metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Helsingin yliopiston digitaalinen

Dracocephalum moldavica L. and *Melissa officinalis* L.: Chemistry and Bioactivities Relevant in Alzheimer's Disease Therapy

Keyvan Dastmalchi

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 1at Viikki Infocenter (Viikinkaari 11) on November 21st, 2008 at 12 noon.

Helsinki 2008

Supervisors: Professor Raimo Hiltunen
Division of Pharmaceutical Biology
Faculty of Pharmacy
University of Helsinki
Finland

Dr. Damien Dorman
Division of Pharmaceutical Biology
Faculty of Pharmacy
University of Helsinki
Finland

Reviewers: Professor Kalevi Pihlaja
Department of Chemistry
University of Turku
Finland

Professor Seppo Lapinjoki
Department of Pharmaceutical Chemistry
Faculty of Pharmacy
University of Kuopio
Finland

Opponent: Professor Michael Heinrich
Centre for Pharmacognosy and
Phytotherapy
The School of Pharmacy
University of London
United Kingdom

© Keyvan Dastmalchi
48/2008
ISBN 978-952-10-5111-1 (paperback)
ISBN 978-952-10-5112-8 (PDF)
ISSN 1795-7079
http://ethesis.helsinki.fi
Yliopistonpaino
Helsinki 2008

To my dear mother and my late grandmother Ashraf

TABLE OF CONTENTS

ACKNOWLEDGEMENTS **6**

LIST OF ORIGINAL PUBLICATIONS **8**

LIST OF ABBREVIATIONS **9**

ABSTRACT **11**

1. INTRODUCTION **13**

2. REVIEW OF THE LITERATURE **15**

2.1. OXIDATIVE STRESS AND ANTIOXIDANTS 15
2.1.1 Oxidative Stress *15*
2.1.2 Antioxidants *16*
2.2. ALZHEIMER'S DISEASE 17
2.2.1. Background *17*
2.2.2. Antioxidants and Alzheimer's Disease Therapy *18*
2.2.3 Acetylcholinesterase Inhibition and Alzheimer's Disease Therapy *19*
2.3 MEDICINAL PLANTS 19
2.3.1. Dracocephalum moldavica *20*
2.3.2. Melissa officinalis *21*
2.4 METHODOLOGIES FOR ANTIOXIDANT SCREENING 22
2.4.1. Competitive Methods *23*
2.4.2. Non-Competitive Methods *30*
2.4.3. Overall Prospective *33*

3. AIMS OF THE STUDY **35**

4. EXPERIMENTAL **36**

4.1. CHEMICALS AND REAGENTS 36
4.2. BOTANICAL MATERIAL 36
4.3. EXTRACTION TECHNIQUES 36
4.3.1. Hydrodistillation *36*
4.3.2. Soxhlet Extraction *36*
4.3.3. Medium Pressure Solid Liquid Extraction *37*
4.4. CHROMATOGRAPHIC TECHNIQUES 37
4.4.1 High Performance Liquid Chromatography-Photodiode Array Analysis *37*
4.4.2. Fractionation *39*
4.4.3. Liquid Chromatography-Diode Array Detector-Electrospray Ionisation-Mass Spectrometry *40*
4.5. ANTIOXIDANT METHODS 41
4.5.1. Iron(III) Reduction *41*
4.5.2. Iron(II) Chelation *41*
4.5.3. 1,1′-Diphenyl-2-picrylhydrazyl Free Radical Scavenging *41*
4.5.4. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonate) Cation Free Radical Scavenging *42*

4.5.5. Peroxyl Free Radical Scavenging (β-Carotene-linoleic Acid Bleaching Inhibition) *43*
4.5.6. Superoxide Anion Free Radical Scavenging *43*
4.5.7. Hydroxyl Free Radical Scavenging *44*
4.5.8. Nitric Oxide Free Radical Scavenging *45*
4.6. MISCELLANEOUS METHODS 46
4.6.1. Acetylcholinesterase Inhibitory Screening *46*
4.6.2. Statistical Analysis *47*

5. RESULTS AND DISCUSSION 48

5.1. *DRACOCEPHALUM MOLDAVICA* 48
5.1.1. Yield and Phenolic Composition *48*
5.1.2. Antioxidant Properties *50*
5.2. *MELISSA OFFICINALIS* 56
5.2.1. Yield and Phenolic Composition *56*
5.2.2. Antioxidant Properties *57*
5.2.3. Acetylcholinesterase Inhibition *62*
5.2.4. Bioactivity Guided Fractionation *63*

6. CONCLUSION 65

7. REFERENCES 67

8. APPENDICES 80

ACKNOWLEDGEMENTS

This work was carried out at the Division of Pharmaceutical Biology, Faculty of Pharmacy, University of Helsinki during the years 2004-2008.

My sincere gratitude goes to my supervisor, Professor Raimo Hiltunen, Dean of the Faculty of Pharmacy, for his supervision throughout my research. He was kind enough to allow me access the facilities and equipments present in the Faculty of Pharmacy.

I am highly indebted to my other supervisor, Dr. Damien Dorman, for his guidance and support. Working with him has been a memorable experience. To me he is also a good friend, whom I consider his friendship a valuable asset.

Special thanks are due to Professor Heikki Vuorela, Vice Dean of the Faculty of Pharmacy, for helping me overcome administrative hurdles that many doctoral students face during their studies.

I consider myself lucky for having worked with excellent co-authors like Dr. Into Laakso, Professor Velimatti Ollilainen, Dr. Muberra Koşar, Prof. Jari Yli-Kauhaluoma, Dr. Yusrida Darwis, Päivi Järvinen, Petri Lackman and Gustav Boije af Gennäs. Their contributions are gratefully acknowledged.

Mikka Ollin, Mikael Husu, Mar Santamaria, Elisenda Sabate and Tarja Hiltunen have provided me with valuable technical assistance, which I deeply appreciate.

I would also like to express my gratitude towards Professor Kalevi Pihlaja and Professor Seppo Lapinjoki for carefully reviewing my thesis and providing me with valuable comments and suggestions.

My sincere thanks are due to Professor Pia Vuorela, Dr. Tuulikki Seppänen-Laakso, Dr. Päivi Tammela, and Dr. Joni Alvesalo for sharing their knowledge and experiences with me.

I would also like to thank my colleagues who provided a congenial atmosphere for me to perform my research.

I am grateful to my dear friends from all over the world especially Nenad, Heikki W., Kevin, Vlasta, Jenny and Leon for their kind support and encouragement during my studies.

Words fail to express my gratitude to Aino, whose support at the critical moments of my research work has been instrumental to my success.

I am not sure if any slightest way I will be able to repay my parents', Asghar and Zahra, kindness. My sincere thanks go to my brothers, Bahram and Mehran, who were always encouraging me and praying for my success.

Research Foundation of University of Helsinki, Chancellor of University of Helsinki, Graduate School in Pharmaceutical Research and Yliopiston Apteeki are acknowledged for their financial support. I would also like to acknowledge the Institute of Medicinal Plants, Iranian Academic Center for Education, Culture and Research, Tehran, Iran for providing me with the plant material.

LIST OF ORIGINAL PUBLICATIONS

This dissertation is derived from the following publications, referred to in the text by the Roman numerals I-V.

I. Dastmalchi, K.; Dorman, H. J. D.; Vuorela, H.; Hiltunen, R. Plants as potential sources for drug development against Alzheimer's disease. *International Journal of Biomedical and Pharmaceutical Sciences* **2007**, *1*(2), 83-104.

II. Dastmalchi, K.; Dorman, H. J. D.; Koşar, M.; Hiltunen, R.; Chemical composition and *in vitro* antioxidant evaluation of a water-soluble Moldavian balm (*Dracocephalum moldavica* L.) extract. *Food Science and Technology* **2007**, *40*(2), 239-248.

III. Dastmalchi, K.; Dorman, H. J. D.; Laakso, I.; Hiltunen, R. Chemical composition and antioxidative activity of Moldavian balm (*Dracocephalum moldavica* L.) extracts. *Food Science and Technology* **2007**, *40*(9), 1655-1663.

IV. Dastmalchi, K.; Dorman, H. J. D.; Oinonen, P. P.; Darwis, Y.; Laakso, I.; Hiltunen, R. Chemical composition and *in vitro* antioxidant activity of a lemon balm (*Melissa officinalis* L.) extract. *LWT-Food Science and Technology* **2008**, *41*(3), 391-400.

V. Dastmalchi, K.; Ollilainen, V.; Dorman, H. J. D.; Oinonen, P. P.; Hiltunen, R. Acetylcholinesterase-inhibitory guided fractionation of *Melissa officinalis* L. *Bioorganic and Medicinal Chemistry*, in review.

Reprinted with the permission of the publishers.

LIST of ABBREVIATIONS

AAPH	2,2′-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) diammonium salt
$ABTS^{\bullet +}$	2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate) diammonium cation free radical
ACh	acetylcholine
AChE	acetylcholinesterase
ACN	acetonitrile
AD	Alzheimer's disease
AGE	Advanced glycation end-product
AMVN	2,2′-azobis(2,4-dimethylvaleronitrile)
Anon	anonymous
aq.	aqueous
AscAE	ascorbic acid equivalence
ATCI	acetylthiocholine iodide
auf	absorbance unit frequency
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
BuChE	butyrylcholinesterase
n-BuOH	*n*-butanol
CH_2CL_2	dichloromethane
CH_3COOH	acetic acid
CNS	central nervous system
DAD	diod array detector
$DPPH^{\bullet}$	1,1′-diphenyl-2-picrylhydrazine
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
EtOAC	ethyl acetate
Eur. Ph.	European pharmacopeia
FDA	Food and Drug Administration
HPLC	high performance liquid chromatography
HWE	hot water extract
ISI®	International Science Index®
KOH	potassium hydroxide
KH_2PO_4	potassium dihydrogen phosphate
LC	liquid chromatography
MPSLE	medium pressure solid liquid extraction
$MgCl_2.6H_2O$	magnesium chloride hexahydrate
MS	mass spectrometer
NADH	nicotinamide adenine dinucleotide

NBT	nitroblue tetrazolium
NaOH	sodium hydroxide
NaH_2PO_4	sodium dihydrogen phosphate
NMR	nuclear magnetic resonance
$NO^{\bullet}$	nitric oxide free radical
$O_2^{\bullet-}$	superoxide anion free radical
$^1O_2$	singlet oxygen
ORAC	oxygen radical absorbance capacity
$HO^{\bullet}$	hydroxyl free radical
$ONOO^{\bullet}$	peroxynitrate free radical
PBS	phosphate buffer saline
PDA	photodiode array detector
PE	petroleum ether
$RO^{\bullet}$	alkoxyl free radical
$ROO^{\bullet}$	alky peroxyl free radical
ROS	reactive oxygen species
RNS	reactive nitrogen species
SE	Soxhlet extractions
SEs	Soxhlet extracts
TCA	trichloroacetic acid
TBA	2-thiobarbituric acid
TBARS	2-thiobarbituric acid reactive substances
TEAC	Trolox Equivalent Antioxidant Capacity
TFA	trifluroacetic acid
TRAP	total radical-trapping antioxidant parameter
PhE	physostigmine equivalence
PHY	physostigmine
UV	ultra violet
Vis	visible
XOD	xanthine oxidase

ABSTRACT

Oxidative stress has been proposed to play a cardinal role in the aetiology and pathogenesis of Alzheimer's disease (AD). Therefore, antioxidants have been studied for their therapeutic potential in AD therapy. However, there is still a need for novel sources of antioxidants.

Recently, there has been an increased interest in aromatic and medicinal plants as sources of natural antioxidants. The plants *Dracocephalum moldavica* L. and *Melissa officinalis* L., which belong to the family Lamiaceae, have been used in Iran for their culinary usefulness and medicinal properties. Therefore, extracts of the plants were screened for antioxidant properties in a battery of *in vitro* assays. The plant extracts demonstrated a wide range of antioxidant activities.

Furthermore, because it was important to determine which constituents present within the extracts may contribute to the observed activity, compositional fingerprint analyses were carried out using HPLC-PDA techniques. The extracts were found to contain polyphenolic compounds such as hydroxylated benzoic and cinnamic acid derivatives and flavonoids. Rosmarinic acid was the most abundant constituent in both plants.

The plant *M. officinalis* has been used since antiquity in the treatment of cognitive dysfunction. The plant was recently assessed for its clinical efficacy against AD and was found to be effective in the management of mild to moderate AD patients. According to a review carried out by the author, screening of medicinal plants for bioactivities relevant in the treatment AD can provide useful leads in the discovery of drugs against AD. Therefore, in addition to antioxidant evaluation, *M. officinalis* was screened for another bioactivity relevant to AD therapy, *viz*. acetylcholinesterase (AChE) inhibition. The plant extract

showed AChE inhibitory activity, which formed the basis for activity guided fractionation.

The extract was fractionated using semipreparative scale HPLC fractionation. Fractions were subsequently subjected to AChE inhibitory screening. Most of the fractions demonstrated inhibitory activity and were proved to be significantly ($P < 0.05$) more potent than the crude extract. This is an indication of the complex nature of potential interactions between various components within the extract. The contents of the most potent fraction were tentatively identified as a mixture of *cis*- and *trans*-rosmarinic acid using LC-DAD-MS and NMR techniques.

There is a need to further investigate the efficacy of these chemical constituents in *in vivo* AD models. The remaining potent fractions should be analysed further to determine the identity of their chemical constituents and the possibility of a correlation existing between antioxidant activity and AChE inhibition should be investigated.

The current study showed the multifaceted nature of antioxidant action of *D. moldavica* and *M. officinalis*. Furthermore, the latter demonstrated AChE inhibitory activity. A review of the potential benefits of ethnopharmacological screening of plants in AD therapy was made and the importance of bioactivity guided screening and fractionation of medicinal plants was highlighted. It can be concluded that *M. officinalis* is a potential source for discovery and development of drugs against AD.

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder characterised by impairment in learning and memory followed by more global cognitive deficits and behavioural disturbances (*i.e.* depression, agitation and psychosis), which becomes progressively more severe. Due to the debilitating nature of the disease, an enormous social and financial burden is placed on society. The significance of AD is further compounded as the prevalence is expected to quadruple by 2050 (Brookmeyer *et al.* 2007). Currently there is no cure for the disorder and much of the treatments available have been able to only delay the progression of the disease or provide symptomatic relief for a short period of time. Therefore, there is a need to discover novel molecular templates for effective drugs against AD.

Plants may be the answer as they have been used in traditional medicinal systems for the treatment of cognitive dysfunction. The strategy used for the discovery of new drugs based on the screening of plants having medicinal uses relevant to the treatment of a particular disease, is referred to as ethnopharmacological screening. This approach has been rewarding as it has resulted in the identification of compounds which are currently either in clinical use or templates for further drug discovery, *e.g.* galantamine (Houghton *et al.* 2005), huperazine A (Houghton *et al.* 2005) and PHY (Orhan *et al.* 2006).

One of the pathological hallmarks of AD is oxidative stress and antioxidant therapy has been one of the major lines of pharmacological intervention studied in the treatment of the disease (Dastmalchi *et al.* 2007a). Therefore, there is a search for novel antioxidants with emphasis being placed on medicinal, aromatic and edible plants.

Plants belonging to the family Lamiaceae have been used in the Mediterranean region and South West Asia because of their culinary usefulness and medicinal properties. Many of these plants have not been

investigated fully for their antioxidant activities. Very often plants exert their effect via a variety of different mechanisms, this is especially true in the case of antioxidant activity (Houghton *et al.* 2007). Therefore, in order to characterise the antioxidant properties of the plants, they are often screened in a battery of *in vitro* assays.

