ANALYSIS OF SELECTED MEDICINAL PLANTS AS ANTIOXIDANTS WITH THERAPEUTIC POTENTIAL FOR TREATING DISEASES RELATED TO FREE RADICAL DAMAGE

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ABSTRACT

Oxidative damage is implicated in the pathogenesis of a number of diseases. Scientific research shows positive links between accumulated free radical damage and age-related diseases such as atherosclerosis and osteoarthritis. There is great interest in the possibility that the antioxidant potential of plant-derived compounds such as flavonoids may reduce the risk of developing these conditions. The aim of this study was to evaluate the antioxidant activity of selected non-food plants, traditionally used by herbalists in their treatment of osteoarthritis, using crude plant extracts and herbal tinctures, the most commonly used form of plant extract. As herbalists traditionally argue that herbs used in combinations or formulae will increase in efficacy when used together, an exploratory study was further carried out to investigate whether the antioxidant activity of two herbs tested in combination was greater than the sum of both herbs tested singly.

Eight plants were selected for phytochemical analysis and investigation for antioxidant activity, based on discussions with clinic supervisors from four herbal medicine training clinics and a review of patient's case notes. The prescriptions from a pilot study investigating outcomes for the herbal treatment of osteoarthritis were used as selection criteria.

Chromatographic analysis of each plant by TLC, HPLC and GCMS confirmed the presence of a number of flavonoids reported in the literature and of other compounds which were not possible to identify. Previous studies have established that certain flavonoids *in vitro* can exert pro-oxidant or antioxidant effects according to the concentration and presence of transition metal ions such as copper and iron. In view of the pro-oxidant effects observed for some extracts during biochemical analysis, metal analysis by ICP was carried out on the selected plant material to test for the presence of selected metal ions known to catalyse free radical reactions. ICP analysis showed the presence of most of the selected metals in all the plant samples.

Several pathways, by which flavonoids and other plant phenolics may exert their effects on chemical oxidation have been identified, one of which is their free radical scavenging capacity to halt the propagation stage of lipid peroxidation. Since lipid

peroxidation is implicated in the pathogenesis of osteoarthritis, assays to measure this *in vitro* were investigated and the following two assays selected:- lipid peroxide assay using the ferric thiocyanate method for the detection of peroxides and an assay using the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) an established method for investigating the potential free radical scavenging activity of plant extracts. The lipid peroxide assay and method of analysis was re-evaluated and a standardised procedure established.

All eight crude plant extracts showed marked antioxidant activity in both assays. Results for the crude plant extract in the lipid peroxide assay varied according to concentration, with 0.1% w/v giving the best results. The crude plant extracts in almost all cases seemed to be more active as antioxidants than tinctures (fluid extracts). When combinations of crude plant extracts were tested in pairs for antioxidant activity, results demonstrated synergy from five of the pairs and antagonism from three, approximately one third of the possible 28 two-herb combinations tested. The synergistic interactions observed could form the foundation for the future development of an antioxidant formula to offset the effects of free radical damage.

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PREFACE

This study was carried out at Middlesex University in the Herbal Medicine Department, part of the Human and Healthcare Sciences Academic Group. I am indebted to Middlesex University for my research studentship award, without which this research project would not have been possible. I am also grateful for the fieldwork expenses awarded which enabled me to carry out High Performance Liquid Chromatography at the Jodrell Laboratory, Royal Botanic Gardens, Kew.

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ABBREVIATIONS

General abbreviations

AD	Alzheimer's disease
ATP	Adenosine triphosphate
BHT	Butylated hydroxy-toluene
CVD	Cardiovascular disease
DAD	Diode array detector
DNA	Deoxyribonucleic acid
DPPH$^{\cdot}$	α,α–diphenyl–β–picrylhydrazyl (free radical)
FRSA	Free radical scavenging activity
GC/MS	Gas chromatography/ Mass spectroscopy
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
ICP	Inductively coupled plasma analysis
IR	Infrared
LDL	Low density lipoprotein
MW	Mann Whitney test
NMR	Nuclear magnetic resonance
OA	Osteoarthritis
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SOD	Superoxide dismutase
TLC	Thin layer chromatography
UV	Ultraviolet

Chemical Formulae

Cu	copper
Cu^{+}	reduced copper ion
Cu^{2+}	oxidised copper ion
EtOH	ethanol
Fe	iron
Fe^{2+}	reduced iron (ferrous) ion
Fe^{3+}	oxidised iron (ferric) ion
$FeCl_2$	ferrous chloride
$FeCl_3$	ferric chloride
H	hydrogen
HCl	hydrochloric acid
H_2O	water
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
$LOO^{\cdot}$	peroxyl radical
$R(C)OO^{\cdot}$	peroxyl radical
$RO^{\cdot}$	alkoxyl radical
LOOH	lipid hydroperoxide
R(C)OOH	hydroperoxide
MeOH	methanol
$NO^{\cdot}$	nitric oxide radical
$NO_2^{\cdot}$	nitrogen dioxide radical
ONOO-	peroxynitrite
$^1O_2$	singlet oxygen
$O_2^{\cdot -}$	superoxide anion
O_2	oxygen
O_3	ozone
OH group	hydroxyl group
$OH^{\cdot}$	hydroxyl radical

Units of Measurement

nm	nanometers
R_f value	Rate of flow i.e. distance moved by solute divided by distance moved by solvent front in TLC
t_R	Retention time in chromatography
v/v	volume/volume i.e. percent volume of a constituent in 100ml of volume
w/v	weight/volume i.e. weight in volume, as the number of grams of constituent in 100ml solution

GENERAL INTRODUCTION

Over the last few decades, research into the role and involvement of oxygen and nitrogen-generated free radicals in the pathogenesis of many of the degenerative diseases of ageing has advanced, with the establishment of a greater body of evidence in regard to the number of essential biological functions associated with these species and their specific contribution to disease pathology. A fine biological balance exists between the normal physiological formation of reactive oxygen and nitrogen species and their removal. An excess of oxidative stress can lead to the oxidation of lipids, DNA and proteins, which is associated with cell injury. Oxidation of the polyunsaturated fatty acids in cell membranes by reactive oxygen species can lead to a biologically significant process implicated in many cell and tissue abnormalities known as lipid peroxidation. The uncontrolled reaction of lipid peroxidation in cellular and sub-cellular membranes can cause or amplify pathological phenomena in degenerative diseases such as atherosclerosis, cataracts, cancer and osteoarthritis (Scott, 1995; Behl, 1999).

There is evidence that a diet high in antioxidants can favourably influence the primary 'intrinsic' ageing process, as well as many of the secondary age-associated pathological processes (Aruoma, 1993). It has been suggested that a high consumption of fruits and vegetables is associated with a lowered risk of degenerative diseases like cancer and atherosclerosis and that dietary flavonoids can repair a range of oxidative radical damage sustained by DNA (Eastwood, 1999; Anderson et al, 2000).

In recent years there has been an increased interest in the use of antioxidants for the treatment of certain conditions. Research to date has focussed mainly on diet-derived antioxidants like vitamins C and E and carotenes (Cao et al, 1998). Some of these studies suggest that combinations of synergistic antioxidants acting by complementary mechanisms are more effective than individual antioxidants. For example, vitamin E alone is relatively ineffective without ascorbic acid (Scott, 1995). Clinically, the current view is that single nutrient therapy is not as effective as multiple nutrient interventions, which are both more effective and safer (Crayhon, 2001). Since it is argued that medicinal herbs contain a multiplicity of chemical compounds which interact synergistically, it is suggested that whole plant extracts could help in alleviating

some of the problems associated with ageing processes which are linked to free radical damage. Consequently there is now a growing interest in the role of plant phenolics, especially flavonoids, which have demonstrated considerable antioxidant activity *in vitro* (Bohm, 1998). Although some isolated plant antioxidants can exhibit potent effects *in vitro*, it is not known whether whole herb extracts can replicate this action. Herbalists generally believe in plant synergy, i.e. that the active constituents are balanced within the plant and are made more (or less) powerful by the numerous other substances present.

Herbal remedies have a long history of use in the treatment of chronic age-related conditions like osteoarthritis. Interestingly however, the contribution of their antioxidant activity to the prevention or treatment of these conditions has received little attention. The main body of antioxidant research has been carried out by the Food Industry on foods associated with dietary intake. Research into the therapeutic properties of medicinal plants that do not constitute a normal part of the diet, currently in its infancy, is desirable.

The purpose of this thesis is to investigate a small selection of medicinal plants traditionally used by herbalists in their treatment of chronic degenerative disease, in particular osteoarthritis, with a view to identifying antioxidant activity that may be contributing to their efficacy. The Herbal Medicine Department of Middlesex University recently carried out a pilot outcome study on the treatment of osteoarthritis, mainly with positive results (Bell et al, unpublished work, 1999). Based on this study, *in vitro* investigations were conducted for this thesis on herbs selected from the herbal formulae used in this trial and from those generally used by herbalists for this condition, with a view to the preparation of whole plant extracts, to assess their antioxidant propensity and to identify any phenolic compounds within these extracts which may be contributing to their antioxidant effects.

Synergy is a well known concept in phytotherapy and, although evidence is accumulating to show that it does occur in extracts and mixtures, there is still a paucity of reports in the scientific literature (Williamson, 2001). In addition to the assessment of single crude plant extracts for antioxidant activity, these extracts were also tested in pairs for any demonstrable synergistic interactions.

Tinctures used as herbal medicines are the most widely used pharmaceutical preparation form of plant origin, traditionally used by herbalists in their prescribing (Bilia, 2001). For this reason, as an adjunct to the assessment of the antioxidant activity of the crude plant extracts, a comparison of these extracts with herbal tinctures used therapeutically and prepared commercially by a phytopharmaceutical company was carried out, to assess the comparative antioxidant activity of the two types of extract. Selected phytochemical methods were used to identify the constituents, especially flavonoids, of the crude plant extracts and the tincture (fluid extracts). The resultant understanding of the chemistry involved may we hope lead to the development of more effective treatment strategies.

Due to its biological significance in the pathogenesis of many age related diseases, assessment of antioxidant activity was focused on the propensity of the chosen plant extracts to inhibit lipid peroxidation. Athough flavonoids may exert a number of biological effects, their inhibitory effects on lipid peroxidation are considered to be their primary action (Bors, 1998).

The ability of flavonoids to act as antioxidants or pro-oxidants *in vitro* is now well documented (Kessler et al, 2003). As pro-oxidant activity is often related to the presence of transition metal ions known to catalyse free radical reactions, all the selected plant parts were tested for the presence of metal ions.

Following a review of the literature, the first part of this thesis concerns the selection, extraction and analysis of the identified plant material. The second part covers biochemical analysis by which the antioxidant propensity of the plant extracts were determined, with a view to the future development of an herbal formula based on any synergistic interactions observed between these extracts.

CHAPTER 1: FREE RADICALS, OXIDATIVE STRESS, AGE-RELATED DISEASES AND ANTIOXIDANTS

1.1 FREE RADICALS, ENDOGENOUS DEFENCE AND THE ROLE OF OXIDATIVE STRESS IN HUMAN DISEASE

1.1.1 Origins of the free radical theory

Denham Harman, the pioneer of free radical research, first made the connection between free radical chemistry and ageing in the early 1950's. His initial life span study was presented as an abstract in 1956 at the American Federation of Clinical Research. It showed that 2-MEA (2 - mercaptoethylamine), a radiation protection compound synthesised by the Atomic Energy Commission, could extend life span by decreasing the level of free radical reactions. Little support was generated for his theories at this time.

Harman proposed that a single common process, the production of free radicals, was responsible for ageing and death of all living things (Harman, 1956: 1992). This theory was based on the chemical nature of free radical reactions and their ubiquitous prominent presence in living systems (Harman, 1956).

The free radical theory of ageing cites oxygen-derived free radicals as being responsible for age-associated impairment at cellular and tissue levels. This assumes that cellular ageing is associated with oxidative stress [an accumulation of pro-oxidant molecules in sufficient concentrations to outweigh antioxidant compounds (Sies, 1986)]. The theory, hitherto viewed with some degree of scepticism, gained credibility with the discovery by McCord and Fridovich (1969) of superoxide dismutase (SOD), a natural enzyme that destroys superoxide free radicals in the body. Subsequently, the free radical theory has inspired more research than any other concept in ageing, with evidence accumulating to support Harman's original conception that free radical damage is a major factor

contributing to the ageing process and possibly to many other age-related diseases such as atherosclerosis, cancer, arthritis and neurodegenerative diseases (Beckman and Ames, 1998; Wilcox et al 2004).

40 years after its original conception, Beckman and Ames (1998) in a review entitled 'The free radical theory of ageing matures' systematically reviewed the status of the free radical theory by categorizing the literature in terms of the various types of experiments that have been performed. They reported that a large body of consistent data had been generated to convince scientists that oxidants play an important role in ageing and the aetiology of many degenerative diseases. In the same year, Diplock et al (1998) also published an extensive review critically evaluating the science base that underpinned the argument that oxidative damage is a significant causative factor in the development of human disease and that antioxidants are capable of preventing or ameliorating these disease processes. Diplock et al (1998) concluded that there was sufficient evidence that mechanisms which involve free radicals are implicated at some stage of the development of human diseases, and that maintenance of well-being and health depends on the supply through the diet of antioxidants which modulate free radical processes *in vivo*. With the general acceptance of the theory that many diseases are related to oxidative stress, current research has mainly focused on the role of free radicals and antioxidants in specific diseases such as atherosclerosis (Maron, 2004; Cherubini et al 2005) and Alzheimer's disease (Engelhart et al 2002; Behl, 2005) and the protective effects of various groups of natural antioxidants further outlined in this review.

After a period of flourishing research on oxidants and antioxidants, Azzi et al (2004) suggest that it is now time to critically re-evaluate the evidence. Speculation that many diseases are related to oxidative stress still needs to be supported by more secure data and the hope that antioxidants can prevent or cure a number of pathological situations also requires reconsideration. Azzi et al (2004) propose that antioxidants may only be useful if the molecular mechanism of the oxidative stress situation or imbalance is known especially since antioxidants can protect, or increase injury, depending on the situation. There is still insufficient knowledge on the pro-oxidant, oxidant and antioxidant properties of the various antioxidant supplements and growing evidence that free radicals are not only by-products, but also play an important role in cell signal transduction, apoptosis and infection control (Bonnefoy, 2002; Halliwell, 2005). Such a review has yet to be published.

1.1.2 Reactive oxygen species and free radicals

1.1.2.1 Origins and targets of free radicals

Much has been written over the last twenty years about the origins and nature of free radicals. To summarise, free radicals are atoms or molecules with unpaired electrons which are capable of independent existence. The unpaired electron creates an unstable and highly reactive molecule which, to stabilise itself, will take an electron away from a stable molecule. On the loss of an electron, this previously stable molecule becomes damaged (a free radical), setting up a destructive chain reaction, i.e. one radical begetting another (Fig. 1.1.).

ADDITION	$x^{\cdot} + y \rightarrow [x\text{-}y]^{\cdot}$
HYDROGEN ABSTRACTION	$x^{\cdot} + yH \rightarrow xH + y^{\cdot}$
ELECTRON DONATION	$x^{\cdot} + y \rightarrow y^{\cdot} + x^{+}$
ELECTRON TRANSFER	$x^{\cdot} + y \rightarrow x + y^{\cdot}$
ELECTRON REMOVAL	$x^{\cdot} + y \rightarrow x^{\cdot} + y^{\cdot +}$
TERMINATION	$x^{\cdot} + x^{\cdot} \rightarrow x_2$
	$x^{\cdot} + y^{\cdot} \rightarrow xy$

Figure 1.1 Free radical reactions: how radicals beget radicals (Halliwell et al, 1992). Free radicals are signified by a superscript dot, which indicates the presence of one or more unpaired electrons (Halliwell, 1994).

Damage is permanent. Even if the free radical regains its electron from a stable molecule it does not regain its original form and function (Halliwell et al, 1992). These free radicals are collectively known as Reactive Oxygen Species (ROS) or Oxygen Derived Species and encompass both oxygen radicals and certain non-radicals that are

oxidising agents, or are easily converted into radicals (Aruoma, 1993; Halliwell, 1996). ROS may oxidise cell components such as lipids, nucleic acids (deoxyribonucleic acid and ribonucleic acid), carbohydrates and proteins, changing their structure and causing them to malfunction (Halliwell, 1996; Diplock et al, 1998).

