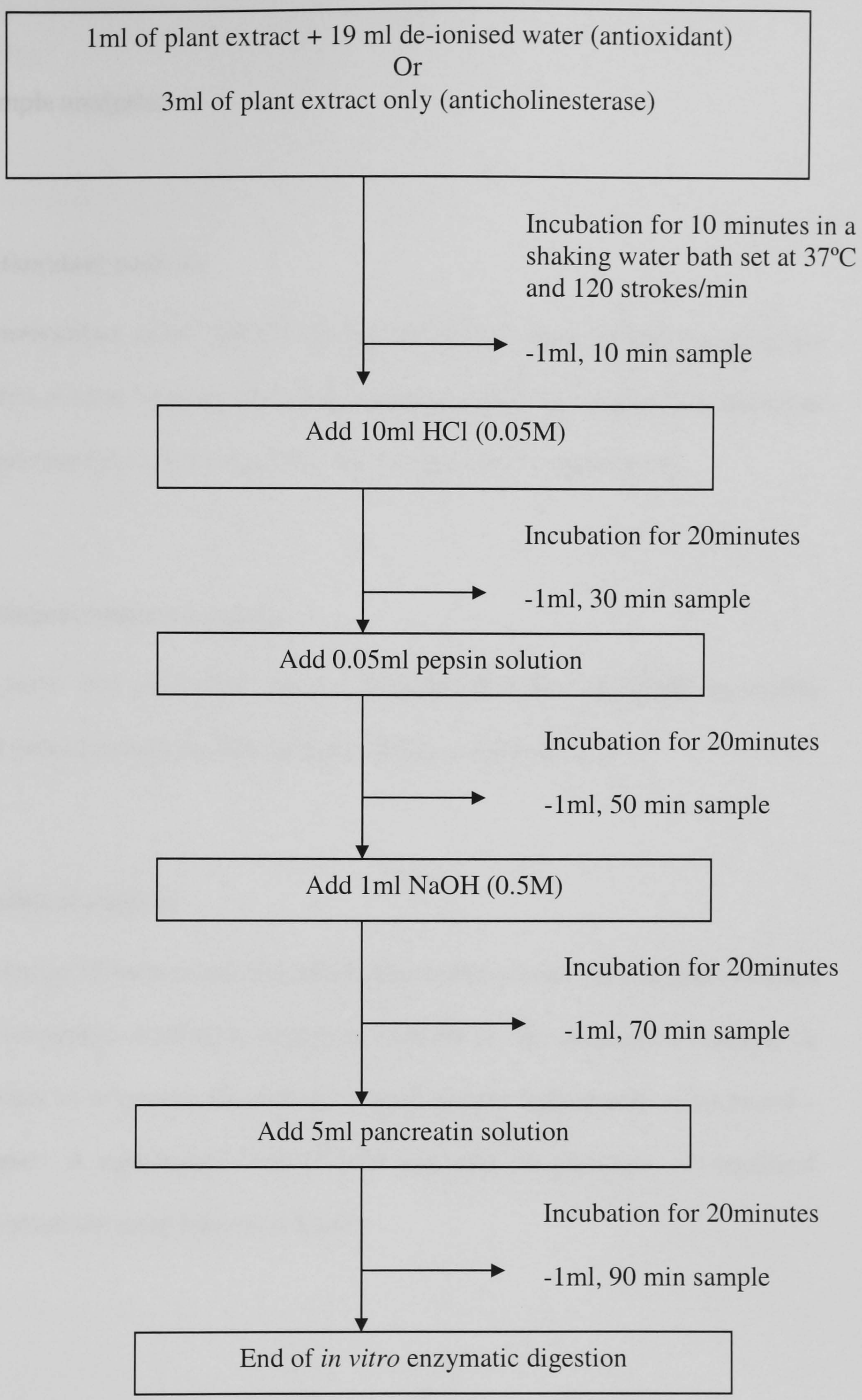


Figure 4.1 Protocol for the *in vitro* enzymatic method.

4.2.4 Sample analysis:

4.2.4.1 Antioxidant analysis:

The antioxidant assays (FRAP; TEAC and DPPH) were carried out using the COBAS Mira clinical analyser (Roche Diagnostics, Welwyn Garden City, Herts) as described previously in sections 2.2.3.1, 2.2.3.2 and 2.2.3.3, respectively.

4.2.4.2 Anticholinesterase analysis:

This assay was preformed using a Titertek Multskan MCC/340 microplate reader. The procedure was as described previously in section 3.2.3.

4.2.5 Statistical analysis

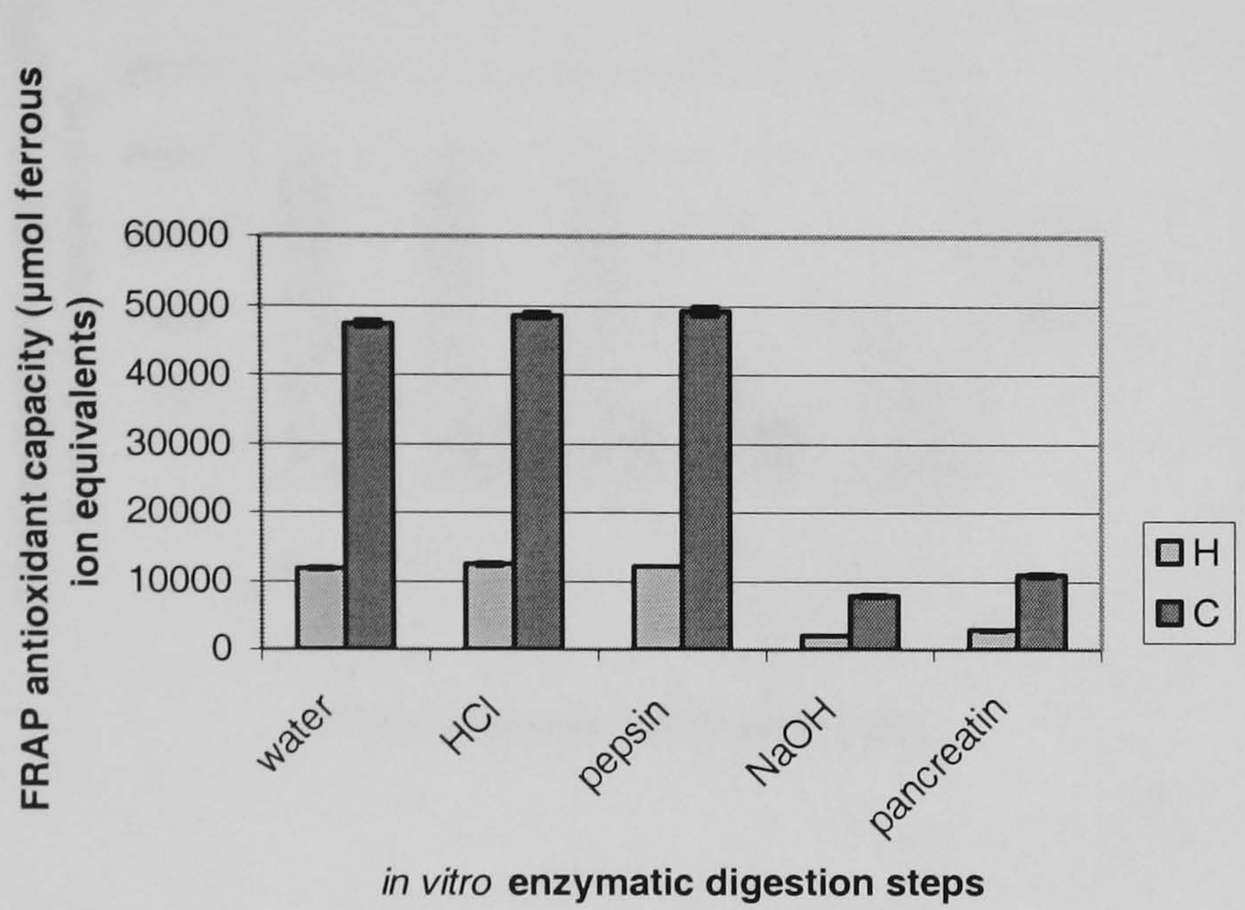
The change of antioxidant and anticholinesterase release for the plant extracts during the enzymatic incubation steps was assessed by the differences between 10 and 90 minutes of enzymatic incubation for each sample individually using paired – sample '*t*-tests'. A significance level of 0.05 was used for each test. All statistical tests were completed using Microsoft Excel.

4.3 Results

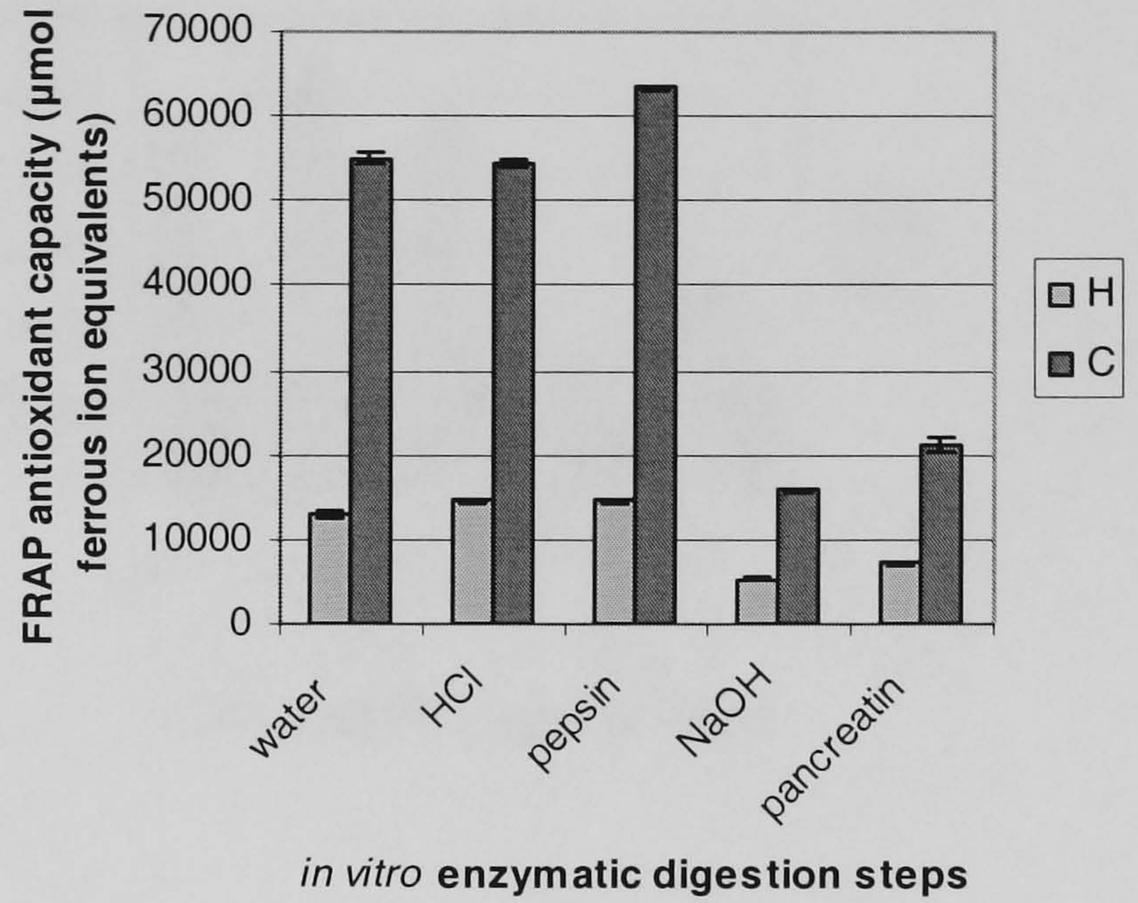
4.3.1 Antioxidant analysis:

4.3.1.1 FRAP assay

The pattern of antioxidant release for the tested plant extracts (hot and cold), green tea, *Myrtus communis*, *Quercus robur, Syzygium aromaticum*, *Olea europaea*, *Matricaria chamomilla*, *Hibiscus sabdariffa*, *Alhagi maurorus*, *Urginea maritima*, *Zingiber officinale* through the whole *in vitro* procedure determined using the FRAP assay is shown in figure 4.2. There was a significant decrease in the total apparent antioxidant capacity at the end of the enzymatic digestion (after 90 min) as compared with the first step (after 10 min) for green tea (A, $p = 0.0001$, both hot and cold), *M. communis* (B, $p = 0.003$, $p = 0.0001$), *Q. robur* (C, $p = 0.01$, $p = 0.0002$), *S. aroaticum* (D, $p = 0.0006$, $p = 0.004$), *O. europaea* (E, $p = 0.001$, $p = 0.02$), *M. chamomilla* (F, $p = 0.0006$ hot only) and *U. maritima* (I, $p = 0.004$, $p = 0.01$). The apparent antioxidant content of *H. sabdariffa* and *A. maurorus* extracts (hot and cold) appeared to increase at the end of the digestion step (after 90 min), although this increase was not statistically significant (figure 4.2, G and H). In contrast, the extracts of *Z. officinale* showed a decrease in the total apparent antioxidant capacity at the end of enzymatic digestion, although the decrease was not significant (figure 4.2, J). Generally, most of the changes in the total antioxidant capacity during the 90 minutes occurred after addition of the sodium hydroxide (NaOH) i.e. when the pH changed from acidic to alkaline (figure 4.2).


FRAP antioxidant capacity (µmol ferrous ion equivalents)
60000
50000
40000
30000
20000
10000
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

A


FRAP antioxidant capacity (µmol ferrous ion equivalents)
70000
60000
50000
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20000
10000
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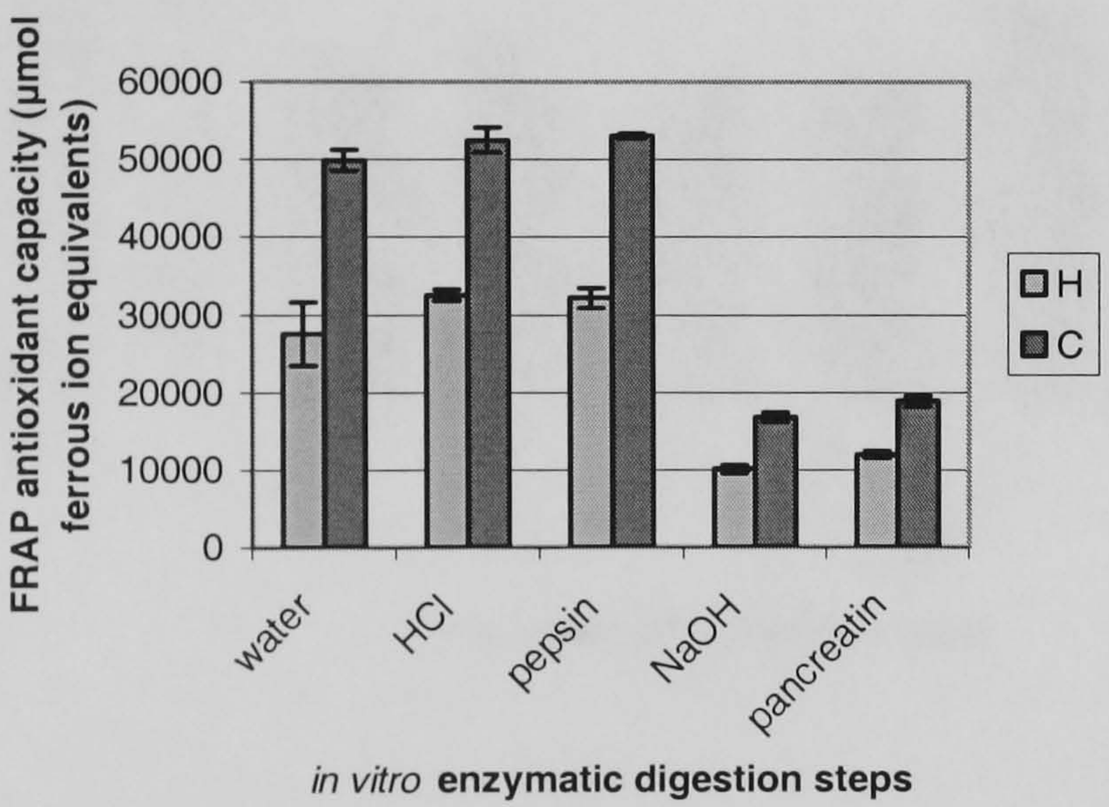
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

B


FRAP antioxidant capacity (µmol ferrous ion equivalents)
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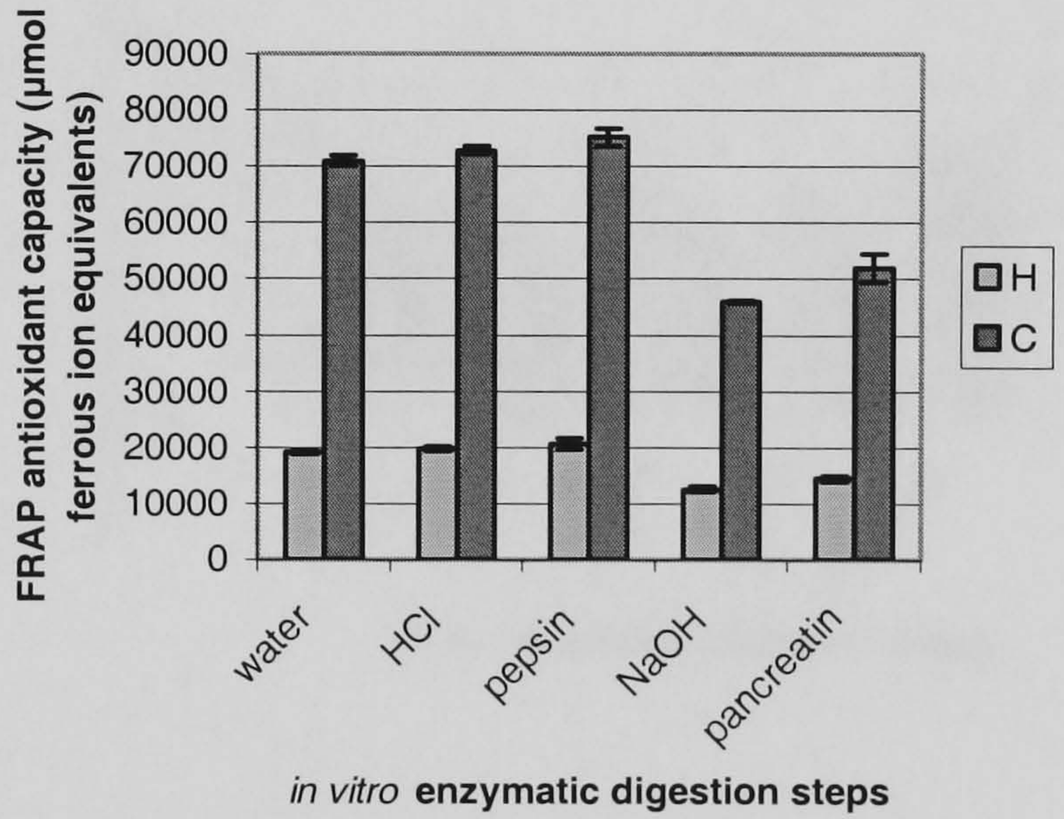
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