Another bioactivity of relevance in the treatment of AD is AChE inhibition. Screening of plant extracts and fractions thereof for this activity has resulted in the identification of AChE inhibitors which are currently either in clinical use or templates for further drug discovery (Dastmalchi *et al.* 2007a). Therefore, AChE inhibitory guided fractionation of Lamiaceae plants, with known antioxidant properties, may have the potential of providing interesting leads in AD drug discovery and development.

2. REVIEW OF THE LITERATURE

2.1. Oxidative Stress and Antioxidants

2.1.1 Oxidative Stress

When living organisms first appeared on Earth, they did so under an atmosphere containing little oxygen (Halliwell 2006). Early life was therefore anaerobic, however, later on the oxygen content started to rise due to the evolution of organisms capable of photosynthesis. They evolved to use sun energy to split water, thereby producing oxygen. As a result of the increase in the oxygen level, most anaerobes did not survive, however, the remaining organisms either retreated to anaerobic environments or adapted antioxidant defences to protect against oxygen toxicity. The later adaptation proved to be a useful path to follow, enabling organisms to utilise oxygen for efficient energy production, employing electron transport chain with oxygen as the terminal electron acceptor. This was needed for the evolution of complex multicellular organisms which have developed systems for delivery of oxygen throughout their bodies.

Any molecular or atomic species containing one or more unpaired electrons is called a free radical (Halliwell 2006). By this definition, even molecular oxygen is a free radical by containing two unpaired electrons. During the course of normal cellular metabolism, oxygen is reduced resulting in the generation of reactive intermediates, known as reactive oxygen species (ROS). These include oxygen radicals and related non-radical species such as H_2O_2. The term reactive species has also been expanded to include reactive nitrogen species (RNS).

ROS and RNS are produced in low to moderate concentrations during normal physiological conditions and are known to possess various physiological roles ranging from cellular signal transduction to defence against pathogens (Valko *et al*. 2007). However, if there is an overproduction of ROS and RNS, oxidative and nitrosative stress will

occur, respectively. These phenomena may occur when the generation of reactive species in the biological system exceeds its ability to neutralise them (Valko *et al*. 2007). The excessive formation of reactive species results in the degradation of cellular components, *viz*., DNA, carbohydrates, proteins and lipids. This will eventually lead to cellular dysfunction and ultimately cell death. That is why oxidative stress has been proposed to play a cardinal role in the pathogenesis of many diseases such as atherosclerosis, diabetes mellitus, certain types of cancer and neurodegenerative disorders such as AD (Dastmalchi *et al*. 2007a; Valko *et al*. 2007). Furthermore, ROS contribute to the deterioration of foodstuffs (Sasaki 1996), cosmetics and pharmaceutical preparations (Branen and Davidson 1997).

2.1.2 Antioxidants

The term antioxidant has been widely used, with its definition being different depending on the field of study. For example, a polymer chemist will define an antioxidant differently from a food technologist. However, a broad definition was suggested by Halliwell and Gutteridge (1995) as "any substance that when present at low concentrations, compared to those of an oxidizable substrate, significantly delays or prevents the oxidation of that substrate". Therefore, based on this definition, only substances capable of protecting biological molecules from oxidative stress can be considered as antioxidants.

A recent literature search performed on the ISI® Web of Knowledge search engine (Thompson Reuters 2008) indicates there has been an exponential increase in the research done on natural antioxidants in particular plant derived antioxidants from aromatic, medicinal and edible plants. It has been suggested by Dastmalchi *et al*. (2008) to be due to three reasons: (i) numerous clinical and epidemiological studies have demonstrated that consumption of fruits and vegetables is

associated with reduced risks of developing chronic diseases such as cancer, cardiovascular disorders and diabetes; (ii) safety consideration regarding the potential harmful effects of the chronic consumption of synthetic antioxidants in foods and beverages; and (iii) the public's perception that natural and dietary antioxidant are safer than synthetic analogues. The result has been increased interest in aromatic and medicinal plants as sources of natural antioxidants.

2.2. Alzheimer's Disease

2.2.1. Background

A reduction in birth rates and increased life expectancy has resulted in a quantitative increase in the mean population age. There has also been an increase in the incidence of age-associated diseases, *e.g.* cancer, cardiovascular disease, neurodegeneration, *etc.* While the aetiology and pathogenesis of many age-related conditions may be affected by lifestyle changes or can be managed through pharmacological intervention, neuro-degenerative disorders are either poorly responsive to such approaches or their progression appears unabatable. Of the neurodegenerative disorders, AD is considered to be responsible for ca. 60% of all dementia in people aged 65 or older (Francis *et al.* 1999). Due to its debilitating nature, an enormous social and economic burden is placed on society. The significance of AD is further compounded as the number of identified cases is estimated to quadruple by 2050 (Brookmeyer 2007).

The pathology of AD is a multifaceted one with several pathogenic pathways believed to contribute to the progression of the disease, *viz.*, senile plaque deposition, neurofibrillary tangle formation, inflammatory cascade, oxidative stress and cholinergic deficit (Small and Mayeux 2005). Based on these pathological hallmarks, several lines of

pharmacological treatments have been investigated, two of which are discussed in the following subsections.

2.2.2. Antioxidants and Alzheimer's Disease Therapy

The vulnerability of CNS to oxidative damage is due to a number of factors such as excessive oxygen uptake and high polyunsaturated lipid content. One of the sources of oxidative stress in AD is the disturbance in metal homeostasis (Sayre *et al*. 2001). Mitochondrial dysfunction is another source of ROS generation (Law *et al*. 2001). It has been proposed that amyloid β peptides in the presence of transition metal ions produces ROS such as $O_2^{\bullet -}$ and H_2O_2 (Varadarajan *et al*. 2000) and microglial activation leads to a massive production of inflammatory cytokines, ROS and RNS, thereby contributing to oxidative and nitrosative damage (Scorer 2001).

Taking into account the various sources of oxidative stress, several antioxidant activities can be suggested such as (i) free radical scavenging *e.g.* vitamins E and C, selegiline and idebenone (Castro *et al*. 2002), (ii) mild to moderate metal chelation *e.g.* clioquinol (Bush 2003; Doraiswamy 2002; Rosenberg 2003), (iii) AGE-inhibition *e.g.* Tenilsetam (Durany *et al*. 1999), (iv) inhibition of membrane lipid peroxidation *e.g.* Lazabemide (Mason *et al*. 2000).

So far, no antioxidant drug has been approved for clinical use, however, clinical studies of antioxidants continue in the hope of finding a suitable treatment in the near future. Examples are *Ginkgo biloba* extract, EGB761 and curcumin isolated from *Curcuma longa* which are in phases III and II of clinical trials, respectively. Some plants and their constituents *e.g.* sinapic acid, rosmarinic acid, salvianolic acids A and B, honkiol and resveratrol which possess potent antioxidant activity have shown biological and pharmacological activities which are relevant to AD therapy (Dastmalchi *et al*. 2007a).

2.2.3 Acetylcholinesterase Inhibition and Alzheimer's Disease Therapy

The selective degeneration of cholinergic neurons in AD results in the loss of all known cholinergic markers, such as choline acetyltransferase, ACh levels and AChE. ACh is associated with cognition and it is the deficit of this neurotransmitter which contributes to cognitive dysfunction. On the other hand, disruption of cholinergic signalling may be contributing to the progression of AD pathology (Lahiri *et al*. 2003). Therefore, restoration of the central cholinergic function may significantly improve cognitive impairment and may inhibit AD progression in patients.

Two types of cholinesterases, AChE and BuChE, are present in a wide variety of tissues. AChE, which is the predominant cholinesterase in the brain, hydrolyzes ACh to choline and acetate, thereby terminating the effect of this neurotransmitter at cholinergic synapses (Small and Mayeux 2005). AChE is, therefore, the target of cholinesterase inhibitors and over the last two decades, AChE inhibition has become the most effective clinical approach to treat the symptoms of AD. However, AChE inhibitors so far have only been useful in the early stages of AD and lose their effectiveness over time. Therefore, there is a need to find more effective AChE inhibitors.

2.3 Medicinal Plants

Plants have been used since antiquity in traditional medicinal systems for the treatment of memory dysfunction. Studies carried out on some plant species have resulted in the identification of compounds which are currently either in clinical use or are templates for further drug discovery, *e.g*. galantamine, an alkaloid isolated from *Galanthus nivalis* L. (Amaryllidaceae). Galantamine was approved by the FDA in 2004 for use as an AChE inhibitor in the treatment of AD (Jones *et al*.

2006). It was the traditional use of *G. nivalis* L. in Bulgaria and Turkey for neurological conditions that led to the development of this drug (Shu 1998). However, opinion about the traditional use of the plant is divided (Heinrich 2004).

The importance of plant-derived compounds in drug discovery is evident from a glance at the Prescription Drug Audit (Jones *et al*. 2006): 35 natural product-related drugs originally discovered from vascular plants were among the 150 top selling drugs in 1993 (Jones *et al*. 2006). The majority of plant-related drugs were discovered from ten plant species, nine of which had been used traditionally for medicinal properties that were related to the current therapeutic indication (Jones *et al*. 2006). The strategy used for the discovery of new drugs based on the screening of plants having medicinal uses relevant to the treatment of a particular disease, is referred to as ethnopharmacological screening (Samuelsson 2004). In a review carried out by Dastmalchi *et al*. (2007a), this approach has been clearly shown to be useful in identifying interesting templates for further drug developmental studies against AD.

2.3.1. Dracocephalum moldavica **L.**

The plant *D. moldavica* L. commonly known as Moldavian balm is a perennial herb belonging to the family Lamiaceae (Labiatae). This plant is native to central Asia and is naturalized in eastern and central Europe. In Iran, it is predominantly found in the north of the country, especially in the Albourz Mountains, where it is known as “Badarshoo”. It is frequently consumed as a food additive (*e.g*. in yogurt) or as an infusion for its organoleptic properties. As a herbal drug, it is used in stomach and liver disorders, headache and congestion (Dastmalchi *et al*. 2007b, 2007c). Very few articles deal with the non-volatile chemistry (Aynehchi *et al*. 1982; Kakasy *et al*. 2002) or antioxidant properties of

Moldavian balm (Povilaityte and Venskutonis 2000; Povilaityte *et al*. 2001).

2.3.2. Melissa officinalis L.

The plant *M. officinalis* L. belongs to the family Lamiaceae and grows widely in central and southern Europe and in Asia Minor (Zargari 1990); however, it is cultivated throughout the world because of it's culinary properties. In Iran, this plant is known locally by the names Badranjbooye, Varangboo and Faranjmoshk and is found in the north, north west and western parts of the country (Anon 2002). It is used in the Iranian traditional system of medicine for the treatment of headaches, flatulence, indigestion, colic, nausea, nervousness, anaemia, vertigo, syncope, malaise, asthma, bronchitis, amenorrhea, cardiac failure, arrhythmias, insomnia, epilepsy, depression, psychosis, hysteria, ulcers and wounds (Zargari 1990; Anon 2002).

Although *M. officinalis* antioxidant studies have been carried out, studies reporting upon the antioxidant activity of polar extracts of the plant are limited (Ivanova *et al*. 2005; Ferreria *et al*. 2006; Triantaphyllou *et al*. 2001; Venkutonis *et al*. 2005). As natural antioxidants can exert their effect via a variety of different mechanisms, it is important to assess fully their different modes of action and this is not possible by conducting only a few assays (Part 1999). Therefore, as a part of our on going antioxidant research on spices and herbs, an aqueous ethanol extract of the plant was screened for its antioxidant properties in a battery of *in vitro* assays. Furthermore, because it is important to determine the constituents present in the extract, which may be contributing to the activity, a compositional fingerprint analysis was carried out using high performance liquid chromatography coupled to photodiode array detector (HPLC-PDA).

2.4 Methodologies for Antioxidant Screening

Antioxidants offer their protective action via a variety of different mechanisms: (i) scavenging ROS/RNS (iii) metal chelation (iv) induction of antioxidant enzymes and (v) inhibition of oxidative enzymes. The latter two types of mechanisms are not covered as they are out of the scope of this current study.

Antioxidants may respond in a different manner to different radicals and oxidant sources. For example, phenolic compounds are stronger scavengers of alkyl peroxyl radicals compared to that of carotenes; however, the later are stronger scavengers of singlet oxygen (Prior *et al.* 2005).

In vitro antioxidant methods can be divided into two general categories: (i) competitive and (ii) non-competitive methods (Magalhães *et al.* 2008). In the former type, an antioxidant sample has to protect a substrate from ROS/RNS-mediated degradation. The antioxidant activity is estimated based on the quantification of a substance that facilitates the analytical measurement referred to as the probe (Magalhães *et al.* 2008). Very often in competitive methods the probe is the substrate or its oxidised form. However, the probe can also be a compound that is added at the end of the assay to measure the quantity of remaining substrate or the reactive species. Furthermore, activity of the antioxidant sample depends on (Magalhães *et al.* 2008): (i) the rate of reaction between the antioxidant sample and the ROS/RNS, (ii) the rate of reaction between the substrate and the reactive species and (iii) the ratio of the antioxidant sample and substrate concentrations.

The following are the requirement of this group of assays (Magalhães *et al.* 2008): (i) the substrate/probe must react with the ROS/RNS at low concentrations, (ii) the spectroscopic change between the oxidized and native forms of the probe should be large enough to maximize sensitivity, (iii) no radical chain reaction beyond

substrate/probe oxidation should take place and (iv) the antioxidant should not react with the substrate.

Non-competitive methods are based on the assessment of the scavenging ability of the antioxidant sample against ROS/RNS in the absence of a substrate. In some of the assays the reactive species will also probe the reaction.

2.4.1. Competitive Methods

Alkyl Peroxyl Free Radical Scavenging Assays

The harmful health effects of $ROO^{\bullet}$ (Valko *et al*. 2007) and its role in oxidative degradation of foods and cosmetics (Laguerre *et al*. 2007) makes its scavenging significantly important. The result has been development of analytical techniques for the assessment of $ROO^{\bullet}$ scavenging (Roginsky *et al*. 2005, Laguerre *et al*. 2007).

$ROO^{\bullet}$ scavenging assays follow a competitive approach. The methods measure the ability of the samples to scavenge $ROO^{\bullet}$ by hydrogen transfer reactions. The assay system consists of three components: (i) thermolabile compound which yields carbon centered free radicals that reacts rapidly with oxygen resulting in the formation of $ROO^{\bullet}$ free radicals, (ii) a substrate and (iii) the antioxidant sample. The antioxidant and the substrate compete in order to react with the free radicals (Magalhães *et al*. 2008).

The ORAC method, which was originally developed by Cao *et al*. (1993), is an example of a $ROO^{\bullet}$ scavenging assay. The principle behind the assay is based on the reaction of $ROO^{\bullet}$ with a fluorescent probe to form a non-fluorescent product. The $ROO^{\bullet}$ is generated by a free radical source *e.g.* AAPH or AMVN. β-Phycoerythrin from *Prophyridium cruentum*, was initially used as the fluorescent substrate/probe which after $ROO^{\bullet}$ scavenging formed a non-fluorescent product (Cao *et al*.

1993). The decay in the fluorescence was monitored throughout the assay and its inhibition by the antioxidant sample was an indication of $ROO^{\bullet}$ scavenging effect of the sample (Prior *et al*. 2005). Batch to batch variation, photobleaching and interaction with polyphenols by non-specific protein binding were the drawbacks associated with the use of β-phycoerythrin (Prior *et al*. 2005; Huang *et al*. 2005). To overcome these limitations a synthetic compound, fluorescein, was used instead of β-phycoerythrin (Ou *et al*. 2001).