Biological membranes comprise the most common target for auto-oxidative and oxidative tissue injury and disease. A free radical attacks membrane lipids by removing a hydrogen atom from a polyunsaturated fatty acid (PUFA) side chain, leaving a carbon centred radical (CH-). Since a hydrogen atom has only one electron, removing it leaves behind an unpaired electron on the carbon. This combines with molecular oxygen, yielding a highly reactive peroxyl radical ($COO^{\cdot}$). By abstracting a hydrogen, peroxyl radicals are capable of attacking adjacent fatty acids, generating new carbon centred radicals, resulting in a chain reaction, known as lipid peroxidation (Santanam et al, 1998) (Fig.1.2). The cell membrane is destabilised by the accumulation of lipid hydroperoxides (-COOH), leading to interrelated derangement of cell metabolism, including DNA strand breakage, alterations in gene expression, damage to membrane ion transporters (resulting in "free" iron and copper ions) and other specific proteins, plus further peroxidation of lipids. This results in the production of mutagenic products, which burden the immune system, whilst threatening it by compromising immune cell function by similar damage (Halliwell, 1984:1994; Snowdon et al, 1996). The production of hydrophilic lipid hydroperoxides in the hydrophobic regions of phospholipids will increase the permeability of the membrane and a damaged membrane allows easy entrance to bacteria and viruses (Lin, 1993, p19). Trans metal ions are important catalysts of free radical reactions and the release of "free" metal ions from sequestered sites can promote the formation of hydroxyl radicals (Halliwell, 1987). Peroxyl radicals can attack not only lipids but also membrane proteins. They may also oxidise cholesterol (Halliwell, 1994).

There is growing evidence that excessive production of free radicals can cause or exacerbate many human diseases (Halliwell, 1992; McCall and Frei, 1999).

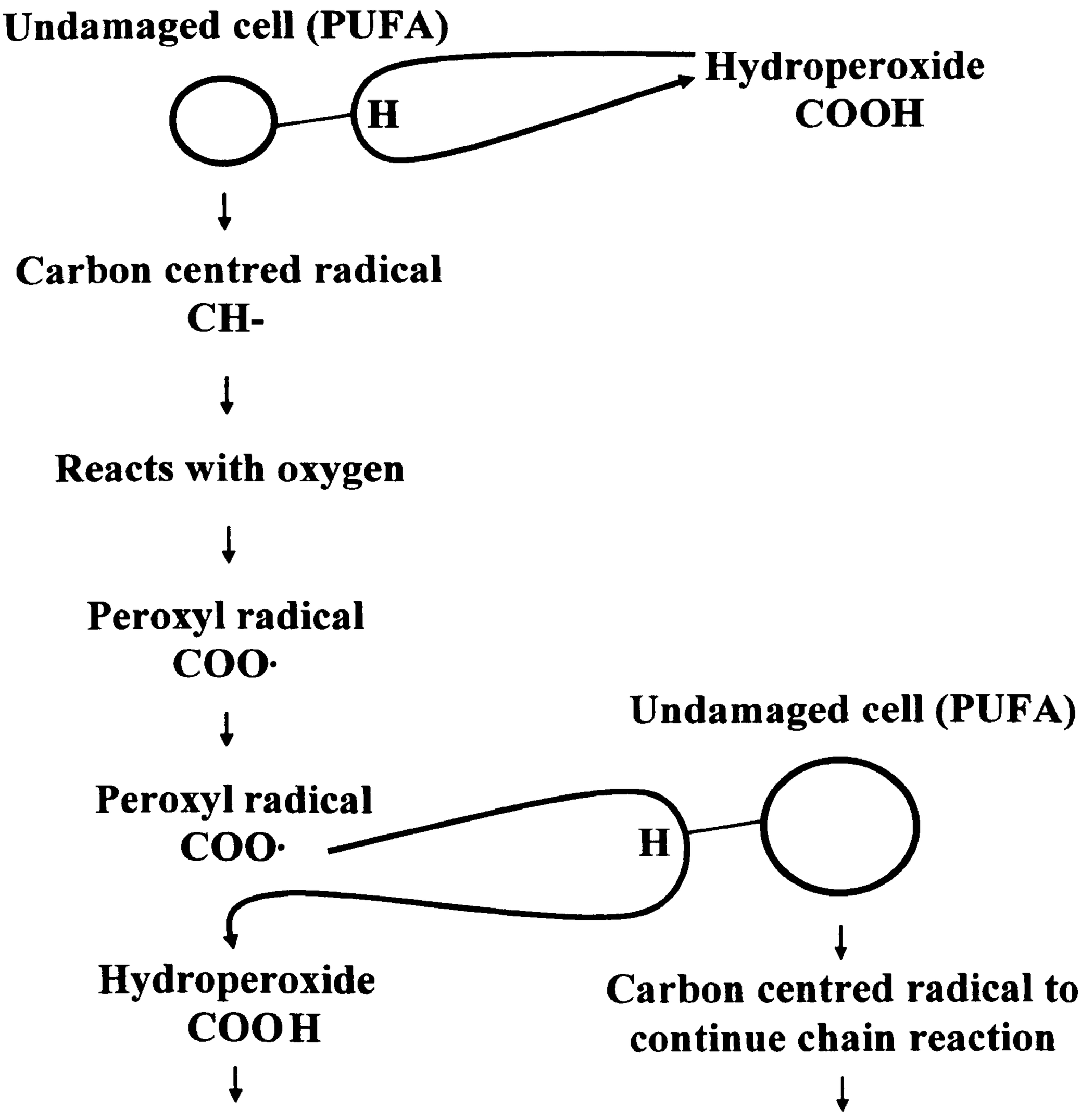


Figure 1.2 An outline of the mechanism of lipid peroxidation adapted from Halliwell (1994)

1.1.2.2 Sources of free radicals

Free radicals emanate from the environment, from other free radicals in chain reactions and from many normal biological processes *in vivo.*

Free radical reactions are initiated continuously in cells and tissues in the body from both enzymatic and non-enzymatic reactions. Enzymatic reactions serving as sources of free radical reactions include those involved in phagocytosis, prostaglandin biosynthesis and in the Cytochrome P450 system. Free radicals also arise in the non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionising radiation (Beckman and Ames, 1998; Diplock et al, 1998).

Sources of free radicals from the environment include tobacco smoke, ozone derived from air pollution, automobile exhaust emissions, excessive radiation, pesticides, deep-fried foods, hydrogenated oils and toxic metals which we inhale or digest (Pryor et al, 1995; Diplock et al, 1998). The destructive free radical nitrogen dioxide ($NO_2^{\cdot}$) for example, which is the result of a reaction between nitric oxide ($NO^{\cdot}$) and oxygen (O_2), is formed in cigarette smoke and vehicle exhaust and has been implicated in respiratory illnesses and irreversible lung damage (Pryor and Stone, 1993; Halliwell, 1994; Van de Vilet and Cross, 2000).

1.1.2.3 Formation of reactive oxygen species and actions *in vivo*

A number of ROS are responsible for oxidative damage in the human body. Some of the more physiologically relevant free radicals include hydroxyl ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$), superoxide anion ($O_2^{\cdot -}$), peroxyl ($RO_2^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$), and the non-radicals, hydrogen peroxide(H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), ozone (O_3) and peroxynitrite (ONOO-) which, although not technically oxygen free radicals, are included in this family due to their radical-forming capacity.

Superoxide anion radical ($O_2^{\cdot -}$), the one electron reduction product of oxygen (Halliwell, 1994), is the most important source of initiating radicals *in vivo* formed from the reduction of molecular oxygen by many physiological processes either deliberately or accidentally. Phagocytic cells like macrophages deliberately produce superoxide to help

inactivate viruses and bacteria (Babior et al, 1973; Halliwell, 1994). Accidental production appears to be a chemical accident of which leakage from the mitochrondria during electron chain transport is one of the most important sources (Halliwell, 1994). Another source is 'autoxidation' (a spontaneous oxidation reaction in which a molecule reacts with oxygen via a free radical, self catalysed route). The development of rancidity in fatty foods is an everyday example. Superoxide radicals can damage red blood cells, cause lung damage and degrade synovial fluid, possibly leading to arthritis (Lin, 1993, p76; Halliwell, 1994). Superoxide can also produce other ROS by a variety of reactions in biological systems (Beckman and Ames, 1998).

Hydrogen peroxide (H_2O_2), a non-radical derivative of oxygen, is present in tap water, expired air, tea and coffee and edible oil (Arumoa, 1993). It is continuously produced *in vivo* as a consequence of many physiological processes including phagocyte activity, peroxisomal fatty acid metabolism and as a result of normal aerobic respiration where mitochondria consume oxygen, reducing it by sequential steps to produce water (H_2O). Hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot -}$) are inevitable by products of this latter process due to leakage of partially reduced oxygen molecules (Ames, 1993).

When superoxide ($O_2^{\cdot -}$) radicals react with hydrogen peroxide (H_2O_2) in metal catalysed processes (Fenton reaction: iron catalysed Haber Weiss reaction), the highly reactive oxygen centred hydroxyl radical ($OH^{\cdot}$) is formed (Beckman and Ames, 1998). This is a deadly free radical that can attack all molecules in the human body. Damage includes cell membrane destruction and genetic mutation (Lin, 1993, p76; Snowdon et al, 1996). These radicals account for a large part of the damage done by ionising radiation (Halliwell, 1994). *In vivo* any $OH^{\cdot}$ produced reacts at or close to its site of formation. The extent of the damage done therefore depends on where it is formed (Halliwell, 1984).

Neither superoxide ($O_2^{\cdot -}$) nor hydrogen peroxide (H_2O_2) alone can attack DNA or initiate lipid peroxidation (Halliwell, 1994). Interest has been mainly focused therefore on the ability of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) to generate the dangerous $OH^{\cdot}$ species.

Singlet molecular oxygen ($^1O_2$), another highly reactive non-radical resulting from energy transfer, is thought to be formed *in vivo* for instance, from exposure to sunlight

or ozone (O_3) (Beckman and Ames, 1998; Diplock et al, 1998). Singlet oxygen can damage lipids, DNA and RNA. β carotene is a known quencher of singlet oxygen and is often prescribed for people who are abnormally photosensitive (Pearson and Shaw, 1982, p.422).

Nitric oxide ($NO^{\cdot}$), only recently recognised as a biologically significant free radical, is a messenger molecule, generated from the amino acid L-arginine that participates in a broad range of physiologic processes such as vasodilation, bronchodilation, neurotransmission and microbial-host defence (Moncanda and Higgs, 1993; Van de Vilet and Cross, 2000). Nitric oxide ($NO^{\cdot}$) contributes to the tissue injury mechanism but in excess it is highly cytotoxic (Beckman and Ames, 1998).
Peroxynitrite (ONOO-) a powerful oxidant which can cause lipid peroxidation is formed when Nitric oxide ($NO^{\cdot}$) reacts with superoxide ($02^{\cdot-}$), (Beckman and Ames, 1998; Van de Vilet and Cross, 2000).

1.1.2.4 Oxidants generated *in vivo* via mitochondrial electron transport

The mitochondria are a principal source of endogenous oxidants (Beckman and Ames, 1998). Mitochondrial respiration utilises oxygen to produce ATP via several reactions including the electron transport system. It appears however, that mitochondria electron transport leaks a small amount of electrons and one-electron reduction of oxygen to form $O_2^{\cdot-}$ occurs. The spontaneous and enzymatic dismutation of $O_2^{\cdot-}$ produces H_2O_2, thus a significant by-product of the actual sequence of oxidation-reduction reactions may be the generation of $O_2^{\cdot-}$ and H_2O_2 (Beckman and Ames, 1998).

In regard to ageing, Harman postulated that mitochondria might be largely responsible for changes usually attributed to the intrinsic ageing process. He considered that ageing changes associated with the environment and disease can be viewed as resulting from varying degrees of interaction between the environment and free radical reactions of mitochondrial and non-mitochondrial origin (Harman, 1972). This theory is still under investigation with a great deal of uncertainty still remaining in regard to the mechanisms, quantity and meaning of mitochrondrial $O_2^{\cdot-}$ generation *in vivo* (Beckman and Ames, 1998; Nohl et al, 1996). The fact that mitochondria posses their own genetic material (mt DNA) and that they lack mt DNA repair enzymes and protective histones

makes them one of the prime targets for the ROS generated continuously by the mitochrondria (Sastre et al, 1999). Collins, (1999) in a review on oxidative DNA damage, antioxidants and cancer states however, that the amount of mitochondrial DNA (mt DNA) is so small relative to the DNA of the nucleus that its contribution to the whole is negligible (Collins, 1999). The mitochondrial damage caused by ROS and concomitant decline in ATP synthesis seems to play a key role not only in ageing but also in the fundamental cellular process of apoptosis (Miquel, 2002). Scientists appear to be in agreement that as the site of respiration, mitochondria must have a particularly high concentration of reactive oxygen and damage to their DNA might have important consequences in cellular ageing (Kowald, 2001; Wei et al, 2001).

1.1.2.5 Transition metals

Transition metal ions are important in the production of ROS. The ability of metal ions to donate and accept single electrons is the basis for the formation and propagation of many ROS. Both copper and iron gain or lose electrons during redox reactions, cycling from reduced to oxidised forms and back. Most iron in the body is stored in the oxidised (ferric form).

Fe^{2+} (reduced iron) $\rightleftharpoons$ Fe^{3+} (oxidised iron)

Cu^{+} (reduced copper) $\rightleftharpoons$ Cu^{2+} (oxidised copper)

In the presence of transition metal ions H_2O_2, which is continuously produced *in vivo*, easily breaks down to produce the $OH^{\cdot}$ radical. This is known as the Fenton reaction, which can also occur with copper and some other metal ions and is usually written as follows (Halliwell et al, 1992).

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$$

Many antioxidants, including vitamin C and some flavonoids can provide the electron source for the redox recycling of transition metal ions which can lead to the formation of $OH^{\cdot}$ by the Fenton reaction (Boik, 2001, p.168).

Transition metal ions such as iron and copper can also participate in the production of the $OH^{\cdot}$ radical by catalysing the steps in the Haber-Weiss reaction shown below, an interaction between H_2O_2 and $O_2^{\cdot -}$ (Boik, 2001, p.169).

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{Fe} O_2 + OH^{\cdot} + OH^{-}$$

Metal sequestration is an important part of extracellular antioxidant defence. As much iron or copper as possible in the human body is bound to transport proteins or functional proteins such as transferrin or haemoglobin (iron) and ceruloplasm and albumin (copper). Metals bound to these proteins are inactive or only weakly active in catalysing $OH^{\cdot}$ production. Although the availability of iron and other transition metals to stimulate radical reactions *in vivo* is very limited, cellular injury appears to increase the availability of metal ions by possibly interfering with their storage vacuoles or by causing vacuole lysis thus exacerbating damaging radical reactions (Halliwell, 1984). There is established evidence for the occurrence of the Fenton and Haber Weiss reactions *in vitro*. Although there is evidence supporting these mechanisms *in vivo* variations in the actual chemistry is likely to occur (Boik, 2001, p.169; Liochev, 1996).

1.1.3 Endogenous protection

Since free radicals are intermediates in many normal and necessary metabolic processes, we have evolved endogenous antioxidant defence mechanisms to protect us against their tissue-damaging effects. They include enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidases which metabolise superoxide, hydrogen peroxide and lipid peroxides, amino acids and proteins, e.g. glutathione, bilirubin, ubiquinol (coenzyme) and uric acid (Heng-long et al, 2000) and copper and iron transport to avoid Fenton chemistry (Ames et al, 1993; Halliwell, 1996). These are supplemented by repair antioxidants based on proteases, lipases, transferases and DNA repair enzymes which are able to repair the damage caused by unscavenged ROS (Pietta, 2000). Antioxidant nutrients like β carotene (provitamin A), vitamin C and vitamin E and non-nutrient compounds like plant flavonoids, although not made in the body, contribute to its antioxidant defences (Knight, 2000). Trace elements from the diet like selenium, copper and zinc are essential co-factors of a number of enzymes with antioxidant function. Selenium is a vital component of glutathione peroxidase and zinc and copper

are components of SOD (Boik, 2001, p.163-4 and p.171). In healthy individuals the natural endogenous antioxidant defences are generally adequate to neutralise stray radicals, but with age, concentrations of these decrease, leading to increased amounts of free radical damage and chronic "age related" disease like atherosclerosis, Alzheimer's disease, arthritis, cataracts and cancer. Indeed, excessive oxidative stress may result at any age, causing serious damage to health when antioxidant defences are unable to cope with the production of free radicals from physiological stress or from the action of certain toxins (Halliwell, 1994; Spiteller, 2001a/ b).