C


FRAP antioxidant capacity (µmol ferrous ion equivalents)
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70000
60000
50000
40000
30000
20000
10000
0
water
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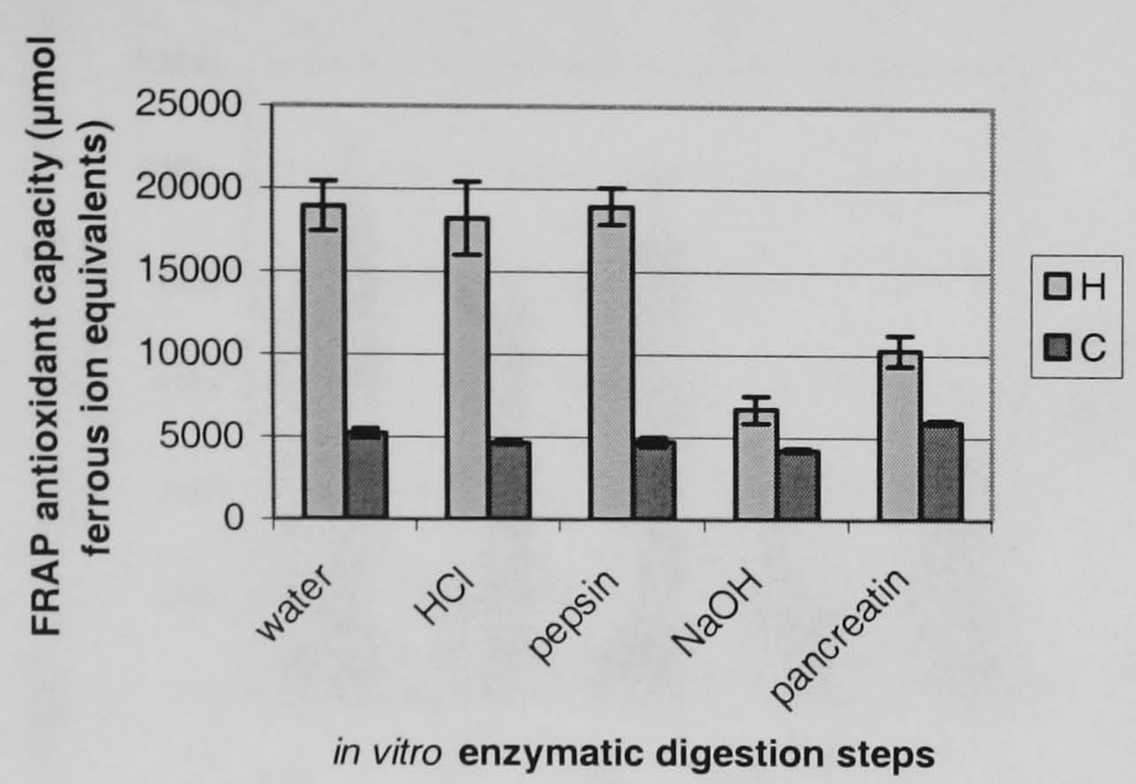
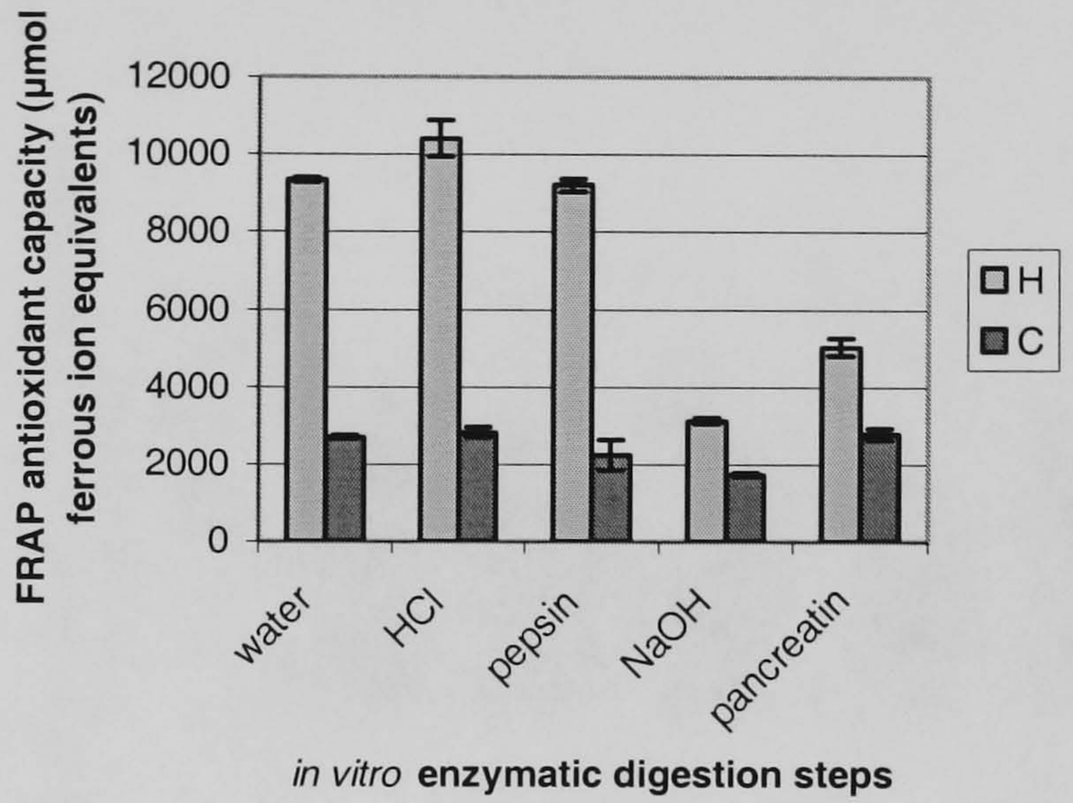
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

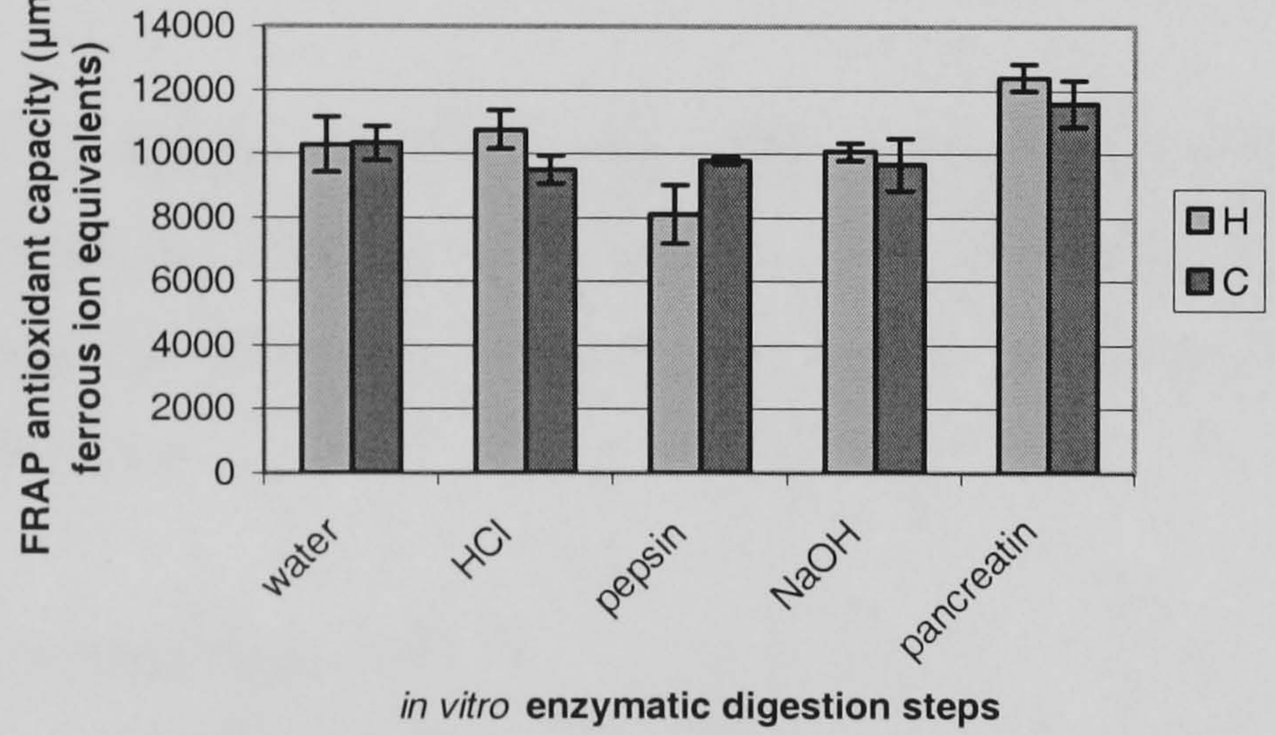
D


FRAP antioxidant capacity (µmol ferrous ion equivalents)
25000
20000
15000
10000
5000
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

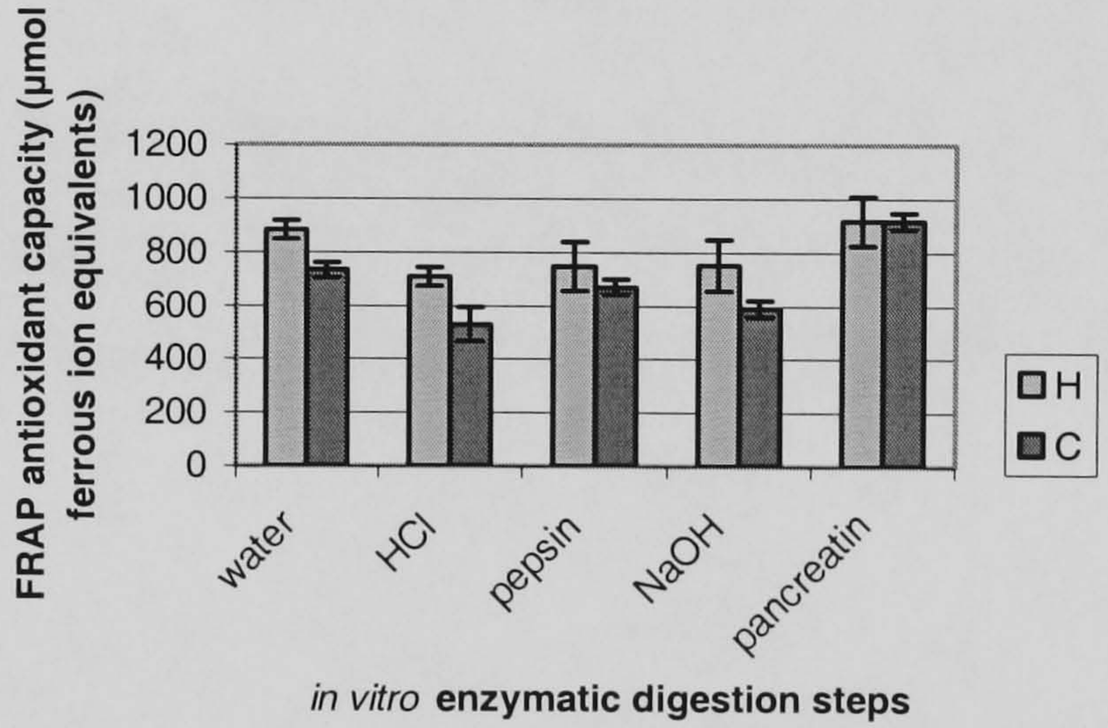
E


FRAP antioxidant capacity (µmol ferrous ion equivalents)
12000
10000
8000
6000
4000
2000
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

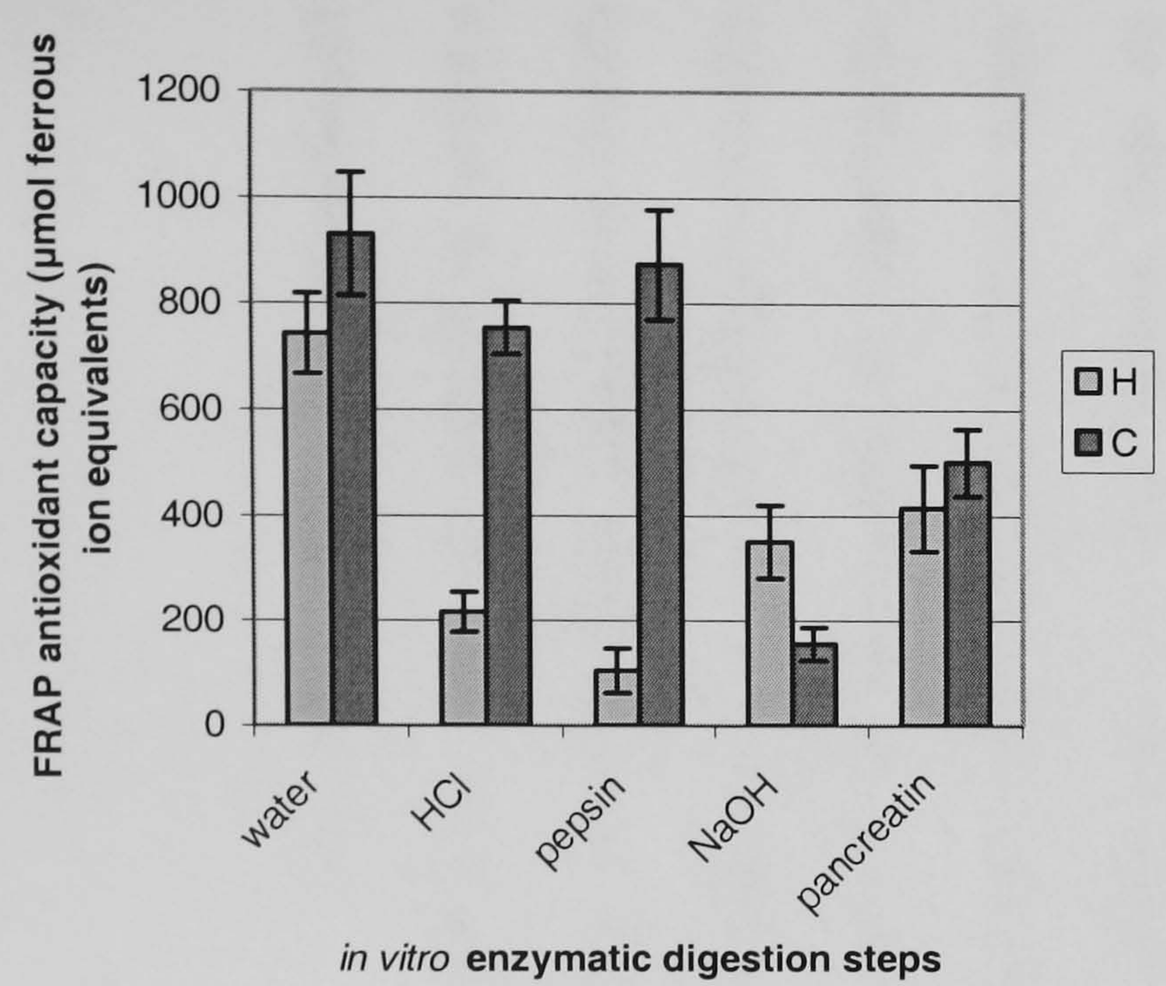
F


FRAP antioxidant capacity (µmol ferrous ion equivalents)
14000
12000
10000
8000
6000
4000
2000
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

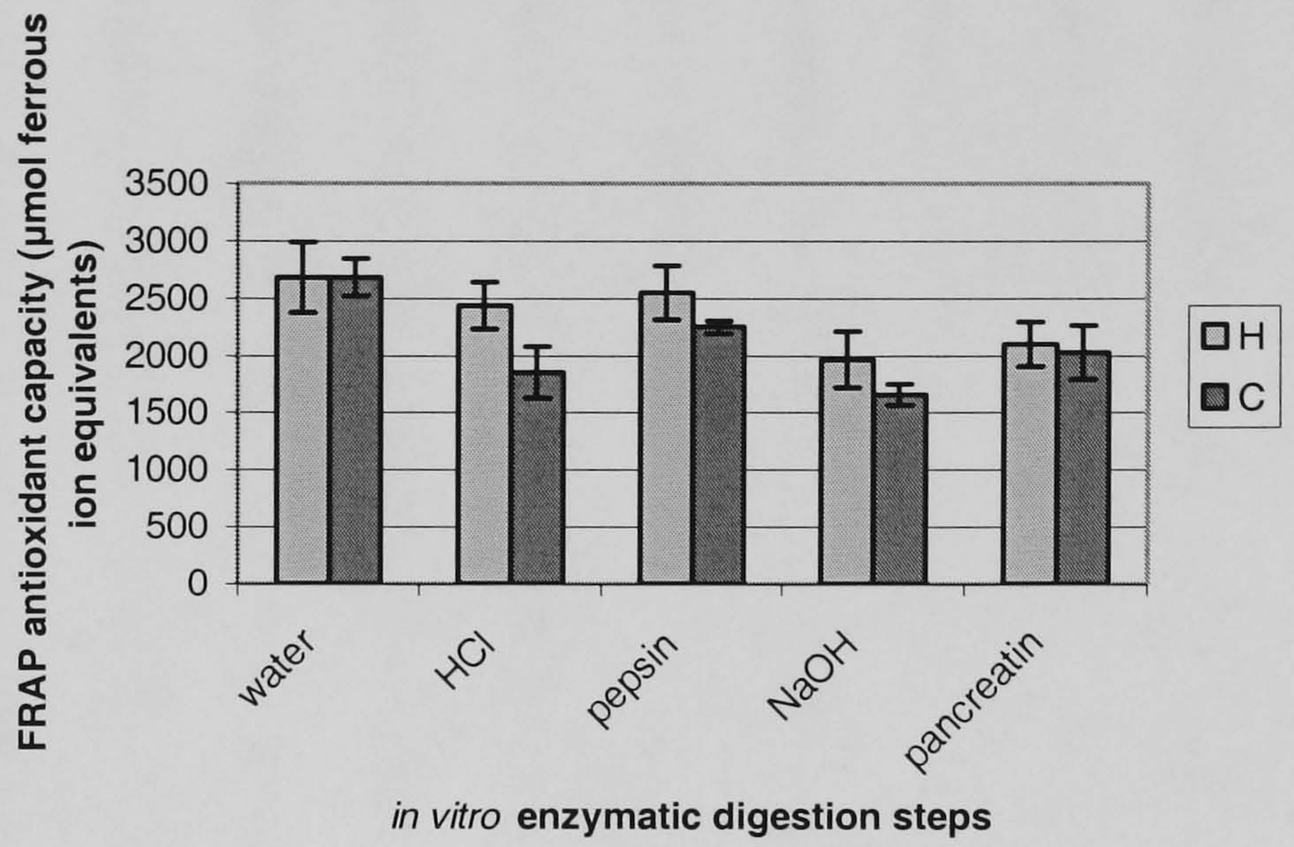
G


FRAP antioxidant capacity (µmol ferrous ion equivalents)
1200
1000
800
600
400
200
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

H



I



J

Figure 4.2. Total antioxidant capacity of selected Libyan medicinal plant extracts (hot and cold) meausured by FRAP assay (µmol ferrous ion equivalents) during *in vitro* enzymatic digestion steps (A) Green tea (B) *Myrtus communis* (C) *Quercus robur* (D) *Syzygium aroaticum* (E) *Olea europaea* (F) *Matricaria chamomilla.*(G) *Hibiscus sabdariffa* (H) *Alhagi maurorus* (I) *Urginea maritima* (J) *Zingiber officinale*.