One of the advantages of this assay is its ability to assess both lipophilic and hydrophilic $ROO^{\bullet}$ scavengers (Huang *et al*. 2002; Prior *et al*. 2003). This can be done by altering the free radical source or by changing the solvent (Prior *et al*. 2003; Naguib *et al*. 2003). Another advantage of the method is that it can readily be automated for use in high throughput screening procedures (Cao *et al*. 1995; Ou *et al*. 2001; Huang *et al*. 2002).

The reaction in the ORAC assay is temperature sensitive, thus temperature variation across the wells in the microplate can result in intra-assay variability (Prior *et al*. 2003). Fluorometers are not readily available in analytical laboratories which, is another demerit of this assay. Furthermore, oxidation of the substrate/probes in the assay does not necessarily mimic oxidative stress in a biological medium (Frankel *et al*. 2000).

The TRAP assay was introduced by Wayner *et al.* (1985) for evaluating antioxidant activity of human plasma. The original method used human plasma as the substrate and oxygen consumed in the oxidation of plasma material was used as the probe molecule (Wayner *et al.* 1985). The assay is based on the measurement of the lag time that corresponds to the time between the beginning of the assay and the beginning of the oxidation of the plasma (Magalhães *et al*. 2008). Due

to the shortcomings associated with detection of oxygen consumption, modifications to the assay were made and substrates/probes such as β-phycoerythrin (DeLange *et al*. 1989) or ABTS (Bartosz *et al*. 1998) are currently used. $ROO^{\bullet}$ scavenging by these substances results in spectroscopic changes which, are monitored optically or flurometrically.

The major drawback of the assay is the presence of too many endpoints, thus making it difficult to compare data between laboratories (Prior *et al*. 2005). The use of the lag phase in the measurement of antioxidant activity is based on the assumption that all antioxidants show a lag phase; however, not every antioxidant possesses an obvious lag phase (Prior *et al*. 2005). Moreover, the activity after the lag phase is totally ignored. The assay is time consuming and requires a high degree expertise to perform (Prior *et al*. 2005). The assay, like that of the ORAC assay, does not simulate oxidative stress in a biological medium (Frankel *et al*. 2000).

Another $ROO^{\bullet}$ scavenging assay is the β-carotene-linoleic acid bleaching inhibition assay. This method was initially developed by Marco (1968) and later modified by Miller (1971). In the assay heat induced oxidation of linoleic acid results in the formation of the free radicals, which attack β-carotene, leading to the loss of conjugation and colour (Liyana-Pathirana *et al*. 2006). Therefore, the inhibition of β-carotene bleaching is considered as a measure of antioxidant acitivy (Liyana-Pathirana *et al*. 2006).

Cell membranes are rich in unsaturated lipids such as linoleic acid; therefore, it has been argued that antioxidant samples capable of preventing β-carotene bleaching may have the ability to prevent *in vivo* lipid peroxidation (Liyana-Pathirana *et al*. 2006).

The advantages associated with the assay are (Laguerre *et al*. 2007) (i) sensitivity, (ii) relatively rapid reaction rate, (iii) simplicity, (iv)

generation of visible results and (v) applicability to thin layer chromatographic method.

One of the drawbacks associated with the assay is that the substrate is oxidised under non-controlled conditions which makes it impossible to obtain reproducible data (Roginsky *et al*. 2005). Some researchers have tried to overcome this by using free radical sources *e.g.* AAPH instead of heat (Roginsky *et al*. 2005). Another drawback is interference from compounds which absorb within the β-carotene spectral window can hamper measurements (Roginsky *et al*. 2005). It is not easy to interpret the results because β-carotene is itself an antioxidant (Roginsky *et al*. 2005).

Superoxide Anion Free Radical Scavenging Assays

Most of the analytical methods used for assessment of $O_2^{\bullet-}$ scavenging activity use the XOD/hypoxanthine system which at pH 7.4 generates the free radical (Lee *et al*. 2002). This radical can also be generated by the non-enzymatic reaction of phenazine methosulphate in the presence of NADH (Ewing and Janero 1995). In both these radical generating systems, $O_2^{\bullet-}$ reduction of NBT results in the formation of formazan, which is spectrophotometrically monitored at 560 nm (Aruoma *et al*. 1993; Ewing and Janero 1995). Antioxidants compete with NBT, which is both a probe and the substrate, for $O_2^{\bullet-}$ scavenging. Another probe which has also been used is cytochrome c. Its constituent iron(III) is reduced to iron(II) which can be monitored at 550 nm (Aruoma *et al*. 1993). Cytochrome c reacts more rapidly with $O_2^{\bullet-}$ than NBT, therefore, it is more difficult for the antioxidant sample to inhibit cytochrome c reduction. This is an example how the choice of probe can influence evaluation of antioxidant activity (Magalhães *et al*. 2008).

All variations of the assay suffer from the drawback that the samples may provide false positive results by interfering with $O_2^{\bullet-}$

generation (Magalhães *et al*. 2008). An example is inhibition of the enzyme xanthine oxidase by the sample suspected of antioxidant activity. The samples should also be checked if they reduce NBT or cytochrome c (Magalhães *et al*. 2008).

Hydroxyl Free Radical Scavenging Assays

In the $HO^{\bullet}$ scavenging assays the iron(III) ions and ascorbic acid system results in the generation of a constant flux of $HO^{\bullet}$ free radicals which will attack 2-deoxy-D-ribose or brain phospholipids depending on the probe used and form a series of degradation products (Halliwell *et al*. 1987; Dastmalchi *et al*. 2007b). These products, which are known as TBARS, react with TBA to give a pink chromogen (Halliwell *et al*. 1987). As a result of the competition between the antioxidant sample and the substrate for the generated free radicals, the formation of TBARS is inhibited. There are, however, cases in which compounds investigated for their activity inhibit $HO^{\bullet}$ generation by (i) scavenging $HO^{\bullet}$ precursors, *e.g.* H_2O_2, or (ii) chelating free iron(III) ions related to $HO^{\bullet}$ formation (Magalhães *et al*. 2008).

Therefore, in the non-site specific 2-deoxy-D-ribose assay, the performance of the antioxidant samples is assessed in the presence of EDTA. This allows the evaluation of a potential antioxidant activity unrelated to transition metal ion chelation (Magalhães *et al*. 2008). In the presence of EDTA, iron(III) ions are no longer bound to the 2-deoxy-D-ribose and therefore $HO^{\bullet}$ damage is non-site specific and inhibition of TBARS is due to direct $HO^{\bullet}$ scavenging action (Dastmalchi *et al*. 2007b; Magalhães *et al*. 2008).

Hydrogen Peroxide Scavenging Assays

One of the most common methods in assessing H_2O_2 scavenging is based on its intrinsic absorption within the UV region which was first

developed by Beers *et al*. (1952). As the concentration of H_2O_2 decreases the absorbance at 230 nm will also decrease. Sometimes the samples also absorb within this region, thus there is a need for blank measurements. However there are still demerits which affect accuracy and precision of the assay. In the case of strong background absorption it will be very difficult to investigate any change in the absorption. The other shortcoming is that absorption of the sample may change after the reaction, thus making the blank measurements irrelevant (Magalhães *et al*. 2008).

Fluorometric methods have also been used for measuring H_2O_2 scavenging activity. An example is the one employing homovanillic acid, which upon oxidation with H_2O_2 is converted to its fluorescent biphenyl dimmer. H_2O_2 scavenging activity results in fluorescence decay (Pazdzioch-Czochra *et al*. 2002). Horseradish peroxidase has been used as a source of H_2O_2 generation in the assay and its inhibition or interference with its activity results in erroneous results (Magalhães *et al*. 2008).

Singlet Oxygen Scavenging Assays

Singlet oxygen is molecular oxygen in an excited state without unpaired electrons. Therefore, despite being a powerful oxidising agent it is not considered as a free radical (Halliwell 2006). $^1O_2$ emits characteristic light during its decay from a high energy state to a lower one, known as phosphorance at 1270 nm. $^1O_2$ scavenging of some of the compounds has been assessed using this property (Wilkinson *et al*. 1995). However, usually the intensity of phosphorance is insufficient to provide reproducible quantitative information (Magalhães *et al*. 2008).

A more sensitive approach using the fluorescence probe *tetra*-*tert*-butylphthalocyanine copper was developed (Fu *et al*. 1997). This

method has been used for the estimation of rate constants of many $^1O_2$ scavengers.

Nitric Oxide Free Radical Scavenging Assays

$NO^•$ plays an important role in the regulation of a variety of physiological and pathological processes *e.g.* AD (Valko *et al*. 2007; Varadarajan *et al*. 2000; Law *et al*. 2001; Moncada *et al*. 1991; Tamir and Tannenbaum 1996). Assessment of $NO^•$ scavenging activity can be done in a number of ways. One of the approaches is based on the use of amperometric measurement of $NO^•$ scavenging of sulphur containing compounds using $NO^•$ sensors (Vriesman *et al*. 1997). The radical is added to the buffered solution of the scavenger and its concentration is monitored during the assay. There is a linear relationship between natural logarithm of the $NO^•$ levels and time. Another approach for the determination of $NO^•$ scavenging uses the Griess reaction. In these types of assays, the remaining $NO^•$ is converted into an azo-derivatives of nitrite, which are measured at 540 nm (Marcocci *et al*. 1994; Dastmalchi *et al*. 2008).

Peroxynitrate Free Radical Scavenging Assays

One of the assays used in assessing $ONOO^{•-}$ scavenging is based on tyrosine nitration. Tyrosine upon reaction with $ONOO^{•-}$ forms 3-nitrotyrosine which is measured using HPLC-PDA (Magalhães *et al*. 2008; Pollards *et al*. 2006). In the presence of an $ONOO^{•-}$ scavenger the formation of 3-nitrotyrosine is inhibited which is estimated in terms of percentage of inhibition (Pollards *et al*. 2006).

2.4.2. Non-Competitive Methods

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonate) Cation Free Radical Scavenging Assay

This assay is also referred to as the TEAC assay. This assay involves the generation of $ABTS^{\bullet+}$, which has absorption maxima at 414, 645, 734 and 815 nm (Re *et al*. 1999). Among them, 734 nm is the most commonly used wavelength for monitoring $ABTS^{\bullet+}$ scavenging spectrophotometrically. Reduction of the free radical cation results in discoloration which is a measure of the antioxidant activity (Re *et al*. 1999). There have been a number of modifications done to the assay. In the original method metmyoglobin and H_2O_2 were used to generate ferrylmyoglobin, which then reacted with ABTS to form $ABTS^{\bullet+}$ (Miller and Rice-Evans 1993). The antioxidant sample was added in the reaction medium before $ABTS^{\bullet+}$ was formed. This presented a problem in that the antioxidant reacted with the oxidizing reagent before the complete formation of $ABTS^{\bullet+}$ had occurred which led to an overestimation of antioxidant activity (Strube *et al*. 1997). In later methods the antioxidant sample to be tested was added after the generation of $ABTS^{\bullet+}$ (Re *et al*. 1999). An example is the assay employed in the current study. After the free radicals are generated by the action of potassium peroxosulphate on ABTS, the antioxidant sample is added to the reaction mixture. The scavenging ability of the sample is estimated in terms of percentage inhibition of $ABTS^{\bullet+}$ absorbance at 734 nm relative to the reactivity of Trolox as reference standard under the same condition (Dastmalchi *et al*. 2007b,c; Dastmalchi *et al*. 2008).

The simplicity of the assay accounts for its wide application in many laboratories as a routine screening method. In addition to this merit the assay can be used over a wide pH range (Lemanska *et al*.

2001) and due to $ABTS^{•+}$ solubility in aqueous and organic phase both hydrophilic and hydrophobic samples can be evaluated for their activities (Re *et al.* 1999). The assay has the potential to be automated and adapted to microplates (Erel 2004).

The $ABTS^{•+}$ is not a physiological radical, thus the assay is not representative of a biological system. Another limitation of the assay is that any compounds having lower redox potential than $ABTS^{•+}$ is capable of reducing it, thus most phenolics can reduce $ABTS^{•+}$. Furthermore, as TEAC values are determined at a specific time interval, the results may not be reflective of the true antioxidadnt activity of a sample as antioxidants are capable of both fast and slow reactions (Prior *et al.* 2005).

1,1′-Diphenyl-2-picrylhydrazyl Free Radical Scavenging Assay

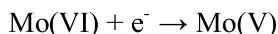
The $DPPH^{•}$ is a stable nitrogen-centred free radical with a deep purple colour, which unlike $ABTS^{•+}$ it is commercially available and does not have to be produced before the assay is carried out. Reduction of $DPPH^{•}$ by the antioxidant sample results in a change of colour from purple to pale yellow (Magalhães *et al.* 2008; Prior *et al.* 2005). The scavenging activity is usually determined in organic media (normally in MeOH) by measuring the change in the absorbance at 515-518 nm until the absorbance remains constant (Magalhães *et al.* 2008). The discolouration method was first developed by Brand-Williams *et al.* (1995). Contrary to the initial view the reaction mechanism is based on electron transfer reaction and hydrogen atom transfer is a marginal pathway (Magalhães *et al.* 2008). As it has been demonstrated by Foti *et al.* (2004), $DPPH^{•}$ scavenging activity is strongly influenced by the solvent used and the pH of the reaction mixture. In the version of the assay used in the current study pH is maintained at pH 7.4 by using

Tris-HCl buffer. The results are reported in terms of the concentration of sample required to decrease the initial concentration of $DPPH^{\bullet}$ by 50 % (IC_{50}) (Dastmalchi *et al*. 2007b,c; Dastmalchi *et al*. 2008).

The merits of the assay can be summed up as its simplicity, speed and the requirement of only readily available instruments, *e.g*. a UV-Vis spectrophotometer (Prior *et al*. 2005). However, when a test sample, *e.g*. fucoxanthin, absorbs light within the same region as $DPPH^{\bullet}$, measurement will be difficult to perform (Noruma *et al*. 1997). Another problem is steric accessibility as a result of which small molecules that have easier access to $DPPH^{\bullet}$ will be considered as more active relative to large antioxidant compounds (Prior *et al*. 2005). $DPPH^{\bullet}$ like $ABTS^{\bullet +}$ is not a physiological radical and its bears no similarity with ROS present in biological systems (Prior *et al*. 2005) .

Folin-Ciocalteu Reagent Assay

The original Folin-Ciocalteu method was developed for tyrosine analysis (Folin 1927), which was later on improved by Singleton *et al*. (1999). The improved method has been used for estimation of total phenols. The reagent used is a molybdotungastophosphoric heteropolyanion reagent. The chem-istry behind the assay relies on the electron transfer from phenolic compounds to molybdenum, forming blue complexes that can be detected at 750-760 nm (Singleton *et al*. 1999).



$$Mo(VI) + e^{-} \rightarrow Mo(V)$$

It is believed that molybdenum can be reduced more easily once it is in a complex form. Blue complexes are formed independently of the structure of the phenolic groups. This rules out the possibility of coordination complex formation during the course of the assay (Singleton *et al*. 1999). Gallic acid is generally used as the reference

standard and the phenolic content of the sample is estimated in terms of gallic acid equivalence.

The Folin-Ciocalteu method is simple and can be used in standardising many botanical samples. Excellent correlation between the assay and other electron transfer methods *e.g.* $DPPH^{\bullet}$ has been established (Roginsky *et al.* 2005). The assay suffers from certain drawbacks. A number of interfering substances such as sugars, aromatic amines, sulphur dioxide, ascorbic acid, Cu(II) and Fe(III) react with Folin-Ciocalteu reagent to give false positive results (Prior *et al.* 2005; Singleton *et al.* 1999).