1.1.4 Oxidative stress, age-related diseases and antioxidants

There is now evidence that mechanisms that involve free radicals are implicated at some stage of the development of many human diseases (Diplock et al, 1998; Tiwari, 2001). The following are several of the chronic disease conditions associated with advancing age where research has implicated free radical involvement in their aetiology and pathogenesis.

1.1.5 Cardiovascular disease

Degenerative heart and blood vessel disease is one of the most common and serious effects of ageing. The gradual build up of atheromatous material in plaques in the walls of coronary arteries is initially asymptomatic but may lead to angina. The plaques may rupture and thromboses become superimposed. This is the usual basis for the acute syndromes of unstable angina and myocardial infarction. If these plaques calcify, arteriosclerosis occurs, plus the risk of cerebral infarction (stroke), leading to severe disability or death (Brown, 1996, p.187-196).

1.1.5.1 The role of lipid peroxidation in atherosclerosis

A major development in cardiovascular disease was the finding that oxidation reactions played a central role in atherogenesis involving lipids in the arterial wall and serum that yield peroxides and other substances (Harman, 1992; Ames et al, 1993). Fatty streaks

formed in the subendothelial space by lipid deposits are one of the earliest lesions of atherosclerosis (Matsuoka, 2001). Lipids, as already outlined, are especially susceptible to free radical attack. Low-density lipoproteins (LDLs) comprised of lipids and proteins carry the majority of the cholesterol in the blood. ROS interact with the lipid component of the LDL particle, peroxidatively modifying it. Oxidised LDLs are cytotoxic and in quantity infiltrate and help to sustain an inflammatory reaction in the arterial vessel wall (Estubauer et al, 1991; Basu, 1999; Cherubini et al, 2005). Oxidised LDLs are more readily taken up by macrophages to form foam cells which play an important role in forming the atheromatous plaque. Foam cells may also come from medial smooth muscle cells that migrate to the intima of the arterial wall where they proliferate and contribute to the formation of the plaque, along with oxidation products including damaged lipoproteins and the cholesterol they are carrying. Much of the lipid in atheroscleroic plaques is composed of LDL particles (Mason, 1997; Diplock et al, 1998; Cherubini et al, 2005). The plaque continues to proliferate by a number of complex mechanisms that include large accumulations of extracellular lipids and connective tissue changes involving the deposition of collagen and elastin. Cholesterol, LDLs and other lipids deposited into the injured area become oxidised further increasing free radical activity and resulting in adherence of platelets to plaques (Kritchersky and Kritchersky, 1999). These attract red blood cells that haemolyse, leaking iron and copper, which are powerful autoxidation catalysts (Herbert et al, 1996; Parke, 1999). With the increase in size of the plaque, blood flow can become seriously compromised initiating thrombosis and aneurysm formation (Basu, 1999; Reed, 2002).

1.1.5.2 Effects of antioxidants on cardiovascular disease

Much research has been carried out in regard to the relationship between cardiovascular disease (CVD) and antioxidant status. Antioxidants may have a role to play in the atherosclerotic component of CVD by protecting both PUFAs and LDL cholesterol from oxidation. Epidemiological studies suggest preventive effects towards atherogenic lesions to be associated with an increased intake of lipophilic antioxidants such as vitamin E, which appears to inhibit the oxidation of LDL (Mason, 1997; Cherubini et al, 2005). Low serum levels of antioxidant vitamins and minerals like selenium are inversely correlated with an increased risk for developing atherosclerosis (Kok, 1991; Diplock, 1993;1998; Rimm et al, 1993). Established research carried out on the relationship between diet and disease has indicated that a large number of non-nutrient

components like plant flavonoids may also be contributing to antioxidant activity (Hirano et al, 2001; Vinson et al, 2001; Maron, 2004). However, because research has focused mainly on dietary sources of flavonoids, like fruit and vegetables, which contain other potentially protective antioxidant substances like micronutrients (vitamins and minerals), it has been difficult to state with certainty that flavonoids play a protective role against CVD. At least three studies however, have suggested a role for flavonoids in the prevention of coronary heart disease, (Hertog et al, 1993:1995; Keli et al, 1996) and research on some medicinal plants have established positive anti-atherogenic effects due to their flavonoid constituents.(Miquel et al, 2002; Zhang et al, 2001).

1.1.5.3 Beneficial effects of flavonoids against cardiovascular disease

The use of the plant Hawthorn *(Crataegus oxyacantha)* as a cardiovascular medicine is a modern herbal development that began with the discovery of a flavonoid-like complex of oligomeric procyanidins and other flavonoid compounds (Reweski and Lewak, 1967; Loew, 1994). Their potent antioxidant properties, which help to combat the damaging effect of free radicals on the cardiovascular system, assist the reduction of the risk of atherosclerosis (Hertog, 1997; Boughon, 1998; Fuhrman and Aviram, 2001). Two mechanisms have been proposed for their cardioprotective action; direct protection of human LDL from oxidation or indirect protection via the prevention of radical induced oxidation of α-tocopherol, a primary antioxidant known to protect human LDL from oxidative modification (Zhang et al, 2001; Maron, 2004).

Turmeric *(Curcuma longa)*, currently the subject of much research for a wide range of modern day health problems, although revered in India for its therapeutic properties for thousands of years, is a relative newcomer to Western herbal medicine. The anti-atherogenic effects of this plant have recently been demonstrated *in vivo* by the daily intake for 60 days of turmeric equivalent to 20mg of the phenolic antioxidant curcumin by 30 healthy volunteers ranging in age from 40 to 90 years, which resulted in a decrease in total blood lipid peroxides as well as reduced LDL and HDL lipid peroxidation, determined via the measurement of serum lipid peroxide levels from blood tests taken before and after the trial (Ramirez-Bosca et al, 1997).

1.1.6 Cancer

Cancer is the uncontrolled virtually autonomous growth of abnormal cells that can arise in any organ or tissue of the body. A transformed neoplastic or cancerous cell is simply a once-normal cell which continues to grow and multiply without limitation. This may be due to a multiplicity of endogenous and exogenous factors, including oxidative stress, which initiate a change in the cell's DNA, resulting in a tumour. Only transformed cells that escape detection by the immune system have the opportunity to become tumours. The immune system usually isolates and destroys the abnormal cells before they proliferate enough to be noticeable as a tumour. Free radicals can compromise immune cell function reducing immune responses which can allow the abnormal cells to continue growing (Ames et al, 1993; Boik, 2001). The question of why the risk of cancer increases with age is an interesting one. Harman, (1993) suggests that this increase is probably due in part to the increased level of endogenous free radical reactions with advancing age and inadequate antioxidant defences resulting in an increased rate of mutations in proto-oncogenes (involved in normal cell growth and development) and tumour-suppressing genes (suppress cell proliferation) coupled with the progressive diminishing capacity of the immune system to eliminate transformed cells.

1.1.6.1 The role of ROS in carcinogenesis

Carcinogenesis is a complex process with the *in vivo* generation of ROS leading to oxidative DNA damage being a significant contributory factor (Collins, 1999).

A critical factor in mutagenesis is cell division. Cancer is rare in non-dividing cells. When the cell divides damage to the cell DNA can give rise to mutation. Oxidative damage to DNA has been demonstrated *in vitro* and *in vivo* showing that ROS damage to cells may contribute to carcinogenesis in a number of ways (Diplock et al, 1998). They may cause:-

- structural alterations in DNA such as gene sequence amplification (Halliwell, 1996);

- translocations and base pair mutations [the oxidised form of guanine has altered base-pairing properties (Collins, 1999)];

- activation or inhibition of signal transduction pathways - over expression of a growth factor receptor is commonly involved in the majority of squamous cell carcinomas of the lung (Robbins, 1995, p112-3);

- abnormal cell-to-cell communication that allows unrestricted cell proliferation (Halliwell, 1996);

- interference with genes that modulate cell growth preventing programmed cell death by apoptosis or necrosis (Robbins, 1995, p.114);

- damage to proteins such as DNA repair enzymes compromising repair of a mutation once it has occurred. (Wiseman et al, 1995; Wiseman and Halliwell, 1996; Halliwell et al, 1996).

The immune system keeps an ever-present vigil to protect us from invading organisms and remove damaged, aged or altered cells which have the potential to cause cancer. White blood cell membranes, like all cell membranes, are composed of lipids, which are highly susceptible to free radical attack. Numerous links have now been established between free radical reactions and altered immune cell function (Niki et al, 1991).

ROS can decrease the membrane fluidity of white blood cells, significantly reducing their function (Baker and Meydani, 1994). Loss of membrane fluidity has been directly related to the decreased ability of lymphocytes to respond to challenges of the immune system (Bendich 1994; 1999) Free radicals can also damage the DNA of immune cells resulting in mutations and reduced cell function (Fabiani et al, 2001). Ironically, free radical damage forms the basis of some chemotherapy drugs and radiation used in cancer treatment (Cottier et al, 1995). Well-documented side effects like hair loss, reduced immunity and gastro-intestinal disturbances result from the barrage of free radicals that indiscriminately destroy healthy cells as well as malignant ones (Buckman, 1996, p.305).

1.1.6.2 Antioxidants and cancer

Epidemiological data provides strong evidence of a cancer prophylactic effect of high intakes of vegetables, fruits and whole grains containing high levels of antioxidant

micronutrients and phytochemicals (Diplock et al, 1998; Eastwood, 1999; Kaur, 2001; Meydani, 2001). Some naturally-occurring phytochemicals such as phenolic/ polyphenolic compounds, e.g. epigallo catechin gallate from green tea (Bushman, 1998; Nie, 2002), curcumin from turmeric (Nagabhushan, 1992; Sriganth and Premalatha, 1999), genestein from soy and red clover (Lian, 1999; Ren, 2001) and silymarin from milk thistle (Jiang, 2000), may reduce cancer risk according to initial trials (Weiner, 1994; Lamson and Brignall, 1999).

There have been a number of bioassay-guided searches for cytotoxic antitumour agents in plants especially those traditionally used in folk medicine for this purpose (Harborne and Williams, 2000; Williamson, 2001). Hartwell, (1971) in his survey, entitled 'Plants against Cancer' catalogues hundreds of plants from around the world that have historically been used for treating cancer in various cultures. Many chemotherapeutic drugs currently in use were first identified in plants, including taxol, vinblastine and vincristine.

1.1.6.3 A traditional herbal formula used by cancer patients

One of the herbal products most widely purchased by cancer patients is 'ESSIAC', originally developed as a folk medicine by the Native American Ojibway Indians which had particular application to auto-immune diseases. In the early 1920's a native healer passed the formula to a Canadian nurse called Rene Caisse (Essiac is Caisse spelled backwards) who successfully, it is claimed, used it to treat cancer. Nurse Caisse reduced the original formula to what she considered the most important ingredients: burdock root *(Arctium lappa),* turkey red rhubarb *(Rheum palmatum),* slippery elm bark *(Ulmus rubra)* and sheep sorrel *(Rumex acetosella).* No *in vivo* studies or clinical trials using the whole tonic have been reported to date. Evidence of anticancer activity for the Essiac formula is limited to anecdotal reports gathered from people who have used it during the decades of its popularity. *In vitro,* this formula has been shown to have strong antioxidant action and contains trace elements, minerals, and phytoestrogens (Flora Manufacturing & Distributing Ltd., cited in Tamayo, 2000).

Quite a body of *in vitro* studies exists on the individual herbs in ESSIAC or the isolated components of these herbs. Burdock root in particular, contains five flavonoid-type antioxidants and several polyphenols that are more effective as antioxidants than vitamin C (Maruta et al, 1995; Tamayo et al, 2000). Slippery elm bark is reported to

contain high concentrations of antioxidants and sheep sorrel contains several anthraquinones including emodin, rhein, alizarin and aloe emodin which are effective antioxidants and radical scavengers *in vitro* (Malterud et al, 1993). Proponents of the Essiac formula claim that its effect is dependent of the herbs being present in the correct proportions in accordance with the original formulation and that, although difficult to demonstrate, its efficacy may be the synergistic interaction of all four herbal constituents used together that contribute to the treatment effect (Tamayo et al, 2000). Although there are no data on the possible synergistic effect of individual herbs in the final formula, research on the individual herbs may not be applicable to the whole preparation (Snow and Klein, 1999). Several new *in vitro* studies showing the effect of Essiac on a selection of cancerous cell lines indicate that this formula may be able to inhibit tumour growth while enhancing immune response to antigenic stimulation making it especially valuable for immune-suppressed individuals. Further research is still urgently required however, to elucidate *in vivo* activities (Ottenweller et al, 2004; Tai et al, 2004).

1.1.7 Neurodegeneration (Alzheimer's disease)

Alzheimer's disease (AD), one of the 21st century's growing health threats, is a neurogenerative disorder which causes progressive and irreversible decline in the ability to remember, learn, think and reason. Alzheimer's disease is the most common neurological disorder in old age and despite extensive research there still remains no effective treatment or prevention for it (Newton, 1998).

1.1.7.1 The role of ROS in Alzheimer's disease

Due to the economic implications for the Health Care System of the increase in our elderly population, with 1 in 5 over the age of 80 currently affected by Alzheimer's disease, research into the aetiology and pathogenesis of this disease has intensified over the last 15 years. There is now a better understanding of the pathogenesis of this tragic condition and possible areas of research into its prevention (Ferry, 1996).

In the histopathology of AD many signs of oxidative reactions, including lipid peroxidation, can be found leading to an oxidative stress hypothesis (Zhou et al, 1995). Neuropathologically the dementia of Alzheimer's disease is associated with senile plaques, neurofibrillary tangles and loss of nerve cells from the cerebral cortex. There is also a change in the brain's chemistry, i.e. neurotransmitter deficits resulting from the neuronal degeneration.

Senile plaques are spherical structures with a dense core of a protein called β–amyloid, surrounded by the decaying remains of nerve cell terminals (Murphy, 1992). β–amyloid can be neurotoxic and this toxicity is mediated by peroxides and by the lipid peroxidation of membrane lipids leading to lysis of the cell, thus generating an overall oxidative microenvironment for the nerve cells resulting in neuronal loss (Behl, 1999). β–amyloid has been isolated, sequenced and found to be a variable length protein of 40 – 42 amino acids which has a tendency to aggregate into insoluble fibrils which in turn form the basis of plaques (Murphy, 1992; Glenner et al, 1994). Studies have shown that it is a breakdown product of a much larger protein, amyloid precursor protein (APP), which is inserted into the cells outer membrane so that a short sequence protrudes into

the cell and a much longer sequence is left outside. This outside segment is cleaved by enzymes into a number of small segments, later broken down by proteases. One of these segments is β– amyloid, which for unknown reasons is not broken down, but accumulates into plaques (Murphy, 1992; Selkoe, 1998). From his very early studies on mice Harman (1993) postulated that APP was one of the unknown substances from cell surface origin inhibited by antioxidants implicating an oxidative process. He surmised that by slowing down the rate of formation of fibrils they had more time to be proteolysed before aggregating to form amyloid (Harman, 1993). The generation of free radicals by β–amyloid has now been directly demonstrated during research supporting the oxidative stress hypothesis (Harris et al, 1995; Martins et al, 1999). It is considered likely that one or more free radical reactions are involved in the pathogenesis of AD.