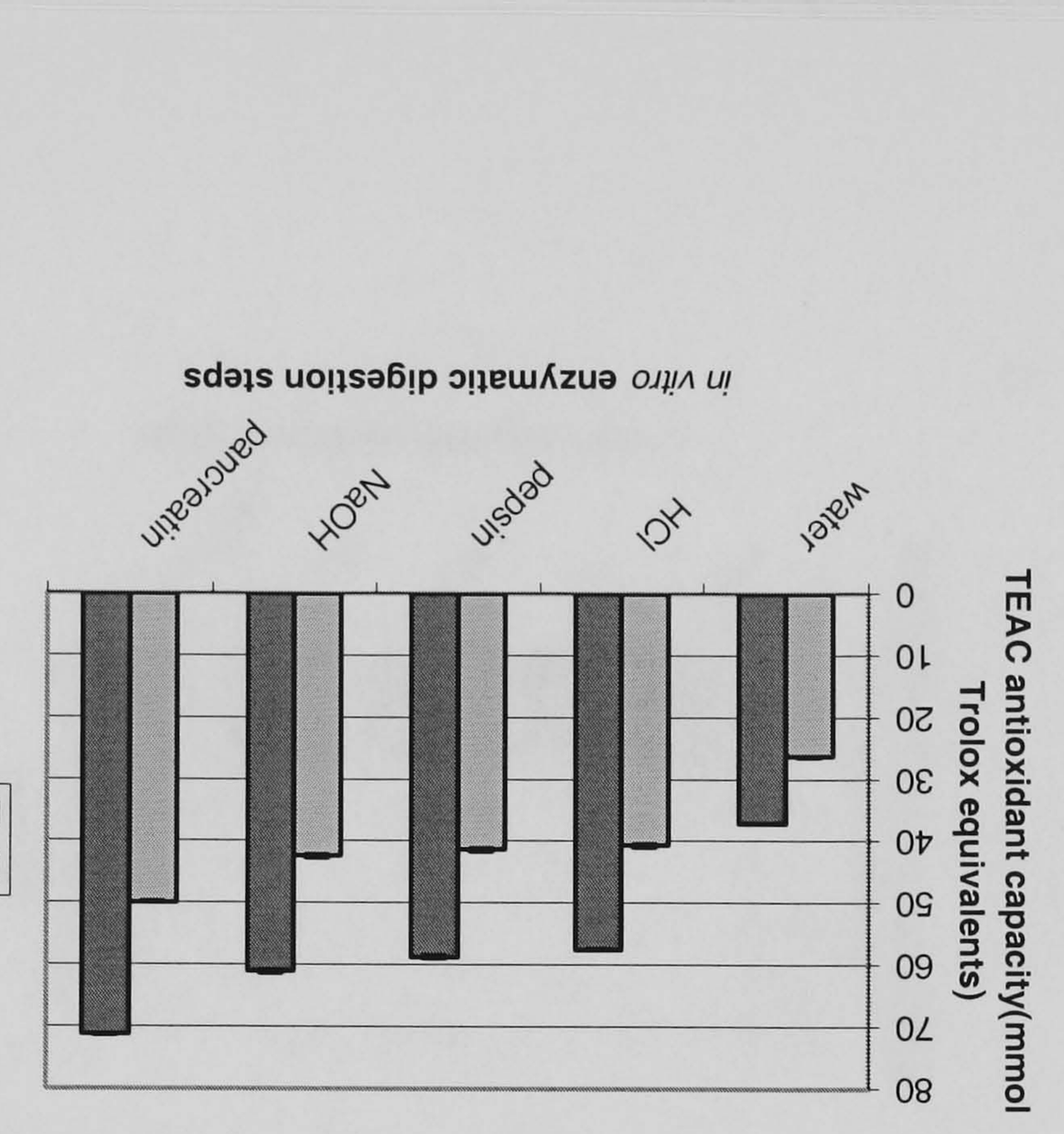
Group (a) plants A-D

Group (b) plants E-G

Group (c) plants H-J

4.3.1.2 TEAC assay

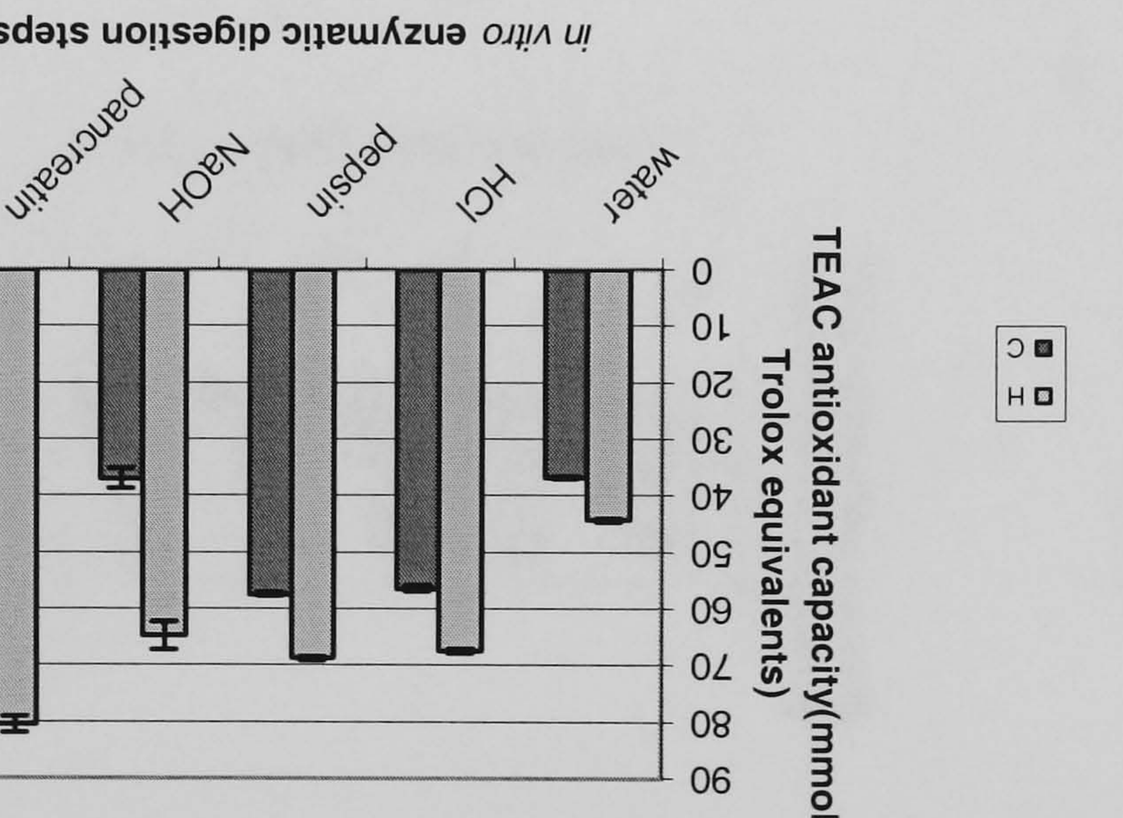
The pattern of antioxidant release for the ten plant extracts examined through the whole *in vitro* procedure determined using the TEAC assay is shown in figure 4.3. The increase in the total antioxidant release was statistically significant during the 90 minutes of the enzymatic digestion for most samples tested by this method including green tea (A, p = 0.01, hot only), *M. communis* (B, p = 0.0003, p = 0.004), *Q. robur* (C, p = 0.008, cold only), *S. aromaticum* (D, p = 0.00006, p = 0.00008), *M. chamomilla* (F, p = 0.05, p = 0.006), *H. sabdariffa* (G, p = 0.007, p = 0.0007), *A. maurorus* (H, p = 0.05, p = 0.01), *U. maritima* (I, p = 0.003, p = 0.001) and *Z. officinale* (J, p = 0.0001, p = 0.005 hot and the cold). In contrast, the hot extract of *O. europaea* showed a significant reduction in the total antioxidant capacity at the end of the enzymatic digestion with p- value less than 0.001, while the apparent antioxidant content of the cold extract appeared to be significantly increased at the end of the digestion steps (p = 0.0007) (figure 4.3, E). Interestingly, it is of note that the plant extracts with low antioxidant activity (group c), showed TEAC values which remained almost constant during the first four steps of the digestion then significantly increased after the addition of pancreatin solution (last step of the digestion) (figure 4.3, H, I and J). The plant extracts with high antioxidant activity (group a) exhibited sharp increases in the antioxidant capacity after adding HCl solution. In general, there was a decrease in the apparent antioxidant capacity after adding the NaOH solution (figure 4.3).


TEAC antioxidant capacity(mmol Trolox equivalents)
in vitro enzymatic digestion steps
water
HCl
pepsin
NaOH
pancreatin
H
C

C

TEAC antioxidant capacity(mmol Trolox equivalents)
in vitro enzymatic digestion steps
water
HCl
pepsin
NaOH
pancreatin
H
C

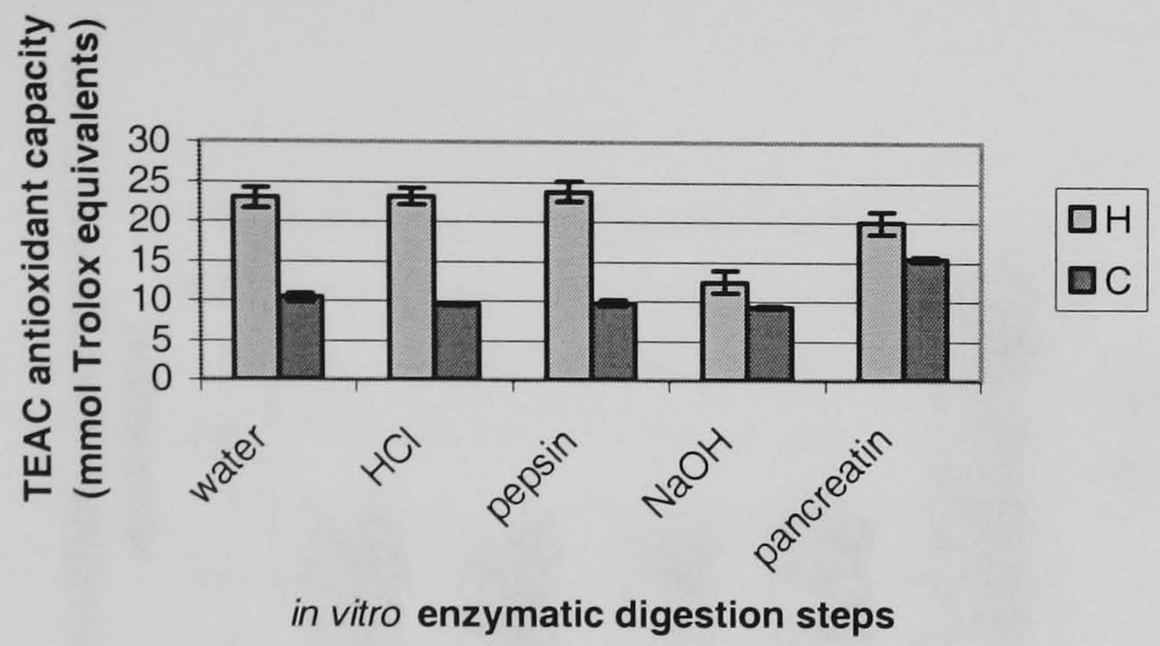
D


TEAC antioxidant capacity(mmol Trolox equivalents)
in vitro enzymatic digestion steps
water
HCl
pepsin
NaOH
pancreatin
H
C

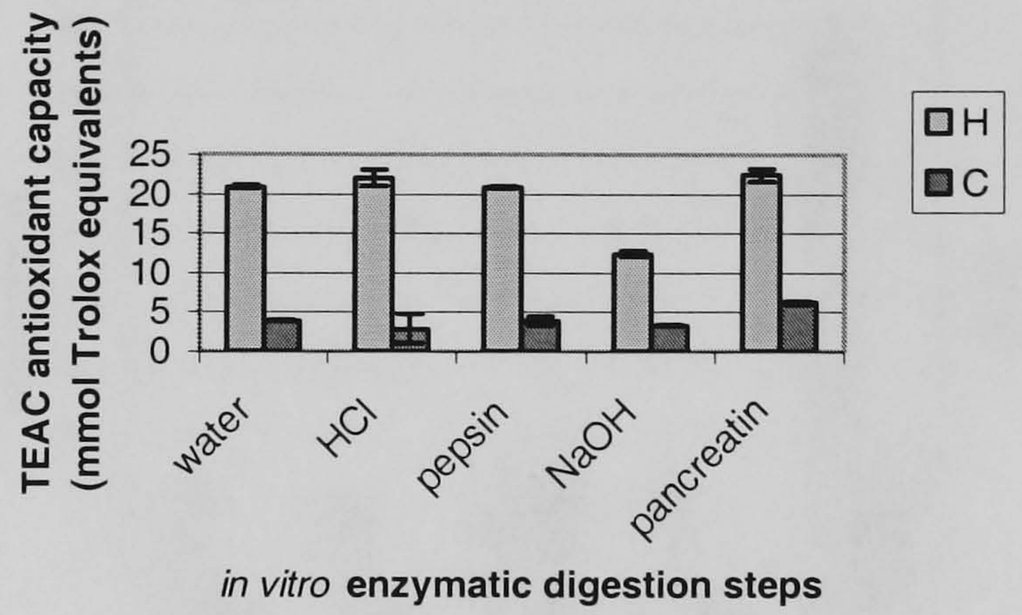
A

TEAC antioxidant capacity(mmol Trolox equivalents)
in vitro enzymatic digestion steps
water
HCl
pepsin
NaOH
pancreatin
H
C

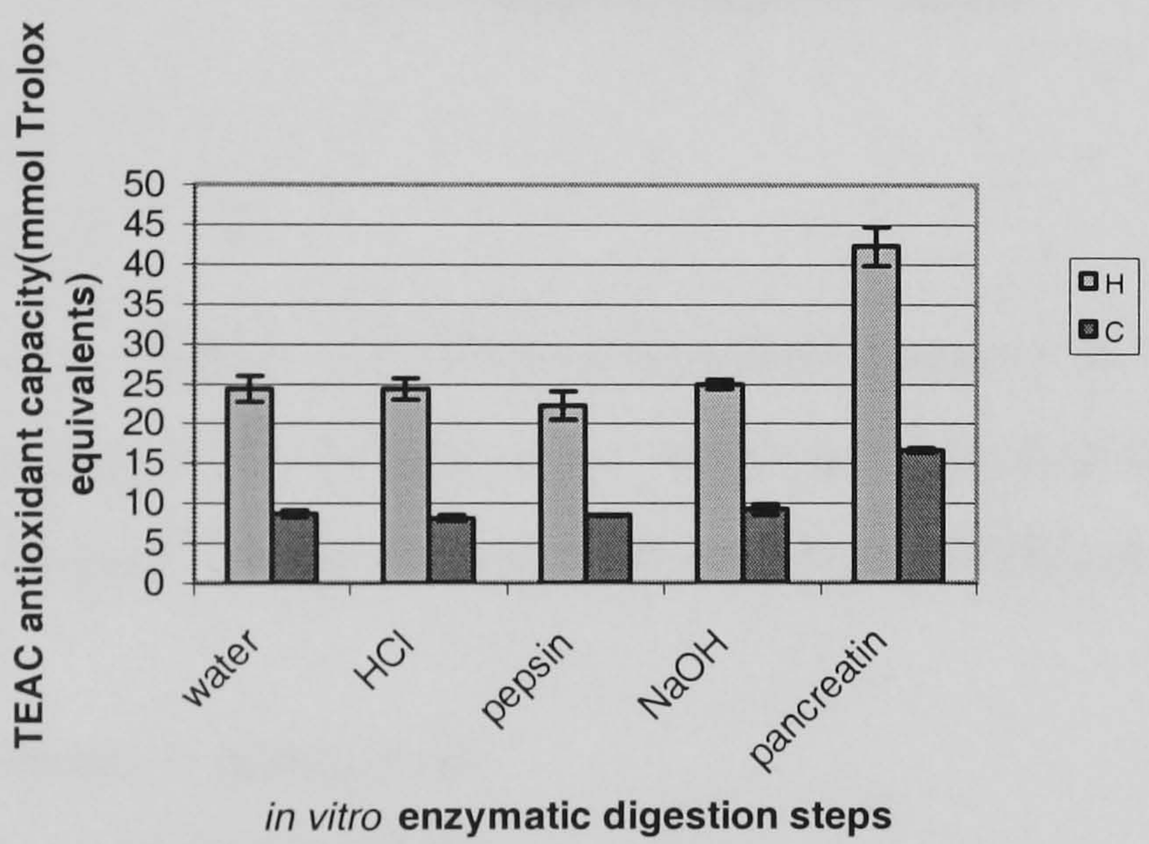
B


TEAC antioxidant capacity (mmol Trolox equivalents)
30
25
20
15
10
5
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

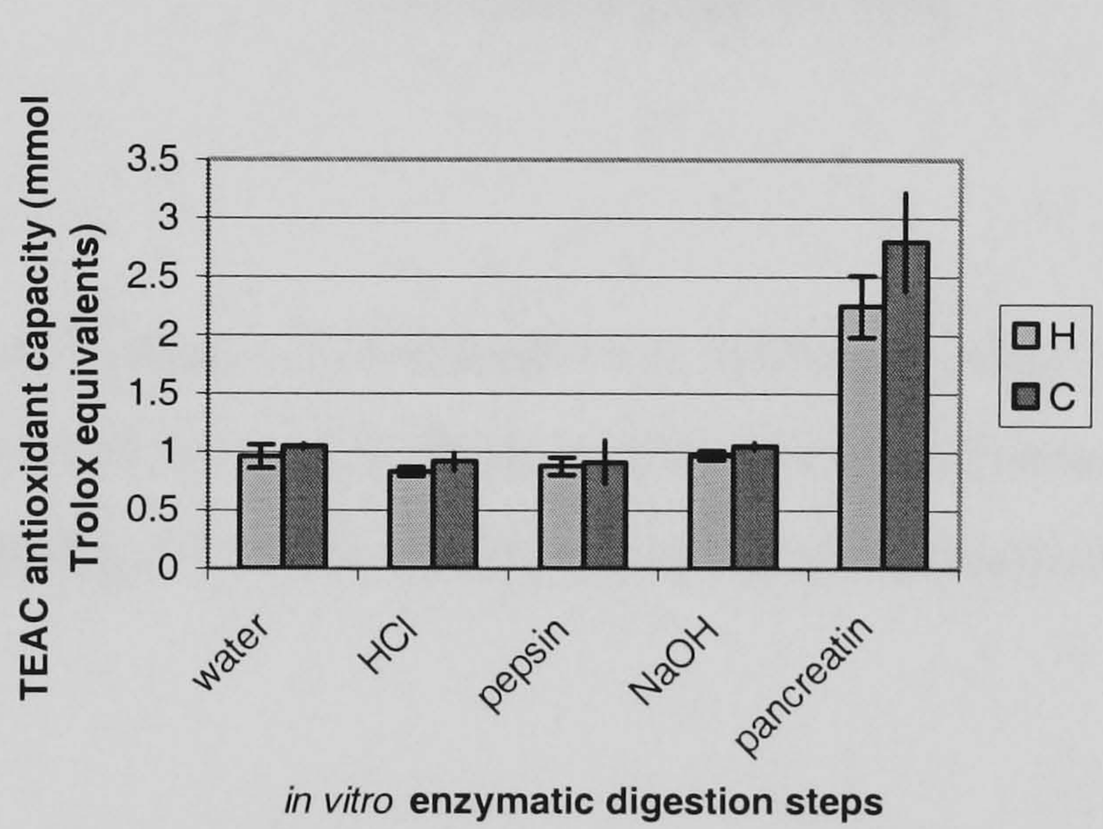
E


TEAC antioxidant capacity (mmol Trolox equivalents)
25
20
15
10
5
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

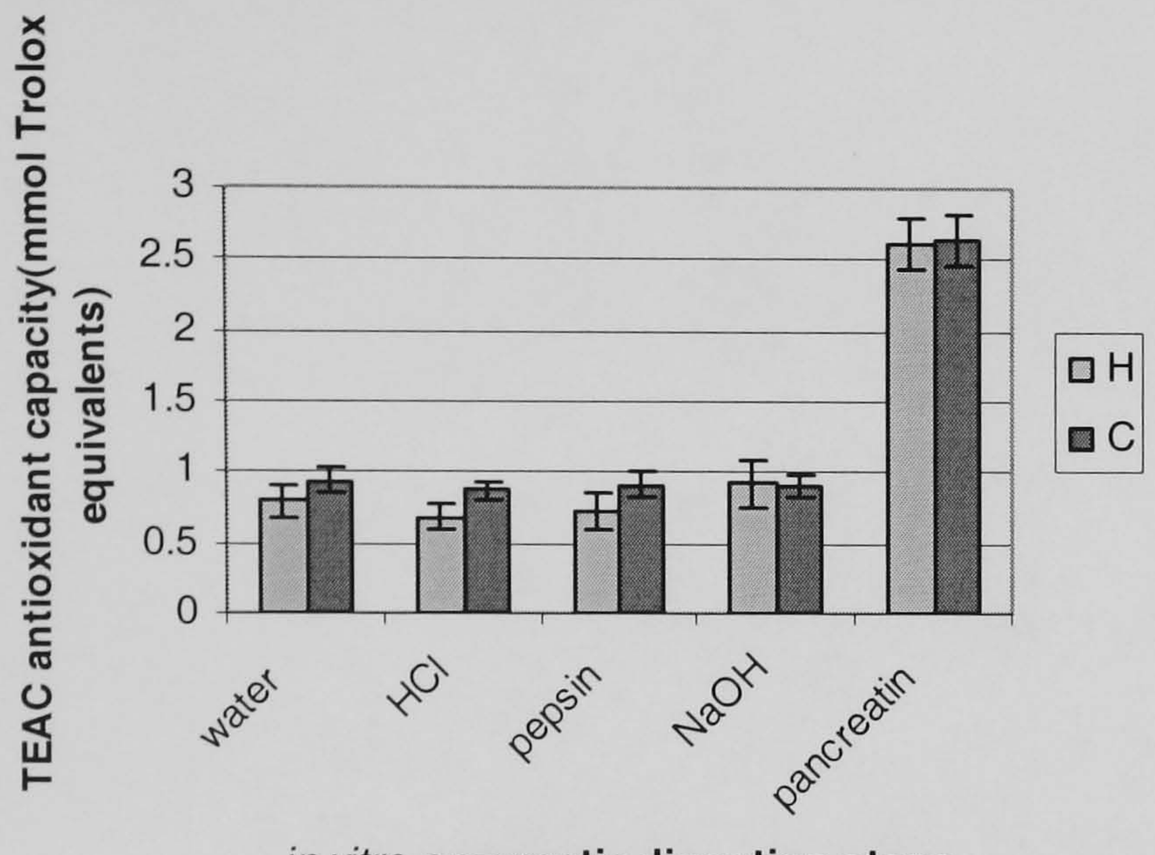
F


TEAC antioxidant capacity(mmol Trolox equivalents)
50
45
40
35
30
25
20
15
10
5
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

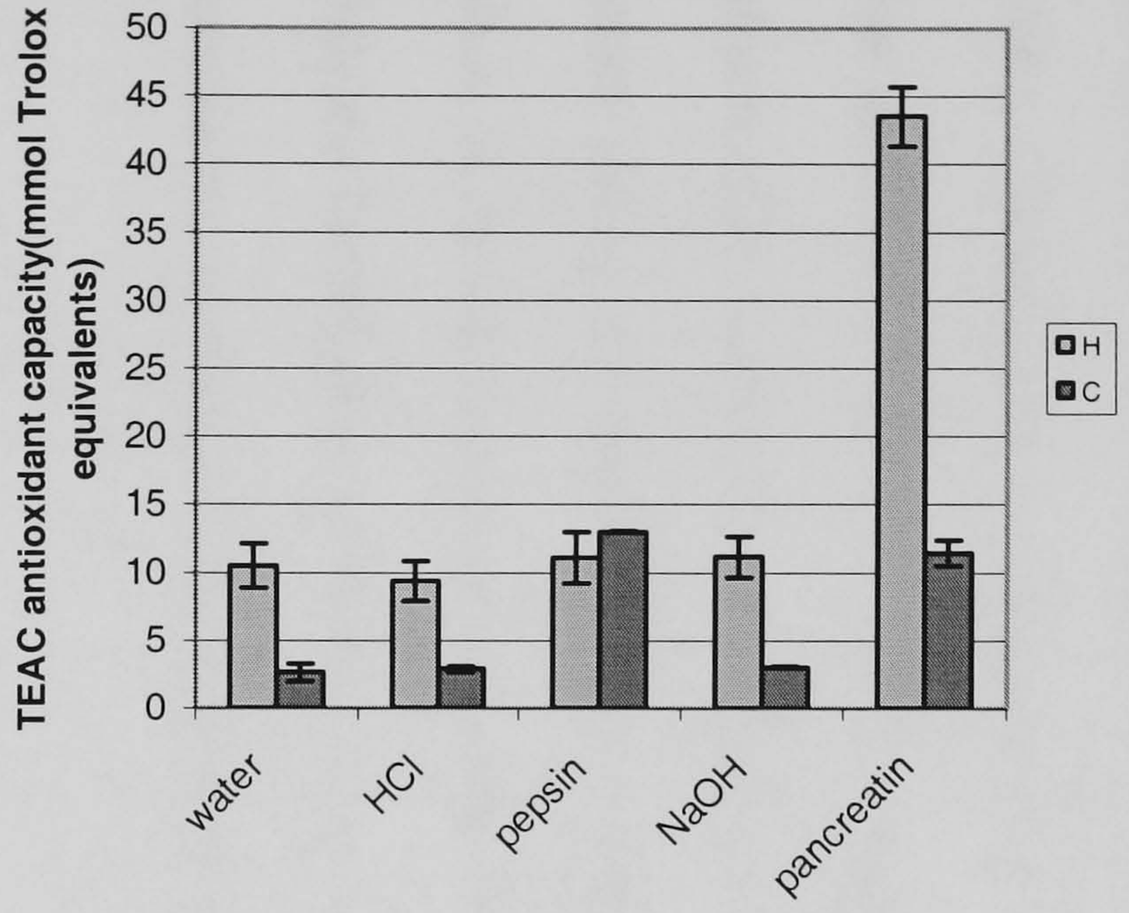
G


TEAC antioxidant capacity (mmol Trolox equivalents)
3.5
3
2.5
2
1.5
1
0.5
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

H



I



J

Figure 4.3. Total antioxidant capacity of selected Libyan medicinal plant extracts (hot and cold) meausured by TEAC (mmol Trolox equivalents) during *in vitro* enzymatic digestion steps (A) Green tea (B) *Myrtus communis* (C) *Quercus robur*(D) *Syzygium aroaticum* (E) *Olea europaea* (F) *Matricaria chamomilla.*(G) *Hibiscus sabdariffa* (H) *Alhagi maurorus* (I) *Urginea maritima* (J) *Zingiber officinale* .