Iron(III) Reduction Assay

The method is based on the reduction of the substrate Fe^{3+} and $Fe(CN)_6^{3-}$ ions and the subsequent formation of Fe^{2+} and $Fe(CN)_6^{4-}$. These species combine to form a Prussian blue complex $Fe_4[Fe(CN)_6]_3$ which is the chromogen (Van-Middlesworth and Cannell 1998). The reducing power of the antioxidant sample is assessed by the change in the absorbance due to the formation of the complex (Oyaizu 1986). Ascorbic acid is used as the standard reference and the activities of the samples are presented in terms of AscAE (Dastmalchi *et al.* 2007b).

2.4.3. Overall Prospective

A large number of assays with different reaction mechanisms, substrates, probes and free radical sources have been discussed. Given the fact that antioxidants act via a variety of different mechanisms it is beneficial to screen them using a battery of different assays in order to characterise them fully.

As our understanding of oxidative stress increases, so does our need for more *in vitro* analytical methods. In designing novel methods, substrates and probes should be chosen that are of biological relevance

and as far as possible related to the disease under investigation. The reaction conditions *e.g.* temperature, pH, concentration and reaction time should reflect the oxidative stress conditions *in vivo*. Other important criteria while designing assays are simplicity, available instrumentation, defined endpoint, adaptability to hydrophilic and hydrophobic conditions and adaptability to high throughput analysis.

3. AIMS OF THE STUDY

The current study was aimed at screening *Dracocephalum moldavica* and *Melissa officinalis* for bioactivities relevant to AD therapy. In order to achieve the stated aim, specific objectives were defined as follows.

Objective:

1. Literature Review: Investigating the Potential of Plants, including *Melissa officinalis* L. in AD (Study **I**)

2. Antioxidant Screening of HWE of *Dracocephalum moldavica* L. (Study **II**)

3. Antioxidant Screening of SEs of *Dracocephalum moldavica* L. (Study **III**)

4. AChE Inhibitory Screening of *Melissa officinalis* (Study **IV**)

5. AChE Inhibitory Guided Fractionation of *Melissa officinalis* (Study **V**)

4. EXPERIMENTAL

4.1. Chemicals and Reagents

Standard compounds, reagents and solvents used in the current study along with their source of purchase are presented in Table 1. Water (HPLC grade) was prepared using a Millipore Plus system.

4.2. Botanical Material

Aerial material of *D. moldavica* was purchased form a herbal products retailer in Tehran, Iran. Air-dried leaves of *M. officinalis* (Specimen No. GeLM175) were obtained from the Institute of Medicinal Plants, Iranian Academic Center for Education, Culture and Research (Halejrad, Iran). The taxonomic identity of the plants was confirmed by a botanist from the department of Cultivation and Development of the Institute of Medicinal Plants (Tehran, Iran).

4.3. Extraction Techniques

4.3.1. Hydrodistillation

This method was used for the preparation of the plant extract used in study **II**. Air-dried plant material was placed in a 500 mL round bottom flask and 250 mL water was added. The flask was connected to Eur. Ph. hydrodistillation apparatus and the water was boiled for 2 h. The volatile oil extracted was discarded and the water extract was separated from the plant material by filtration. The process was repeated with fresh extractant a further two times. The extracts were pooled, filtered, concentrated *in vacuo* (45°C), lyophilised and stored at 4 °C until required for analysis. This extract is referred to throughout the text as the HWE.

4.3.2. Soxhlet Extraction

This method was used for the preparation of the plant extracts used in study **III**. Air-dried plant material was packed into a paper thimble and inserted into a Soxhlet apparatus. The plant material was exhaustively extracted in a sequential manner with 250 mL PE, CH_2Cl_2, ACN, EtOAc, MeOH, *n*-BuOH and H_2O. The organic extracts were dried over anhyd. magnesium sulphate and their solvents were removed *in vacuo* (40°C). The water extract was concentrated *in vacuo* (40 °C) and lyophilised. All extracts were stored at 4°C until required for analysis.

4.3.3. Medium Pressure Solid Liquid Extraction

This method was used for the preparation of the plant extract used in studies **IV** and **V**. The air-dried leaves of the plant were extracted with 45 % (v/v) aq. ethanol using medium pressure solid liquid extraction. The column used was manufactured by Büch (Büch Laboratorium Tecknic Ag, Switzerland) and the ratio of material to the extractant was 1:5.4 (w/v). The incubation period and the time between the runs was 3 h and each run took 45 min at a flow rate of 3 mL/min. The plant material was extracted in two batches of 200 g and 250 g and the number of runs were 8 and 10, respectively. Finally, the extracts from all the runs were pooled and concentrated *in vacuo* at 45 °C, lyophilized and stored at 4 °C until required for analysis.

4.4. Chromatographic Techniques

4.4.1 High Performance Liquid Chromatography-Photodiode Array Analysis

Method A

The chromatographic method used in study **II** was as follows. The HPLC system (Shimadzu LC 10 A*vp*) consisted of an in-line degasser, pump and controller coupled to a SPD-M10 A*vp* PDA detector

equipped with an automatic sampler interfaced to PC running Class VP chromatography manager software (Shimadzu). Separations were performed on a 250 x 4.6 mm i.d., 5 μm particle size, reverse-phase Discovery-C18 analytical column (Supelco) operating at room temperature. Detection was carried out with a sensitivity of 0.1 auf between the wavelengths of 200 to 550 nm. Elution was done by using a binary non-linear gradient of 5% aq. formic acid (solvent A) and ACN (solvent B). The changes in the mobile phase composition during each chromatographic run can be seen in Table 2.

All extracts and standards were dissolved in 70% aq. ACN at a concentration of 1 mg/mL and 10 mg/mL, respectively. The concentration used for the calibration of reference compounds was 0.00-0.10 mg/mL. All standard and sample solutions were injected in quadruplicate.

Method B

The chromatographic method used in study **III** was as follows. The HPLC system (Waters 600E) consisted of an in-line degasser, pump and controller coupled to a 991 photodiode array detector equipped with a 717 automatic sampler (20 μL injection volume) interfaced to a PC running PDA 991 software (Waters Corp., Milford, MA). Separations were performed on a 250 × 4.6 mm i.d., 5 μm reverse-phase Hypersil BDS-C18 analytical column (Agilent Technologies). Detection was carried out with a sensitivity of 0.1 auf between the wavelengths of 200 and 550 nm. Elution was done by using a ternary nonlinear gradient of the solvent mixture MeOH/H_2O/CH_3COOH (10:88:2, v/v/v) (solvent A), MeOH/H_2O/CH_3COOH (90:8:2, v/v/v) (solvent B) and MeOH (solvent C).

Stock solutions of the extract and standards were prepared in 70% aq. MeOH to final concentrations of 1 and 10 mg/mL, respectively. The

concentration range used for calibration of the standard compounds was 0.01-0.10 mg/mL. All standard and sample solutions were injected in duplicate.

Method C

The chromatographic method used in study **IV** was as follows. The instrumentation was the same as described in method **B**. Detection was carried out with a sensitivity of 0.1 aufs between the wavelengths of 200 and 550 nm. Elution was done by using a binary non-linear gradient of the solvent mixture 0.2 % aq. TFA (solvent A) and MeOH (solvent B). Composition of the mobile phase changed according to Table 4. Components were identified by comparison of their retention times to those of authentic standards under identical analysis conditions and by comparison of their UV spectra with an in-house PDA-library.

Stock solutions of the extract and standards were prepared in 45% aq. ethanol and 70% aq. methanol to final concentrations of 1 and 10 mg/mL, respectively. The concentration range used for calibration of the standard compounds was 0.001-0.10 mg/mL. The standards and samples were injected in duplicate.

4.4.2. Fractionation

Chromatographic based fractionation was performed in study **V** using a HPLC system (Waters 2545) consisting of a binary gradient module and active flow splitter coupled to a PDA detector (Waters 2996), a mass spectrometer (Waters), a make up pump (Waters 515) and a sample manager (Waters 2767) interfaced to a PC running MassLynx 4.1 software (Waters). Separations were performed using Supelco C18 discovery column (25 cm x 10 mm, 5 µm) along with a Supel guard discovery C18 precolumn (1 cm x 10 mm, 5 µm) and a binary non-linear gradient of 0.02% aq. TFA (Solvent A) and MeOH

(Solvent B). The composition of the mobile phase was changed as described in Table 5. The injection volume of the extract was 3 mL at a concentration of 5 mg/mL. UV monitoring was carried out at 280 nm and the fractionation was time based as shown in Table 6.

4.4.3. Liquid Chromatography-Diode Array Detector-Electrospray Ionisation-Mass Spectrometry

The chromatographic method used in study **V** was as follows. The HPLC system (Agilent 1100 series) consisted of an automatic sampler, binary pump, an online degasser, heated column compartment, coupled to a DAD detector (Agilent 1100 series) interfaced to a PC running HP ChemStation Plus A.07.01. software (Agilent Technologies). Separation was performed using Luna C18(2) (150 mm x 1 mm, 5 µm, Phenomenex Ltd.). Elution was carried out using binary non-linear gradient of 0.02% aq. TFA (Solvent A) and 0.02% TFA in MeOH (Solvent B). The mobile phase composition changed according to Table 7. The column was maintained at a constant temperature of 30 °C. The injection volume of the fraction was 3 µL. UV monitoring was done at 280 nm and 360 nm channels.

Mass spectrometric analysis was carried out using an Esquire-LC quadruple ion trap mass spectrometer equipped with an electrospray interface (Bruker Daltonics, Berman, Germany) controlled using Esquire-LC NT 3.1 (Bruker Daltonics) software. Electrospray ionization was performed in both negative and positive modes. When using negative mode scan range of 100-700 m/z, capillary exit voltage of 85 volts and Trap drive value of 36 were used. During positive mode ionization a scan range of 100-700 m/z, capillary exit voltage of 35 and a trap drive value of 43 was used. Helium was used as the collision gas.

4.5. Antioxidant Methods

4.5.1. Iron(III) Reduction

The ability of the plant extracts to reduce iron(III) to iron(II) ions was assessed in studies **II**, **III** and **IV** by the method described by Oyaizu (1986). Briefly, 1 mL sample was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL 1% (w/v) potassium hexacyanoferrate solution. After 30 min at 50 °C, 2.5 mL 10% (w/v) TCA was added and the mixture was centrifuged for 10 min (2000 rpm). Finally, a 2.5 mL aliquot was mixed with 2.5 mL ultra-pure water and 0.5 mL 0.1% (w/v) iron(III) chloride and the absorbance was recorded at 700 nm. Values are presented as ascorbic acid equivalents (AscAE, µmol ascorbic acid/g sample).

4.5.2. Iron(II) Chelation

The ability of the plant extracts to chelate iron(II) ions was assessed in studies **II** and **IV,** by the method described by Carter (1971). Briefly, to a 200 µL of dissolved extract was added 100 µL (2.0 mM) iron(II) chloride tetrahydrate and 900 µL methanol. After a 5 min incubation, the reaction was initiated by the addition of 400 µL (5.0 mM) ferrozine. After a further 10 min incubation period, the absorbance at 562 nm was recorded. The percentage chelating activity was calculated using Eq. 1 and the concen-tration at which the extract exerts 50% of its effect (EC_{50}) was estimated by a linear regression algorithm.

$$\text{Percentage Chelation} = \left[\frac{\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right)}{\text{Abs}_{\text{control}}}\right] x100 \quad \textbf{(Eq. 1)}$$

4.5.3. 1,1′-Diphenyl-2-picrylhydrazyl Free Radical Scavenging

The ability of the plant extracts to scavenge $DPPH^{\bullet}$ free radicals was assessed in studies **II**, **III** and **IV** by the method described by

Gyamfi *et al.* (1999). Briefly, a 50 μL aliquot of dissolved extract was mixed with 450 μL Tris–HCl buffer (50 mM, pH 7.4) and 1.0 mL (0.1 mM) $DPPH^{\bullet}$ dissolved in methanol. After a 30 min reaction period, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using Eq. 2 and the concentration of the extract at which it exhibits 50% inhibition (IC_{50}) was estimated using a non-linear regression algorithm.

$$\text{Percentage Inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \text{x}\,100 \qquad (\textbf{Eq. 2})$$

4.5.4. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonate) Cation Free Radical Scavenging

The ability of the plant extracts to scavenge $ABTS^{\bullet+}$ free radical scavenging was assessed in studies **II**, **III**, **IV** by the method described by Re *et al.* (1999). Briefly, the $ABTS^{\bullet+}$ radical was generated by reacting a 7 mM ABTS aqueous solution with potassium peroxosulphate (2.45 mM, final concentration) in the dark for 12–16 h, at ambient temperature, and adjusting the $Abs_{734\ nm}$ to 0.700 (± 0.020) with ethanol. The samples were diluted, such that, a 15 μL sample when added to 1.485 mL $ABTS^{\bullet+}$ resulted in a 20–80% inhibition of the blank absorbance. After 1.485 mL $ABTS^{\bullet+}$ solution was added to 15 μL sample, the absorbance at 734 nm was recorded 1 min after initial mixing and subsequently at 5 minutes intervals (40 min, *in toto*). The results are expressed as the Trolox equivalent antioxidant capacity (TEAC, mM Trolox) at different time intervals.

4.5.5. Peroxyl Free Radical Scavenging (β-Carotene-linoleic Acid Bleaching Inhibition)

The ability of the plant extracts to inhibit the bleaching of the β-carotene-linoleic acid emulsion was assessed in studies **III** and **IV** using a modification of the method described by Koleva *et al*. (2002). Briefly, 0.2 mg β-carotene dissolved in 1 mL chloroform, 20 mg of linoleic acid and 200 mg of Tween 20 were transferred into a round-bottom flask. Once the chloroform had been removed under nitrogen, 50 mL distilled H_2O was added and the resulting mixture was vigorously stirred for 30 minutes. Thereafter, 5 mL aliquots of this emulsion were transferred to tubes containing either 200 μL of dissolved extract (1 mg/mL) or 200 μL of positive controls (1 mg/mL). After mixing, the absorbance (Abs_0) at 470 nm was recorded. The remaining samples were placed in a water bath at 50 °C for a period of 2 h. Thereafter, the absorbance of each sample was remeasured at 470 nm (Abs_{120}). The data (n = 2) are presented as antioxidant activity % (AA%) values, calculated using Eq. 3.

$$AA\% = \left[1 - \frac{(Abs_{0\ sample} - Abs_{120\ sample})}{(Abs_{0\ control} - Abs_{120\ control})} \right] x\ 100 \qquad \textbf{(Eq. 3)}$$

4.5.6. Superoxide Anion Free Radical Scavenging

The ability of the plant extracts to scavenge superoxide anion radicals was assessed in studies **II** and **IV** by the method described by Lee *et al*. (2002). Briefly a 100 μL aliquot of dissolved sample was taken, to which it was added: 100 μL (30 mM) Na_2EDTA, 100 μL (3 mM) hypoxanthine in 50 mM NaOH and 200 μL (1.42 mM) NBT in NaH_2PO_4-NaOH buffer (50 mM, pH 7.4). After a 3 min incubation period at room temperature, 100 μL (0.5 U/mL) XOD in the NaH_2PO_4-NaOH buffer was added followed 2.4 mL NaH_2PO_4-NaOH buffer. The

resulting solution was incubated at room temperature for 20 min and the absorbance at 560 nm was measured. The absorbance was also measured at 293 nm to detect if the extract inhibited uric acid generation. Once it was confirmed that uric acid formation is not inhibited, then the percentage inhibition at 560 nm was calculated using Eq. 2 and IC_{50} values were estimated using a non-linear regression algorithm.