1.1.7.2 Antioxidants and Alzheimer's disease

Antioxidant supplementation has naturally formed the basis of much research, with preliminary evidence suggesting that antioxidants may have a protective effect against the development of AD (Flynn and Ranno, 1999; Behl, 2005). The antioxidant herb Ginkgo biloba and vitamin E have both demonstrated the ability to help relieve some of the symptoms of AD in the early stages and slow its progression (Wolff, 2001; Grundman, 2000). Several studies involving the daily administration of Ginkgo biloba to mildly-demented patients resulted in measurable improvements in memory, attention and mood according to caregivers compared to those taking a placebo (Hofferberth, 1994; Kanowski et al, 1997; Le Bars et al, 1997; Kanowski and Hoerr, 2003). Due to the nature of AD, Ginkgo biloba may be able to slow the progression of early stage disease, but it is considered unrealistic to expect significant results in more advanced patients (Wolff, 2001).

1.1.8 Osteoarthritis

Osteoarthritis, a joint disease strongly associated with ageing and probably one of the commonest disorders to affect homo sapiens, continues to baffle scientists as to its precise causation and pathophysiology.

The modern view of OA is that it is neither a disease nor a single condition. It is now viewed as the *dynamic repair* process of synovial joints triggered by a variety of mechanical, metabolic or constitutional insults (Doherty et al, 2001). Sometimes a clear cause such as trauma may be apparent (secondary OA) but more often these insults remain unclear (primary OA). All the tissues of the joint such as cartilage, bone, synovium, ligament and muscle, depend on each other for health and function. Insult to one impacts on the others, resulting in a common OA phenotype affecting the whole joint (Doherty et al, 2001).

1.1.8.1 The role of ROS in osteoarthritis

Free radicals are now being considered as one of the contributory factors to the development of OA (Ames et al, 1993; Tiku et al, 2000). ROS and the products of their reactions were shown by early research to decrease the fluidity of synovial fluids, consequently reducing their function (Merry et al, 1989: Merry, 1991). Excessive free radicals in the synovial fluid can destroy the synovium, causing loss of joint fluid and support between bones. The resulting inflammation can trigger an inflammatory response resulting in the generation of more free radicals exacerbating the disease process (Henrotin et al, 1992).

More recent studies report high levels of superoxide radicals in the exudates of patients with active synovitis, and evidence of oxidative DNA damage to cartilage and lipid peroxidation of chrondrocytes, fuelling the current suggestion that free radical damage may be the molecular mechanism of OA (Zhu et al, 1998; Kucera et al, 1998; Tiku et al 2000). Tiku et al (2000) suggests that naturally occurring radicals like superoxide and nitric oxide, shown to be produced by chrondrocytes, could combine, resulting in the formation of peroxynitrite, a powerful oxidant known to initiate lipid peroxidation. Collectively, chrondrocyte lipid peroxidation plays both a physiological and pathological role in cartilage. Thus, a state of uncontrolled oxidative stress leading to

unbridled oxidation of cartilage collagen could make it more brittle and prone to mechanical fatigue failure, thereby instituting osteoarthritis.

1.1.8.2 Antioxidants and osteoarthritis

The role for dietary antioxidants in the prevention of OA is still unclear. In the Framington knee OA cohort study, vitamin C was shown to reduce the progression and development of knee pain, but there was no evidence to suggest that increased dietary intake of antioxidants is generally protective against the incidence of knee OA (Felson et al, 1995; McAlindon et al, 1996).

Herbal remedies have a long history of use in the treatment of 'arthritis' (Meletis, 1999; Aleves-Avela, 2001). The efficacy of many of these traditional remedies such as devil's claw *(Harpagophytum procumbens),* turmeric *(Curcuma longa)* and ginger *(Zingiber officinale)* on both the progression and pain associated with OA are now being confirmed by research. Proposed mechanisms of action all include antioxidant activity (Grant, 2000; Leblan, 2000; Marcus and Suarez-Almazor, 2001).

OA is not exclusive to humans. It can also affect other animals including dogs. A large

pharmaceutical

for arthritic dog

longa and Curc

in vitro (Grant and Schneider, 2000; Phytopharm, 2001). Although evaluation of treatments for canine OA is difficult, results of a randomised, double blind, placebo-controlled study using this remedy to treat 61 dogs, reported an improvement in at least 56% of those treated with turmeric extracts (Phytopharm, 2001).

1.1.9 Reactive oxygen species and human disease

As research into the role and involvement of oxygen and nitrogen-generated free radicals in the pathogenesis of many human diseases advances, a greater body of evidence is being established in regard to the number of essential biological functions associated with these species and their specific contribution to disease pathology. A

fine biological balance exists between the normal physiological formation of ROS/RNS and their removal. An excess of oxidative stress can lead to the oxidation of lipids, proteins and nucleic acids which is associated with cell injury. The biochemistry of oxidative pathobiology is complex. Some disease processes like carcinogenesis can be directly caused by ROS damage to biological molecules, whereas in others, like rheumatoid arthritis and ulcerative colitis where the disease process is characterized by inflammation, oxidative stress is not the cause of, but may be a contributory factor to the disease pathology by perpetuating tissue injury (Halliwell et al, 1992).

Besides playing a role in the pathogenesis of many 'age related' disorders, recent research suggests that free radicals may be implicated in a considerable number of other disorders. The following is a short representative list of human diseases in which oxidative mechanisms has been suggested may play an important role.

Table 1.1 Range of human diseases in which oxidative damage have been claimed to play a role in the pathogenesis

Category:	***Examples:***
Visual disorders	Cataract and age-related macular degeneration (Diplock et al, 1998)
Neuronal diseases	Parkinson's disease (Jenner et al, 1992), schizophrenia (Smythies, 1998; Reddy and Yao, 1999)
Shock	Septic, haemorrhagic and burnshock are all associated with severe oxidative stress and depletion of antioxidant defences (Goode and Webster, 1993)
Respiratory diseases	Asthma, lung cancer, cystic fibrosis, especially during exacerbations, exposure to environmental pollutants (O_3, NO_2, SO_2, auto exhaust), emphysema (Van der Vliet and Cross, 2000)
Digestive system diseases	Inflammatory bowel disease, ulcerative colitis (Grisham, 1993)
Muscular Skeletal	Rheumatoid arthritis (Halliwell et al, 1992)
Infectious diseases	AIDS, malaria (Halliwell and Gutteridge, 1999; Mollace et al, 2001)
Endocrine disease	Diabetes (Paolisso et al, 1995)

1.2 THERAPEUTIC USE OF ANTIOXIDANTS

1.2.1 Introduction

The accepted definition of an antioxidant is a molecule which, when present in small concentrations compared to the biomolecules they are supposed to protect, can prevent or reduce the extent of oxidative destruction of biomolecules (Halliwell 1990). In recent years there has been an increased interest in the application of antioxidants to medical treatment as information is constantly gathered linking the development of human diseases to oxidative stress. Because free radicals play many important physiological functions however, if antioxidants were stored in the body in such levels that all free radicals were neutralized many important body functions might fail.

1.2.2 Mechanisms of action

Two principle mechanisms of action have been proposed for antioxidants (Vaya and Aviram, 2001). The first is a chain-breaking mechanism, by which the primary antioxidant donates an electron to the free radical present in the system (e.g., lipid radical) forming a new radical, more stable than the initial one. Primary antioxidants include compounds such as flavonoids, tocopherol and ascorbic acid. The second mechanism involves removal of ROS initiators (secondary antioxidants) by quenching chain-initiating catalysts. This second mechanism can be accomplished by deactivation of high-energy species like $O2^{\cdot-}$, absorption of UV light, chelations of metal catalysing free radical reactions, or by inhibition of peroxidases, such as xanthine oxidase or lipoxygenases (Vaya and Avrim, 2001). Any compound that can react with the initiating radical, inhibit the initiating enzyme, or reduce the O_2 level without generating ROS, can be considered as a secondary antioxidant.

1.2.3 Antioxidants and lifespan

Scientists have revealed an interesting relationship between longevity and factors of ageing in humans and other mammals. The level of antioxidants detected in different mammals is directly related to the species' expected life spans. Humans, who were found to have the highest level of antioxidants, had the longest life expectancies of all mammals (Cutler, 1991).

Experimental studies have also shown that the life span of laboratory animals can be prolonged with antioxidants (Short et al, 1997). In some of his early studies involving mice, Harman, (1968) demonstrated that by feeding weaned mice on diets containing 0.5 – 1% antioxidants their life span was increased 30-40%. He equated this in human terms to raising the human life span from 73 to 95 years (Harman, 1968; Pearson and Shaw, 1982, p.116). He used synthetic antioxidants such as Sevitoquin (commonly used as a stabiliser in chicken food) and butylated hydroxy-toluene (BHT) an antioxidant food additive. His choice of these substances led to some scepticism towards the relevance of his work. At this time Harman's free radical theory of ageing was still a relatively new concept and his initial research with synthetic, rather than recognised dietary antioxidants did not achieve a convincing outcome. It has also been suggested that the taste or smell of the synthetic antioxidants may have reduced the animals' food intake, which is known to retard ageing in mammals (Scott, 1995). Harman (1987) observed quite early on that an antioxidant capable of inhibiting essential free radical reactions would become toxic beyond some level of intake, e.g. BHT is toxic in the mouse when added to the diet at levels above about 0.5% by weight (Horrum, Harman and Tobin, 1987). Studies on the lifespan of mice receiving various supplement antioxidants have continued. Unfortunately, no lifespan studies appear to have been carried out on popularly available antioxidants such as proanthocyanins, lycopenes, bioflavonoids and other antioxidants from plant sources (Donaldson, 2005).

1.2.4 Epidemiological studies

Research to date has centred on diet-derived antioxidants such as vitamins C and E and carotenes. In a review by Diplock et al, (1998) it was concluded that although

epidemiological studies generally support the hypothesis that these vitamins may play a beneficial role in reducing the risk of several chronic age-related diseases, findings for human intervention studies are inconsistent. Many synthetic antioxidants, although more efficient *in vitro* than biological antioxidants of the body defence system, produce unwanted side reactions unrelated to their biological functions (Rekka and Kourounakis, 1991). β carotene for example can both inhibit or exacerbate the growth of cancer cells, depending upon dosage (Lowe et al, 1999; Wang and Russell, 1999). A large human intervention trial carried out on smokers to investigate the efficacy of β carotene and alpha-tocopherol supplementation in reducing the incidence of lung and other cancers produced unexpected results. Not only did the smokers show no benefit, but also an 18% increase in the incidence of lung cancer was observed among those who only received β carotene (Omenn et al, 1994:1996; Collins, 1999). β carotene in its unoxidized form appears to be an anti-carcinogen, but its oxidised products appear to facilitate carcinogenesis. The carcinogenic response in lung tissue to high dose β carotene observed in the trial is thought to be caused by the instability of β carotene in the ROS rich environment of the lungs, particularly in cigarette smokers (Crayhon, 2001; Wang and Russell, 1999). This is especially possible because smoke decreases tissue levels of other antioxidants such as ascorbic acid and alpha-tocopherol, which normally have a stabilising effect on the unoxidised form of β carotene (Crayhon, 2001).

Dr. Jeff Evans oncologist, Cancer Research Campaign, University of Glasgow points out that even in successful human trials using β carotene supplementation, protection lasts only as long as the supplement continues and ends on its discontinuation (Evans, 2001). In respect of cancer therefore, for which there is a large body of research, it would seem that antioxidant vitamins used alone especially in doses considerably higher than normal dietary intake can produce beneficial, detrimental or insignificant results depending on the circumstances (Boik, 2001, p.189).

Results from epidemiological studies involving increased fruit and vegetable intake are generally more conclusive, appearing to confirm their protective function against various diseases such as cardiovascular disease and cancer and their contribution to the promotion of good health generally (Eastwood, 1999). This protective effect may be attributed to a variety of constituents, including not only a combination of vitamins,

minerals and fibre, but numerous phytochemicals, including flavonoids (Aruoma, 1993; Eastwood, 1999).

The electron transfer studies carried out by Anderson et al., (2000) support the possibility that dietary flavonoids, in addition to being effective scavengers of free radicals, can repair a range of oxidative radical damage sustained by DNA by a mechanism of H atom transfer from the flavonoids to free radical sites on DNA which result in the fast chemical repair of some of the oxidative damage resulting from $OH^{\cdot}$ radical attack. As oxidative DNA damage is considered to be a pathogenic event in the induction of many cancers for example, a reduction in the rate of such damage would probably result in a reduced risk of cancer, postulating the beneficial protective role of fresh fruit and vegetables.

1.2.5 Dietary supplementation with antioxidant enzymes

The therapeutic benefits of oral supplementation with isolated antioxidant enzymes are mixed. For example, SOD (superoxide dismutase) taken as a nutritional supplement is ineffective due to the destruction of the enzyme during the digestive process (Dev-Marderoscan, 2001, p.605-6). The results for Coenzyme Q10 [2,3 dimethoxy-5 methylbenzoquinone (often abbreviated to CoQ10)] a naturally occurring fat soluble quinine taken as a food supplement, are more promising (Lamson and Brignall, 1999; Dev-Marderoscan, 2001, p.605-6). Morton et al, (1957) introduced the name ubiquinone, meaning the ubiquitous quinone because it is ubiquitous in eukaryotic cells. Mellors and Tappel, (1966) showed that in its reduced form, *in vitro* it was an effective antioxidant against lipid peroxidation in membranes. Ubiquinone is found in foods, particularly organ meat, but cooking and processing methods tend to destroy it and, whilst it can be made in the body, production declines with age. Professor B. Ames from the Department of Chemistry, University of California, Berkeley suggests that optimal levels of dietary ubiquinone/ubiquinol could be important in many of the degenerative diseases of ageing (Ames et al, 1993; Burke et al, 2001).

1.2.6 The synergistic approach

Single nutrient therapy has comprised the bulk of all nutrient intervention studies because generally the aim of research is to isolate the activity of nutrients so that their individual roles in deficiency states and disease prevention can be established. Clinically however, it is now being established that when synergistic nutrient interventions are compared to single nutrient interventions, the combined nutrient approach is both more effective and safer (Crayhon, 2001).

Intriguing results from some studies suggest that combinations of synergistic antioxidants acting by complementary mechanisms are more effective than individual antioxidants (Scott, 1995). For example Vitamin E alone is relatively ineffective without ascorbic acid (Scott, 1995). Vitamin C and other antioxidants are known to enhance iron absorption (Yang et al, 1999) and zinc is a nutrient that can be considered as synergistic with almost every other nutrient. A lack of zinc can lead to a lack of absorption of other nutrients especially fat soluble vitamins like β carotene and alpha-tocopherol.

The effect of the carotenoid lycopene when used alone or in association with other antioxidants on the growth of two different human prostate carcinoma cell lines (androgen insensitive DU-145 and PC-3) has been studied (Pastori et al 1998). Lycopene alone was not a potent inhibitor of prostate carcinoma cell proliferation however, in combination with alpha-tocopherol at physiological concentrations (> 1μM and 50μM respectively) exhibited a strong inhibitory effect on prostate carcinoma cell proliferation, which reached values close to 90% inhibition (Crayhon, 2001; Pastori et al, 1998). Supplementation with a single antioxidant therefore may be counter-productive because it could result in less efficient control of oxidation.

Based on this knowledge it could be postulated that medicinal plants, which contain a multiplicity of chemical compounds possibly working synergistically, could help in alleviating some of the problems associated with ageing processes linked to free radical damage. Although there is little documented evidence of plant synergy, one example comes from investigations on the anti-malarial herb Qing Hao *(Artemisia annua)* which suggest that the flavonoids it contains, especially casticin and artemitin, can enhance the *in vitro* activity of the main anti-malarial constituent, artemisinin (Williamson, 2001).

1.2.7 Phenolic compounds as antioxidants

1.2.7.1 Introduction

Though in its infancy, there is now a growing research interest in the role of plant phenolics, especially flavonoids, as antioxidants *in vitro* (Bohm et al, 1998). As well as being widely distributed in the fruits and vegetables of our diet flavonoids now seem to be the active constituents in numerous medicinal plants used in herbal medicine traditions throughout the world.

Clearly a better understanding of the role played by free radicals in the pathogenesis of disease will lead to a better understanding of the way to control excessive free radicals with antioxidants. The tissue specificity of certain antioxidant compounds in medicinal plants is already being established indicating a need for more research and the development of more specific antioxidant treatments, e.g. Milkthistle (*Silybum marianum)* and the liver (Morazzoni and Bombardelli, 1995).