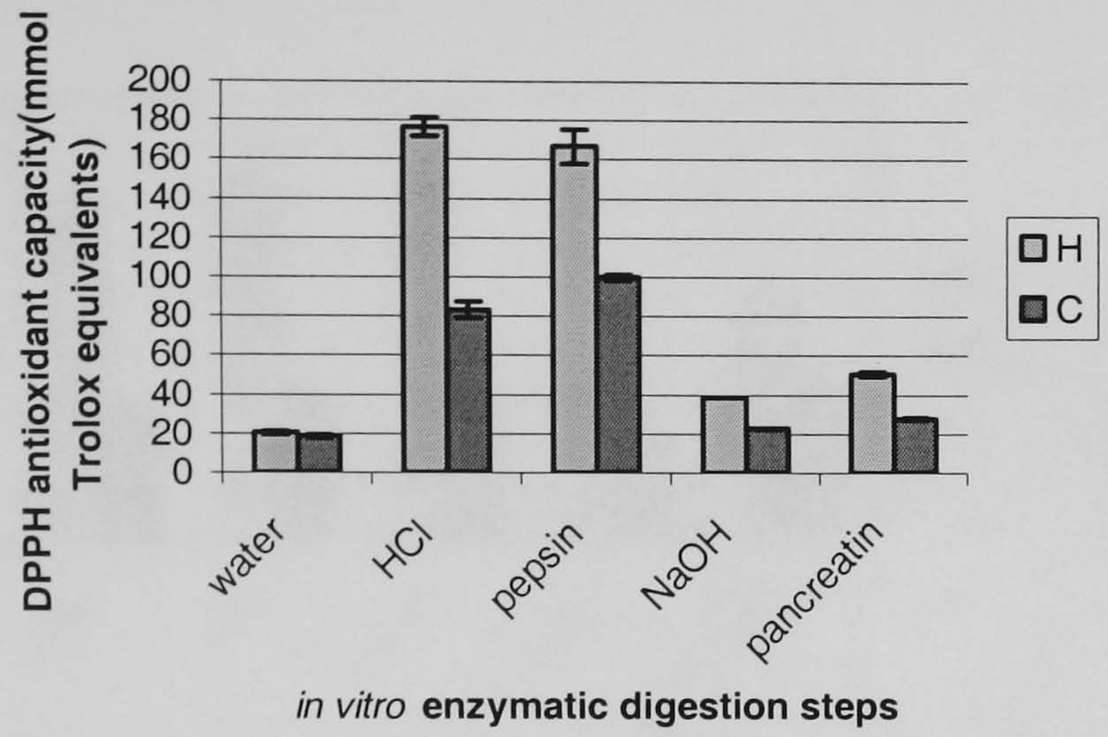
Group (a) plants A-D

Group (b) plants E-G

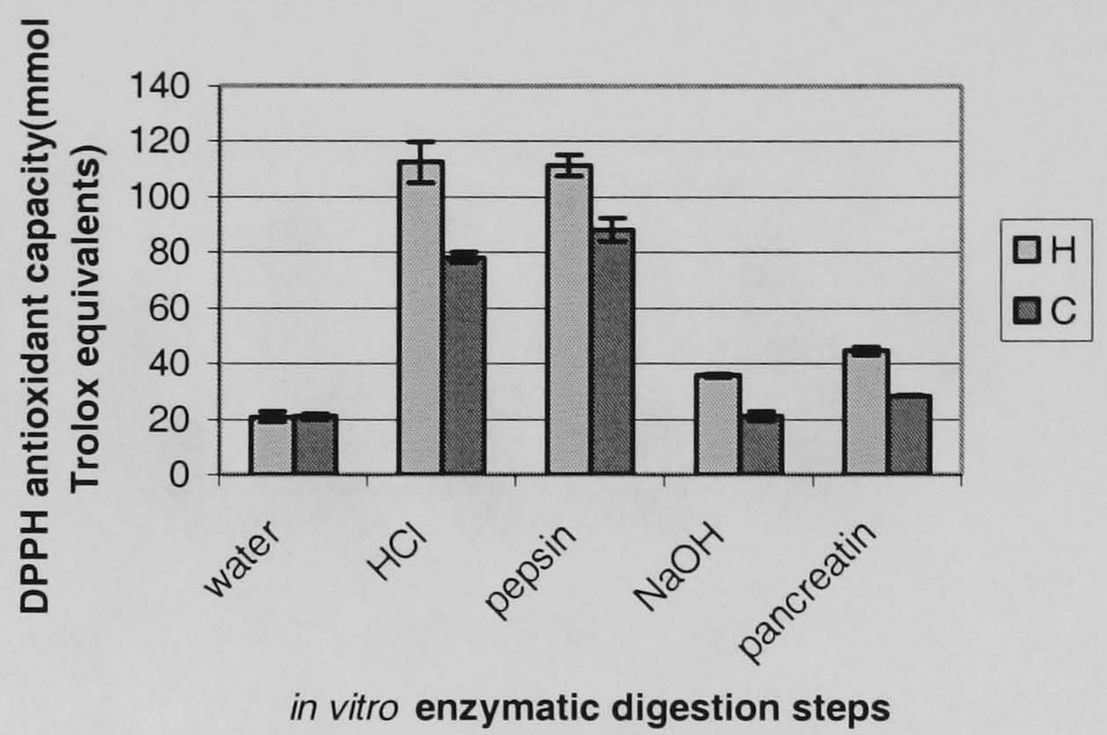
Group (c) plants H-J

4.3.1.3 DPPH assay

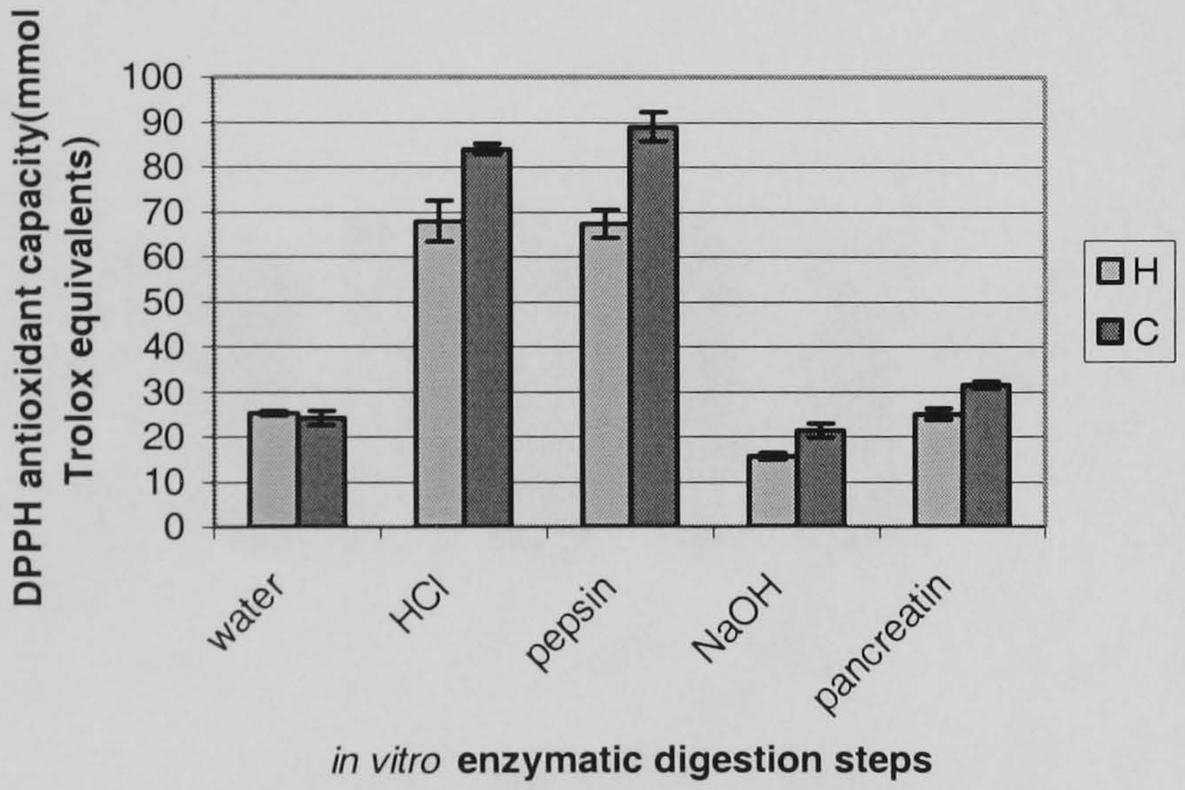
The pattern of antioxidant release for the ten plant extracts examined through the whole *in vitro* procedure determined using the DPPH assay is shown in figure 4.4. There was a significant increase in the total apparent antioxidant capacity for hot and cold extracts of green tea (A, p = 0.0001, 0.0008), *M. communis* (B, p = 0.003, p < 0.006), *Q. robur* (C, p = 0.03, cold only), *S. aromaticum* (D, p < 0.007, C only), *M. chamomilla* (F, p = 0.05, 0.03), *A. maurorus* (H, p = 0.02, p<0.01), *U. maritima*(I, p = 0.01, < 0.004) and *Z. officinale* (J, p = 0.007, cold only) at the last step of the *in vitro* enzymatic digestion. The scavenging activity of the hot and the cold water extracts of *H. sabdariffa* and *O. europaea* against DPPH radicals showed a constant pattern during the digestion steps except the final panceatin step where the DPPH values of the hot extract of *O. europaea* was significantly decreased (E, p = 0.008), while the DPPH value of the hot extract of *H. sabdariffa* was increased, although this increase was not statistically significant (Figure 4.4, E and G).


DPPH antioxidant capacity(mmol Trolox equivalents)
200
180
160
140
120
100
80
60
40
20
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

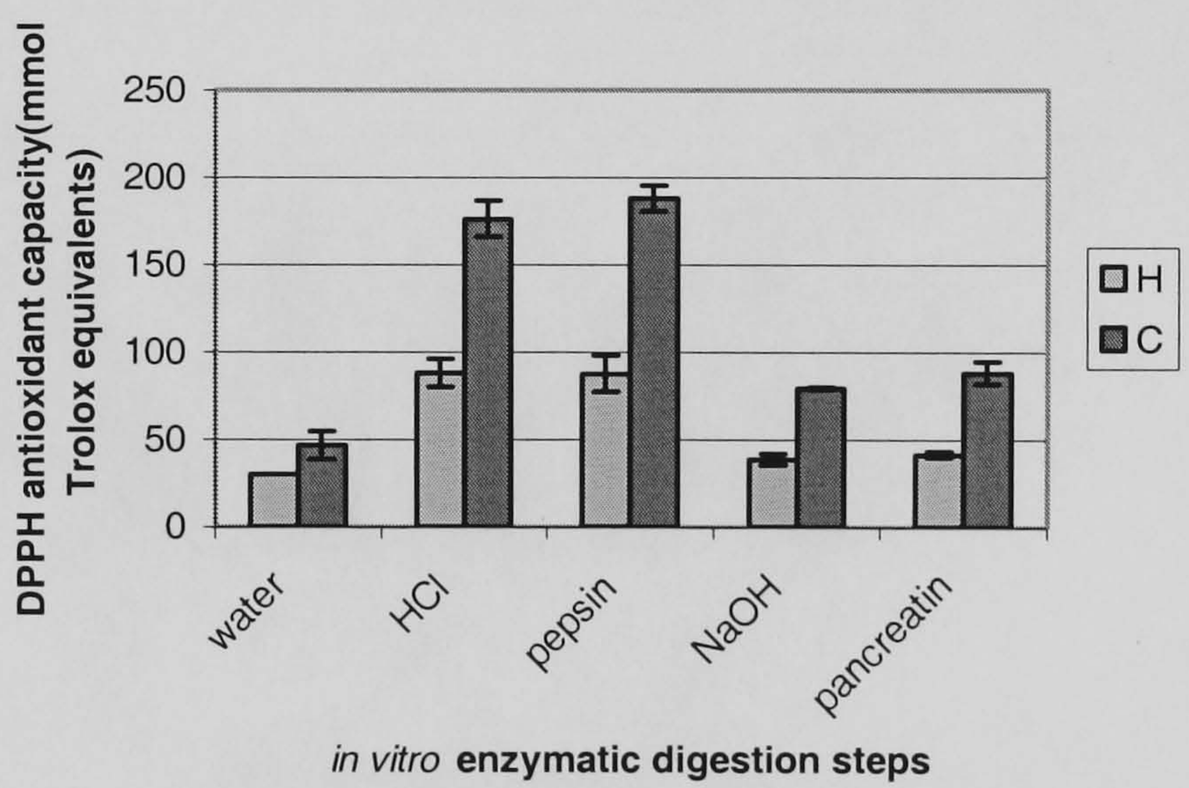
A


DPPH antioxidant capacity(mmol Trolox equivalents)
140
120
100
80
60
40
20
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

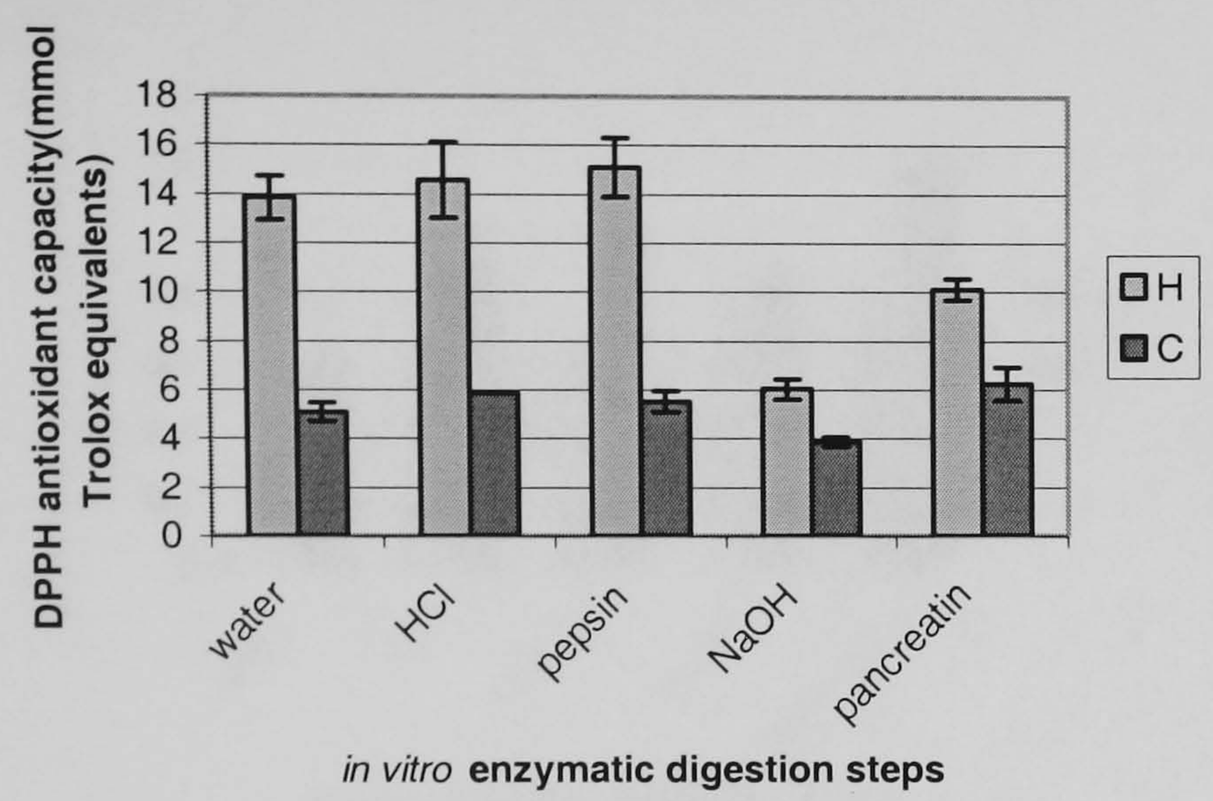
B


DPPH antioxidant capacity(mmol Trolox equivalents)
100
90
80
70
60
50
40
30
20
10
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

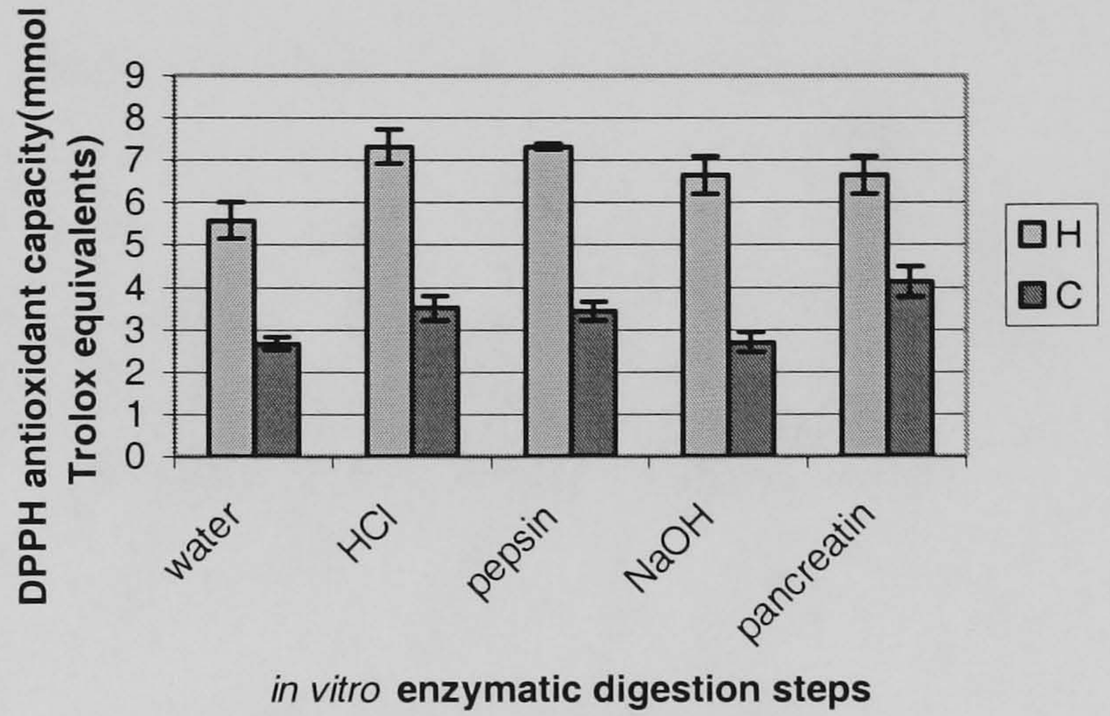
C


DPPH antioxidant capacity(mmol Trolox equivalents)
250
200
150
100
50
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