4.5.7. Hydroxyl Free Radical Scavenging

Ascorbate-Iron(III)/EDTA-H_2O_2-Catalysed2-Deoxy-D-ribose Degradation

The ability of the plant extract to inhibit hydroxyl radical-mediated 2-deoxy-D-ribose degradation was assessed in study **II** by the method described by Halliwell *et al*. (1987). The reaction mixture contained 500 µL of extract dissolved in 50 mM KH_2PO_4-KOH buffer (pH 7.4), 100 µL of 28 mM 2-deoxy-D-ribose in KH_2PO_4-KOH buffer, 200 µL of a premixed 100 µM $FeCl_3$, and 104 mM Na_2EDTA (1:1 v/v) solution, 100 µL of 1.0 mM hydrogen peroxide and 100 µL of 1.0 mM aq. ascorbic acid. Tubes were vortexed and incubated at 37 °C for 60 min. Thereafter, 50 µL of 2% (w/v) BHT was added to each tube followed by 1 mL of 2.8% (w/v) TCA and 1 mL of 1% (w/v) TBA (in 0.05 M NaOH). The samples were vortexed and heated in a water bath at 100 °C for 20 min. The reaction was stopped by placing the test tubes in an ice-H_2O bath for 5 min. To each tube was added 2 mL of *n*-butanol and the mixture was vigorously vortexed. After centrifugation, the extent of oxidation was estimated from the absorbance of the organic layer at 532 nm. The percentage inhibition was calculated using equation 2 and the IC_{50} values were estimated using a non-linear regression algorithm.

Ascorbate-Iron(III)-Catalysed Phospholipid Degradation

The ability of the plant extract to inhibit hydroxyl radical-mediated phospholipid degradation at physiological pH was assessed in study **II** by the method described by Aruoma *et al*. (1997). Bovine brain extract was mixed with 10 mM PBS (pH 7.4) and sonicated in an ice bath until an opalescent suspension was obtained containing 5 mg/mL phospholipid liposomes. The liposomes (0.2 mL) were combined with 0.5 mL of PBS buffer, 0.1 mL of 1 mM $FeCl_3$, and 0.1 mL of sample dissolved in an appropriate solvent. The peroxidation was initiated by adding 0.1 mL of 1 mM aq. ascorbic acid. The mixture was incubated at 37 °C for 60 min. After incubation, 50 µL of 2% (w/v) BHT (in ethanol) was added to each tube, followed by 1 mL of 2.8% (w/v) TCA and 1 mL of 1% (w/v) TBA in 0.05 M NaOH. The solutions were heated in a water bath at 100 °C for 20 min. The resulting $(TBA)_2$-MDA chromogen was extracted into 2 mL of *n*-butanol and the extent of peroxidation was determined in the organic layer at 532 nm. The percentage inhibition was calculated using equation 1 and IC_{50} values were estimated using a non-linear regression algorithm.

4.5.8. Nitric Oxide Free Radical Scavenging

The ability of the plant extract to scavenge nitric oxide free radicals was assessed in study **IV** using a modification of the method described by Marcocci *et al*. (1994). Briefly, a 0.5 mL aliquot of extract (1 mg/mL) or positive control (1 mg/mL) dissolved in KH_2PO_4-KOH (50 mM, pH 7.4) was mixed with 0.5 mL of (10 mM) sodium nitroprusside solution. The mixture was incubated at 37 °C for 2.5 hours under normal light conditions. After incubation the sample was placed in dark for 20 minutes. Thereafter, 1 mL of Griess reagent (0.1% *N*-(1-naphtyl)ethylenediamine and 1% (w/v) sulfanilamide dissolved in 2% (w/v) aqueous H_3PO_4) was added and the absorbance was taken after 40

minutes at 546 nm. The percentage inhibition was calculated using Eq. 2.

4.6. Miscellaneous Methods

4.6.1. Acetylcholinesterase Inhibitory Screening

The ability of the plant extract to inhibit AChE was assessed in study **V** using a 96 well plate format by the method described by Oinonen *et al*. (2006). Twenty five microlitres of plant extract dissolved in 50 mM Tris buffer (pH 8) was added to the wells in a 96 well plate, followed by 25 μL of 15 mM ATCI in H_2O, 125 μL of 3 mM DTNB in 50 mM Tris buffer containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$ (pH 8) and 50 μL of 50 mM Tris buffer containing 0.1% (w/v) BSA. The absorbance at 405 nm was measured ten times during ten minutes (spontaneous hydrolysis) using a Victor2 multilable counter (Perkin Elmer Life and Analytical Sciences/Wallac Oy, Turku, Finland). Then, 25 μL of 0.226 U/mL AChE in 50 mM Tris buffer containing 0.1% (w/v) BSA was added to start the reaction and the measurement was repeated. The absorbance readings were corrected by subtracting from them the spontaneous hydrolysis values. AChE inhibitory effect of the sample was estimated in terms of percentage inhibition, which is percentage change in absorbance compared to the control using Eq. 4. PHY was used as the reference standard. Concentration-response curves for the sample and PHY were plotted using percentage inhibition values vs. concentration. The potency of the extract was calculated from the standard response curve of PHY using linear regression analysis. The potency values were expressed as PHY equivalents (PhE, μg PHY/mg (dry wt.) extract).

$$\text{Percentage Inhibition} = \left[1 - \frac{\left(\text{Abs}_{1\ \text{control}} - \text{Abs}_{t\ \text{control}}\right)}{\text{Abs}_{1\ \text{sample}} - \text{Abs}_{t\ \text{sample}}}\right] x100 \quad \textbf{(Eq. 4)}$$

Where $\text{Abs}_{1\ \text{control}}$ is the absorbance of the control sample at 1 minute, $\text{Abs}_{t\ \text{control}}$ is the absorbance of the control sample at t minutes, $\text{Abs}_{1\ \text{sample}}$ is the absorbance of control at 1 minute and $\text{Abs}_{t\ \text{sample}}$ is the absorbance of control at t minutes.

4.6.2. Statistical Analysis

Data were presented as mean values ± 95% confidence interval. Analysis of variance was performed using ANOVA procedures. Significant differences between means were deter-mined by Tukey's pairwise comparison test at a level of $P < 0.05$. Microsoft Office Excel 2003, Sigmaplot 2001 and Minitab 10.5 software were used for the statistical analysis.

5. RESULTS AND DISCUSSION

5.1. *Dracocephalum moldavica*

5.1.1. Yield and Phenolic Composition

During hydrodistillation of *D. moldavica*, volatile aroma constituents were removed from the plant material and polar constituents remained in the hot water extract (HWE). The yield of the HWE (496 mg/g) from study **II** was higher than SE yields obtained in study **III**. The yields of SEs range from 3.7 mg/g (EtOAc and *n*-BuOH extracts) to 109.2 mg/g (MeOH extract), and increasing in the order EtOAc and *n*-BuOH (no significant difference, $P > 0.05$) < ACN < CH_2Cl_2 < PE < H_2O < MeOH.

The total phenolic content of the extracts, as estimated by the Folin-Ciocalteu method, ranged from non-detected amounts (PE extract) to 488.4 ± 1.8 mg gallic acid/g (MeOH extract). The total phenol content of the extracts increased in the order PE < CH_2Cl_2 < ACN < EtOAc < H_2O < *n*-BuOH < HWE < MeOH. It can be clearly seen that the extraction yields did not follow the order of total phenolic contents. This is due to the fact that plant extracts, especially of low polarity *e.g.* PE and CH_2Cl_2, extracts containing phytochemicals other than phenolics such as fatty acids and waxes.

Dracocephalum species are known to contain a range of secondary metabolites, such as terpenoids, flavonoids, both glycosides and aglycones, and hydroxybenzoic and hydroxycinnamic acids (Gohari *et al.*, 2003; Saeidnia *et al.* 2005). In study **II** using HPLC-PDA method A, phenolic compounds in the HWE were classified and quantified as hydroxycinnamic acids and flavonoids, respectively. The components identified, in their increasing order of abundance, were luteolin < luteolin-7-*O*-glucoside < apigenin < ferulic acid < rosmarinic acid < caffeic acid. This is in agreement with previous studies whereby caffeic

acid derivatives, *i.e.* caffeic and rosmarinic acids, and flavonoids were identified as the principal components of *D. moldavica* (Gu *et al*. 2004) and other *Dracocephalum* species (Gohari *et al*., 2003).

The total phenolic content of the extract (145.59 ± 0.62 mg/g) determined in study **II** by HPLC-PDA method A was similar to the value determined by Folin-Ciocalteu method (145.3± 18.9 mg/g).

In study **III** using HPLC-PDA method B, phenolic compounds in the SEs were classified and quantified as hydroxybenzoates, hydroxycinnamates and flavonoids. The components identified, in their increasing order of abundance, were apigenin < *p*-coumaric acid < quercetin and caffeic acid < ferulic acid < apigenin-7-*O*-glucoside < chlorogenic acid < rosmarinic acid. This is in general agreement with previously published qualitative-quantitative analyses of Lamiaceae species (Shan *et al*. 2005; Triantaphyllou *et al*. 2001). Rosmarinic acid was present in all the extracts with the exception of PE and CH_2Cl_2 extracts, however, the *n*-BuOH and MeOH extract had the highest content. The content of rosmarinic acid in the alcoholic extracts (*n*-BuOH and MeOH) was even higher compared to that found in the HWE. This can be explained in terms of high polarity of the solvents and their ability to penetrate cell membranes and extract intracellular material.

The total phenolic content of the extracts determined in study **III** by HPLC-PDA method B increased in the order PE < CH_2Cl_2 < EtOAc < ACN < H_2O < MeOH < *n*-BuOH was different from the trend observed in case of Folin-Ciocalteu reagent method. The total sum of phenolic contents of the extracts (476.59 ± 25.22 mg/g) determined by HPLC-PDA method B was also different from the value determined by Folin-Ciocalteu method (848.5 ± 3.12 mg/g). These results show the limitation of the Folin-Ciocalteu method. As previously mentioned,

interfering substances such as sugars react with Folin-Ciocalteu reagent to produce false positive results. It can also be seen from the results, that the solvent *n*-BuOH is even more effective than MeOH for the extraction of phenolic compounds when a Soxhlet apparatus is used. This may be due to absence of extensive intermolecular hydrogen bonding, thus it is easier for phenolic compounds to dissolve in *n*-BuOH.

Based on the results presented, a scheme can be designed for the optimum extraction of phenolic compounds from *D. moldavica* aerial material using a Soxhlet apparatus. The plant material can be defatted by initial extraction with PE followed by CH_2Cl_2, then the phenolic components can be extracted with *n*-BuOH.

5.1.2. Antioxidant Properties

Iron(III) to iron(II) reduction

Study **II**

The HWE of *D. moldavica* demonstrated the ability to reduce iron(III) to iron(II) ions. The reducing power of the extract and positive controls increased in the order HWE < Pycnogenol (a standardized extract of *Pinus pinaster* Aiton) < BHT < BHA < ascorbic acid. The HWE was less active than the positive controls. The fact that the extract has a lower reducing power than the controls may be due to the lower concentrations of electron donating constituents.

Study **III**

The SEs of *D. moldavica* demonstrated the ability to reduce iron(III) ions. The reducing power of the extracts and positive controls increased in the order PE < CH_2Cl_2 < EtOAC and *n*-BuOH (no significant difference, $P > 0.05$) < MeOH < ACN < quercetin < ascorbic acid < gallic acid. The reducing power order is different from the phenolic content trend estimated by the HPLC-DAD analysis. This is

due to the fact that substances other than polyphenolic compounds contribute to reduction of iron(III) ions.

Iron(II) chelation

The HWE of *D. moldavica* demonstrated the ability to chelate iron(II) ions in study **II**. The extract was a stronger iron(II) chelator than either ascorbic acid, BHT, BHA or Pycnogenol. EC_{50} values could not be estimated for the aforementioned positive controls due to their poor activity in this assay. However, the iron(II) chelating activity of the extract is less than that of EDTA.

By chelating transition metals the extract demonstrated the ability to prevent these species from participating in metal-catalysed initiation and hydroperoxide decomposition reactions (Gordon 1990). This is termed as a secondary antioxidant effect (Gordon 1990).

Transition metal ions have been proposed to contribute to the oxidative damage in neurodegenerative disorders like AD (Varadarajan *et al*. 2000; Bush 2003). One of the lines of treatments currently under investigation is selective low affinity binding of transition metals, known as metal protein attenuation (Doraiswamy 2002; Rosenberg 2003). Therefore, if the plant extract will also display mild chelating activity in *in vivo* studies then it can be of therapeutic potential in the treatment of AD.

DPPH$^{\bullet}$ radical scavenging

Study **II**

The HWE of *D. moldavica* demonstrated the ability to scavenge DPPH$^{\bullet}$. The scavenging activity of the extract and the positive controls increased in the order of HWE < Pycnogenol and ascorbic acid (no significant difference, $P > 0.05$) < BHA. The scavenging activity of the samples in this assay can be due to either electron and/or hydrogen

donating effects. In some cases the samples may take part in both these kind of reactions (Magalhães *et al*. 2008), which may explain the reason for the observed difference in the order of DPPH$^{\bullet}$ scavenging activity compared to iron(III) reducing power.

Study **III**

All the SEs of *D. moldavica* at a dose of 1 mg/mL, with the exception of the PE and CH_2Cl_2 extracts, demonstrated the ability to scavenge DPPH$^{\bullet}$. The scavenging activity of the extracts and the positive controls increased in the order *n*-BuOH < ACN < quercetin < MeOH < ascorbic acid and gallic acid (no significant difference, $P > 0.05$). All the extracts with the exception of the MeOH extract were less active than the positive controls. The MeOH extract was a stronger DPPH$^{\bullet}$ scavenger than quercetin.

The activity of SEs in the DPPH$^{\bullet}$ assay can be due to both electron and hydrogen donating effects. Both reaction mechanisms can contribute to the formation of hydrazine; however, iron(III) reduction assay is based only on an electron transfer mechanism. This can be the reason for the difference in the order of scavenging activity and iron(III) reducing power (Magalhães *et al*. 2008).

ABTS$^{\bullet+}$ radical scavenging

Study **II**

The HWE of *D. moldavica* demonstrated the ability to scavenge ABTS$^{\bullet+}$. The scavenging activity of the extract and the positive controls at 1 minute increased in the order HWE and BHT (no significant difference, $P > 0.05$) < ascorbic acid < BHA. The activity order changed afterwards to HWE < BHT < ascorbic acid < BHA. The order remained the same throughout the assay. This shows that BHT is slower acting antioxidant compared to the plant extract. The activity of the

extract and the control with the exception of BHT remained constant and uninterrupted throughout the assay.

Study **III**

The SEs of *D. moldavica* demonstrated the ability to scavenge $ABTS^{\bullet+}$ except for the PE and CH_2Cl_2 extracts. The initial scavenging effect, $t = 0$, of the extracts and the positive controls increased in the order *n*-BuOH < ACN and MeOH < H_2O < EtOAc < ascorbic acid < quercetin < gallic acid. The order of activity after 5 minutes changed to *n*-BuOH < ACN < MeOH < H_2O < EtOAc < ascorbic acid < quercetin < gallic acid. The hierarchy of activity thereafter remained the same throughout the remaining assay run. There were gradual but significant changes in the activities of MeOH, H_2O, EtOAc and BuOH extracts, however, the activity of ACN extract remained the same. This is an indication that the former extracts are slower acting antioxidants in this assay. Among the positive controls, it was only quercetin whose activity continued to increase up to 15 minutes. Unlike in the $DPPH^{\bullet}$ assay none of the SEs were more effective than the positive controls. The $ABTS^{\bullet+}$ scavenging results suggest that, with the exception of PE and CH_2Cl_2 extracts, components present within the extracts should have the ability to protect biological material from free radical-mediated oxidative damage. The importance of free radical scavenging in the prevention of oxidative stress is due to its potential therapeutic application in the treatment of disorders such as AD. Therefore, the free radical scavenging properties of SEs were further investigated.