The Naturopath Leon Chaitow, proposes that taking antioxidant supplements which the body should be producing itself may just result in the down regulation of production, thereby defeating the object of supplementation (Chaitow, 1993). Thus, the author feels that antioxidants from medicinal plants may provide the vital key to unlocking the preventive and therapeutic potential for antioxidants that researchers are still seeking. Further, if the body does not recognise the unique plant antioxidants as part of its normal metabolism, it may still continue to manufacture its own antioxidants, which will work alongside the plant antioxidants to control free radical reactions.

1.2.7.2 Flavonoids

Although the interest in flavonoids from a physiological viewpoint was first initiated over 60 years ago, the full potential of this diverse group of compounds is still to be realised, particularly from non-nutrient sources like medicinal plants. Resurgence of interest in holistic medicine and media-generated 'scare stories' about drug side effects, coupled with scientific evidence that diets rich in fruit and vegetables may be protective against some chronic diseases, has rekindled interest in 'herbal medicines' with terms like 'bioflavonoids', 'proanthocyanins' and ' high potency flavonoids' being touted

commercially as panaceas for ageing and disease prevention. Little research has been published on specific flavonoids such as proanthocyanins or on studies related either to absorption or antioxidant activity of these compounds *in vivo* in man. Most manufacturers' claims are extrapolations of the general benefits of flavonoids and proanthocyanins, one of which may be antioxidant activity.

1.2.7.3 History and origins

The flavonoids were first isolated in the 1930's by Albert Szent-Gyorgyi, the Nobel Laureate who discovered Vitamin C. From his dietary experiments on guinea pigs, fed with a scorbutogenic diet, he discovered that by feeding them daily with an additional supplement isolated from lemons which he called 'citrin' they could be kept alive longer (Fairbairn, 1959). 'Citrin' was subsequently found to consist of a mixture of the flavanones hesperitin and eriodictyol glucoside, compounds belonging to a group of substances known as bioflavonoids or just flavonoids (Evans, 1996). Szent-Gyorgyi found that 'citrin' decreased capillary permeability and fragility in ways Vitamin C alone could not and the name vitamin P was proposed (Fairbarn 1959). This term was subsequently dropped because the flavonoids ultimately did not meet the definition of a vitamin, as no deficiency symptoms could be provoked.

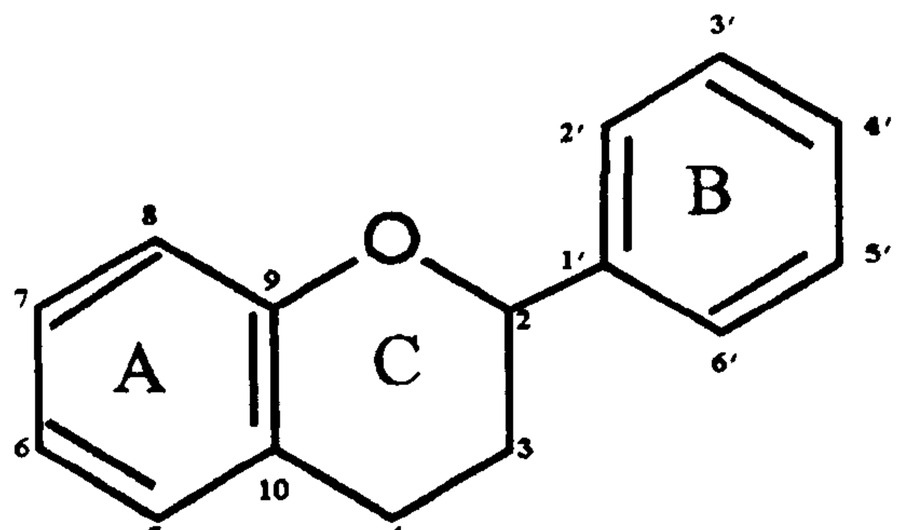
Flavonoids are a class of phenolic compounds present in most plants concentrated in the seeds, fruit skin or peel, bark and the flowers. They are the products of secondary plant metabolism biosynthesised via the shikimic, mevalonate and phenylpropanoid pathways (Duthrie and Crozier, 2000) that assist the plant's survival against harmful threats such as microbial invasion, insect and mammalian herbivory and UV radiation. Flavonoid pigments give plants their characteristic flower colour which acts as a vital attractant for pollinating insects. Protection however, particularly to UV-B (280-315nm) radiation, is conferred by the leaf flavonoids located at the upper surface of the leaf in the epidermal cells hidden by chlorophylls that have UV absorbing properties and are able to scavenge UV-generated ROS (Harborne and Williams, 2000). The intracellular flavonoids are thought to filter out UV-A (315-350nm) radiation (Harborne and Williams, 2000). The flavonoid content of plants can vary considerably and is influenced by many factors, including species, variety, light, and degree of ripeness, processing and storage. There can be surprisingly wide variations in the flavonol content of seeming similar produce (Duthrie and Crozier, 2000).

In resisting UV-A radiation and other insults such as wounding or infection, plants show significant increases in flavone or flavonol synthesis, constituents which are capable of free radical scavenging, a phenomena currently being investigated by scientists, who by stressing the plants during growth, hope to increase the levels of these constituents in medicinal plants to increase their pharmacological potency (Harborne and Williams, 2000).

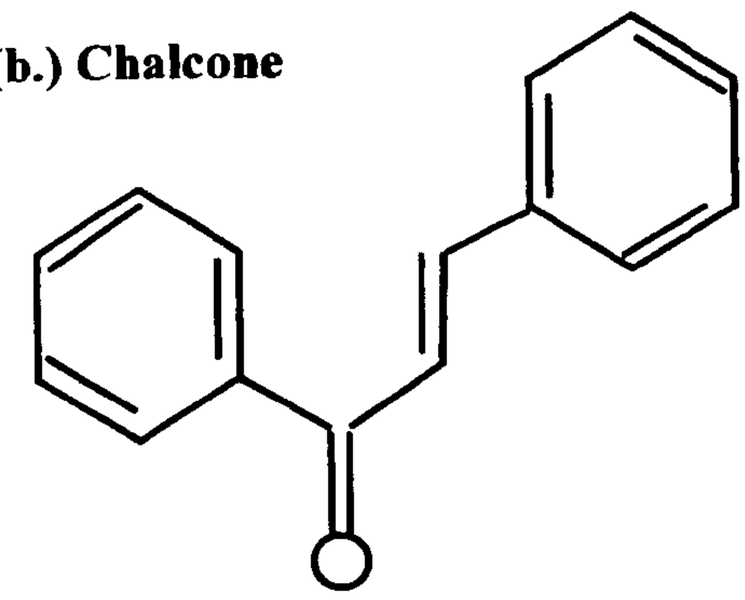
1.2.7.4 Structures and properties

The flavonoids are characterized by a basic C_6-C_3-C_6 (flavan) carbon skeleton: two benzene rings (A and B) linked through an oxygen-containing pyran or pyrone ring (C) [Fig. 1.3a]. The various flavonoids differ in regard to the number and position of hydroxyl, or substituted hydroxyl groups in the aromatic rings, and the extent and character of oxidation in the middle C_3 portion of the molecule (Fairbairn, 1959). Individual compounds within a class differ in the pattern of substitution of the A and B rings. Six main groups of flavonoid aglycones are recognised based on the flavan skeleton, these are chalcones [Fig. 1.3b], flavonols [Fig. 1.3c], flavones [Fig. 1.3d], flavanones [Fig. 1.3e], isoflavonoids [Fig. 1.3f] and anthocyanins [Fig. 1.3g] and they are found in a wide variety of fruits, vegetables and many medicinal plants (Evans, 1996). Other flavonoid classes of particular interest to this study include dihydroflavonols and proanthocyanidins (condensed tannins).

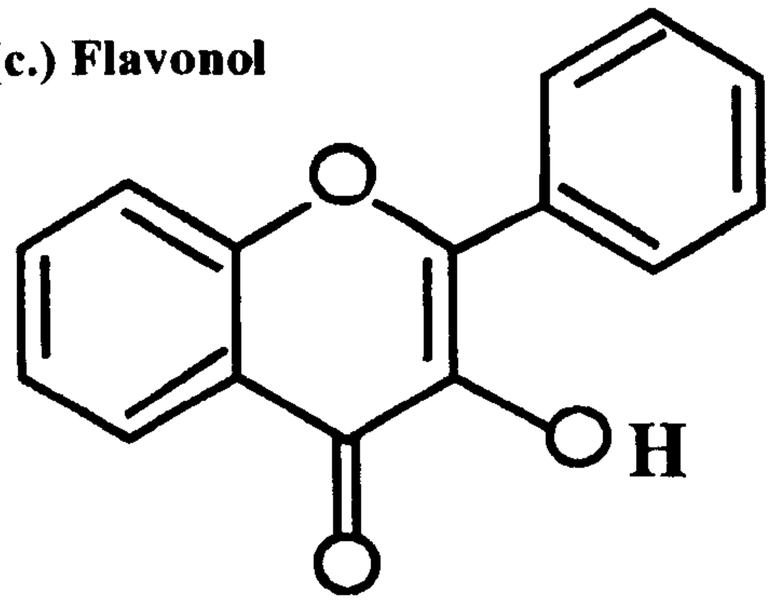
(a.) Basic Flavonoid Structure



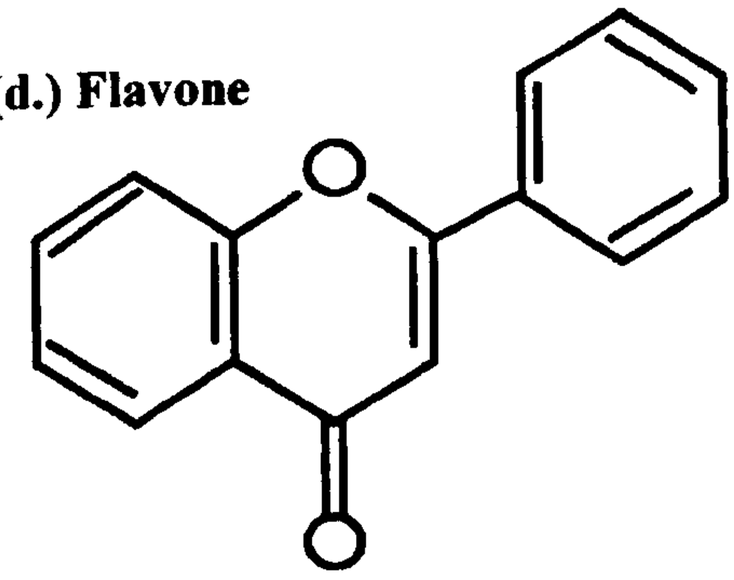
(b.) Chalcone



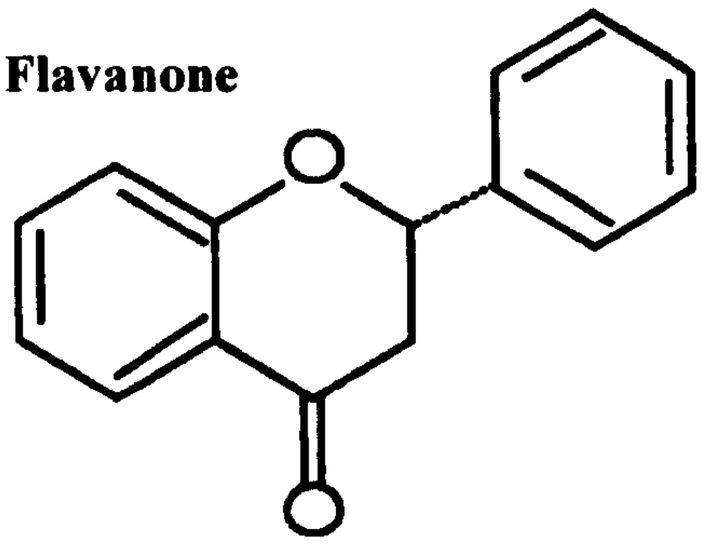
(c.) Flavonol



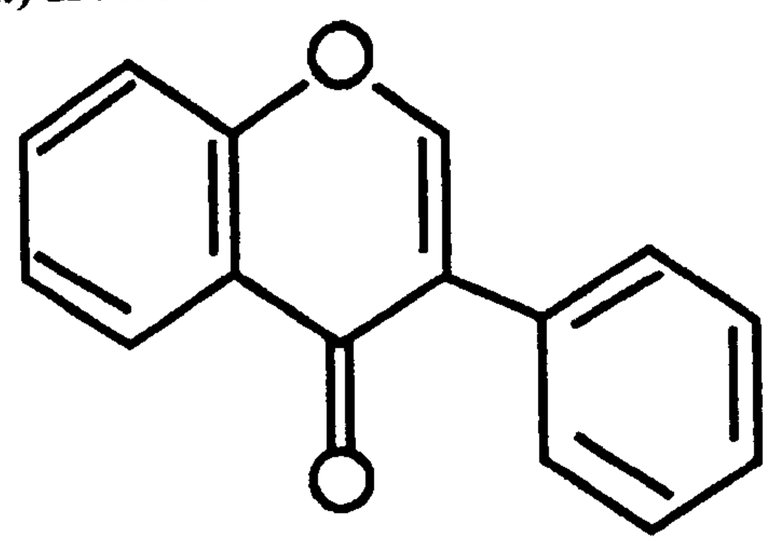
(d.) Flavone



(e.) Flavanone



(f.) Isoflavone



(g.) Anthocyanidin

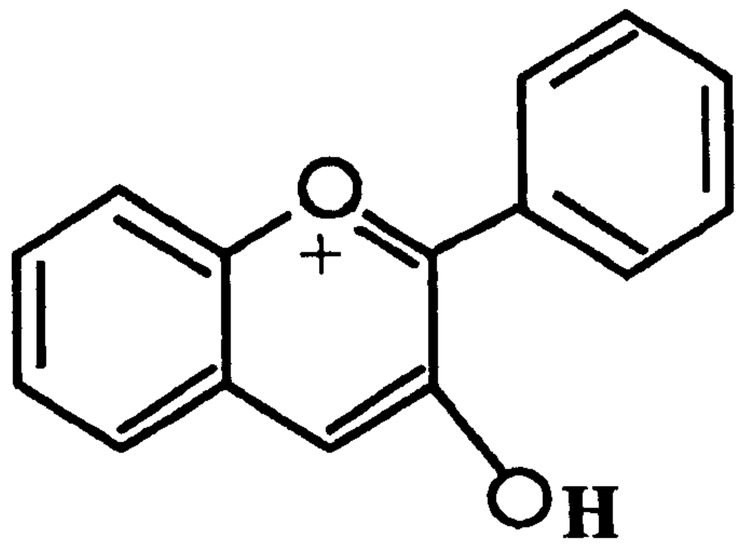


Figure 1.3 Examples of flavonoid types based on the structure of the basic flavonoid nucleus as shown in (a.)

Flavonoids usually occur in actively metabolising plant tissues in combination with sugars as glycosides (a sugar portion linked to a non-sugar molecule called the aglycone) with a changeable spectrum of glycosidation. Although all glycosides contain a sugar unit, generally the pharmacological action of the glycoside is due to its aglycone, which varies widely in their chemical constituents. The sugars found in glycosides are mainly monosaccharides such as glucose, rhamnose, galactose and xylose (Rice-Evans et al, 1996). A glycoside molecule may contain just one, several of the same, or several different monosaccharide units. The glycoside rutin for example contains one rhamnose and one glucose molecule called rutinose. When there are two or more monosaccharides in a glycoside molecule, the sugars are mainly linked together in a di-, tri- or polysaccharide chain linked directly to the aglycone. The preferred glycosylation site on the flavonoids is the 3 position and less frequently the 7 position (Rice-Evans et al, 1996). The most common linkage between the sugar and the aglycone is a hemiacetal bond formed between the sugar and hydroxyl group of the flavonoid aglycone. Most of the plant glycosides known are of this type and are designated as O-glycosides. Other glycosides do occur however, whereby the sugar is linked to the thiol (sulfhydryl) group (S-glycosides); an amino group (N-glycosides); or by a carbon-to-carbon bond (C-glycosides). Quite a number of C-glycosylated flavonoids occur naturally. The flavone apigenin can occur with glucose at C6 (isovitexin); C8 (vitexin) or at both C6 and C8 (vicenin). The nature and number of saccharide-units in the sugar- portion of a glycoside affect the degree of polarity of the glycoside and consequently its solubility in and partition between different solvents. Glycosides are generally only very slightly soluble in water, but are more soluble in ethanol, methanol, or a mixture of water with ethanol or methanol.