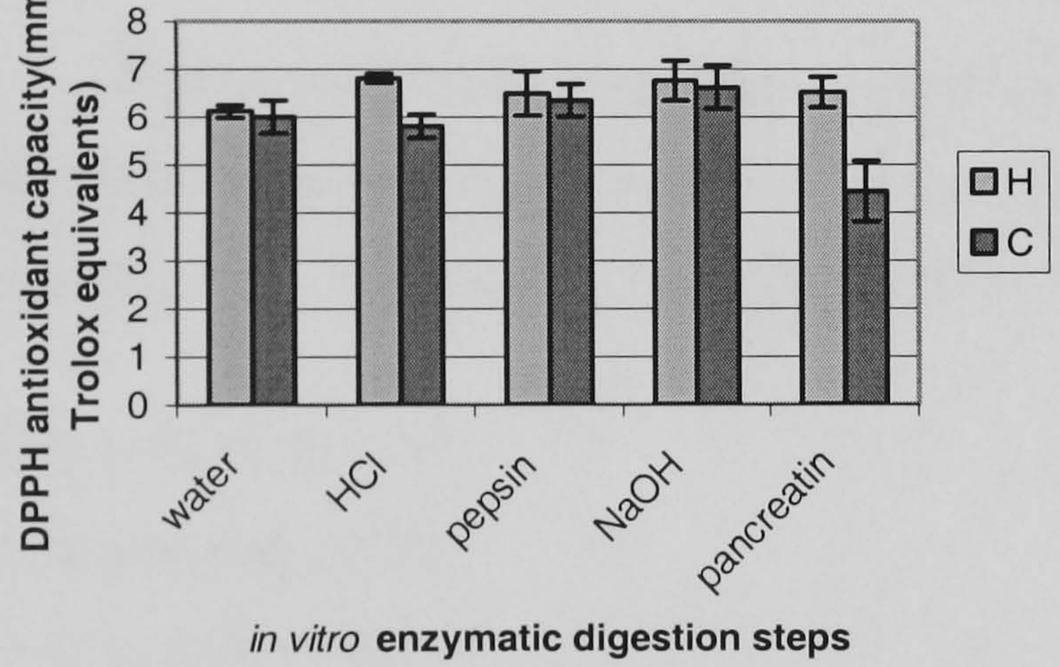
D


DPPH antioxidant capacity(mmol Trolox equivalents)
18
16
14
12
10
8
6
4
2
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

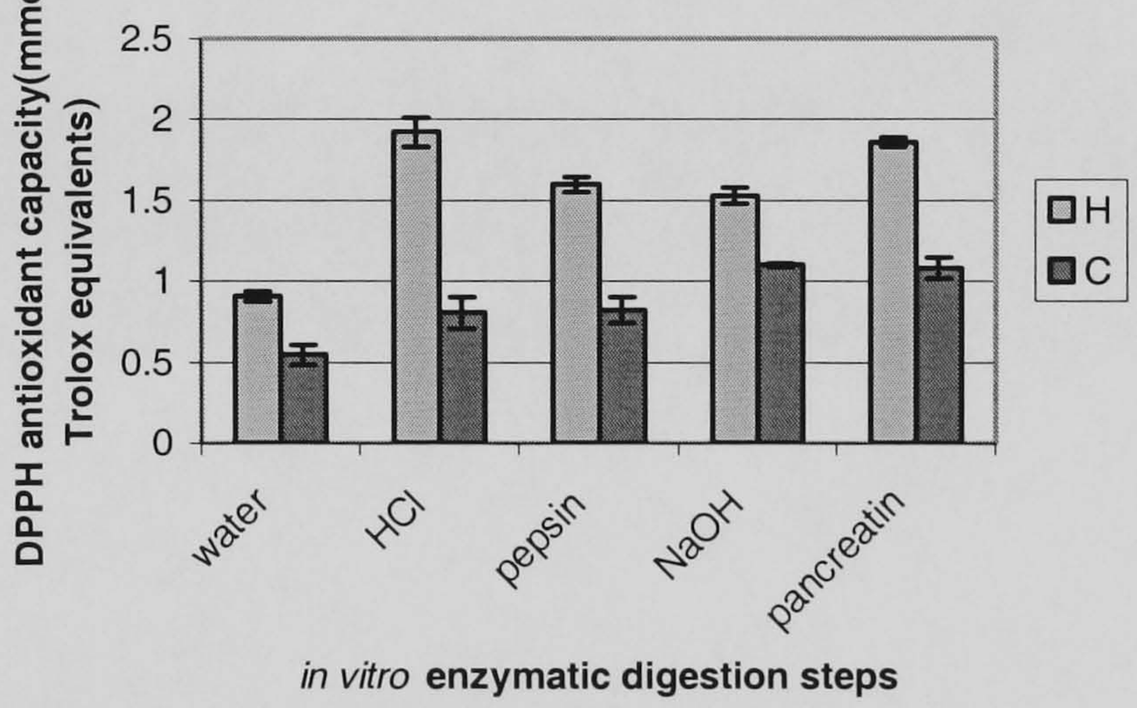
E


DPPH antioxidant capacity(mmol Trolox equivalents)
9
8
7
6
5
4
3
2
1
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

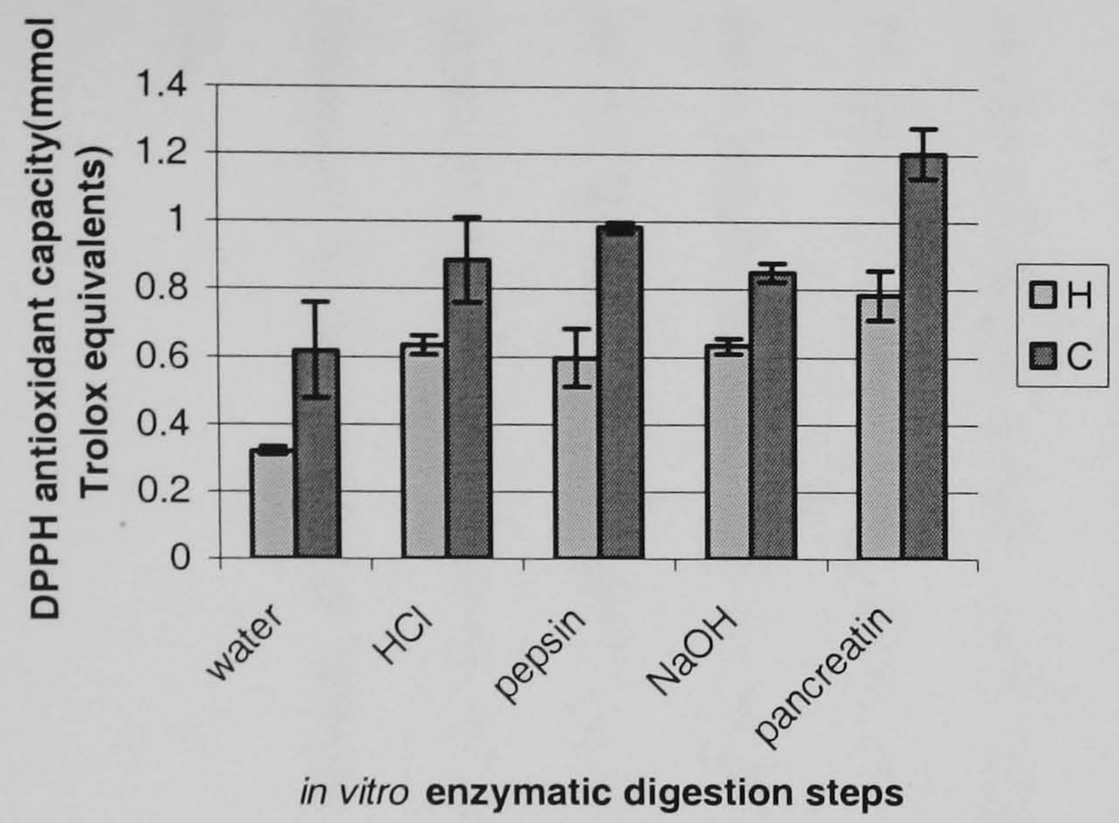
F


DPPH antioxidant capacity(mmol Trolox equivalents)
8
7
6
5
4
3
2
1
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

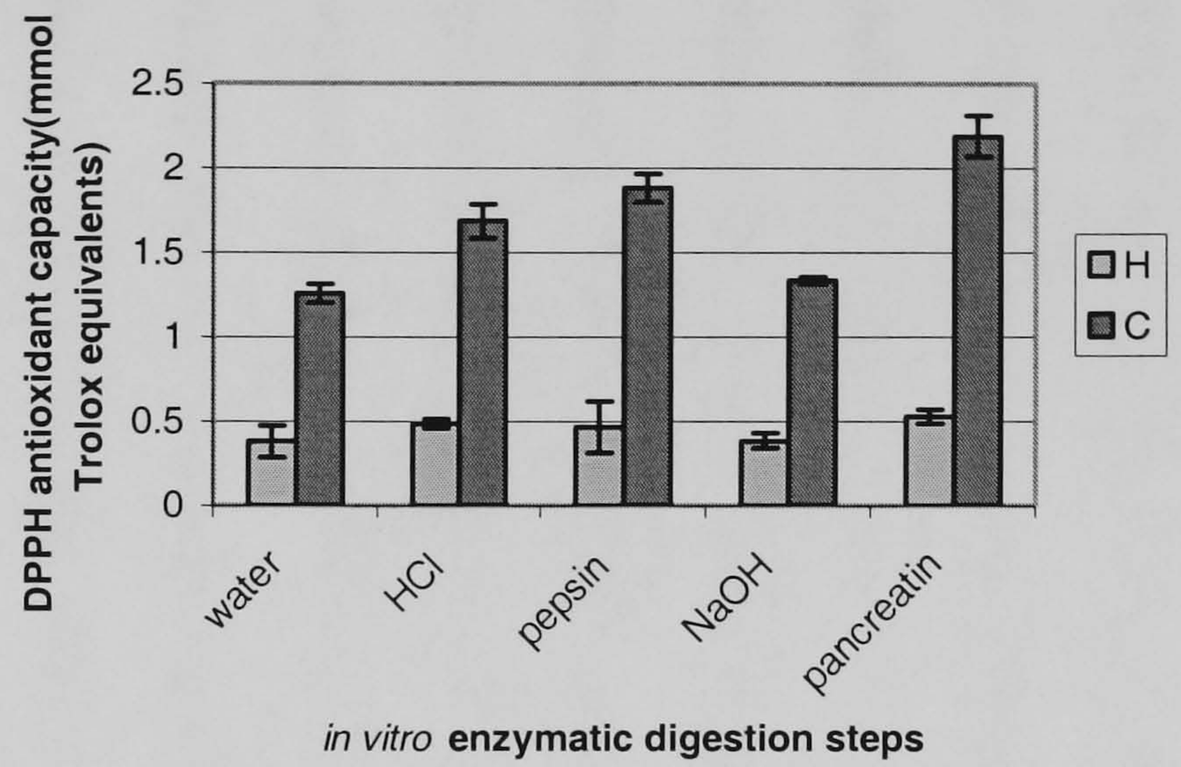
G


DPPH antioxidant capacity(mmol Trolox equivalents)
2.5
2
1.5
1
0.5
0
water
HCl
pepsin
NaOH
pancreatin
H
C
in vitro enzymatic digestion steps

H



I



J

Figure 4.4. Total antioxidant capacity of selected Libyan medicinal plant extracts (hot and cold) meausured by DPPH (mmol Trolox equivalents) during *in vitro* enzymatic digestion steps (A) Green tea (B) *Myrtus communis* (C) *Quercus robur* (D) *Syzygium aroaticum* (E) *Olea europaea* (F) *Matricaria chamomilla.*(G) *Hibiscus sabdariffa* (H) *Alhagi maurorus* (I) *Urginea maritima* (J) *Zingiber officinale.*

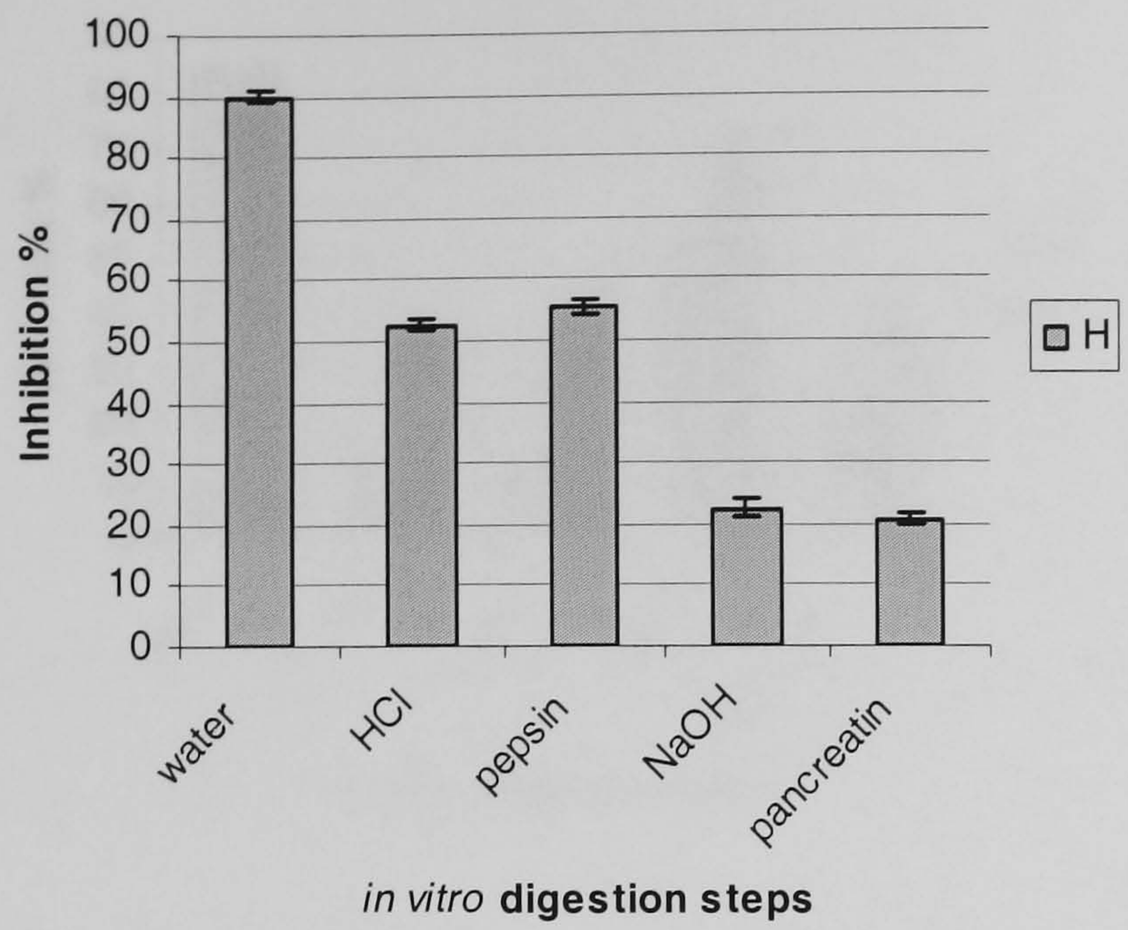
Group (a) plants A-D

Group (b) plants E-G

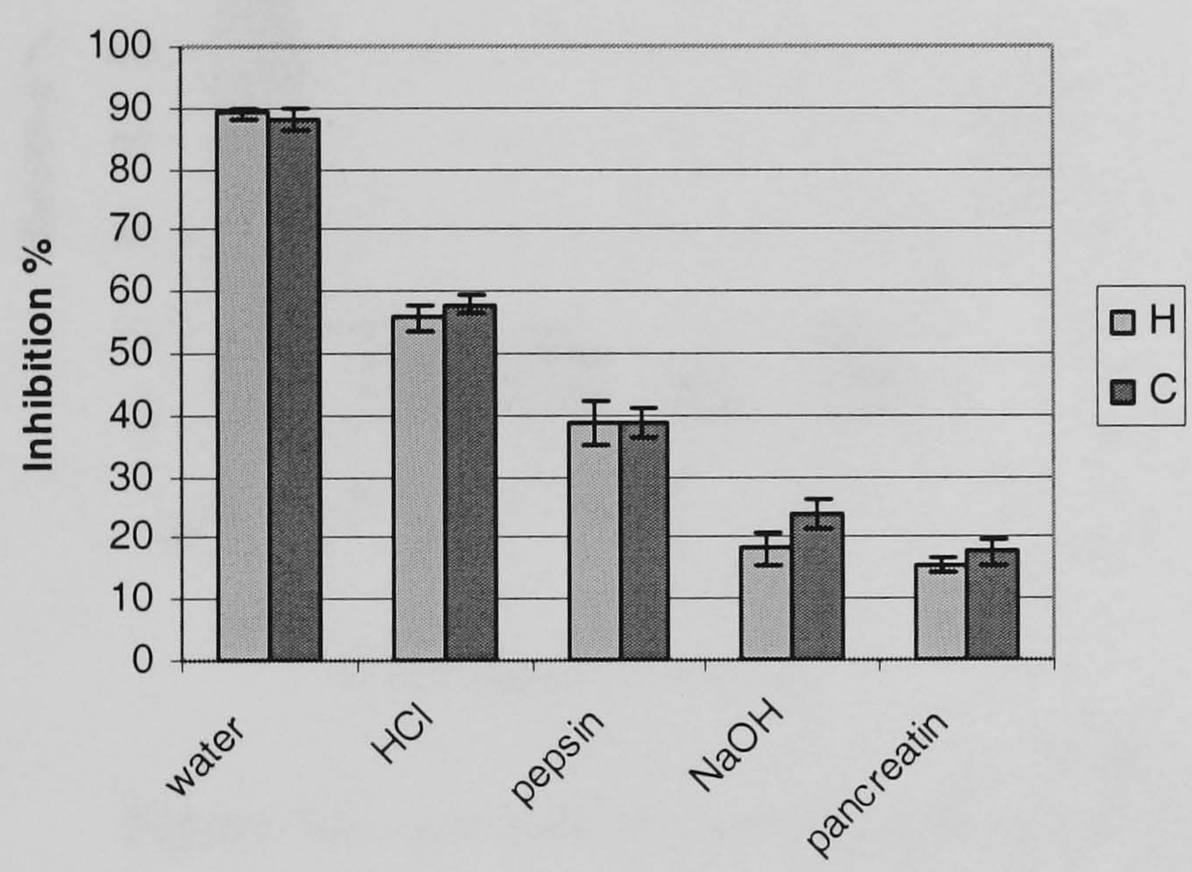
Group (c) plants H-J

4.3.2 Anticholinesterase analysis:

The pattern of anti-BuChE activity for green tea (A) and *Q. robur* (B) extracts and anti-AChE activity for *Urginea maritima* (A) and *Syzygium aromaticum* (B) extracts through the whole *in vitro* procedure are shown in figure 4.5 and figure 4.6 respectively. The results show the gradual decrease of the inhibition of human BuChE enzyme activity during the enzymatic steps for green tea (from 89.95 to 20.54 %) and *Q. robur* extracts (from 89.04 to 15.52% and from 88.12 to 17.80 % for hot and cold extracts respectively) (figure 4.5, A and B). In contrast to these results, the inhibition activity against BuChE enzyme completely disappeared following the addition of HCl solution (the second step of the digestion) for all the remaining samples tested (results not shown). Similarly, most of the plant extracts that were tested against AChE showed no activity after adding HCl solution (*M. communis* the hot and the cold extracts and the hot extract of green tea) or NaOH solution (the cold extract of green tea) (results not shown). Exceptions to this are shown in figure 4.6. The inhibition activity of the hot and the cold extracts of *U. maritima* during the enzymatic steps was more varied and showed a sharp decrease in the inhibition activity after adding HCl solution (from 83.33 to 12.77 % and from 83.10 to 37.16% for hot and cold extracts respectively) (Figure 4.6, A). The activity then increased after adding NaOH solution (from 9.44 to 48.97 % and from 41.21 to 69.59 % for hot and cold extracts respectively) and finally the activity decreased again at the last step (figure 4.6, A). The *S. aromaticum* extracts exhibited a gradual decrease in the inhibition activity during each step of *in vitro* digestion (figure 4.6, B).



A



B

Figure 4.5. Inhibition of human BuChE enzyme during *in vitro* enzymatic digestion steps by (A) Green tea (B) *Q. robur* extracts.