Alkyl peroxyl free radical scavenging (β-carotene-linoleic acid bleaching inhibition)

The SEs of *D. moldavica* demonstrated the ability to inhibit the bleaching of β-carotene by scavenging of $ROO^{\bullet}$ radicals generated from the thermal autoxidation of linoleic acid in study **III**. The scavenging

activity of the extracts and positive controls increased in the order MeOH < PE, CH_2Cl_2, *n*-BuOH and ACN (statistically insignificant, $P > 0.05$) < *n*-BuOH and H_2O (no significant difference, $P > 0.05$) < H_2O and EtOAc (no significant difference, $P > 0.05$) < BHA. None of the extracts were as effective as the control.

The difference between this assay and the pervious ones are: (i) the utilisation of a medium consisting of aqueous and lipid phases and (ii) assessment of $ROO^{\bullet}$ scavenging activity which is of physiological relevance.

It has been proposed by some workers (Porter 1986; Frankel *et al.* 1994; Koleva *et al.* 2002) that in this assay non-polar extracts should show higher antioxidant activity than polar extracts. This is explained in terms of a concentrating effect of non-polar compound in the oil/water interphase. This phenomenon is known as the polar paradox. However, contrary to the expectations this phenomenon was not observed when screening the SEs. This anomaly has been explained by Koleva *et al.* (2003) by saying that this phenomenon is not observed in case of all extracts.

Superoxide anion radical scavenging

The HWE of *D. moldavica* demonstrated the ability to scavenge $O_2^{\bullet -}$ in study **II**. The scavenging activity of the extract and the positive controls increased in the order HWE < Pycnogenol < gallic acid. The results show that the extract has $O_2^{\bullet -}$ scavenging activity which can be of potential health interest as it may be effective in reducing elevated levels of $O_2^{\bullet -}$ during oxidative stress. Oxidative stress mediated by $O_2^{\bullet -}$ is believed to be involved in the pathogenesis of AD (Valko *et al.* 2007; Varadarajan *et al.* 2000). Therefore, $O_2^{\bullet -}$ scavenging activity is considered a relevant to AD therapy. However, there is a need to

investigate the ability of the extract to scavenge ROS *in vivo* using AD models.

Prior to the screening of the extract and control substances for $O_2^{\bullet-}$ scavenging, any potential inhibitory effect they might have upon the enzyme xanthine oxidase was determined by measuring the presence of uric acid a byproduct of xanthine oxidase-mediated generation of $O_2^{\bullet-}$. This was to ensure that any positive effect observed for the extract or control substances was due to $O_2^{\bullet-}$ scavenging and not to the inhibition of $O_2^{\bullet-}$ formation due to xanthine oxidase inhibition. Neither the extract nor the control substances inhibited the xanthine oxidase enzyme.

Ascorbate-iron(III)/EDTA-H_2O_2-catalysed 2-deoxy-D-ribose degradation

The HWE of *D. moldavica* demonstrated the ability to protect 2-deoxy-D-ribose from oxidative degradation by scavenging $HO^{\bullet}$ in study **II**. The scavenging activity of the extract and the positive controls increased in the order HWE and Pycnogenol (no significant difference, $P > 0.05$) < gallic acid.

In this assay $HO^{\bullet}$ is generated by Fenton chemistry using iron(III) ions, ascorbic acid and hydrogen peroxide. By premixing the reagents EDTA and iron(III) together before introducing them into the reaction mixture iron(III) ions are no longer available to attach to the 2-deoxy-D-ribose substrate. As a result, $HO^{\bullet}$ is generated by iron(III), ascorbate and H_2O_2 in "free" solution. Any damage which subsequently occurs to the 2-deoxy-D-ribose substrate is due to a test sample's inability to scavenge $HO^{\bullet}$ free radicals and not because of properties such as transition metal chelation. This form of the assay is known to be non-site specific (Hagerman *et al*. 1998). The significance of this result is that the extract has the potential to protect biological substrates *e.g.* carbohydrates, proteins and DNA from deleterious effect of $HO^{\bullet}$ during

oxidative stress. $HO^{\bullet}$ and other ROS are involved in the oxidative cascade involved in pathogenesis of AD. Therefore, the HWE of *D. moldavica* should be studied further for its ability to scavenge ROS *in vivo* using models simulating AD conditions.

Ascorbate-iron(III)-catalysed phospholipid degradation

The HWE of *D. moldavica* demonstrated the ability to protect bovine brain-derived phospholipid liposomes from oxidative degradation by scavenging $HO^{\bullet}$ in study **II**. The scavenging activity of the extract and the positive controls increased in the order gallic acid < HWE < Pycnogenol < BHA. The HWE was less active compared to Pycnogenol and BHA, however, the extract was a stronger $HO^{\bullet}$ scavenger than the positive control gallic acid. The $HO^{\bullet}$ scavenging ability of the extract at physiological pH and its subsequent prevention of oxidative degradation of phospholipids is of great biological relevance. The importance of this finding can be understood in the light of the fact that there is a high content of polyunsaturated fatty acid rich phospholipids in the brain and $HO^{\bullet}$ mediated lipid peroxidation plays a cardinal role in the pathogenesis of neurodegenerative disorders such as AD. Therefore, in order to investigate the therapeutic potential of the extract's scavenging there is need for further *in vivo* investigation in AD models.

5.2. *Melissa officinalis*

5.2.1. Yield and Phenolic Composition

The yield of *M. officinalis* extract from study **IV** was 308.7 mg/g and its total phenol content was estimated as 268.9 ± 21.3 mg gallic acid/g extract. In study **IV** using HPLC-PDA method C, phenolic compounds in the extract were classified and quantified as

hydroxycinnamic acids and flavonoids, respectively. The components identified, in their increasing order of abundance, were *m*-coumaric acid < naringin < naringenin < eriodictyol-7-*O*-glucoside < caffeic acid < hesperetin < hesperidin < rosmarinic acid.

From the compounds identified, rosmarinic and caffeic acids have already been reported to be present in *M. officinalis* by other researchers (Zgórka and Glowniak 2006; Carnat *et al*. 1998; Ziaková and Brandšteterova 2002, 2003). Although the remaining phenolic components have not been reported previously, their presence in other species of the Lamiaceae family has been confirmed (Dastmalchi *et al*. 2007b, 2007c; Dorman *et al*. 2003a, 2003b). In a number of compositional studies reported on this plant, benzoic acid derivatives, luteolin-7-*O*-glucoside and luteolin-3-*O*-glucuronide have been detected (Carnat *et al*. 1998; Heitz *et al*. 2000). The absence in this sample may be due to a variety of reasons ranging from climate and geography to differences in the specificity of the extraction procedures used (Zgórka and Glowniak 2006; Carnat *et al*. 1998; Ziaková and Brandšteterova 2002 and 2003; Heitz *et al*. 2000).

The total phenolic content of the extract (199.89 ± 0.41 mg/g) determined in study **IV** by HPLC-PDA method C was less than the value determined by Folin-Ciocalteu method (268.9 ± 21.3 mg/g). This may be explained due to the limitation of the latter assay in which interfering substances may participate in the assay.

5.2.2. Antioxidant Properties

Iron(III) to iron(II) reduction

The plant extract demonstrated the ability to reduce iron(III) ions in study **IV**. The reducing power of the extract and positive controls increased in the order *M. officinalis* < BHA < quercetin < ascorbic acid < gallic acid. The results indicate that the reducing power of the plant

extract was less than those of the positive controls. This follows the trend observed in the case of extracts of other Lamiaceae plants (Dastmalchi *et al*. 2007b, 2007c; Dorman *et al*. 2003a, 2003b). The reason is believed to be that the positive controls are present at much higher concentrations than the electron donating components present in the extract.

Iron(II) chelation

The plant extract demonstrated the ability to chelate iron(II) ions in study **IV**. The extract was a better chelator of iron(II) than ascorbic acid, gallic acid or BHA. The chelating activity of EDTA, however, was significantly higher ($P < 0.05$) than that of the plant extract. The relatively mild iron(II) chelating activity of the extract is of great significance.

By chelating transition metals the extract demonstrated the ability to prevent these species from participating in metal-catalysed initiation and hydroperoxide decomposition reactions (Gordon 1990). This is termed as a secondary antioxidant effect (Gordon 1990).

The chelating activity of the extract can be of potential interest in the food industry where transition metal ions by catalysing the initiation and decomposition of hydroperoxides contribute to lipid oxidation which is the main source of degradation of food products (Antolovich *et al*. 2002).

Transition metal ions have been proposed to contribute to the oxidative damage in neurodegenerative disorders like AD (Varadarajan *et al*. 2000; Bush 2003). One of the lines of treatment currently under investigation is selective low affinity binding of transition metals, known as metal protein attenuation (Doraiswamy 2002; Rosenberg 2003). Therefore, if the plant extract will also display mild chelating

activity in *in vivo* studies, it can be of therapeutic potential in the treatment of AD.

DPPH$^{\bullet}$ free radical scavenging

The plant extract demonstrated the ability to scavenge DPPH$^{\bullet}$ in study **IV**. The scavenging activity of the extract and the positive controls increased in the order *M. officinalis* < quercetin < caffeic acid < gallic acid. The extract was less active than the positive controls which may be due to the fact that antioxidant components in the extract are present at lower concentrations compared to antioxidant positive controls. The same trend in DPPH$^{\bullet}$ has been observed in other Lamiaceae plants. Based on the DPPH$^{\bullet}$ scavenging data, the extract may have the ability to scavenge free radicals generated during oxidative stress. This phenomenon is one of the pathological hallmarks of AD. Therefore it was important to fully characterise the antioxidant properties in other assays.

ABTS$^{\bullet+}$ free radical scavenging

The plant extract demonstrated the ability to scavenge ABTS$^{\bullet+}$ in study **IV**. The scavenging activity of the extract and the positive controls at 1 min increased in the order *M. officinalis* < quercetin < caffeic acid < BHA < gallic acid. However, the order of activity changed after 10 min to *M. officinalis* < quercetin and caffeic acid (not significantly different, $P > 0.05$) < BHA < gallic acid. The scavenging activity of the extract increased up to 30 minutes after which there was no significant change in the activity ($P > 0.05$). This clearly indicates that the extract was a slow acting free radical scavenger; however, throughout the assay its scavenging activity was less than those of the controls. The TEAC value calculated for *M. officinalis* is different from that recently reported by Ivanova *et al*. (2005). The reason for the

difference in the TEAC values can lie in the different extraction procedures of the plant. The higher TEAC value reported for the water infusion of the plant can be due to the higher content of phenolic substances which have been extracted.

The $ABTS^{\bullet+}$ scavenging results suggest that the components present within the extract should have the ability to protect biological material from free radical-mediated oxidative damage. The importance of free radical scavenging in the prevention of oxidative stress is due to its potential therapeutic application in treatment of disorders such as AD. Therefore, the free radical scavenging properties of the extract was further investigated in different assay conditions.

Alkyl Peroxyl Free radical Scavenging (β-Carotene-linoleic Acid Bleaching Inhibition)

The plant extract demonstrated the ability to inhibit bleaching of β-carotene by scavenging of $ROO^{\bullet}$ radicals generated from thermal autoxidation of linoleic acid in study **IV**. The scavenging activity of the extracts and positive controls increased in the order gallic and caffeic acids (statistically insignificant, $P > 0.05$) < *M. officinalis*, quercetin and BHA (statistically insignificant, $P > 0.05$).

The strong antioxidant effect of the less polar compounds, quercetin and BHA, and the plant extract, which is of a heterogeneous nature, may be explained by the 'polar paradox' phenomenon as described by Frankel *et al*. (1994). It has been proposed that less polar antioxidants exhibit stronger antioxidant properties in oil-in-water emulsions because they get concentrated at the oil-water interphase, thus protecting the lipids from oxidation. On the other hand, the polar antioxidants remain diluted in the bulk (aqueous) phase and are hence less effective in protecting the lipids.

The high radical scavenging activity of *M. officinalis* extract may be of biological relevance as it may prevent oxidation of lipid components such as linoleic acid within cell membranes (Liyana-Pathirana *et al.* 2006). Therefore, the plant extract may prove to be useful in inhibiting progression of oxidative stress mediated pathogenesis of diseases such as AD.

Superoxide anion radical scavenging

The plant extract demonstrated the ability to scavenge $O_2^{\bullet -}$ in study **IV**. The scavenging activity of the extract and the positive controls increased in the order *M. officinalis* < caffeic acid < gallic acid. Quercetin had a poor scavenging activity and its IC_{50} could not be determined. The fact that the extract is less active than the controls can be explained in terms of the concentration of radical scavenging constituents.

The results show that the extract has $O_2^{\bullet -}$ scavenging activity which can be of potential health interest as it may be effective in reducing the level of $O_2^{\bullet -}$ elevated during oxidative stress. $O_2^{\bullet -}$-mediated oxidative stress is believed to be involved in the pathogenesis of AD (Valko *et al.* 2007, Varadarajan *et al.* 2000). Therefore, the scavenging activity is considered relevant to AD therapy. However, there is a need to investigate the ability of the extract to scavenge ROS *in vivo* using AD models.

Prior to the screening of the extract and control substances for $O_2^{\bullet -}$ scavenging, any potential inhibitory effect they might have upon the enzyme xanthine oxidase was determined by measuring the presence of uric acid, a byproduct of xanthine oxidase-mediated generation of $O_2^{\bullet -}$. This was to ensure that any positive effect observed for the extract or control substances was due to $O_2^{\bullet -}$ scavenging and not the inhibition of

$O_2^{\bullet-}$ formation due to xanthine oxidase inhibition. Neither the extract nor the control substances inhibited the enzyme xanthine oxidase.

Nitric oxide ($NO^{\bullet}$) radical scavenging

The plant extract demonstrated the ability to scavenge $NO^{\bullet}$ in study **IV.** The scavenging activity of the extract and the positive controls increased in the order *M. officinalis* < caffeic acid < gallic acid. Quercetin did not exhibit any scavenging activity. The fact that quercetin is not capable of scavenging physiological radicals such as $O_2^{\bullet-}$ and $NO^{\bullet}$ should be studied further.

The $NO^{\bullet}$ scavenging activity of the extract is of potential health interest as it has been proposed that $NO^{\bullet}$ plays an important role in the progression of many diseases and pathological conditions such as septic shock, atherosclerosis, ischemia reperfusion, neurodegenerative disorders like AD, cancer and diabetes (Valko *et al*. 2007; Varadarajan *et al*. 2000; Law *et al*. 2001; Moncada *et al*. 1991; Tamir and Tannenbaum 1996). Therefore, this scavenging activity can also be considered of significant relevance to AD therapy. However, as mentioned above, the extract has to be investigated further *in vivo* using AD models.

5.2.3. Acetylcholinesterase Inhibition

The plant extract demonstrated the ability to inhibit AChE in study **V**. The extract and the positive control, PHY, inhibited AChE in a similar fashion throughout the assay period. Since the inhibitory activities of PHY and *M. officinalis* extracts were highest at the end of the assay, AChE inhibition at 10 min was used as the basis for estimation of potency. The potency of the extract at 10 min, calculated in terms of PhE, was 1.72 ± 0.16 μg PHY/mg.