The most common flavonoid found in food is the flavonol quercetin, present in onions, olives, tea, wine, apples and many other fruits and vegetables (Noroozi et al, 2000). It is also found in medicinal plants like Hawthorn, Ginkgo and German Chamomile (Barnes et al, 2002). Any one flavonoid aglycone can occur in a single plant in several glycosidic combinations. Over one hundred different glycosides of quercetin are known, the commonest of which are the 3-O-glycosides quercetrin containing rhamnose as its sugar-portion and rutin that contains the disaccharide rutinose. Most flavonoids are glycosylated in their natural dietary form (Formica and Regelson, 1995) but due to this structure they cannot be absorbed from the small intestine. No enzymes that can split the glycosidic bonds are secreted into the gut or present in the intestinal wall.

Hydrolysis only occurs in the colon by cecal microflora that are capable of releasing the aglycone from its sugar. Furthermore, the aglycones can then undergo further breakdown by a process known as C-ring fission to give a variety of phenolic products. The manner in which the aglycone or its breakdown products transfer across the gut wall is still unclear and research on the metabolism of flavonoids particularly in humans is still sparse (Hollman, 1997). In a normal diet flavonoids are routinely consumed daily, with an estimated total consumption varying from 20mg to1g (Hertog et al, 1993). Table 1.2 shows a representative list of some other well known flavonoids and their dietary sources.

Table 1.2 Sources of some well known compounds found in the different classes of flavonoids

Compound:	***Type:***	***Occurrence:***
Naringenin, Hesperidin	Flavanones	Citrus fruits (Rice-Evans, 2001).
Taxifolin	Dihydroflavonol	Milkthistle (Barnes et al, 2002).
Apigenin, Luteolin	Flavones	Parsley, celery and Roman chamomile (Evans, 1996).
Cyanidin, Malvidin	Anthocyanidins	Dark red fruit berries such as strawberries, cherries, purple grapes and hawthorn (Rice-Evans, 2001).
Catechin, Epicatechin, Epigallocatechin	Proanthocyanidin	Barks such as willow and cinnamon. Lime and hawthorn flowers. Green tea and red wines (Hertog and Katan, 1998).
Genistein, Daidzein	Isoflavonoids	Legumes, soya beans and red clover (Evans, 1996).
Quercetin, Kaempferol, Myricetin, Isorhamnetin	Flavonols	Various fruits and vegetables (mainly found in the skin). Green and black tea and red wine (Duthrie and Crozier, 2000).

Flavones and flavonols represent about 80% of known flavonoids (Bruneton, 1995, p.268). Flavones occur most frequently as glycosides located within the vacuole of the plant cells. Flavonols are a class of flavones in which the 3-position of the pyran ring is

occupied by a hydroxyl group. Flavones typically occur as 7-glycosides, whereas flavonols are usually present as 3-glycosides. For identification purposes flavonols absorb ultraviolet at higher wavelengths than flavones. Flavanones are formed biosynthetically from chalcones. They occur in a range of glycosidic forms with the sugar usually being attached to the 7 hydroxyl. Only a limited number of flavanones are currently known (Harborne and Baxter, 1999). Dihydroflavonols, often referred to as flavanon-3-ols, are formed by oxidative addition of a hydroxyl group to the 3-position of a flavanone. Taxifolin (dihydroquercetin), one of the commonest dihydroflavonols, is closely related in structure to the common flavonols (Harborne & Baxter, 1999). Anthocyanidins are coloured sap pigments related in structure to flavones. The anthocyanidin aglycone is referred to as an anthocyanidin which in plants is almost always found as the more stable glycosylated derivative known as an anthocyanin. The sugars are commonly at the C3 position or more rarely the C5 position. These sugars may be modified by acylation with acids such as *p* coumaric, caffeic and acetic acid (Bruneton, 1995, p.303). Proanthocyanidins also known as condensed tannins, are colourless substances with polymeric flavan-3-ol structures (catechins) and/ or flavan-3,4-diols (leucoanthocyanidins) linked by carbon-carbon bonds usually from the 8-position of one unit to the 4-position of the next unit (Harborne and Baxter, 1999). The term proanthocyanidin is derived from the acid catalysed oxidation reaction that produces red anthocyanidins on hot acid treatment. Production of anthocyanidins in this way is the main method of detecting these colourless substances in plants (Walker, 1975; Harborne, 1984).

1.2.7.5 Flavonoids as antioxidants

Plant flavonoids are multifunctional and may exert cell protective mechanisms by more than one biochemical mechanism. They can act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers and, in some cases, metal-chelating properties have been proposed (Boik, 2001, p.255; Rice-Evans et al, 1996). Many flavonoids have been shown in studies to be potent antioxidants capable of quenching $HO^{\cdot}$ radicals, superoxide anions, and lipid peroxidation (Duthrie and Crozier, 2000). They may also however, exert a number of other biological affects including vasodilatory, antibacterial, antihepatotoxic, anti-allergenic, anti-inflammatory, anti-spasmolytic and anti-tumour activities which may be relevant to their overall effects on human health (Harborne and Williams, 2000). It is considered that the value of most

flavonoid medicinal plants rests not on the flavonoid fraction alone but on a complex mixture of chemically different compounds. Other components may either directly contribute or play an adjuvant role that strengthens the action of the flavonoids (Pietta, 1998).

Several pathways whereby flavonoids and other plant phenolics may exert their antioxidant effects on chemical oxidation have now been identified, the most important of which is likely to be their free radical scavenging capacity to halt the domino effect of a free radical chain reaction in lipid peroxidation by donating an electron to the peroxyl radical of a fatty acid, and thus halting propagation (Bors et al, 1998; Terao and Piskula, 1998). They may work synergistically with other vitamin antioxidants potentiating their actions. For example, *in vitro* studies have shown that rutin potentiates vitamins C and E when used in combination, yielding a more potent radical scavenging action (Negre-Salvayne et al, 1991). Flavonoids may act as antioxidants by influencing enzyme systems such as superoxide dismutase, catalase and glutathione peroxidases in various tissue sites (Weiner, 1994). Animal studies using silymarin and silybin extracts from *Silybum marianum* have demonstrated a stimulatory effect on superoxide dismutase activity and increase in the amount of total glutathione in the liver, intestines and stomach of normal healthy rats (Valenzuela et al, 1989; Morazzoni and Bombardelli, 1995). Further, they may also prevent the generation of the highly reactive hydroxyl radical by Fenton type reactions via their ability to sequester transition metal ions (Halliwell et al, 1995; Diplock et al, 1998). *In vitro* studies involving this final pathway have led to possible concerns of pro-oxidant effects *in vivo* whereby flavonoids could also generate free radicals in reactions with some free transition metal ions (Halliwell and Gutteridge, 1986; Halliwell,1996; Cao et al, 1997). Flavonoids can reduce CU^{2+} to CU^{+} and Fe^{3+} to Fe^{2+} hence allowing the formation of initiating radicals. Although metal ions in the body are largely sequestered in forms unable to catalyse free radical reactions, injury to tissue may release iron or copper (Halliwell et al, 1992) and catalytic metal ions have been measured in atherosclerotic lesions (Smith et al, 1992) leading to concerns that under certain circumstances flavonoids could also generate free radicals in reactions with free transition metal ions. Most antioxidants i.e. isolated compounds like vitamin C and quercetin that have been tested, exhibit both antioxidant and pro-oxidant effects *in vitro,* depending on conditions that include the concentration of the antioxidant, the presence of other antioxidants, and the presence of iron and copper ions (Boik, 2001, p.189). In an *in vitro* study the

flavonoid compounds apigenin and luteolin acted as antioxidants at low iron concentrations but as pro-oxidants at high iron concentrations (Sugihara et al, 1999). There is much *in vitro* evidence to support the antioxidant activity of flavonoids, but their effects *in vivo* are still unclear and mainly speculative. The *in vivo* antioxidant activity of flavonoids is largely dependent on their bioavailability. This in turn is dependent on a number of factors including their release and stability in the gut, glycosylation, absorption through the intestinal wall, liver metabolism and accessibility to the tissue target site.

Taking current knowledge into account, i.e. that flavonoids may exert antioxidant protection by more than one biochemical mechanism, the use of total extracts or combinations of herbs in formulations may provide an important advantage, the possibility of additive or synergistic interactions that could target a range of cell protective antioxidant mechanisms. Safety is a further advantage, because the use of combinations allows lower and safer doses of each compound to be used.

Synergy is comparatively difficult to measure and documented examples are scarce. Implicated instances, however are becoming more common. In a recent study, workers reporting the unnaturally high antioxidant activity of a liquorice extract compared to isolated compounds from the plant suggested that the activity of licorice extracts is due to the contribution from a number of components, especially flavonoids (Gordon and An, 1995).

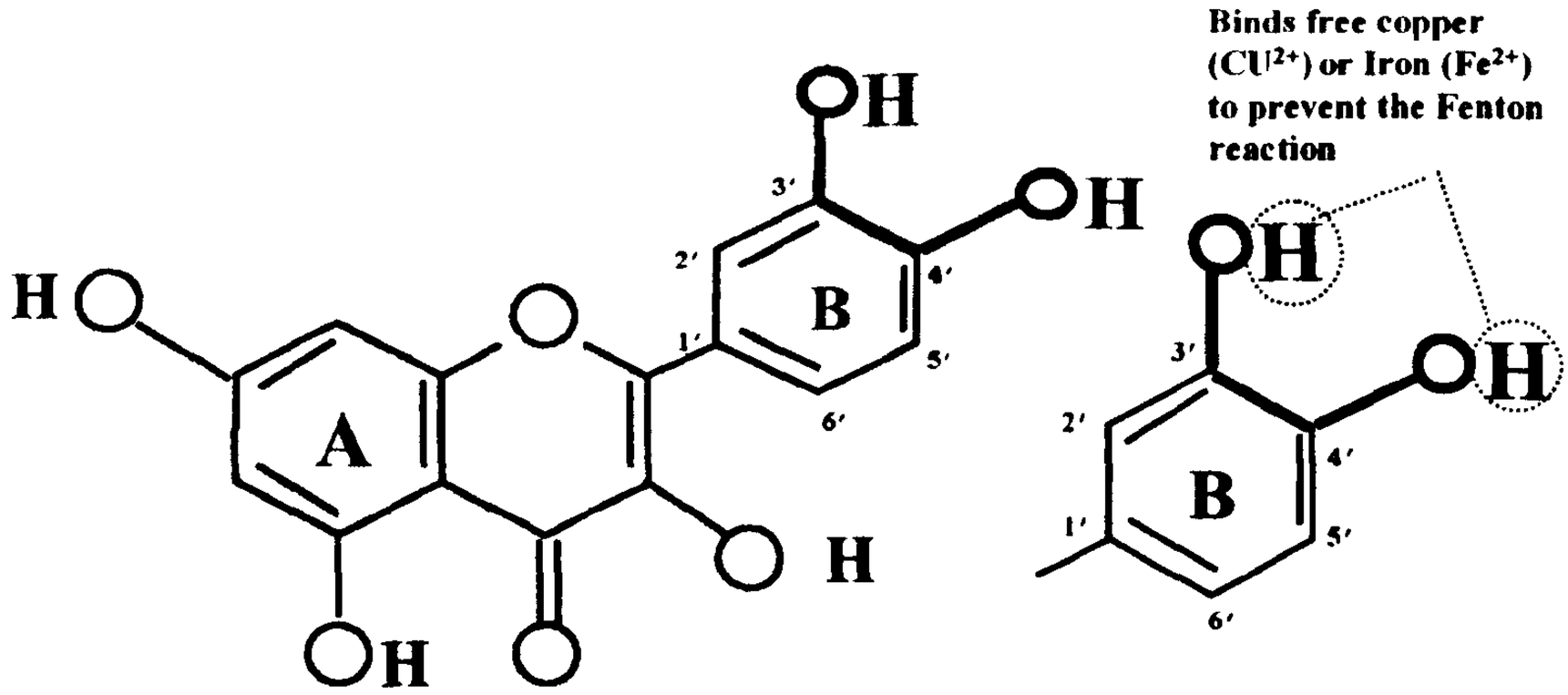
Various studies have established the structure-antioxidant activity relationships of the flavonoids, the chemistry of which is fully outlined in Bors et al, (1990). To briefly summarise, the structural features of flavonoids necessary for the optimum free radical scavenging are as follows illustrated in Fig. 1.4. (Bors et al, 1990).

1. The presence of two adjacent hydroxyl groups on the B ring. The O-dihydroxy (catechol) structure for electron delocalisation is the obvious radical target site for all flavonoids with a saturated 2,3-bond.

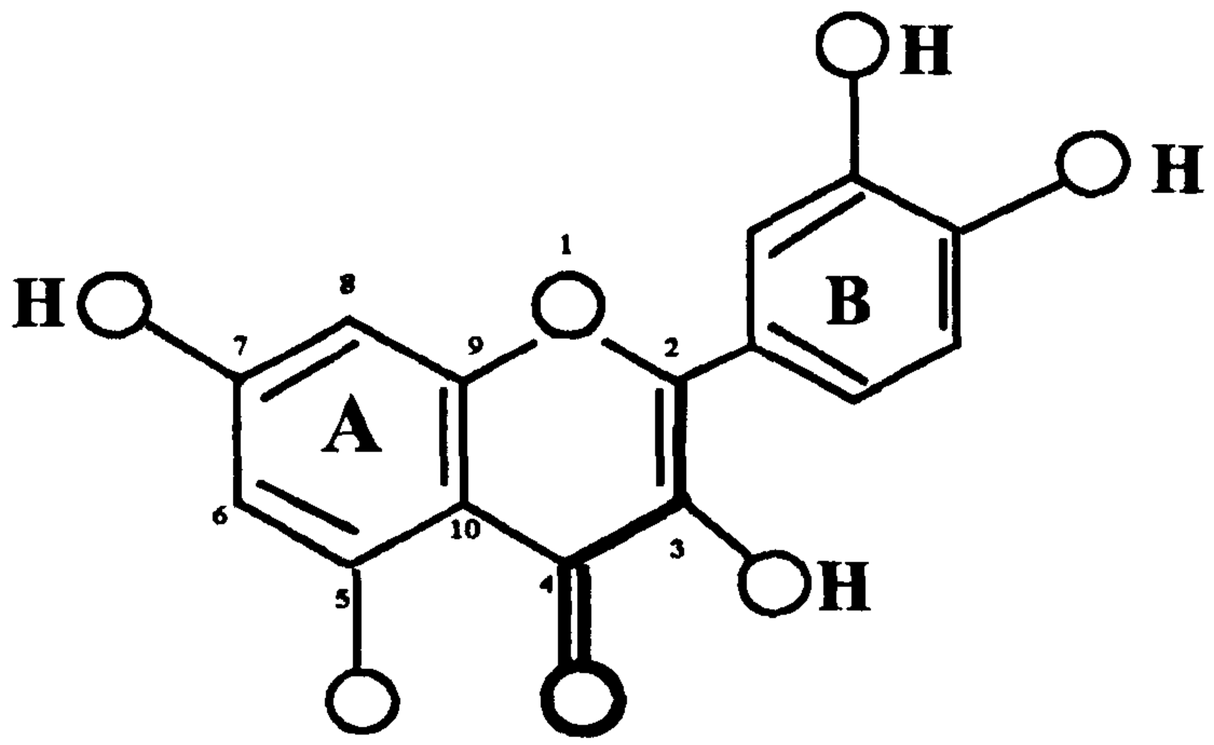
2. The 2,3 double bond in conjugation with a 4-oxo function in the C-4 position providing electron delocalisation from the B-ring.

3. The presence of an OH group at positions C-3 and C-5 to provide hydrogen bonding to the keto group for maximum radical scavenging potential and strongest radical absorption (kinetically, the 3- and 5- OH groups are equivalent owing to their H-bonds with the keto group).