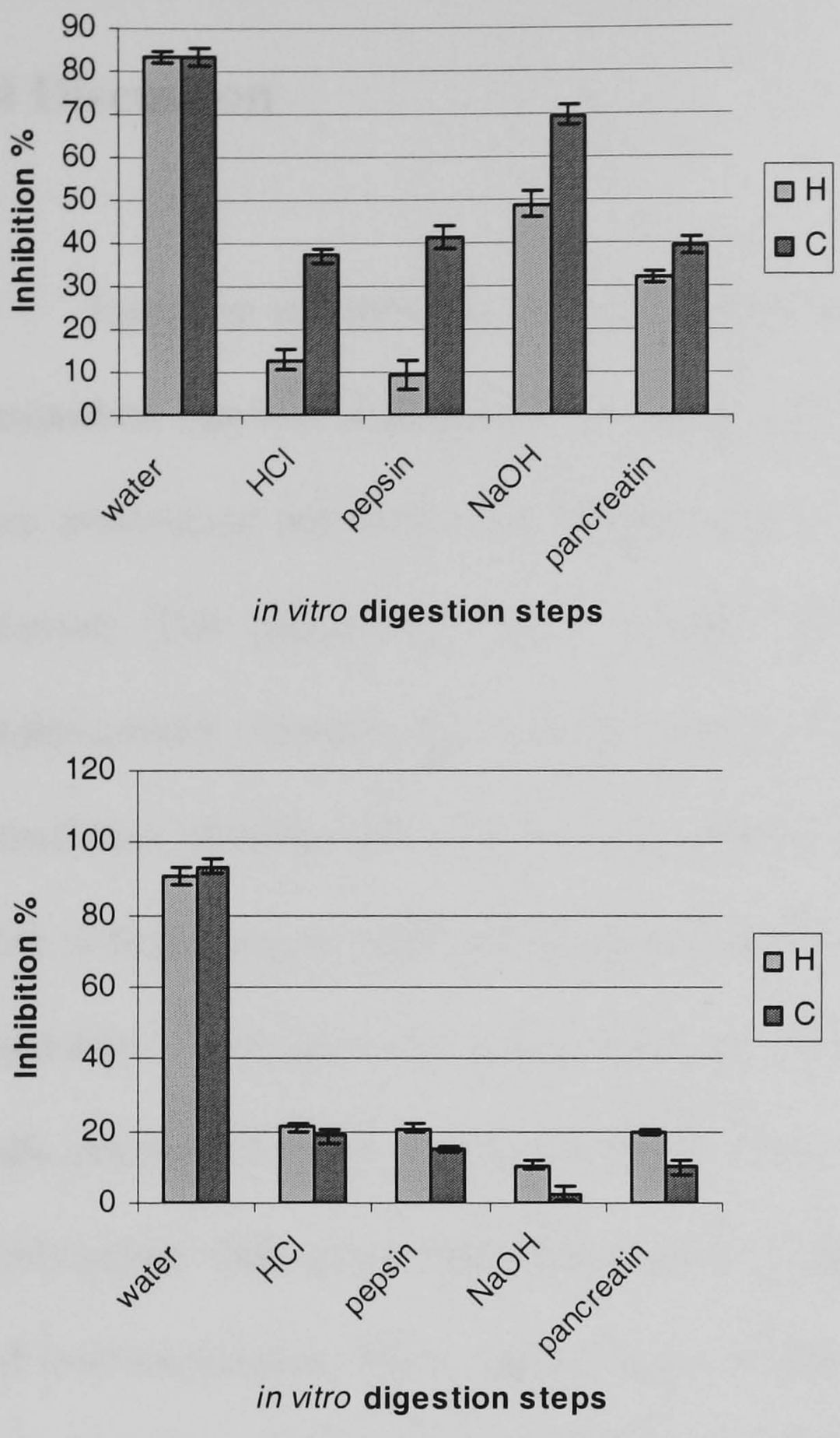


Figure 4.6. Anti-AChE activity during *in vitro* enzymatic digestion steps for (A) *Urginea maritima* (B) *Syzygium aromaticum* extracts.

4.4 Discussion

Intensive research has been conducted over the last 10 years to promote the antioxidant nutritional medicine in therapeutic fields. Plant extracts now known to have antioxidant activities were traditionally used to strengthen the natural immune defenses. The protective effects of diets rich in fruit and vegetables against cardiovascular disease and certain cancers have been attributed partly to the antioxidant vitamins and carotenoids contained therein (Serrano et al., 2005). Recent work is beginning to highlight the potential role of the phenolic components of fruit, vegetables, beverages and grains. The flavonoids, phenylpropanoids and phenolic acids present in these foods and drinks may act as antioxidants, or may act in combination with other constituents such as glucosinolates, indoles, dithiolthiones, and isothiocyanates. These agents may constitute alternative mechanisms that may contribute to the anticarcinogenic or cardioprotective actions. Many *in vitro* studies demonstrate the free radical scavenging properties of dietary phenolic phytochemicals. These valuable compounds might be destroyed in the gastrointestinal tract and lose their activity even before they are absorbed. In the present work, the medicinal plant species were selected in accordance with the results of the previous experiments (Chapter 2 and Chapter 3). According to the antioxidant activity of the plants, they were divided into three categories: group (a), high antioxidant; group (b), moderate antioxidant and group (c), low antioxidant. Four plant species from group (a) *Camellia sinensis; Myrtus communis*; *Quercus robur* and *Syzygium aromaticum* and three from (b) *Olea europaea; Matricaria chamomilla* and *Hibiscus sabdariffa* and (c) *Alhagi maurorum; Urginea maritime*

and *Zingiber officinale* were selected for the experiments of antioxidant release during *in vitro* digestion. Only the plant extracts that represented acceptable high anticholinesterase activity were chosen for *in vitro* antiochinesterase release experiments.

Within biological systems there are various sources of antioxidant including enzymes (such as superoxide dimutase, glutathione peroxidise and catalase), large molecules (like albumin and ferritin), small molecules (uric acid and polyphenols), some hormones (melatonin and estrogen) and molecules of dietary origin such as vitamin C, carotenoids, flavonoids, etc. Furthermore, both oxidants and antioxidants may have different chemical and physical features and antioxidants themselves may respond in different manners to different radical or oxidant sources. Consequently, the use of more than one assay (FRAP, TEAC and DPPH) to assess the antioxidant activity is appropriate. In addition, these assays themselves are sensitive and responsive to different antioxidant molecules. Thus in a complex system (such as the digestive tract or blood) a combination of different assays should provide a balanced evaluation of antioxidant capacity or potential.

As shown in figure 4.2 the apparent antioxidant remained constant under acidic conditions and following partial protein hydrolysis then decreased under the alkaline conditions. This shows that the stability of some phenolic compounds is highly influenced by the pH and the intestinal medium produces considerable changes. These results were consistent with those reported by Martinez-Ortega et al.

(2001) and Serrano et al. (2001, 2007). This means that potentially most antioxidants would be available in the first stage of the digestion process, and numerous studies have shown that the bioavaliability of bioaccessible antioxidant in the small intestine can be very low, for instance, for polyphenols (Clifford, 2004) and for β- carotene (Serrano et al., 2005, Granado et al., 2006, Novotny et al., 2005, McDougall et al., 2005). In the case of *H. sabdariffa* and *A. maurorus* extracts (hot and cold) the increased FRAP values at the end of the digestion step (after 90 minutes) (figure 4.2, G and H) could be explained by the ability of plant phenols to complex with protein by hydrogen bonding and the subsequent release of these antioxidants during protein hydrolysis (Duodu et al., 2003, McDougall et al., 2005). In general, the initial release of antioxidant in water confirms the presence of water soluble compounds which are probably in a free form within these extracts.

In contrast, the results obtained by the TEAC assay (figure 4.3) and the DPPH assay (figure 4.4) showed significant release of antioxidant capacity at the final step of the *in vitro* digestion procedure (after 90 min) in most extracts tested. Many flavonoids occur in plants in the glycosidic form and need to be heated with HCl to release the free flavonoids (Harborne, 1984). For instance, there are more than a hundred different glycosides of quercetin alone which have been described. In addition, it was clearly demonstrated that some polyphenols are normally only detected after acid or alkaline hydrolysis of plant tissue (Harborne, 1984). This could explain the high release of antioxidant capacity after addition of the HCl in all plant extracts with high antioxidant activity (group a) (figure 4.3, A, B, C and D and figure 4.4, A, B, C and D). On the other hand, the plant extracts representing the moderate

and low antioxidant activity (group b and c) exhibited approximately the same pattern in the increased release of antioxidant activities (which was constant during the acidic conditions, partial protein hydrolysis and under the alkaline conditions) after the addition of pancreatin. This suggests that some antioxidants may have been bound or associated with the carbohydrate fraction of the soluble plant material and there is the potential for the release of antioxidants and increased availability for absorption in different regions of the intestine as well as providing protection from oxidative damage within the gut lumen and intestinal mucosa itself (Nagah and Seal, 2005, Serrano et al., 2007, Record and Lane, 2001). In general, the results suggest that the variation in antioxidant activity detected by the three assays are due to the structure and chemistry of the individual polyphenols, their ability to chelate metal and scavenge free radicals as well as the chemistry involved in the antioxidant analysis. No data has been found in the literature regarding the *in vitro* digestion of these plant extracts tested herein (except for green tea) (Record and Lane, 2001). Thus this is the first report and there is, therefore, scope for much further investigation of these plant species.

Numerous plants and their constituents with pharmacological activity that are traditionally used for the treatment of cognitive disorders including anticholinesterase have been reviewed (Howes and Houghton, 2003, Perry et al., 2003, Howes et al., 2003). Plant alkaloids such as galantamine are best known for inhibiting cholinesterase enzymes. However, recent publications demonstrate new classes of cholinesterase-inhibiting phytochemicals such as terpenoids, especially monoterpenes such as 1,8-cineole and camphor (Perry et al., 2000, Perry et al., 2003, Greenberg-Levy et al., 1993) and coumarins (Howes and Houghton, 2003). Such findings indicate that plants may yield novel ChE inhibitors from these classes. It is

well established that in the majority of cases alkaloids contain at least one nitrogen atom. The compounds are generally basic, and this means that salt formation can occur in the presence of acidic conditions. However, the use of very low pH such as in the stomach may result in hydrolysis of ester groups if these are present in the alkaloids. This could explain the loss of inhibitory activity of most of the plant extracts against human AChE and BuChE enzymes during the *in vitro* digestion procedure. For example, as shown in Figure 4.5 and Figure 4.6 the inhibition activities were gradually decreased after addition of the HCl solution (i.e. at the acidic conditions). To the best of the knowledge of the author, there are no previous reports dealing with anticholinesterase activity of plant extracts during *in vitro* digestion.

In summary, these plants are rich in interesting mixtures of polar polyphenols with potential antioxidant activity. Some of these plant extracts proved to be resistant to gastrointestinal treatment and released more antioxidants. The apparent variation in the stability of these extracts under gastrointestinal conditions seems to be highly depending on the nature and chemistry of the polyphenols that exist in the extract. The anticholinesterase data indicate that these plant extracts are very susceptible to digestive proteolysis and therefore they do not display any activity against human AChE and BuChE enzymes. Further detailed studies including fractionation, biochemical analysis, toxicity and metabolism are needed to better evaluate the potential use of these extracts.

Chapter 5 Screening of antioxidant and anticholinesterase potential of selected traditionally used cacti and succulents.

5.1 Introduction

Cacti are a group of plants found in habitats which experience regular dry periods and make up a coherent botanical family, the Cactaceae (Haustein, 1991). Typical cacti are xerophytes, mostly with succulent columnar or spherical stems bearing leaves which are rudimentary or modified into spines, and areoles, which are modified side shoots found in the leaf axils and are diagnostic structures for the family. The Cactaceae are an almost exclusively American taxon with a centre of distribution in Mexico, and are found as far north as Canada and south as Patagonia. A few epiphytic cacti have been found in Africa, but these are generally believed to have arrived there as a result of human or animal activity (Rowley, 1978).

Xerophytes (cacti and other succulent plants) are found in regions such as mountains, deserts, semi-deserts and arid scrublands which feature a definite dry season during which the plant becomes dormant. Time and the ecological changes which have driven the evolution of xerophytes have resulted in such plants having a range of structural and temporal features which enable them to utilize habitats of high environmental stress. Most cacti grow in areas with low rainfall, long dry periods and intense sunlight. Certain highly characteristic adaptations make it possible for them to endure these extreme environmental conditions without damage (Rowley, 1978).

Adaptations observed in xerophytes to the pressures of harsh, seasonally arid environments include morphological adaptations to accommodate water-storage tissues and reduced surface to volume ratios of the plants, seen in the thickened globular or thick columnar habits of many xerophytes. Particularly cacti possess a great reduction in the number of stomata in the epidermis as compared with mesophytic species. In addition, many other adaptations are found such as the recessing of stomata or development of coverings of hair or scales by stomata (resulting in decreased water loss). Also secretion of waxes by the outer epidermis, absence of leaves or their reduction to spines (which act as condensation foci and deter herbivores) and Crassulacean acid metabolism (CAM) which enables the plants to fix carbon at night when water loss can be minimised are considered as potential mechanisms to survive (Haustein, 1991, Rowley, 1978).

A small number of species of cacti are used in traditional Native American medicine systems as pain killers, especially for the skin to treat burns and other wounds, where they are thought to be able to speed up the healing process and reduce the infection risk, and as ritual intoxicants and hallucinogens ('entheogens') (Wu et al., 2006). Numerous cacti contain a variety of alkaloids. In addition, an effective heart stimulant is extracted from the stems of *Selenicereus grandiflorus* and used mainly in England and North America (Haustein, 1991). Furthermore, their juices are used as beverages or used in food flavourings and colouring (Kuti, 2004). It is well established that *Aloe vera* extracts possess different biological activities such as anti-inflammation, anti-cancer, anti-diabetes and macrophage activation (Reynolds and Dweek, 1999). Cactus pear fruits (*Opuntia ficus-indica*) attract great

interest because of their nutritional and antioxidant properties (Kuti, 2004). A recent study has confirmed the antioxidant properties of *Aloe vera* (Wu et al., 2006).

Interest in antioxidants has grown during the late 20th and early 21st centuries because of their putative anti-ageing properties and the needs of industries which use natural products and to prolong storage times and shelf-lives of their products. Xerophytes could reasonably be thought of as possible sources of known and novel antioxidants; sources conceivably richer than might be found in plants native to more mesic habitats. These plants may have other uses yet to be described in this comparatively little-known area of ethnobotany. Consequently, there has been an increased interest to discover and search for "new" compounds with an antioxidant potential from natural sources. Therefore, a range of xerophytic species (*Senecio saginata, Echinocereus cucumis, Ephedra nootkatensis, Opuntia stricta, Euphorbia mammillaris* and *Euphorbia inermis*) were assayed for their antioxidant and anticholinesterase activities.

5.2 Materials and methods

5.2.1 Plant materials:

5.2.1.1 Plant collection and identification:

The plant species used in this experiment (Table.5.1) were obtained from the permanent plant collection of Moorbank Botanical Garden, University of Newcastle upon Tyne, UK and verified by Dr G. Wake.

5.2.1.2 Plant extract:

Plant extracts in either hot or cold water (hot means freshly boiled water and cold means room temperature water) were prepared as follows:

5.2.1.2.1 Hot water extract:

One gram of each fresh shoot material was homogenised (ULTRA-TURRAX® T25 basic, IKA®-Werke) with boiling de-ionized water (1:20 w/v) in a conical flask and shaken for 30 minutes at 150 strokes per minute (Grant Instruments Cambridge Ltd, Cambridge, UK). The extracts were filtered through Whatman filter papers (1 circle 70mm Ø) then the filtrates were centrifuged (2500×g, 10 min) (Mistral 3000i centrifuge, MSE scientific Instruments, Crawley, UK) to remove insoluble matter, and the supernatants retained to be used for antioxidant and anticholinesterase analysis

5.2.1.2.2 Cold water extract:

One gram of each fresh shoot material was homogenised (ULTRA-TURRAX® T25 basic, IKA®-Werke) with cold de-ionized water (1:20 w/v) in a

conical flask and shaken for 24 hours at 150 strokes per minute (Grant Instruments Cambridge Ltd, Cambridge, UK). The extracts were filtered through Whatman filter papers (1 circle 70mm Ø) then the filtrates were centrifuged (2500×g, 10 min) (Mistral 3000i centrifuge, MSE scientific Instruments, Crawley, UK) to remove insoluble matter, and the supernatants retained for antioxidant and anticholinesterase analysis.

Because natural drying takes so long, weighed fresh material was used for extraction and a weighed small sample of tissue was freeze dried to constant weight giving a dry weight. This was used to calculate the dry weight of the tissue used in extract preparations.

Table 5.1 A list of the selected cacti and succulents used in this study.
http://herb.umd.umich.edu/

Scientific name	Original location	Medical uses
Senecio saginata	Yemen	Bleeding, high blood pressure, diabetes.
Senecio saginata	S. Africa	Bleeding, high blood pressure, diabetes
Echinocereus cucumis	Mexico	Urinary tract infection
Ephedra nootkatensis	N. America	Asthma , bronchitis
Opuntia stricta	Argentina	Diabetes, Cholesterol, immune system.
Euphorbia mammillaris	S. Africa	Colds, asthma, bronchitis, stomach
Euphorbia inermis	S. Africa	Colds, asthma, bronchitis, stomach

5.2.2 Chemicals:

The chemicals used in these experiments, acetylthiocholine iodide (ATChI), butyrylthiocholine iodide (BuTChI), 5,5′-dithiobisnitrobenzoic acid (DTNB), butyrylcholinesterase (BuChE. E.C.3.1.18, from human serum), acetylcholinesterase (AChE, E. C.3.1.1.7, from human erythrocytes), sodium bicarbonate, 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) powder $ABTS^{+}$, potassium persulphate, (+/-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), ferrous sulphate, ferric chloride, 2,4,6 tripyridyl-s-triazine (TPTZ), 1,1′-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma Chemical Company, UK. Buffers and other chemicals were of analytical grade. All other solvents, salts and reagents were obtained from VWR International, Country Durham, UK.

5.2.3 Sample analysis:

5.2.3.1 Antioxidant analysis:

The antioxidant assays (FRAP, TEAC and DPPH) were carried out using the COBAS Mira clinical analyser (Roche Diagnostics, Welwyn Garden City, Herts), as previously described in chapter 2 sections 2.2.3.1, 2.2.3.2, 2.2.3.3, respectively.

5.2.3.2 Anticholinesterase analysis:

This assay was preformed using a Titertek Multiskan MCC/340 microplate reader. The procedure was previously described in 3.2.3.

5.2.4 Statistical analysis:

The experiments were carried out in triplicate. The results are given as mean ± standard deviation (SD). The data of antioxidant activity for each assay were analysed by one-way analysis of variance (ANOVA), and for comparison with green tea extract (standard antioxidant reference), the Dunnett's post –test was used which is designed to compare several treatments with one control treatment (Ljubuncic et al., 2005, Arredondo et al., 2004). A difference was considered statistically significant when $p< 0.05$. All statistical tests were completed using Minitab version 14.0 and Microsoft Excel.

5.3 Results

5.3.1 Antioxidant analysis:

5.3.1.1 FRAP assay

In this study, the water phase antioxidant activity of plant extracts (hot and cold) produced from different cacti and succulents in comparison with the well established antioxidant properties of green tea was tested. As shown in Table 5.2 and Table 5.3, the FRAP values varied from 855 to 170569 and 572 to 145147 (µmol Fe^{2+} E/g dried weight) for hot and cold water extracts, respectively. The hot and cold extracts of *Euphorbia mammillaris* showed very high FRAP values as compared with green tea (highly significant, p values less than 0.0001), fourteen and three times higher for hot and cold extracts respectively (figure 5.1). The *Echinocereus cucumis* extracts exhibited moderate activity with FRAP values of 10860 and 41982 (µmol Fe^{2+} E/g dried weight) for hot and cold extracts respectively. The remaining plant extracts (hot and cold) measured by this method showed a low activity.