AChE inhibition is the most widely studied and effective clinical approach to treat the symptoms of AD (Dastmalchi *et al*. 2008). Therefore, the activity of the extract is of great relevance in AD therapy. In addition, the extract also demonstrated a wide range of antioxidant activities which are also considered relevant in AD therapy. Therefore, there was a need for activity guided fractionation of the extract in order to identify components contributing to the activity.

5.2.4. Bioactivity Guided Fractionation

The crude *M. officinalis* extract was fractionated into twelve fractions (F1-F12) on a time-based scheme in study **V** (Table 6, Fig 1). All fractions, with the exception of fractions F10-F12, inhibited AChE activity. The PhE values at 10 min were used for estimating the potency of the fractions. Fractions F2-F9 were more potent than the crude extract (1.72 ± 0.16 µg PHY/mg), which is indicative of the complex nature of possible interactions amongst the compounds within the extract. Antagonistic and synergistic effects between various constituents within plant extracts are responsible for extracts showing lower or higher bioactivity compared to their fractions (Dewick 1997).

Fraction F8 was identified as the most potent fraction (25.36 ± 1.63 µg PHY/mg), whilst the least potent fraction was identified as fraction F1 (0.55 ± 0.03 µg PHY/mg). Interesting, the least potent fraction (F1) had the highest inhibitory activity (44.35 ± 1.31 %). This shows the importance of using PhE as opposed to percentage inhibition for potency estimation. In calculating the former, the yield of the sample being screened is taken into account, however, that is not the case when estimating the latter.

The most active fraction was further analysed for its composition using LC-DAD-ESI-MS analysis. Based on the retention characteristics, mass and UV spectral data, the components present in fraction F8 were

identified as apigenin-hexoside, rosmarinic acid and two rosmarinic acid derivatives. However, there are limitations associated with this analytical method which are (i) possible fragmentation of ions before reaching ion trap, (ii) suppression of ionisation by the constituents present in the plant extract and (iii) if the constituents fall outside the chemical class for which the MS parameters have been optimised they will not be detected. Due to these limitations there is a need for NMR studies of the extract. The presence of *cis*- and *trans*-rosmarinic acid isomers have been confirmed. Another chemical constituent present in the fraction is suspected to be close derivatives of rosmarinic acid.

6. CONCLUSION

Recently, there has been an exponential growth in interest in natural antioxidants, in particular plant-derived antioxidants from aromatic, medicinal and edible plants. The plants *D. moldavica* L. and *M. officinalis* L., which belong to the family Lamiaceae, have been used in Iran because of their culinary usefulness and medicinal properties. Therefore, extracts of the plants were screened for antioxidant properties in a battery of *in vitro* assays. The plant extracts demonstrated a wide range of antioxidant properties, some of which were relevant to AD therapy. These results warrant further investigation of the plant extracts for their ability to prevent oxidative stress in *in vivo* models of AD. The wide spectrum of antioxidant activity of the extract is a proof that natural antioxidants exert their effect *via* multiple mechanisms.

The compositional fingerprint analysis of the plant extracts indicates that polyphenolic compounds present in the plants are in the form of hydroxylated benzoic and cinnamic acid derivatives and flavonoids. These components are proven antioxidants; therefore, it is possible they are contributing to the activity of the extracts. However, as seen from the β-carotene-linoleic acid bleaching inhibition results, there are also non-polar components which are contributing to the antioxidant activities of the plant extracts. Rosmarinic acid was the most abundant constituent in both the plants. This feature is shared with many Lamiaceae plants. *n*-BuOH was found to be the most effective solvent for the extraction of polyphenolic compounds from *D. moldavica*. The least effective solvents were PE and CH_2Cl_2.

A review of the published biological, pharmacological and clinical studies show that plant extracts traditionally used in the treatment of cognitive dysfunction have provided novel molecular templates for effective drugs against AD. Therefore, it can be concluded that the

screening of plants for bioactivities relevant to AD therapy may provide useful leads in the discovery of drugs against the disease.

Based on its ethnomedical use and recent success in a clinical study, *M. officinalis*, in addition to antioxidant evaluation, was screened for another bioactivity relevant to AD therapy, *viz*., AChE inhibition. The plant extract was capable of AChE inhibition, which formed the basis of activity guided fractionation of the crude extract.

The majority of the fractions (F1-F9) obtained from the semi-preparative scale HPLC fractionation of the plant extracts demonstrated AChE inhibitory activity with fractions F2-F9 proving to be significantly ($P < 0.05$) more potent than the crude extract. This is an example of antagonism between phytochemical constituents present within the plant extract. Fraction F8 was the most potent fraction. To resolve the identity of the components within the fraction, it was submitted to further analysis using LC-DAD-MS and NMR techniques, which resulted in the elucidation of *cis*- and *trans*-rosmarinic acid isomers.

There is a need to further investigate the efficacy of these chemical constituents in different AD models. Compositional analysis of the remaining potent fractions should be carried out in order to determine the identity of their chemical constituents. Furthermore, there is a need to investigate the possibility of a correlation existing between antioxidant and AChE inhibitory activities.

7. REFERENCES

Anon, *Iranian Herbal Pharmacopoeia*, Ministry of Health Publication, Tehran, 2002, *1*, pp-141–121

Antolovich M, Prenzler P, Pastalides E, Donald S (2002) Methods for testing antioxidant activity. *The Analyst* **127**, 183-198

Aruoma OI, Murcia A, Butler J, Halliwell B (1993) Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *Journal of Agricultural and Food Chemistry* **41**, 1880-1885.

Aruoma OI, Spencer J, Warren D, Jenner P, Butler, J, Halliwell B (1997). Characterization of food antioxidants, illustrated using commercial garlic and ginger preparations. *Food Chemistry* **60**, 149–156

Aynehchi Y, Sormaghi MHS, Amin GH, Soltani A, Qumehr N (1982). Survey of Iranian plants for saponins, alkaloids, flavonoids and tannins. II. *International Journal of Crude Drug Research* **20**, 61-70

Bartosz G, Janaszewska A, Ertel D, Bartosz M (1998) Simple determination of peroxyl radical-trapping capacity. *Biochemsitry and Molecular Biology International* **46**, 519-528

Beers RF, Sizer IW (1952) A spectroscopic method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biology Chemistry* **195**, 133-140

Brand-Williams W, Cuvelier ME, Berest C (1995) Use of a free radical method to evaluate antioxidant activity. *Food Science and Technology* **28**, 25-30

Branen AL, Davidson PM (1997) Use of antioxidants in self-preserving cosmetic and drug formulations. *Cosmetic Science and Technology Series* **16**, 159-179

Bush AI (2003) The metallobiology of Alzheimer's disease. *Trends in Neuroscience* **26,** 207-214

Cao G, Alessio HM, Cutler RG (1993) Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine* **14**, 303-311

Cao G, Verdon CP, Wu AH, Wang H, Prior R (1995) Automated assay of oxygen radical absorbance capacity with COBAS FARA II. *Clinical Chemistry* **41,** 1738-1744

Castro A, Conde S, Rodriguez-Franco MI, Martinez A (2002) Non-cholinergic pharmacotherapy approaches to the future treatment of Alzheimer's disease. *Mini Reviews in Medicinal Chemistry* **2**, 37-50

Carnat AB, Carnat D, Lamaison J-L (1998) The aromatic and polyphenolic compositions of lemon balm (*Melissa officinalis* L. Subsp. officinalis) tea. *Pharmaceutica Acta Helvetiae* **72**, 301-305

Carter P (1971) Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). *Analytical Biochemistry*, **40**, 405-458

Dastmalchi K, Dorman HJD, Vuorela H, Hiltunen R (2007a) Plants as potential sources for drug development against Alzhiemr's disease. *International Journal of Biomedical and Pharmaceutical Sciences*,**1**, 83-104

http://www.globalsciencebooks.info/Journals/IJBPS.html

Dastmalchi K, Dorman HJD, Koşar M, Hiltunen R (2007b) Chemical composition and *in vitro* antioxidant evaluation of a water soluble Moldavian balm (*Dracocephalum moldavica* L.) extract. *Food Science and Technology* **40**, 239-248

Dastmalchi K, Dorman HJD, Laakso I, Hiltunen R (2007c) Chemical composition and antioxidant activity of Moldavian

balm (*Dracocephalum moldavica* L.) extracts. *Food Science and Technology* **40***,* 1655-1663

Dastmalchi K, Dorman HJD, Oinonen PP, Darwis Y, Laakso I, Hiltunen R (2008) Chemical composition and *in vitro* antioxidant activity of a lemon balm (*Melissa officinalis* L.) extract. *Food Science and Technology* **41**, 391-400

DeLange RJ, Glazer AN (1989) Phycoerythrin fluorescence-based assay for peroxyl radicals: a screen for biologically relevant protective agents. *Analytical Biochemistry* **28**, 300-306

Dewick PM (1997) Tumor inhibitors from plants. In: Evans WC (Eds) Trease and Evans' Pharmacognsy, W.B. Saunders Company, London, UK pp 409-425

Doraiswamy PM (2002) Non-Cholinergic strategies for treating and preventing Alzheimer's disease. *CNS Drugs* **16**, 811-824

Dorman HJD, Bachmayer O, Koşar M, Hiltunen R (2003a) Antioxidant properties of aqueous extracts from selected Lamiaceae species grown in Turkey. *Journal of Agricultural and Food Chemistry* **51**, 4563-4569

Dorman HJD, Koşar M, Kahlos K, Holm Y, Hiltunen R (2003b) Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties, and cultivars. *Journal of Agricultural and Food Chemistry* **51**, 4563-4569

Durany N, Münch G, Michel T, Riederer P (1999) Investigations on oxidative stress and therapeutical implications in dementia. *European Archives of Psychiatry and Clinical Neuroscience* **249**, S68-S73

Erel O (2004) A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry* **37**, 277-285

Ewing JF, Janero DR (1995) Micorplate superoxide dismutase employing nonenzymatic superoxide generator *Analytical Biochemistry* **232**, 243-248

Ferreria A, Proença C, Serralheiro MLM, Araújo MEM (2006) The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plant from Portugal. *Journal of Ethnopharmacology* **108**, 31-37

Fillit HM (2000) The pharmacoeconomics of Alzheimer's disease *The American Journal of Managed Care* **22**, S1139-S1148

Folin O (1927) Tyrosine and tryptophan determination in proteins. *Journal of Biological Chemistry* **73**, 627-649

Foti MC, Daquino C, Geraci J (2004) Electron transfer reactions of cinnamic acids and their methyl esters with $DPPH^{\bullet}$ radical in alcohol solutions. *Journal of Organic Chemistry* **69**, 2309-2314

Francis PT, Palmer AM, Snape M, Wilcock GK (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. *Journal of Neurology Neurosurgery an Psychiatry* **66**, 137-147

Frankel EN, Huang S-W, Kanner J, German JB (1994) Interfacial phenomena in the evaluation of antioxidants: bulk oils vs emulsions. *Journal of Agricultural and Food Chemistry* **42**, 1054–1059

Frankel EN, Meyer AS (2000) The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants *Journal of the Science of Food and Agriculture* **80**, 1925-1941.

Fu YL, Krasnovsky CS, Foote CS (1997) Quenching of Singlet Oxygen and Sensitized Delayed Phthalocyanine Fluorescence. *Journal of Physical Chemistry A* **101**, 2551-2554

Gohari AR, Saeidnia S, Matsuo K, Uchiyama N, Yagura T, Ito M, Kiuchi F, Honda G (2003). Flavonoid constituents of *Dracocephalum kotschyi* growing in Iran and their trypanocidal activity. *Natural Medicines* **57**, 250-252

Gordon M (1990) The mechanism of antioxidant action in vitro. In: Hudson, BJF (Ed.) *Food Antioxidants*. Elsevier Applied Science, London pp.1-18

Gu HF, Chen RY, Sun YH, Lui F (2004) Studies on chemical constituents from herb of *Dracocephalum moldavica*. *Zhongguo Zhong Yao Za Zhi* **29**, 232-234

Gyamfi MA, Yonamine M, Aniya Y (1999) Free-radical scavenging action of medicinal herbs from Ghana: *Thonningia sanguinea* on experimentally-induced liver injuries. *General Pharmacology* **32**, 661–667

Hagerman AE, Riedl KM, Jones GA, Sovik KN, Ritchard PW, Hartzfeld TL, Riechel J (1998) High molecular weight polyphenolics (tannins) as biological antioxidants *Journal of Agricultural and Food Chemistry* **46**, 1887-1892

Halliwell B (2006) Reactive species and antioxidants. Redox Biology is a fundamental theme of Aerobic life. *Plant Physiology* **141**, 312-322

Halliwell B, Gutterdge JMC (1995) The definition and measurement of antioxidants in biological systems. *Free Radical Biology & Medicine* **18**, 125-126

Halliwell B, Gutteridge JMC, Aruoma OI (1987) The deoxyribose method: A simple "test tube assay" for determination of rate constants for reactions of hydroxyl radicals. *Analytical Biochemistry* **165**, 215-219

Haung D, Ou B, Hampsch-Woodill M, Flanagan J, Prior RL (2002) High-throughput assay of oxygen radical absorbance capacity

(ORAC) using a multichannel liquid handling system coupled with a micorplate fluorescence reader in 96- well format. *Journal of Agricultural and Food Chemistry* **50**, 4437-4444

Haung D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry* **53**, 1841-1856

Heinrich M, Teoh HL (2004) Galantamine from snowdrop- the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *Journal of Ethnopharmacology* **92**, 147-162

Heitz A, Carnat A, Fraisse D, Carnat A-P, Lamaison J-L (2000) Luteolin 3-glucuronide, the major flavonoid from *Melissa officinalis* subsp. *officinalis. Fitoterapia* **71***,* 201-202

Houghton PJ, Howes M-J, Lee CC, Steventon G (2007) Uses and abuses of in vitro tests in ethnopharmacology: Visualizing an elephant. *Journal of Ethnopharmacology* **110***,* 391-400

Houghton PJ, Howes M-J (2005) Natural products and derivatives affecting neurotransmissions relevant to Alzheimer's and Parkinson's disease. *Neurosignals* **14**, 6-22

Ivanova D, Gerova D, Chervenkov T, Yankova T (2005) Polyphenols and antioxidant capacity of Bulgarian medicinal plant. *Journal of Ethnopharmacology* **96**, 145-150

Jones WP, Chin Y-W, Kinghorn AD (2006) The role of Pharmacognosy in Modern Medicine and Pharmacy. *Current Drug Targets* **7**, 247-264

Kakasy AZ, Lemberkovics E, Kursinszki L, Janicsak G, Szoke, E. (2002) Data to the phytochemical evaluation of Moldavian dragonhead (*Dracocephalum moldavica* L., Lamiaceae). *Herba Polonica* **48**, 112-119

Koleva II, van Beek TA, Linssen JPH, de Groot A, Evstatieva, LN (2002) Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochemical Analysis* **13**, 8-17

Koleva II, Linssen JPH, van Beek TA, Evstatieva LN, Kortenska V, Handjieva, N (2003). Antioxidant activity screening of extracts from *Sideritis* species (Labiatae) grown in Bulgaria. *Journal of the Science of Food and Agriculture* **83**, 809-819

Lahiri DK, Farlow MR, Sambamurti K, Greig NH, Giacobini E, Schneider LS (2003) A critical analysis of new molecular targets and strategies for drug development in Alzheimer's disease. *Current Drug Targets* **4**, 97-112

Laguerre M, Lecomte P, Villeneuve P (2007) Evaluation of the ability of antioxidants to counter lipid oxidation: Existing methods, new trends and challenges *Progress in Lipid Research* **46**, 244-282

Law A, Gauthier S, Quirion, R. (2001) Say NO to Alzheimer's disease: the putative links between nitric oxide and dementia of the Alzheimer's type. *Brain Research Reviews* **35**, 73-96