1. Two adjacent hydroxyl groups on the B-ring



2. A 2,3 double bond conjugated with the 4 oxo group



3. The presence of an OH group at positions number 3 and 5 with oxo function

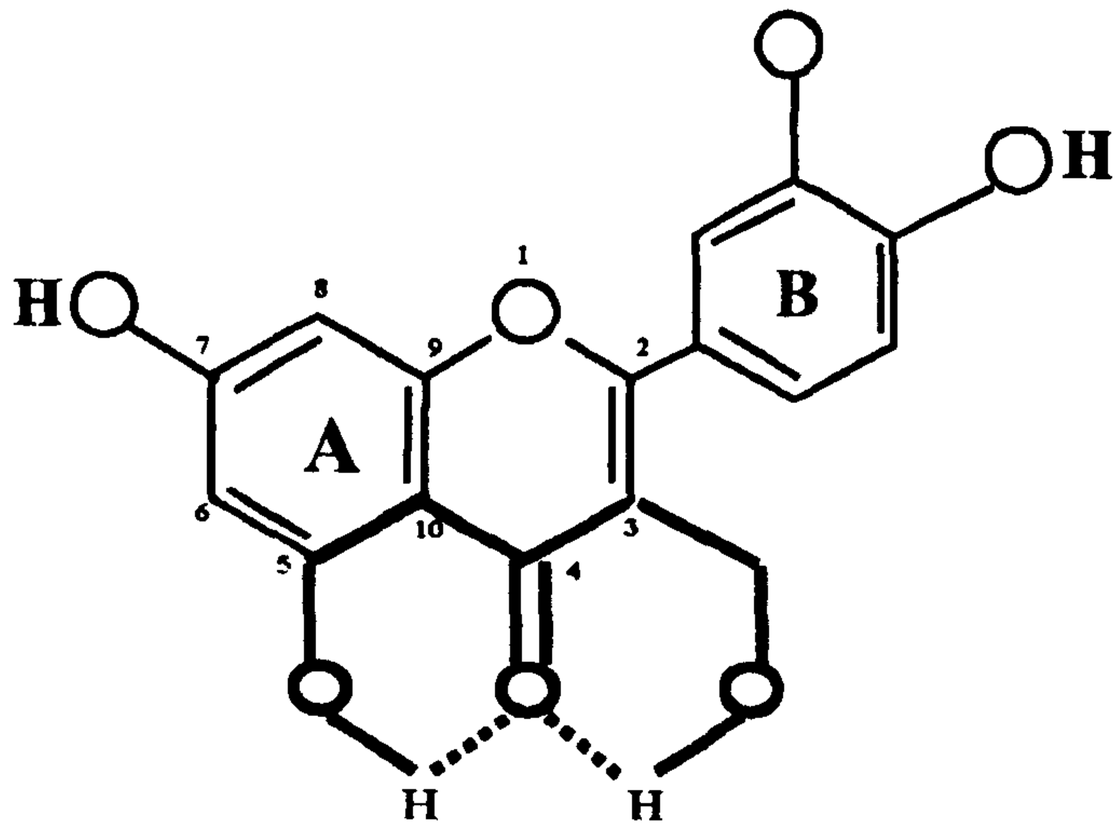


Figure 1.4 The major determinates for effective radical scavenging of flavonoids (Bors 1990)

Rice-Evans et al, (1996) shows that the flavonol quercetin satisfies all the above criteria and is a more effective antioxidant than catechin for example, a proanthocyanidin that only satisfies requirement 1.

1.2.7.6 Flavonoids in human health

In addition to the potential health beneficial properties of the flavonoids and polyphenolic compounds found in dietary fruit and vegetables, more recent research has discovered rich sources of antioxidant compounds from medicinal plants, which exert valuable tissue-specific effects.

The seed of milk thistle *(Silybum marianum)* for example, contains a number of flavonolignans including silymarin (a mixture of silybin, silychristin and silydianin) with antihepatoxic properties which, among other mechanisms, inhibit liver damage by scavenging free radicals and increasing glutathione levels (Fraschini et al, 2002). Preliminary *in vitro* studies indicate that in addition to its hepato-protective qualities milk thistle may also have an application in the prevention and treatment of certain cancers (Jiang, 2000). A number of flavonoids and other compounds have been isolated from the leaves of Ginkgo biloba which are believed to work synergistically as free radical scavengers (Kleijnen, 1992; Miller, 1998b). An important area of research relates to Ginkgo biloba in the prevention and treatment of vascular diseases and brain function. Excessive oxidation and cell damage is associated with Alzheimer's disease, as already outlined in section 1.1.7. In an intent-to-treat analysis 0f 2020 patients with various stages of dementia, Ginkgo biloba was found to decrease the Alzheimer's disease assessment scale (cognitive subscale score 1.4 points better than placebo), suggesting that Ginkgo biloba was capable of stabilising and perhaps improving cognitive performance in patients with dementia (Le Bars et al, 1997). The diterpene ginkgolides and sesquiterpene bilobalides isolated from Ginkgo appear to be exclusive to this species, possessing a structure unique in the vegetable kingdom. These compounds may work synergistically with the Gingko flavones (Kleijnen, 1992). As plants such as these do not constitute a normal part of the diet, they would need to be taken as supplements.

Hundreds of plants have been studied and found to have antioxidant activity *in vitro.* However, very few tissue-specific effects for plant based medicines containing

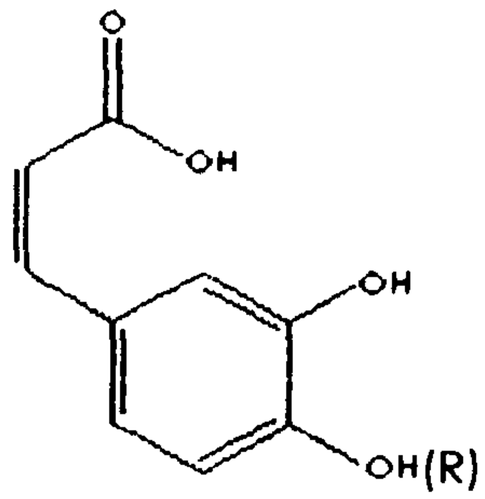
flavonoids have been established. Many herbal medicines traditionally used to provide protection against chronic diseases contain flavonoids, but their antioxidant potential has yet to be investigated. In herbal medicine the term herbs is used loosely to refer not only to herbaceous plants as defined by the botanical term, but also to bark, roots, leaves, seeds, flowers and fruit of trees, shrubs and woody vines.

Flavonoids have become a priority of herbal medicine research, the current focus of which is their antioxidant activity for the development of defence strategies against ROS induced illness and preventive measures for maintaining and improving overall health.

1.2.7.7 Hydroxycinnamic acids

In addition to the flavonoids, further phenolic compounds with antioxidant activity of potential interest in this study are derivatives of cinnamic acid; e.g caffeic acid and chlorogenic acid (Rice-Evans and Miller, 1996b). Known as hydroxycinnamic acids these compounds are a group of phenylpropanoid (C6-C3) derivatives present in our daily diet in fruit and vegetables and many plants used in phytomedicine. They are essentially present as combined forms, usually esters, of four basic molecules: *p*-coumaric, caffeic, ferulic and sinapic acids [Fig. 1.5] (Macheix and Fleuriet, 1998). Caffeic acid occurs regularly as the quinic acid ester chlorogenic acid (3-caffeoylquinic acid). Hydroxycinnamic acids frequently esterify the hydroxyl group of many secondary metabolites such as flavonoids and anthocyanins (Bruneton, 1995, p.214).

The antioxidant activity of hydroxycinnamic acids has been fully established and may contribute to the protective function of increased fruit and vegetable intake against the incidence of diseases such as cardiovascular disease and cancer, as found in epidemiologic studies (Radtke et al, 1998). Due to the abundance and diversity of the hydroxycinammic acid derivatives, they are almost ubiquitous in plants with *p*-coumaric acid being the most widely distributed (Harborne, 1984, p.46). Phenolic acids are rarely listed in food composition tables and there is no dietary intake data available (Radtke et al, 1998; Firuzi et al, 2003).



Caffeic acid (R) = OH as shown

p-coumaric acid (R) = H

Ferulic acid (R) = OCH_3

Figure 1.5 Chemical structures of some common hydroxycinnamic acids.

1.3 MEASUREMENT OF ANTIOXIDANT ACTIVITY

1.3.1 Introduction

In order to investigate the antioxidant potential of the selected plant extracts, the current literature was reviewed for established methods used for the *in vitro* measurement of the products of oxidation associated with the pathological processes outlined in sections 1.1.4-1.1.8. Various methods using animal tissue or chemical assays have been developed for measuring the antioxidant activity of plants *in vitro,* mainly by the food industry seeking safe effective antioxidants from natural sources to prevent deterioration of foods. For this purpose antioxidants are classified into two groups; primary or chain-breaking antioxidants and secondary antioxidants, depending on their mechanism of action. Primary or chain-breaking antioxidants (free radical inhibitors) and peroxide decomposers react with lipid peroxy radicals to convert them to stable products. Secondary antioxidants, like oxygen scavengers, remove ROS/RNS initiators by quenching chain-initiating catalysts (Duh, 1998). As already outlined in section 1.2.2, antioxidants may have more than one mechanism for their effect on biological systems. Antioxidant function *in vivo* is much more complicated than a single free radical scavenging process and the interpretation of results obtained from *in vitro* measurements of antioxidant activity of a compound or crude plant extract must be dealt with caution as their antioxidative effect may vary considerably with the method and conditions used. Selection of the assay to be used should be based on the intended application of the antioxidant.

1.3.2 Methods to examine antioxidant activity in vitro

Methods to examine antioxidant activity of a sample can generally be divided into two major categories:

1. Measuring its ability to donate an electron or hydrogen atom to a specific ROS or other electron acceptor.

2. Testing its ability to remove any source of oxidative initiation such as chelation of transition metal ions or inhibition of enzymes.

The presence of lipid peroxides in certain tissues has been demonstrated in association with several pathological conditions associated with ageing in 1.1.4. Due to its biological significance, assays to assess lipid peroxidation are the most appropriate to determine the antioxidant activity of the crude plant extracts and herbal preparations in this study. The assays most commonly used for measurements of lipid peroxidation will be briefly reviewed.

1.3.3 Measurement of peroxides

Many *in vitro* methods for detecting antioxidant activity use the ability of an antioxidant to retard the oxidation of a PUFA such as linoleic acid ($C_{18}H_{32}O_2$) exposed to oxidative stress, determined by exposing the linoleic acid to heat, oxygen, light or free radical generators. Several products are generated from each PUFA during lipid peroxidation. Measurement of these oxidation products enables assessment at different stages of the oxidative pathway.

1.3.4 Products of lipid peroxidation

The mechanism of lipid peroxidation (further outlined in Chapter 3) can be divided into three phases, initiation, propagation and termination. Initiation usually involves hydrogen abstraction from a C – H bond on carbon atoms adjacent to double bonds in a PUFA, forming a carbon-centred radical. During the propagation stage this carbon radical is rearranged to a conjugated diene followed by reaction with oxygen to produce a highly reactive peroxyl radical ($LOO^{\cdot}$) which simultaneously propagates the reaction by hydrogen extraction from another PUFA to form a lipid hydroperoxide (LOOH). Lipid hydroperoxide is the first, comparatively stable, product of the lipid peroxidation reaction (Abuja and Albertini, 2001).

In the presence of transition metal ions such as copper, iron or cobalt (Barber and Berheim, 1967) the rate of lipid peroxidation can be increased or re-initiated by radicals

generated from LOOH which breaks down to form a lipid alkoxyl radical ($RO^{\cdot}$) that is more reactive than the initial hydroperoxide. Alkoxyl radicals can continue the chain reaction of lipid peroxidation, or it can be hydrolysed to form aldehydes. Lipid hydroperoxides in the presence or absence of catalytic metal ions (M) give rise to a variety of aldehydes many of which can be used to assess the degree of lipid peroxidation. The general equation suggested for the metal catalysis of the initiation reaction is as follows:-

$$M^{N+} + O_2 \rightarrow M^{(M+1)+} + O_2^-$$

The O_2^- can act as initiator of the autoxidation reaction by several different mechanisms.

Two mechanisms are proposed for the metal catalysed breakdown of hydroperoxides:-

$$M^{N+} + LOOH \rightarrow M^{(N+1)+} + RO^{\cdot} + OH^-$$

$$M^{(M+1)+} + LOOH \rightarrow M^{N+} + RO_2^{\cdot} + H^+$$

1.3.5 Current *in vitro* methods for the measurement of antioxidant activity in lipid peroxidation

The following assays are commonly used to measure the antioxidant activity of natural and synthetic antioxidants.

1.3.5.1 Conjugated diene assay

During the propagation stage of lipid peroxidation the carbon radical is usually stabilized via an allylic shift to form a conjugated diene which strongly absorbs light at a wavelength of 234nm (Abuja and Albertini, 2001).

This method allows dynamic quantification of the formation of conjugated dienes during linoleic acid oxidation by measuring the absorbance of light at 234nm. The

amount of conjugated dienes produced is directly proportional to the amount of hydroperoxides formed as the major initial product of the lipid peroxidation reaction.

1.3.5.2 Lipid peroxide assay

Conceptually, one of the simplest methods is the measurement of lipid hydro-peroxides formed during linoleic acid oxidation. This can be measured by various colorimetric methods involving either a colour change brought about by the oxidising action of the peroxide directly on the chromogen, or by the oxidising action of the peroxide on some intermediate that then reacts with the chromogen to affect a colour change (Barber and Berheim, 1967). Several of the more widely used methods involve some modification of the iodometric procedure which is based on the ability of peroxides to oxidise iodide salts and liberate the iodine quantitatively (Lea, 1931). Alternatives include the use of thiocyanate (Glavind and Hartmann, 1955) which involves the oxidation of ferrous iron to ferric and xylenol orange (FOX) which also involves ferrous oxidation in the formation of a coloured adduct (Wolff, 1994; Nourooz-Zadeh, 1999). The amount of lipid hydroperoxides accumulated first reaches a maximum and declines thereafter while forming aldehydes.

1.3.5.3 Thiobarbituric acid reactive substances assay (TBARS)

The thiobarbituric acid test is one of the oldest and most frequently used tests for measuring lipid peroxidation. Lipid hydroperoxides in the decomposition phase give rise to a variety of products including aldehydes such as hexanal, malondialdehyde (MDA) and 4-hydroxynonenal. This assay is based on the detection of a stable product which is formed between aldehydes and thiobarbituric (TBA) in the aqueous phase (Halliwell and Chirico, 1993).

1.3.5.4 Quantitative measurement of lipid hydroperoxides

The most direct method for the quantitative determination of lipid hydroperoxide products is HPLC. This method is specific and does not require any derivatization, unlike the more sensitive GC-MS methods which involves esterification and silylation. HPLC determination is best achieved following partitioning of the hydroperoxide into a

polar solvent, which achieves a primary separation between the less polar triacylglycerol and cholesterol hydroperoxides and the more polar free fatty acids and phospholipid hydroperoxides (Rice-Evans et al, 1991).

Chemiluminescence is a collective term, which includes the emission of light by molecules which have been excited to a higher energy level as a result of a chemical reaction (Abuja and Albertini, 2001). Chemiluminescence-based detection is gaining popularity as a very sensitive analytical tool particularly for monitoring the process of lipid peroxidation (Diplock et al, 1998; Abuja and Albertini, 2001).

1.3.5.5 Reaction with 1,1-Diphenyl-2-picryl-hydrazl (DPPH)

The DPPH assay measures the free radical scavenging capacity of a compound. Because DPPH is known to abstract the labile hydrogen atom of chemical compounds the antioxidant that can scavenge the DPPH radical is expected to depress lipid peroxidation.

In the presence of an antioxidant which can donate an electron to DPPH, the purple colour which is typical to free DPPH radical decays, and the change in absorbancy at 517nm is followed spectrophotometrically (Blois, 1958). This assay is useful for comparing the reduction potential of unknown materials like plant extracts.

1.3.6 *In vitro* assays for the general measurement of antioxidant activity

1.3.6.1 Cyclic voltametry

This technique developed to measure the antioxidant activity of tissue or plasma has been adapted by Chevion et al (1999) for evaluation of the antioxidant capacity of edible plants as reflected by their low-molecular-weight antioxidants (LMWA). The method is based on measurement of the reductive capacity of the various LMWA components of a plant extract either singly or as mixtures of compounds, indicating their ability to donate electrons. The total reducing power of a plant, without the

necessity to measure the specific antioxidant capacity of each component alone, can be evaluated.