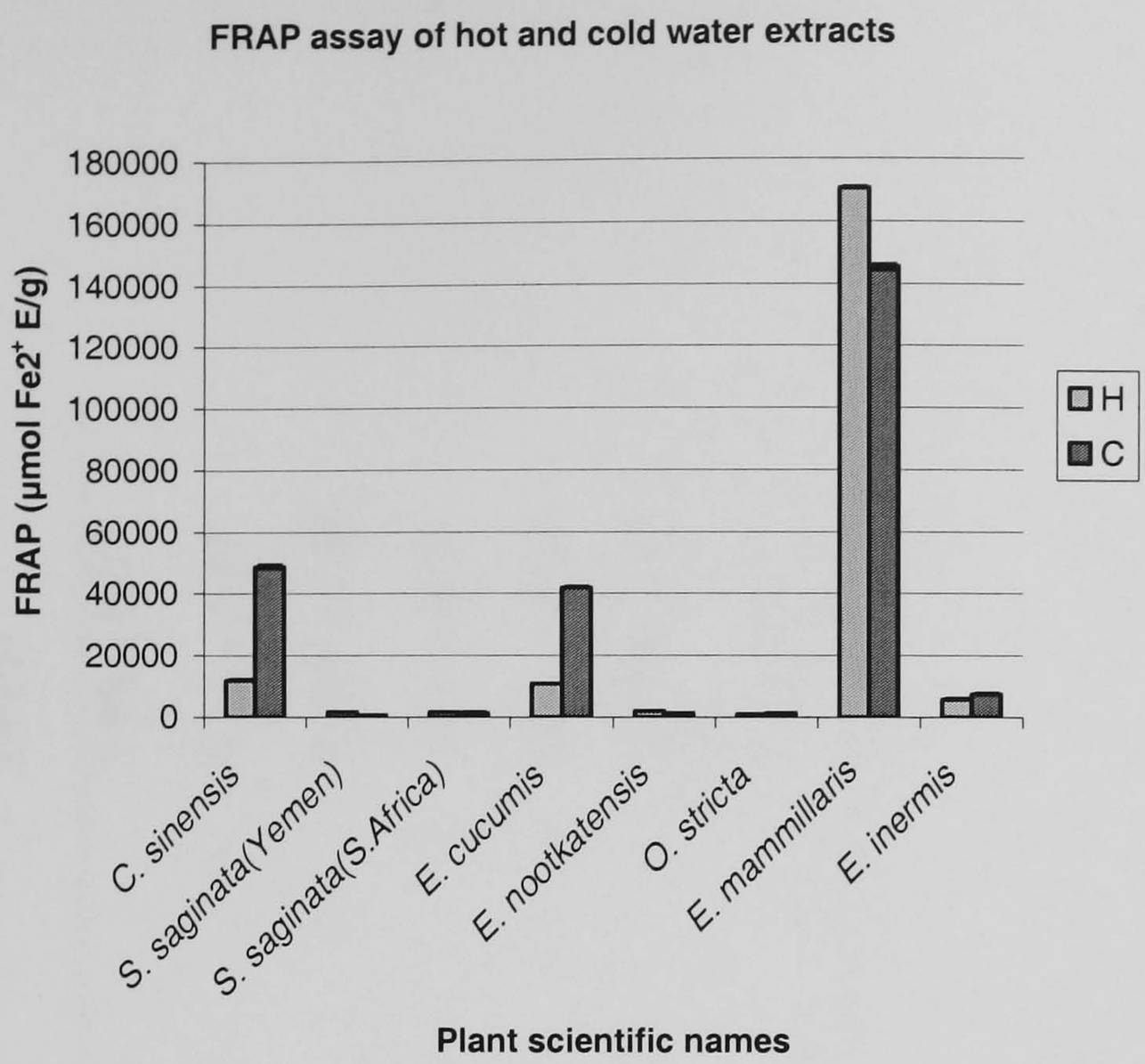


Figure 5.1 FRAP assay of hot and cold extracts.

Table 5.2. Water soluble antioxidant capacity of cacti and succulents hot water extracts.

Scientific name	FRAP	TEAC	DPPH
Senecio saginata (Yemen)	1704 ± 32	2.39 ± 0.07	3.99 ± 0.07
Senecio saginata (S.Africa)	1550 ± 17	2.01 ± 0.05	3.18 ± 0.27
Echinocereus cucumis	10860 ± 80	17.66 ± 0.06	11.95 ± 1.05
Ephedra nootkatensis	2059 ± 67	4.07 ± 0.05	2.61 ± 0.16
Opuntia stricta	855 ± 27	1.19 ± 0.04	6.64 ± 0.04
Euphorbia mammillaris	170569 ± 262*	39.06 ± 0.11	18.50± 0.48
Euphorbia inermis	5933 ± 117	5.40 ± 0.03	5.11 ± 0.22

Data expressed as mean ± SD, (n = 3)

FRAP (μmol Fe^{2+} E/g dried weight).

TEAC (mmol Trolox E/g dried weight).

DPPH (mmol Trolox E/g dried weight).

* Extremely statistically significantly higher than the green tea (the standard antioxidant), $P<0.0001$.

Table 5.3. Water soluble antioxidant capacity of cacti and succulents cold water extracts.

Scientific name	FRAP	TEAC	DPPH
Senecio saginata (Yemen)	572 ± 18	1.17 ± 0.02	3.52 ± 0.18
Senecio saginata (S.Africa)	1531 ± 29	1.75 ± 0.02	3.11 ± 0.11
Echinocereus cucumis	41982± 124	18.54 ± 0.03	42.51 ± 1.41*
Ephedra nootkatensis	1248 ± 25	2.49 ± 0.02	2.70 ± 0.06
Opuntia stricta	1224 ± 56	1.61 ± 0.02	6.79 ± 0.11
Euphorbia mammillaris	145147±78*	38.69 ± 0.06*	30.91 ± 0.53*
Euphorbia inermis	7619 ± 89	6.92 ± 0.09	7.62 ± 0.09

Data expressed as mean ± SD, (n = 3)

FRAP (μmol Fe^{2+} E/g dried weight).

TEAC (mmol Trolox E/g dried weight).

DPPH (mmol Trolox E/g dried weight).

* Extremely statistically significantly higher than the green tea (the standard antioxidant), $P<0.0001$

5.3.1.2 TEAC assay

The water soluble antioxidant activities of hot and cold extracts produced from the selected cacti and succulents were studied in comparison with green tea, the established antioxidant reference. The results obtained by this assay are summarised in Table 5.2 and Table 5.3, and figure 5.2. The antioxidant power of cold extracts of *Euphorbia mammillaris* was found to be higher than that of green tea ($p < 0.0001$), while the hot extract of this cactus exhibited a TEAC value (39.06 mmol Trolox E/g) which was almost the same as that obtained from the green tea (39.46 mmol Trolox E/g). Both extracts of *Echinocereus cucumi* exhibited moderate ABTS scavenging potential, although this potential was not as high as that for green tea (figure 5.2). Low antioxidant activities (values lower than 7 mmol Trolox E/g), were determined for the remaining plant extracts.

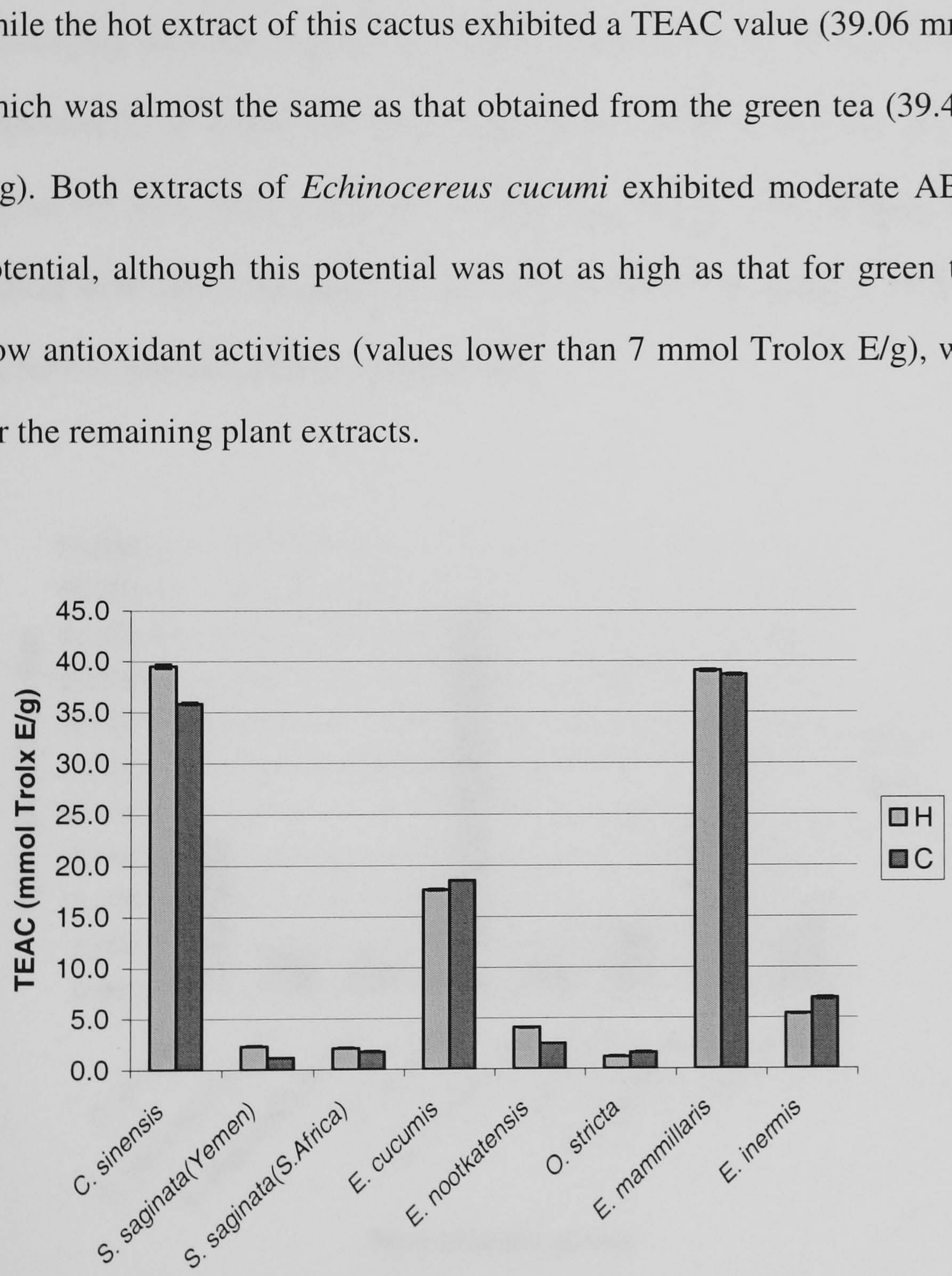


Figure 5.2 TEAC assay for hot and cold plant extracts.

5.3.1.3 DPPH assay

Results of DPPH reduction by extracts are shown in Table 5.2 and Table 5.3. As can be seen in figure 5.3, the cold extracts of *Euphorbia mammillaris* and *Echinocereus cucumi* exhibited high DPPH radical scavengening activities which were significantly higher than that of the positive control ($p<0.0001$). The hot *Euphorbia mammillaris* and *Echinocereus cucumis* extracts also showed moderate scavenging activities against the DPPH free radical (18.50 and 11.92 mmol TE/ g, respectively) as compared with the green tea (19.43 mmol TE/ g) (figure 5.3). The remaining plant extracts showed a slight scavenging activity against the DPPH free radical with values ranged from 2.61 to 6.64 mmol TE /g and 2.70 to 7.62 mmol T E / g for hot and cold extracts respectively.

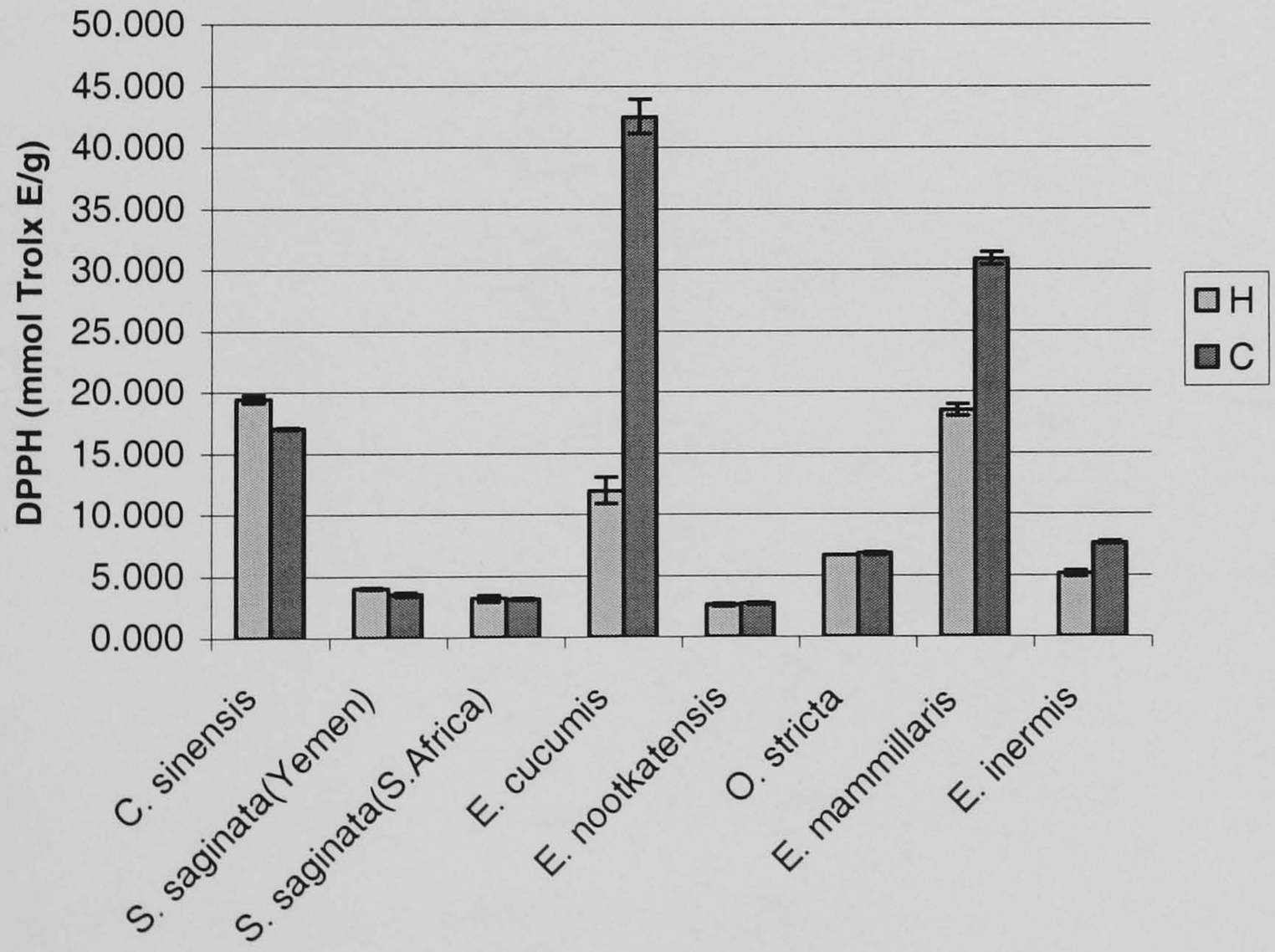


Figure 5.3 DPPH assay for hot and cold extracts.

5.3.2 Anticholinesterase analysis:

The inhibitory activities of hot and cold extracts of selected cacti and succulents were tested against both AChE and BuChE enzymes. None of the plant extracts tested showed inhibitory activity against either enzyme (results not shown).

5.4 Discussion

Extremes of temperature, harsh soil chemistry alone or in combination with osmotic effects caused by water insufficiency might reasonably be expected to result in promotion of undesirable protoplasmic chemistry events such as molecular breakage and generation of reactive free radicals at high rates. Therefore, strong defence systems are expected to exist in xerophytes to deal with such events and conditions. Such enhancement could reasonably be imagined to include high-activities of systems which deal with problems such as DNA repair, photorespiration, wound repair, and neutralisation of oxidative free-radicals.

In this study, six medicinal cacti and succulent species (*Senecio saginata; Echinocereus cucumis; Ephedra nootkatensis; Opuntia stricta; Euphorbia mammillaris* and *Euphorbia inermis*) with traditional uses for the treatment of various disorders, where the free radicals are thought to be implicated were investigated. The extracts from these plants were screened for their antioxidant activity using the three most popular methods (FRAP, TEAC and DPPH) which had been used previously in this thesis in the assessment of medicinal plants of Libyan origin. The results (Table 5.2, 5.3) show that of these extracts, the crude extracts (hot and/or cold) of *E. cucumis* and *E. mammillaris* exhibited the highest antioxidant activity in all assays used in this study (FRAP, TEAC and DPPH). As shown in figure 5.3, the cold extracts possessed higher activity than those obtained using hot water. The differences in the activity of these extracts (the cold and the hot) can be explained by the loss of certain phytoconstituents which are probably because of the difference in temperature of the extraction procedures (Velioglu et al 1998; Gazzani

et al 1998; Sun & Ho 2005), and possible breakdown or chemical modification of these compounds during the intial extraction with boiling water. Furthermore, the length of the extraction process could be considered as a critical factor in explaining possible differences between the results of hot and cold extracts. The hot extraction is a much shorter extraction, so although there is potential for rapid solubilisation, thereafter the plant material is only in the water for short time (30 min). In contrast, the cold extraction leaves the material in the water for a long time (24 h). This may mean that eventually even poorly soluble materials may be extracted from the plant material. In another words, the soaking temperature and soaking time that were applied for these extract preparation have a vital effect on antioxidant activities of these cacti. Similarly, a previous study by Zhou et al. (2000) showed that antioxidant activities of teas are highly dependent on their soaking temperature and time.

Low antioxidant activities of the remaining plant extracts could be explained by the following reasons. Firstly the plants used in this study were grown at Moorbank Botanical Garden and they might not have been exposed to high stress levels which would be found in the wild environment as described in the introduction (section 5.1). Secondly they are perhaps healthy plants and may not need to produce high antioxidant levels. Finally the extraction procedure of water–heavy tissues may not be ideal. Rather than looking for reasons that perhaps the antioxidant activity of cacti was suppressed in some way, an alternative hypothesis could be that cacti do not produce antioxidants which can be detected by the FRAP, TEAC or DPPH methodology as perhaps they do not, after all, produce antioxidants in high quantities. The results were consistent no matter which assay was employed (FRAP, TEAC and DPPH). This is an interesting observation as most plants previously tested

showed varying antioxidant activities depending upon the assays used (Katalinic et al., 2006, Miliauskas et al., 2004a, Serrano et al., 2007, Nagah and Seal, 2005). For example, Miliauskas et al. (2004b) showed that extract of *Echinacea purpurea* exhibited apparently higher antioxidant capacity using the ABTS test than when the same extract was assayed using DPPH assay.

Although the cacti contain a lot of alkaloids they did not show any inhibitory activities either against human AChE or against BuChE enzymes. There appears to be no direct correlation between antioxidant and anticholinesterase activities in cacti. These results are consistent with those of Godkar et al. (2006), who reported that seed oil of *Celestrus panicultus* exhibits a potent antioxidant capacity in the DPPH assay and showed no inhibitory activity against AChE at all. This will be discussed further in the general discussion (section 6).

In conclusion, the data on extraction procedures and antioxidant activity assessement obtained in these experiments single out *E. cucumis* and *E. mammillaris* as the most promising sources of natural antioxidants. Purification and identificaltion of the active compounds in these cacti is required for a better understanding of the protective mechanisms involved and for possible thereapeutic application.

Chapter 6 General Discussion

Free radicals can be defined as any molecules or molecular fragments which contain one or more unpaired electrons (Halliwell et al., 1995, Halliwell and Gutteridge, 1999, Parsons, 2000, Halliwell, 2000). These molecules of unpaired electrons usually confer a considerable degree of reactivity upon free radicals. Those derived from oxygen represent the most vital class of such species generated in living systems (Valko et al., 2006). Reactive Oxygen Species (ROS) are highly reactive and potentially damaging transient chemical species created in all cells through various physiological and biochemical process (such as activation of phagocytes, mitochondrial respiration, and biosynthesis of endoperoxide) as undesirable metabolic by-products of normal aerobic metabolism (Rice-Evans and Miller, 1994, Parke et al., 1991, Rice-Evans, 2000). In addition to ROS from endogenous sources, ROS can also be produced by a host of exogenous processes such as environmental agents (pollutant gases) (Scott, 1997, Valko et al., 2006). Free radicals have the potential to damage intracellular organelles and components such as nucleic acids, lipids and proteins on which normal cell function depends. Free radical oxidation damages the outer membrane of a cell, and then continues into the cell where DNA and intracellular molecules may be affected. Thus, if the damage is to DNA, any damage to the cell will be replicated each time during cell division (Valko et al., 2004, Halliwell and Gutteridge, 1999, Hillestrom et al., 2006). Molecular mechanisms of ROS toxicity are mainly through oxidation of vital thio - compounds to disulphate, loss of tissue GSH (glutathione), impairment of energy generation (ATP, NADH, and NADPH), oxidation of cytoplasm, inhibition of Ca^{2+} transport

and electrolyte homeostasis, DNA cleavage and the initiation and promotion of mutations and carcinogenesis (Parke, 1999).

Research in recent years has shown the importance of ROS in degenerative processes related to aging (Ames et al., 1993) and diseases such as coronary heart disease, chronic renal failure, diabetes mellitus, immune dysfunction, atherosclerosis, cancer and neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Halliwell, 1994, Arts and Hollman, 2005, Ames, 1983, Arredondo et al., 2004, Young and Woodside, 2001, Halliwell, 1996, Halliwell, 2002, Halliwell et al., 1992, Chisolm and Steinberg, 2000, Morello et al., 2002, Basu et al., 1999, Wiseman and O'Reilly, 1997, Vanharanta et al., 2003).