Lee J-C, Kim H-R, Kim J, Jang Y-S (2002) Antioxidant property of an ethanol extract of the stem of *Opuntia ficus-indica* var. *saboten*. *Journal of Agricultural and Food Chemistry* **50**, 6490-6496

Lemanska K, Szymusiaka H, Tyrakowaska B, Zeilinski R, Soffer AEMF, Rietjens IMCM (2001) The influence of pH on antioxidant properties and the mechanisms of antioxidant action of hydroxyflavones. *Free Radical Biology & Medicine* **31,** 869-881

Liyana-Pathirana CM, Shahidi F (2006) Antioxidant properties of commercial soft and hard winter wheats (*Triticum aestivum* L.)

and their milling fractions. *Journal of the Science of Food and Agriculture* **86**, 477-485

Magalhães LM, Segundo MA, Reis S, Lima JLFC (2008) Methodological aspects about *in vitro* evaluation of antioxidant properties. *Analytical Chemica Acta* **613**, 1-19

Marco GL (1968) A rapid method for evaluation of antioxidants. *Journal of the American Oil Chemists' Society* **45**, 594-598

Marcocci L, Maguire JL, Droy-Lefaix MT, Packer L (1994) The nitric oxide scavenging properties of ginkgo biloba extract Egb 761. *Biochemical and Biophysical Research Communications* **201**, 748-755

Mason RP, Olmstead EG, Jacob RF (2000) Antioxidant activity of the monoamine oxidase B inhibitor Lazabemide. *Biochemical Pharmacology* **60**, 709-716

Miller HE (1971) A simplified method for the evaluation of antioxidants. *Journal of the American Oil Chemists' Society* **48**, 91

Miller NG, Diplock AT, Rice-Evans C, Davis MJ, Gopinathan V, Milner A (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science* **84**, 407-412

Moncada S, Palmer, RMJ, Higgs EA (1991) Nitric oxide: Physiology, pathology and pharmacology. *Pharmacological Reviews* **43**, 109-142

Naguib YMA (2000) Antioxidant activities of astaxanthins and related carotenoids. *Journal of Agricultural and Food Chemistry* **48**, 1150-1154

Noruma T, Kikuchi M, Kawakami Y (1997) Proton-donative antioxidant activity of fucoxanthin with 1,1-diphenyl-2-

picrylhydrazyl (DPPH). *Biochemistry and Molecular Biology International* **42**, 361-370

Oinonen PP, Jokela JK, Hatakka AI, Vuorela PM (2006) Linarin, a selective acetylcholinesterase inhibitor from *Mentha arvensis*. *Fitoterapia* **77**, 429-434

Orhan G, Orhan I, Şener B (2006) Recent developments in natural and synthetic drug research for Alzheimer's disease. *Letters in Drug Design & Discovery* **3**, 268-274

Ou B, Hampsch-Woodill M, Prior RL (2001) Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry* **49**, 4619-4926

Oyaizu, M (1986) Studies on products of browning reaction. In: Antioxidative activity of products of browning reaction. *Japanese Journal of Nutrition* **44**, 307-315

Part ED (1999) Natural antioxidants not exploited commercially. In: Hudson BJF (Ed.) *Food Antioxidants*, Elsevier Applied Science, London, pp1-18

Pazdzioch-Czochra M, Widenska A (2002) Spectrofluorimetric determination of hydrogen peroxide scavenging activity. *Analytica Chimica Acta* **452**, 177-184

Pollard SE, Gunter GCK, Vauzour D, Vafeiadou K, Tzounis X, Whiteman M, Rice-Evans C, Spencer JPE The reaction of flavonoid metabolites with peroxynitrite. *Biochemical and Biophysical Research Communication* **350**, 960-968

Porter WL (1986). Recent trends in food applications of antioxidants. In: Simic MG, Karel M (Eds.) *Autooxidation in Food and Biological Systems*, Plenum Press, New York, pp. 295-365

Povilaityte V, Venskutonis PR (2000) Investigation of antioxidative activity of purple peril (*Perilla frutescens* L.), Moldavian

dragonhead (*Dracocephalum moldavica* L.) and Roman chamomile (*Anthemis nobilis* L.) extracts in rapeseed oil. *Journal of the American Oil Chemists' Society* **77**, 951-956

Povilaityte V, Cuvelier M-E, Berset C (2001) Antioxidant properties of Moldavian dragonhead (*Dracocephalum moldavica* L.). *Journal of Food Lipids* **8**, 45-64

Prior RL, Hoang H, Gu L, Wu X, Bacchiocca M, Howard L, Hampsch-Woodill M, Huang D, Ou B, Jacob R (2003) Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry* **51**, 3273-3279

Prior RL, Wu XL, Schaich K (2005) Standardized methods for determination of antioxidant capacity and phenolics in food and dietary supplements. *Journal of Agricultural and Food Chemistry* **53**, 4290-4302

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorisation assay. *Free Radicals in Biology and Medicine* **26**, 1231-1237

Rosenberg RN (2003) Metal chelation therapy for Alzheimer's disease. *Archives of Neurology* **60**, 1678-1679

Roginsky V, Eduardo AL (2005) Review of methods to determine chain-breaking antioxidant activity in food. *Food Chemistry* **92,** 235-254

Saeidnia S, Gohari AR, Ito M, Kiuchi F, Honda G (2005) Bioactive constituents from *Dracocephalum subcapitatum* (O. Kuntze) Lipsky. *Zeitschrift für Naturforschung C* **60**, 22-24

Samuelsson G (2004) *Drugs of Natural Origin: A Text Book of Pharmacognosy*, Aotekarsocieteten-Swedish Pharmaceutical

Society, Swedish Pharmaceutical Press, Stockholm, Sweden, pp. 20

Sasaki S, Ohta T, Decker EA (1996) Antioxidant activity of water-soluble fractions of salmon spermary tissue. *Journal of Agricultural and Food Chemistry* **44**, 1682–1686

Sayre LM, Smith MA, Perry G (2001) Chemistry and biochemistry of oxidative stress in neurodegenerative disease. *Current Medicinal Chemistry* **8**, 721-738

Scorer CA (2001) Preclinical and clinical challenges in the development of disease modifying therapies for Alzheimer's disease. *Drug Discovery Today* **6**, 1207-1219

Shan B, Cai YZ, Sun M, Corke H (2005) Antioxidant capacity of 26 spice extracts and charaterisation of their phenolic constituents. *Journal of Agricultural and Food Chemistry* **53**, 7749-7759

Shu Y-Z (1998) Recent natural products based drug development: a pharmaceutical industry perspective. *Journal of Natural Products* **61**, 1053-1071

Singleton VL, Orthofer R, Lamuela-Raventós RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology* **299**, 152-315

Small SA, Mayeux R (2005) Alzheimer's disease and related dementias. In: Rowland LP (Ed) *Merritt's Neurology*, Lippincott Williams & Wilkins, Philadelphia, USA, 107, pp. 771-776

Strube M, Haenen GRRM, Van den Berg H, Bast A (1997) Pitfalls in a method for assessment of total antioxidant capacity *Free Radical Research* **26**, 515-521

Tamir S, Tannenbaum, SR (1996). The role of nitric oxide ($NO^{\bullet}$) in the carcinogenic process. *Biochimica & Biophysica Acta* **1228**, F31-F36

Triantaphyllou K, Blekas, G, Boskou D (2001) Antioxidant properties of water extracts obtained from herbs of the species Lamiaceae. *International Journal of Food Science and Nutrition* **52**, 313-317

Valko M, Leibfritz D, Moncol J, Cronin, MTD, Mazur M, Tesler J (2007) Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cellular Biology* **39**, 44-84

VanMiddlesworth F, Cannell RJP (1998) Dereplication and partial identification of natural products. In: Cannell RJP (Ed) *Methods in Biotechnology 4: Natural Product Isolation*, Humana Press, Totowa, New Jersey, USA pp 291

Varadarajan S, Yatin S, Aksenova M, Butterfield DA (2000) Review: Alzheimer's amyloid β-peptide-associated free radical oxidative stress and neurotoxicity. *Journal of Structural Biology* **130**, 184-208

Venkutonis PR, Gruzdiene D, Trizite D, Trizite G (2005) Assessment of antioxidant activity of plant extracts by different methods *Acta Horticulturae* **677**, 99-107

Vriesman MF, Haenen GRMM, Westerveld GJ, Paquay JBG, Voss HP, Bast A (1997) A method for measuring nitric oxide radical scavenging activity. Scavenging properties of sulphur containg compounds. *Pharmacy World & Science* **19**, 283-286

Wayner DDM, Burton GM, Ingold KU, Locke S (1985) Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation *FEBS Letters* **187**, 33-37

Wilkinson F, Helman WP, Ross AB (1995) Rate constants for decay and reactions of the lowest electronically excited single state of molecular oxygen in solution. An expanded and revised

compilation. *Journal of Physical and Chemical Reference Data* **24**, 663-1021

Zargari AI *Medicinal Plants*, Tehran University Press, Tehran 1990, *4*, 77-81

Ziaková A, Brandšteterova E (2002) Application of different preparation techniques for extraction of phenolic antioxidants from lemon balm (*Melissa officinalis*) before HPLC analysis. *Journal of Liquid Chromatography and Related Technology* **25**, 3017-3032

Ziaková A, Brandšteterova, E (2003). Validation of HPLC determination of phenolic acids present in some Lamiaceae family plants. *Journal of Liquid Chromatography and Related Technology* **26**, 443-453

Zgórka G, Glowniak K (2006). Variation of free phenolics acid in medicinal plants belonging to the *Lamiaceae* family *Journal of Pharmaceutical and Biomedical Analysis* **26**, 79-87

8. APPENDICES

Table 1. List of compound used

Compound	Source
2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonate) diammonium salt	Sigma Chemicals Co.
Acetylcholinesterase	Sigma Chemicals Co.
Acetylthiocholine iodide	Sigma Chemicals Co.
Acetonitrile	Rathburn Chemicals Ltd.
Ascorbic acid	Sigma Chemicals Co.
Apigenin	Extrasynthase
Apigenin-7-*O*-glucoside	Extrasynthase
Butylated hydroxyanisole	Sigma Chemicals Co.
Butylated hydroxytoluene	Sigma Chemicals Co.
Bovine serum albumin	Sigma Chemicals Co.
n-Butanol	Rathburn Chemicals Ltd.
Caffeic acid	Extrasynthase
Chlorogenic acid	Extrasynthase
m-Coumaric acid	Extrasynthase
o-Coumaric acid	Extrasynthase
p-Coumaric acid	Extrasynthase
Dichloromethane	Rathburn Chemicals Ltd.
1,1′-Diphenyl-2-picrylhydrazine	Sigma Chemicals Co.
Dimethyl sulphoxide	Sigma Chemicals Co.
5,5′-Dithiobis(2-nitrobenzoic Acid)	Sigma Chemicals Co.
Ethylenediaminetetraacetic acid	Sigma Chemicals Co.
Eriocitrin	Extrasynthase
Eriodictyol	Extrasynthase
Eriodictyol-7-*O*-glucoside	Extrasynthase
Ethyl acetate	Rathburn Chemicals Ltd.
Ferrozine	Sigma Chemicals Co.

Ferulic acid	Extrasynthase
Folin Ciocalteu reagent	Sigma Chemicals Co.
Gallic acid	Sigma Chemicals Co.
Hesperetin	Extrasynthase
Hesperidin	Extrasynthase
Hydrogen peroxide	Fluka BioChemika
p-Hydroxybenzoic acid	Fluka BioChemika
Hypoxanthine	Sigma Chemicals Co.
Hyperoside	Extrasynthase
Iron(III) chloride	Sigma Chemicals Co.
Iron(II) chloride	Sigma Chemicals Co.
Isorhamnetin	Extrasynthase
Isorhoifolin	Extrasynthase
Kaempferol	Extrasynthase
Kaempferol-7-*O*-glucoside	Extrasynthase
Potassium hydroxide	Merck
Potassium dihydrogen phosphate	Merck
Linoleic acid	Sigma Chemicals Co.
Luteolin	Extrasynthase
Luteolin-7-*O*-glucoside	Extrasynthase
Magnesium chloride hexahydrate	Merck
Magnesium sulphate (anhyd.)	Sigma Chemicals Co.
Methanol	Rathburn Chemicals Ltd.
Sodium chloride	Farmitalia Caro Erba
Disodium ethylenediaminetetraacetate	Sigma Chemicals Co.
Sodium hydroxide	Merck
Sodium dihydrogen phosphate	Merck
Naringenin	Extrasynthase
Naringenin-7-*O*-glucoside	Extrasynthase
Naringin	Extrasynthase
Nitroblue tetrazolium	Sigma Chemicals Co.
Phsophate buffer saline tablets	Sigma Chemicals Co.

Petroleum ether	Rathburn Chemicals Ltd.
Physostigmine	Fluka BioChemika
Potassium peroxosulfate	Sigma Chemicals Co.
Potassium hexacyanoferrate	Fluka BioChemika
Pycnogenol	Biolands Arômes
Quercetin	Extrasynthase
Rhamnetin	Extrasynthase
Rosmarinic acid	Extrasynthase
Sodium carbonate	Merck
TCA	Sigma Chemicals Co.
TBA	Sigma Chemicals Co.
TFA	Fluka BioChemika
Tris HCl	Sigma Chemicals Co.
Tizma base	Sigma Chemicals Co.
Trolox	Sigma Chemicals Co.
Tween 20	Fluka BioChemika
Vanillic acid	Extrasynthase
Vitexin	Extrasynthase
Xanthine oxidase	Sigma Chemicals Co.

Table 2. Mobile phase composition during method A

Time(min)	(A/B)	Flow Rate (mL/min)
0	100:0	1
30	80:20	1
35	80:20	1
45	50.50	1
50	10:90	1
55	100.0	1
60	100:0	1

Table 3. Mobile phase composition during method B

Time(min)	(A/B/C)	Flow Rate (mL/min)
0	85:15:0	0.7
15	70:30:0	0.7
18	60:40:0	0.7
30	60:40:0	0.7
35	0:100:0	0.7
37	0:85:15	0.7
48	0:70:30	0.7
63	85:15:0	0.7

Table 4. Mobile phase composition during method C

Time(min)	(A/B)	Flow Rate (mL/min)
0	80:20	1
5	80:20	1
10	70:30	1
20	55.45	1
25	55:45	1
45	20:80	1
55	20:80	1
60	80:20	1
80	80:20	1

Table 5. Mobile phase composition during HPLC run

Time (min)	(A/B)	Flow Rate (mL/min)
0	90:10	3.5
5	90:10	3.5
15	70:30	3.5
25	57:43	3.5
37	57:43	3.5
55	20:80	3.5
57	20:80	3.5
60	90:10	3.5
72	90:10	3.5

Table 6. Fractionation scheme

Time (min)	Collection Event
4	Start
9	Stop
9	Start
14	Stop
14	Start
19	Stop
19	Start
24	Stop
24	Start
29	Stop
29	Start
34	Stop
34	Start
37	Stop
37	Start
40	Stop
40	Start
45	Stop
45	Start
50	Stop
50	Start
55	Stop
55	Start
60	Stop
60	Start
62	Stop

Table 7. Mobile phase composition during LC-DAD-ESI-MS

Time (min)	(A/B)	Flow Rate (µL/min)
0	95:5	75
5	95:5	75
15	70:30	75
25	55:45	75
30	55:45	75
65	5:95	75
70	5:95	75
75	95:5	75
105	95:5	75

Figure 1. Semi-preparative HPLC based fractionation of the *M.officinalis* extract