Exposure to free radicals from internal and external sources has led organisms to develop a series of biological defence mechanisms against oxidative damage induced by ROS (Valko et al., 2007). The biological defence systems involve preventative mechanisms, repair mechanisms and physical defences. These mechanisms are dependent upon antioxidants. Antioxidants can be defined as "any substance which when present at low concentrations compared with those of an oxidizable substance, significantly delays or prevents a pro-oxidant initiated oxidation of that substrate" (Halliwell, 1995, Kumaran and Joel Karunakaran, 2007). Thus antioxidants play the housekeeper's role, "mopping up" free radicals before they get a chance to cause injury. Under normal physiological conditions, there is a balance between both the activities and intracellular concentration of antioxidants. However, cellular injury resulting from the imbalance between the free radical generating and scavenging system resulting in "oxidative stress", has been implicated

in the pathogenesis of a variety of disorders. Among the complex array of endogenous defences are radical scavenging proteins, enzymes, numerous endogenous antioxidant factors which include coenzyme Q, glutathione (GSH) and other tissue thiols, metal ion sequestering proteins such as oxidizing (Fe^{2+}) to (Fe^{3+}), ROS metabolising enzymes such as superoxide dismutase, and catalase. In addition, there are other important antioxidant compounds including α-tocopherol (vitamin E), ascorbic acid (vitamin C), uric acid, and the histidine- containing dipeptides carnosine and anserine (Hussin et al., 2007). Besides these compounds, there are a variety of plant-derived dietary compounds which act as antioxidants such as carotenoids and flavonoids (Prenesti et al., 2007, Montoro et al., 2005, Yu et al., 2005).

Antioxidant activity cannot be measured directly but rather by the effects of antioxidants in a reaction medium in controlling the extent of oxidation. A wide range of methods using this principle are currently used to assess antioxidant capacity in food, botanicals, nutraceuticals and other dietary supplements. Although there is a great multiplicity of methods used for antioxidant testing, none of them provide an ideal individual, approved, standardized reference method, as both oxidants and antioxidants may have different chemical and physical features. Furthermore, antioxidants may respond in different manners to different radical or oxidant sources (Prior et al., 2005). In general, most of the methods are based on the generation of the radical in the assay and the antioxidant response in the sample against the radical is measured. Antioxidant capacity estimation is assay dependent. The specificity and sensitivity of one method does not lead to complete examination of all phenolic compounds and antioxidants in the extracts. Therefore, a combination

of several methods *in vitro* provides a more reliable assessment of antioxidant activity. The most widely and frequently used assays among these are ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996, Benzie and Strain, 1999) (μmol ferrous ion equivalents); Trolox equivalent antioxidant capacity (TEAC) (Re et al., 1999) (mmol Trolox equivalents) and scavenging activity of diphenyl-2- picryl-hydrazyl (DPPH) radicals (Brand-Williams et al., 1995, Blois, 1958, Koleva et al., 2002) (mmol Trolox equivalents).

The FRAP assay is a method of antioxidant activity evaluation based on redox reactions. It is quick and simple to perform and it is a reasonable screen for the ability to maintain redox status in cells or tissues. Reducing power appears to be related to the degree of hydroxylation and extent of conjugation in polyphenols (Zaporozhets et al., 2004). The FRAP mechanism measures electron transfer. However, it cannot detect compounds that act by radical quenching (hydrogen transfer), such as thiols and proteins (Prior et al., 2005). Thus it is helpful to use FRAP in combination with other methods. The TEAC assay gained popularity because it enables high-throughput screening on potential antioxidant capacity (Van den Berg et al., 1999, Re et al., 1999). This assay assesses the total radical scavenging capacity, based on the ability of a compound to scavenge the stable ABTS radical (Arts et al., 2004a, Arts et al., 2003, Arts et al., 2004b). The DPPH$^{\cdot}$ system is a stable radical-generating procedure. It can be used to assay a large number of samples in a short period of time, and is sensitive enough to detect active compounds even at low concentrations and thus it was used in the present study. In the DPPH$^{\cdot}$ assay hydrogen–donating ability is an index of the primary chain-breaking antioxidant. These antioxidants donate hydrogen to free radicals which are

then converted to non-radical species and thus inhibit the propagation phase of lipid peroxidation (Koleva et al., 2002, Apati et al., 2003). In the results presented in this thesis, there were clear differences in appearent antioxidant capacities for the same samples when measured using different assay systems. Whilst, in many cases the relative responses were similar (i.e. high FRAP values correlated with high TEAC values) this was not always the case. This study, therefore, indicates that it is important to meaure the antioxidant activity by using various radicals and oxidation systems and to take into account both antioxidant and phenolic contents while evaluating the antioxidant potential of plant extracts. The model system consisting of a combination of FRAP, TEAC and DPPH assays can, however, be used to screen large number of sources for their antioxidant capacity because of the relatively rapid through put of samples. Nevertheless, it is important to use an *in vivo* methodology to assess the real potential of an antioxidant to protect cells. Consequently, the present work should be complemented with further investigations.

Herbal medicine still represents a very important phenomenon in traditional cultures. However, the use of pharmaceutical products of plant origin is a growing area in the treatment of several illnesses and competes with popular medicine in many cultures. Furthermore, medicinal plants represent a vast role in the introduction of new therapeutic agents and have received much attention as a source of biologically active substances including antioxidants and anticholinesterase. Therefore, medicinal plants continue to be subjected to extensive screening worldwide in an attempt to develop and identify antioxidative and anticholinesterase compounds and this has become an important issue for pharmaceutical industries worldwide.

In the first experiments, twenty three medicinal plants, native to the Libyan region and traditionally used for the treatment of various disorders where free radicals are thought to be implicated were chosen. The plants tested and their medicinal uses are shown in Table 1.5 (Chapter 1). Based on the traditional way of ingestion of plant- derived antioxidant it was appropriate to study the water-soluble antioxidant capacity and phenolic content of herbal teas prepared from these Libyan medicinal plants. Plant extracts with either hot (freshly boiled water) or cold (room temperature water) were prepared simply in ways which mimic the traditionally used methods in Folk medicine (Triantaphyllou *et. al.*, 2001). The extracts from these plants were screened for their antioxidant activity using the three most popular methods (FRAP, TEAC and DPPH).

The results (Table 2.1, 2.2, 2.3, 2.4, Chapter 2) show that out of these twenty three extracts, the crude extracts (hot and cold) of *M. communis, Q. robur* and *S. aromaticum* exhibited the strongest antioxidant activity in all tests used in this study (FRAP, TEAC and DPPH) and were higher than that of the positive control, green tea, a product for which there is an extensive literature showing antioxidant and anticholinesterase properties. This suggests that for these particular extracts any of these methods will offer a reliable measurement of antioxidant capacity. On the basis of the results of this study, it is clear that these plant extracts have powerful antioxidant activity against various antioxidative systems *in vitro*. Moreover, these plants can be used as easily accessible sources of natural antioxidants for use as possible supplements or in a pharmaceutical application. The extracts that showed high TEAC values could contain substances that have a redox potential lower than

that of the marker radical ABTS$^{\cdot}$. Indeed, many phenolic compounds have low redox potentials and can thus react with ABTS$^{\cdot}$ to remove the singlet electron (Frankel et al 2000; Prior et al 2005).

Numerous studies have suggested that the potent antioxidant activities of green tea (Rechner et al., 2002, Campanella et al., 2003) are due largely to the polyphenolic content of the leaves. Consequently, it was reasonable to determine the total phenolic content in the selected Libyan plants. Again, *M. communis, Q. robur* and *S. aromaticum* showed the highest phenolic content among the examined extracts (figure 2.8, Chapter 2). In addition, the values of FRAP, TEAC and DPPH in both the crude extracts (the cold and the hot) were highly correlated with the content of total phenolics. The results from these experiments are consistent with previous reports in the literature (Gao et al., 2000, Arredondo et al., 2004, Tsai et al., 2002, Tepe et al., 2004, Katalinic et al., 2006, Duan et al., 2006, Sun and Ho, 2005). These authers found a good correlation between antioxidant capacity (by FRAP, TEAC and DPPH) and phenolic contents. For many extracts, phenolic compounds appear to be responsible for the antioxidant potential. In this sense, these plants rich in mixtures of polar phenols with potential antioxidant activity possibly provide a clue for the discovery of new therapeutic strategies in pathologies associated with oxidative stress- induced cell damage. In the case of phenolic compounds, the ability of the phenolics to act as antioxidants depends on the redox properties of their phenolic hydroxyl groups, which allow them to act as reducing agents, hydrogen-donated antioxidants or oxygen quenchers (Rice-Evans and Miller, 1996).

From the results of the antioxidant studies plant extracts were categorized as (a) high antioxidant activity, (b) intermediate activity and (c) low antioxidant activity (see Table 2.4, Chapter 2). Four plants from group (a), three plants from group (b) and three plants from group (c) were selected for further studies. These plants and their categories are shown in Table 6.1.

Table 6.1 List of the selected plants and their categories.

Antioxidant category	Scientific names	Principle constituents	Medicinal uses
(a) high	*Camellia sinensis*	Caffeine, theobromine, many minerals	Stimulant to the CNS, diuretic
	Myrtus communis	Tannins, resins, comphene, myrtenol, cineole, nerol, geraniol	Antidiabetic, astringent, in eczema epilepsy, wound and ulcers.
	Quercus robus	Tannins and quercitrin	Very astringent, used to treat haemorrhoids
	Syzygium aromaticum	Eugenol, α and β cariophyllins	Headaches, respiratory disorders
(b) intermediate	*Olea europaea*	Tannins, glycosides, choline, triterpene	show hypoglycemic activity, increases blood circulation and urine secretion and hypotensive
	Matricaria chamomilla	Tannins, azlene, anthemidine and matricin	Tonic, mild laxative, diuretic, antispasmodic, diaphoretic, carminative, urinary and respiratory antiseptic.
	Hibiscus sabdariffa	Vit. C, malic, tartaric and hibiscic acids, tannins, gossipetin and hibiscin	As a source of vit. C, laxative, diuretic, reduce blood pressure, mild laxative and intestinal antiseptic.
(c) low	*Alhagi maurorum*	Unknown	Diuretic and expectorant, treatment of rheumatism, mild laxative.
	Urginea maritime	Cardiac glycosides (scillaren A, B)	Heart diseases (very low doses)
	Zingiber officiale	Camphene, linalol and gingerol	Cardiotonic, pain relief

Cholinesterases (ChE) are members of the serine hydrolase family, so named because they utilize a serine at the active site. According to substrate specificities and susceptibility to inhibitors, this group of enzymes is divided into two major categories. The first category is plasma butyrylcholinesterase (BuChE) pseudo, or type II cholinesterase (Quinn, 1987, Godkar et al., 2006). This enzyme is found principally in plasma and it acts on a variety of choline phenyl, nitro phenyl and other types of esters. The second category is acetylcholinesterase (AChE), which is also called "red cell", true, specific or type I cholinesterase. AChE exists primarily in nerve cells and muscular junctions but it is also found in other neural and non neural cells. AChE is distinguished from BuChE by its greater specificity for hydrolyzing acetylcholine (ACh), the cationic neurotransmitter (Quinn, 1987, Giacobini, 2004). AChE plays an important role in terminating the nerve impulse in the cholinergic synapses by hydrolysis or breakdown of Ach into acetic acid and choline. Extensive inhibition of this vital enzyme leads to accumulation of the neurotransmitter ACh and enhanced stimulation of postsynaptic cholinergic receptors and prevent the smooth transmission of nerve impulses across the synaptic gap at nerve junctions (Quinn, 1987, Pope et al., 2005). This causes loss of coordination, convulsion and, ultimately, death. Therefore, AChE is a key component of cholinergic brain synapses and neuromuscular junctions (Pope et al., 2005). Because of the essential role that AChE plays in the nervous system, this enzyme has long been an attractive target for the rational design of mechanism-based inhibitors (Giacobini, 2004). Cholinesterase inhibitors have been used in the treatment of human diseases such as Alzheimer's disease (AD), the control of insect pests such as by organophosphate insecticides (OP), and more notoriously as chemical warfare agents. Most uses of cholinesterase inhibitors are based on a common mechanism of action initiated by inhibition of

acetylcholinesterase (AChE) (Pope et al., 2005). The therapy that has been used as a potential strategy to improve AD pathology was mainly by inhibiting AChE (Ezoulin et al., 2005, Ferreira et al., 2006, Howes and Houghton, 2003). It has been suggested that the inhibition of AChE and BuChE enzymes should be one of the objectives in the treatment of cognitive dysfunctions associated with AD (Giacobini, 2004, Okello et al., 2004). A number of novel natural inhibitors of cholinesterases (AChE and BuChE) isolated from different medicinally-important plants has been previously reported. For instance, new natural AChE and BuChE inhibitors were isolated from *Ajuga bracteosa*, *Withania somnifera* and *Sarcococca hookeriana* by Choudhary (2005a, 2005c). Consequently, in the second experiment of this work, the plant species of *Camellia sinensis; Myrtus communis; Alhagi maurorum; Urginea maritime; Olea europaea; Matricaria chamomilla; Hibiscus sabdariffa; Quercus robur; Syzygium aromaticum;* and *Zingiber officinale*, which have various ethnobotanical uses, were examied for their anticholinesterase (AChE and BuChE) activities.

Most the plant extracts analyzed exhibited various inhibitory activities toward both enzymes. According to their activities, plant extracts were divided into four categories (Table 3.3, Chapter 3). The first category includes the plants that did not show any inhibitory activities. The second category includes the plants that showed low activity (1- 25% inhibition); the third category contains the plants that exhibited moderate activity (25-50% inhibition) and the last category represented the plants which possess high activity (50-100% inhibition).

The results for hot extracts of green tea (*Camellia sinensis*) (Table 3.1) were 0.068 ± 0.001mg/ml and 0.066 ± 0.004mg/ml (IC_{50} values). Similar inhibitory activities were obtained from the hot extract of *S. aromaticum* with IC_{50} of 0.075 ± 0.003 mg/ml and 0.116 ± 0.007 mg/ml toward the AChE and BuChE enzymes respectively. Thus the extracts of these plants with single or dual anticholinesterase activity may be the most appropriate for clinical trials among the plant extracts investigated here. To the best knowledge of the auther, all the plant extracts screened and reported in this thesis showing high inhibitory activity (except green tea) are reported here for the first time as cholinesterase inhibitors.

Focusing on the possible correlations between antioxidant and anticholinesterase activities, excluding the *U. maritime* extracts, all the plant extracts that have been identified in this study to possess a noticeable inhibitory activity against either AChE or BuChE or both were found to have a potent antioxidant activity and presented in group (a) (Table 2.4, Chapter 2 and Table 3.3, Chapter 3). These interesting observations are consistent with a recent study by Ferreira et al (2006) who reported that the extracts of *Hypericum undulatum*, *Melissa officinalis*, *Laurus nobilis* and *Lavandula pedunculata* which showed high antioxidant values also possess strong anticholinesterase activities. This may be explained by the presence of components with dual functions. Further studies are needed to fractionate the tested extracts and test each fraction for both activities (antioxidant and anticholinesterase) to confirm this correlation. In contrast, there appears to be no direct correlation between antioxidant and anticholinesterase activities in cacti. These results are consistent with that of Godkar et al. (2006), who reported that seed oil of

Celestrus panicultus exhibited a potent antioxidant capacity using the DPPH assay and showed no inhibitory activity against AChE at all.

There was good agreement between the antioxidant activity (especially for the FRAP assay) and the extraction method used in folklore for most plant species. For instance, *N. oleander, M. chamomilla, T. vulgaris, S. officinalis* and *O. europaea* were suggested to be used only as a hot infusion in the traditional uses. These results showed a higher antioxidant activity for those plants in hot extracts. Similarly, for those suggested to be used as cold infusions such as *Q. robur* and *S. aromaticum*, the highest activities have been found in the cold extracts (El Gadi, 1992). These results suggested that the efficiency of these plants could be explained, at least in part by their antioxidant activity. *M. communis* is reported by Romani et al., (2004) to have the same main polyphenols that exist in green tea, such as galloyl derivatives, and this can explain the high antioxidant capacity of *M. communis*. In addition, Abdel-Wahhab and Aly (2005) have demonstrated that the antioxidant capacity of *S. aromaticum* is due in part to the contribution of aromatic chemicals such as eugenol and eugenol acetate.

The results obtained in this thesis indicate that there is a correlation between the medicinal uses and the antioxidant categories. For instance, most of the plants that grouped in category (a), such as green tea, *S. officinale, Q. robur* and *M. communis* were found to be used as astringents and as stimulants to the central nervous system (CNS). On the other hand, the plants that grouped in category (b) are more varied but in general most of them such as *O. europaea, P. tortousus* and *H. sabdariffa* are used for the treatment of blood pressure and diabetes. The plants of

category (c) such as *Z. officinale* and *U. martima* were found to treat heart diseases or respiratory disorders (see Table 1.5, Chapter 1). There is potential opportunity, therefore, to target particular plant species to particular diseases. This is clearly an area where further study will be important.

Digestion is an initial step involving changes in pH and activity of proteolytic enzymes (Serrano et al., 2007). Some of the antioxidant compounds can be inactivated and digested along the gastro-intestinal tract thus destroying the antioxidant pharmacological properties of these molecules. It is extremely important to evaluate possible changes in antioxidant properties and anticholinesterase activity of these plant extracts during digestion to test if the aqueous extracts will be able to survive in the gut and therefore still be of pharmacological use. According to their antioxidant activity, plants were divided into three categories: group (a), high antioxidant; group (b), moderate antioxidant and group (c), low antioxidant (see chapter 2). Four plant species from group (a), three from group (b) and three from group (c) as shown in Table 6.1 were selected for experiments on antioxidant property changes during *in vitro* digestion. Only those plant extracts that showed acceptable high anticholinesterase activities were chosen for *in vitro* anticholinesterase release experiments (see Chapter 3).

The apparent antioxidant activity (assayed by the FRAP method) for green tea, *M. communis*, *Q. robur*, *S. aromaticum*, *O. europaea*, *M. chamomilla* and *U. maritima* remained constant under acidic conditions and following partial protein hydrolysis then decreased under the alkaline conditions. This suggests that the

stability or antioxidant redox potential of some phenolic compounds is highly influenced by pH and the intestinal medium produces considerable changes. This suggests that most antioxidants would be most available in the first stage of the digestion process. The increased FRAP values at the end of the digestion step (after 90 min) (figure 4.2, G and H) could be explained by the ability of plant phenols to complex with protein by hydrogen bonding and during protein hydrolysis release more antioxidant (Duodu et al., 2003, McDougall et al., 2005). In general, the initial release of antioxidant in water confirms the presence of water soluble compounds which are probably in a free form within these extracts.

The results obtained by the TEAC assay (figure 4.3) and the DPPH assay (figure 4.4) showed significant release of antioxidant capacity at the final step of the *in vitro* digestion procedure (after 90min) in most extracts tested. Many flavonoids occur in plants in the glycosidic form and need to be heated with HCl to release the free flavonoids (Harborne, 1984). In addition, it was clearly demonstrated that some polyphenols are normally detected after acid or alkaline hydrolysis of plant tissue (Harborne, 1984). This could explain the high release of antioxidant capacity after addition of the HCl in all plant extracts with high antioxidant activity (group a) (figure 4.3, A, B, C and D and figure 4.4, A, B, C and D). The extracts which showed high TEAC values could contain substances that have a redox potential lower than that of the $ABTS^{\cdot}$ radicals. Indeed, many phenolic compounds have low redox potentials and can thus react with the $ABTS^{\cdot}$ radical (Frankel et al 2000; Prior et al 2005). In general, it appears clearly that the variation in antioxidant activity detected by the three assays are due to the structure and chemistry of the individual

polyphenols, their ability to chelate metal and scavenge free radicals as well as the chemistry involved in the antioxidant analysis. Thus further structural analysis of these components in the extracts tested will be essential to further explore their potential for use *in vivo*.

Almost all anticholinesterase activity was lost at the HCl stage of the *in vitro* digestion procedure. These results indicate that these extracts are susceptible to digestive hydrolysis and they need to be commercially extracted and processed (for example protected by coverage with proteins or lipids) for their possible therapeutic usage as cholinesterase inhibitors. Further studies to fractionate the active extracts are now needed to identify active bio-ingredient and to establish the exact mechanism of action in order to explain their therapeutic efficacy.

Testing *in vitro* bioavailability of plant extracts is a useful step in predicting *in vivo* bioavailability. Ingestion of plant extracts (as supplements after commercial procedures) may help to prevent *in vivo* oxidation damage which is associated with many diseases including cancer, diabetes and arteriosclerosis. The plants investigated in this study could provide protection against oxidative stress. However, it is not known which of the components of the aqueous extracts are responsible for this phenomenon. A bio-guided fractionation in order to elucidate the active principles should be involved in further investigations which may lead to extraction and production of active compounds in single or combined forms with useful application.

It should be emphasised that this study was focused on the possibility of using these extracts as natural sources for antioxidants and cholinesterase inhibitors which could possibly be used in future therapy. The results obtained here are promising and therefore further investigations should be targeted on such important issue as toxicology, stability processing as commercial products (highest possible activity and absence of smell). The experience with many other antioxidatively-active natural products such as Ginkgo and sage preparations shows that all the above-mentioned steps toward commercial products are time consuming and need detailed follow-up studies. These *in vitro* results provide some scientific validation for the widespread use of these plants in traditional medicine. However, these data represent a first crucial step that could be followed up with further *in vivo* studies.

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