1.3.6.2 Reaction with 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS)

This technique for the measurement of total antioxidant activity measures the relative abilities of antioxidants to scavenge the ABTS radical cation ($ABTS^{\cdot+}$) in comparison with the antioxidant potency of standard amounts of Trolox, a water soluble vitamin E analogue, Results are expressed as "Trolox equivalent antioxidant activity" (TEAC). The TEAC reflects the ability of hydrogen-donating antioxidants to scavenge the ABTS radical cation $ABTS^{\cdot+}$, a blue/green chromogen with characteristic absorption maxima at 645nm, 734nm, and 815nm, compared with that of Trolox . Antioxidants suppress absorbance at 734nm to an extent and on a time scale dependent on antioxidant activity. TEAC is defined as the concentration of Trolox solution with equivalent potential to 1Mm concentration of the compound under investigation (Rice-Evans and Miller, 1996a).

1.3.6.3 Ferric reducing antioxidant power assay (FRAP)

This assay uses antioxidants to reduce ferric ion in a complex with tripyridiltriazine (Fe^3 –TPTZ) to an intense blue ferrous (Fe^2) complex that develops an absorption maximum at 593nm. FRAP values are obtained by comparing the absorbance change at 593nm in test reaction mixtures with those containing ferrous ions in known concentrations. Hence, this test measures the ability of a sample to reduce ferric ion complex. Absorbance changes are linear over a wide concentration range with antioxidant mixtures and with solutions containing one antioxidant in purified form (Benzie and Strain, 1996).

1.4 CONCLUSION

In view of diverse and multitudinous evidence regarding the sources of free radicals, both environmental and endogenous and their destructive effect upon the human body, future research should be weighted towards their prevention. Greater emphasis is needed upon research into environmentally-generated free radicals and how they enter into and affect the body, and also how and if they, in some way, exacerbate the endogenous and possibly benign free radicals, conceivably making them more damaging.

As the greater therapeutic efficacy of many plant extracts over single isolated constituents is recognised, research on isolated phytochemicals may not be the way forward, since the use of individual compounds may not reflect the synergistic action of the whole plant. Future research therefore, should be directed at investigating the therapeutic properties of the whole plant as used traditionally, rather than with isolated plant constituents which in some instances have been shown to cause toxicity, e.g. beta carotene and more recently, the reported hepato-toxic effects of concentrated standardised extracts of *Piper methysticum* (Kava Kava) (Whitton et al, 2003). Research needs to be conducted into the possible benefits of synergy *in vitro* on whole plant extracts and combinations of plant extracts as opposed to isolated constituents.

Furthermore, there is a need for more information regarding the therapeutic roles of medicinal plant constituents, particularly tissue specificity, where the majority of evidence of their efficacy is anecdotal, i.e. *Silymarin marianum* and the liver, *Ginkgo biloba* and the brain and cardiovascular system; *Crataegus* spp. and the heart/circulatory system. Most flavonoid research has been carried out on dietary flavonoids with very few studies on medicinal plants.

A number of different evaluation methods have been developed for assessing the *in vitro* antioxidant effects of flavonoids making comparison of the various studies very difficult. The reason for this is flavonoids do not specifically react with a single ROS, but may act as scavengers of various oxidizing species. Results obtained from *in vitro* measurement of antioxidant activity of a crude plant extract may vary considerably with the conditions and the methods used. Thus, selection of the appropriate assay to be used

should be based on the intended application of the antioxidant. Concentration of the plant extract used in the assay must be consistent for comparison of the antioxidant activity of several different plant samples. The ability of flavonoids to react with metal ions may also render them pro-oxidant. In an *in vitro* study by Cao et al (1997) using three different oxidation systems, flavonoids had potent antioxidant activity against peroxyl radicals and hydroxyl radicals, but were pro-oxidant in the presence of Cu^{2+}. It was presumed that the flavonoids reduced the Cu^{2+} to Cu^{+}, hence allowing the formation of initiating radicals. Studies have also shown that some antioxidants have the potential to act as pro-oxidants depending on final concentration. Thus, a greater degree of standardisation of method is desirable in the future.

The antioxidant activity of a plant *in vitro* may not correspond with effects *in vivo.* Since there is a paucity of information on the bioavailability, absorption and metabolism of flavonoids, this could be due to differences in pharmacokinetics particularly between the different groups (Hollman, 1997; Wiseman, 1999). Most flavonoids enter the diet as glycosides, but the extent of absorption from the diet is largely unknown and may depend on the glycoside present (Bone, 2001). The metabolism of flavonoids is determined by the hydroxylation pattern, thus the final products of digested flavonoids absorbed in the bloodstream may not bear any resemblance to the original crude plant extract, giving conflicting results *in vitro* and *in vivo.*

Moreover, there are very few human intervention studies using antioxidant supplementation, especially in regard to medicinal plants (individual or in combination) that establish cause-and-effect relationships. It is still unclear whether individual antioxidants from dietary or medicinal plants, working alone or in synergy, will improve human health and also at what level of intake. A relationship has been established between dietary intake of fresh fruit and vegetables and lowered risk of cancer (Eastwood, 1999) but no conclusive proof exists of specific antioxidants or exclusive medicinal plant constituents lowering disease-incidence to date.

Finally, in order to facilitate the formulation of prophylactic herbal counter-measures to help maintain and improve good health, a greater understanding of the pathogenesis of certain diseases is required.

CHAPTER 2: THE BASIC PRINCIPALS OF HERBAL MEDICINE AND THE SELECTION OF HERBS FOR THIS STUDY

2.1 THE MEDICINAL USE OF PLANTS

2.1.1. Introduction

Plants have played a significant role in maintaining the health and improving the quality of life for humans over thousands of years. The WHO estimate that 80% of the world's population rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components (Craig, 1999). Many Western drugs were originally derived from plants. Atropine, digoxin, colchinine, taxol and vincristine are examples of isolated plant constituents that have found their way into the prescription drug inventory (Craig, 1999).

There are a number of healthcare approaches involving the use of plants. Medical herbalism, one of the primary complementary approaches to medicine in the U.K., encompasses the use of plant extracts for treatment and prevention of disease within a complete holistic framework of healing. One of the basic principles of herbal medicine is that all the constituents in the whole plant extract work collectively to achieve therapeutic efficacy. There is however, limited experimental and clinical evidence relating to the remedies traditionally used by herbalists or the synergistic interactions of combinations of herbs. As previously outlined, the aims of this study were to investigate a small selection of plants traditionally used by herbalists, including tincture extracts, the most widely-prescribed form of plant medicine in the U.K.

2.1.2 Plants as medicine

Herbal medicine refers to the use of whole plant remedies in the promotion of healing and maintenance of health. Herbal remedies can be made from a combination of herbs

or from a single herb and can be administered to a patient in a variety of forms including tinctures, infusions, oils, flower waters, poultices, suppositories, syrups, liniments and salves. Preparations of single chemicals contained in a plant are not classified as herbal medicines (Ernst, 2004). Although the salicylates from the bark of *Salix alba* (white willow) provided the natural product model for Aspirin, neither synthetic salicylate (acetylsalicylic acid) from which Aspirin is produced, nor the original active principal salicylic acid, a metabolite of salicin can be referred to as Herbal medicines. Isolating the active principals from a plant or synthetically reproducing them may alter the specific mode of action or produce unwanted side effects (Gilbert and Alves, 2003). For example, it has been found that at the doses necessary to produce the desired analgesic effect, willow bark extract containing salicin does not inhibit the cyclooxygenase COX-1 and does not cause gastro-intestinal irritation, both of which are observed with acetylsalicylic acid (Gilbert and Alves, 2003; Vane and Botting, 2003).

2.1.3 Isolation of biologically active constituents

In some cases research has shown that by isolating the pharmacologically active compounds, less activity may be produced than when they are retained in the whole plant extract (Houghton, 2000). Cott and Misra, (cited in Gilbert and Alves, 2003) reported that, in the field of psychotherapeutic plants, substances isolated from *Hypericum perforatum* and *Passiflora incarnata* did not reproduce the activity of the crude extracts of the plants at comparable doses of the isolates, either alone or in combination. The authors concluded that a synergistic effect was involved in which some substances present in the plants could enhance the activity of the so called active principles. These results contribute a scientific basis to the empirical beliefs of Medical Herbalists that not all constituents are medically active, but in many cases are adjuvant substances that may enhance or influence their efficacy. For scientific research the isolation of an active substance is preferred in order to provide an exact dosage and for ease of measuring bioactivity. In many cases the plant preparation as a whole may be traditionally reported as being clinically effective but the pharmacologically active moieties may not be known even if the chemical composition of the plant extract has been elucidated (Wuthold et al, 2004). Examples include *Crataegus spp., Vitex agnus-castus and Passiflora incarnata* (Williamson, 2005).

2.1.4 Formulations and synergy

Traditional herbal practice in many part of the world involves prescribing combinations of herbs with a wide range of actions that concurrently cover several treatment strategies. Rather than focusing on a specific disease pathology, Herbal Practitioners treat holistically with individualised herbal formulae (Williamson, 2001). Combinations provide multiple active constituents working together which may produce additive, or synergistic interactions. Both Chinese Herbal Medicine and Ayuvedic Medicine (the ancient healing system of India) have been using combinations of natural compounds for centuries, if not millennia. Chinese records go back more than 4000 years (Gilbert and Alves, 2003). The value of using combinations is not now confined to herbal medicines and the use of small drug combinations is now routinely used in cancer chemotherapy and the treatment of HIV and hypertension (Wagner, 2004). These combinations usually comprise 3 – 5 agents. Larger combinations are not tested because as the size of the combination increases so does the expense, technical difficulty, potential adverse effects and the difficulty of statistical analysis (Boik, 2001, p.x).

In the field of pharmacology, the study of interactions between constituents of a single herb, between herbs in combination and between herbs and pharmaceutical agents is increasing. An interesting series of studies looking at the combined effects of plant extracts and xenobiotics on liposomal lipid peroxidation was carried out by Popovic et al (1999; 2001). Two of the studies involved the industrial chemical pyralene, a lipid soluble polychlorinated biphenyl (PCB) environmentally damaging to human health and a commonly prescribed antimicrobial drug Ciprofloxacin in combination with either extracts of *Calendula officinalis* (Marigold) or *Taraxacum officinalis* (Dandelion). Various extracts of *Calendula officinalis* or *Taraxacum officinalis* alone or in combination with different doses of Ciprofloxacin or pyralene were used to study their effect on lipid peroxidation induced by Fe^{2+} and ascorbic acid. All the plant extracts tested individually showed antioxidant activity. Pyralene alone exerted a pro-oxidant action which was decreased by combining with both the dandelion and marigold extracts. At lower concentrations, ciprofloxacin alone did not affect lipid peroxidation but at higher concentrations it acted as a pro-oxidant. Both the dandelion and marigold extracts in combination with ciprofloxacin showed a protective effect by decreasing its pro-oxidative action which for marigold was also dose dependent. Wagner (2004)

comments that the gradual renunciation in mainstream medicine of the long-standing reliance on monosubstance therapy in favour of multidrug therapy is one of the new paradigm shifts in current healthcare. Phytomedicines, he postulates, have a good chance of contributing to this strategy.

2.1.5 Evaluating synergy in herbal medicine

The term synergy, as understood by Medical Herbalists in the U.K., applies to the formulation of whole herb combinations, the potency of which either by additive or synergistic interactions is increased. The skill and practice of combining herbs in this way is an expertise that has evolved over many generations. Since synergy can be extremely difficult to prove this knowledge is mainly without scientific basis. Current models for testing synergy are mainly based on the combination of pure compounds. Unlike pharmaceutical drugs where an exact dose of the active principal can be measured, the active principal in a whole plant extract is notoriously variable. To try and eliminate as many variables as possible, the use of plant extracts standardised to known active constituents are generally preferred for scientific validity. Controlled double-blind studies, still widely regarded as the most reliable evidence of objective drug reactions, depend upon consistency. Studies of herbal medicines face fundamental problems of external validity that are less obvious than those for the synthetic drugs that generally can be subjected to much more stringent standardisation because many different types of product can be made in different ways from raw plant material that may itself be inherently variable, for reasons that include geography, geology, weather and genetics, (Chrubasik et al, 2003). In addition, raw plant material may also vary with different harvesting procedures, time of harvest and storage conditions. Different manufacturing methods, i.e. methods of extraction, generate differing constituent profiles with subsequent variations in biological response. The use of isolated constituents and standardised plant extracts does not reflect the ethos of Herbal Medicine practice which is that of wholism, individualised treatment and herbal formulae based on whole plant extracts. This level of complexity presents a challenge to any research carried out into herbal medicine.

2.1.6 Herbal extracts

Tinctures used as herbal medicines are the most widely used form of plant extract traditionally used by Medical Herbalists in their prescribing (Bilia et al, 2001). A tincture is a preparation containing the soluble constituents of a medicinal plant, most often the result of macerating or percolating the plant parts with alcohol/water solutions. Fluid extracts (liquid extracts) are highly concentrated preparations; one part by volume of the preparation is equivalent to one part by weight of the original plant material (1:1). Tinctures are commonly made to a concentration of 1:3, 1:4, 1:5, 1:8 or 1:10. For example, a 1:4 tincture has 1 kg plant material to 4 litres alcohol/water. The strength of alcohol in water also varies depending on the type of constituent to be extracted. Four strengths are used in the British Herbal Pharmacopoeia (1996), 45%, 60%, 70% and 90%. In everyday practice where water alone would be sufficient to extract the medicinal compounds but alcohol is required to preserve the extract, 25% alcohol is often used. Both the British and European Herbal Pharmacopoeias (1996; 1997) contain general monographs about tinctures in which their modes of preparation and the amounts of herbal drugs in the tinctures are specified. The amount of alcohol required to facilitate extraction of the phytocomplex from individual herbs however is not specified, resulting in commercially available tinctures that contain different amounts of alcohol that may produce varying phytochemical profiles.

2.1.7 Defining and demonstrating synergy

Medical herbalists are taught to use polypharmacy, i.e. formulation of a preparation by combining a range of actions using a mixture of herbal tinctures. The synergistic interactions between the numerous constituents are believed to be a vital part of their therapeutic efficacy. It is not known, however, whether potentiation of the active principals, enhanced bioavailability, cumulative effects of the medicine or simply the additive properties of the constituents are responsible for any perceived enhancement of activity (Williamson, 2001; Gilbert and Alves, 2003).

From a scientific view the fundamental problem with synergy is that the concept, by its very definition, lies outside of the current belief that wholes can be understood by the isolation and analysis of their parts. Plant synergy is not considered a rational approach

to the combination of molecules. Numerous mathematical models have been proposed in the quest for a quantitative measurement of synergy, the definition of which tends to be defined by the precise mathematical method used to demonstrate it. Berenbaum, (1989) and Greco et al, (1995) review these methods which, because they were mainly designed to assess the interaction of pharmaceutical drug combinations, do not take into account the multiple compounds, actions, interactions and effects of whole herb preparations and formulae. Williamson (2001) in a review on plant synergy cites the isobole method as proposed by Berenbaum (1989) as the current method of choice. Wagner and Steinke (2004) have successfully employed this method to assess synergy between various mixtures of ginkgolide A and B (constituents of *Ginkgo biloba*) measured using the thrombocyte aggregation assay. Synergy was indicated by the concave-up isobole curve obtained i.e. in the construction of the iso-curve, if the point representing a combination of two substances forms a straight line with single points on the x-y axis which are the doses of the individual substances, there is no interaction. If the point representing the effect of the combination lies below this line, the curve will be concave-up indicating that synergy is present. A point above this line produces a concave-down isobole indicating antagonism (Fig. 2.1). Wagner (2004) comments however, that although this method may be suitable for dose-response investigations with two-component containing mixtures, it is not a plausible method to be applied to herbal extract mixtures which would require detailed *in vitro* or *in vivo* comparative investigations with single constituents or mixtures and extract fractions or whole extracts to be performed.

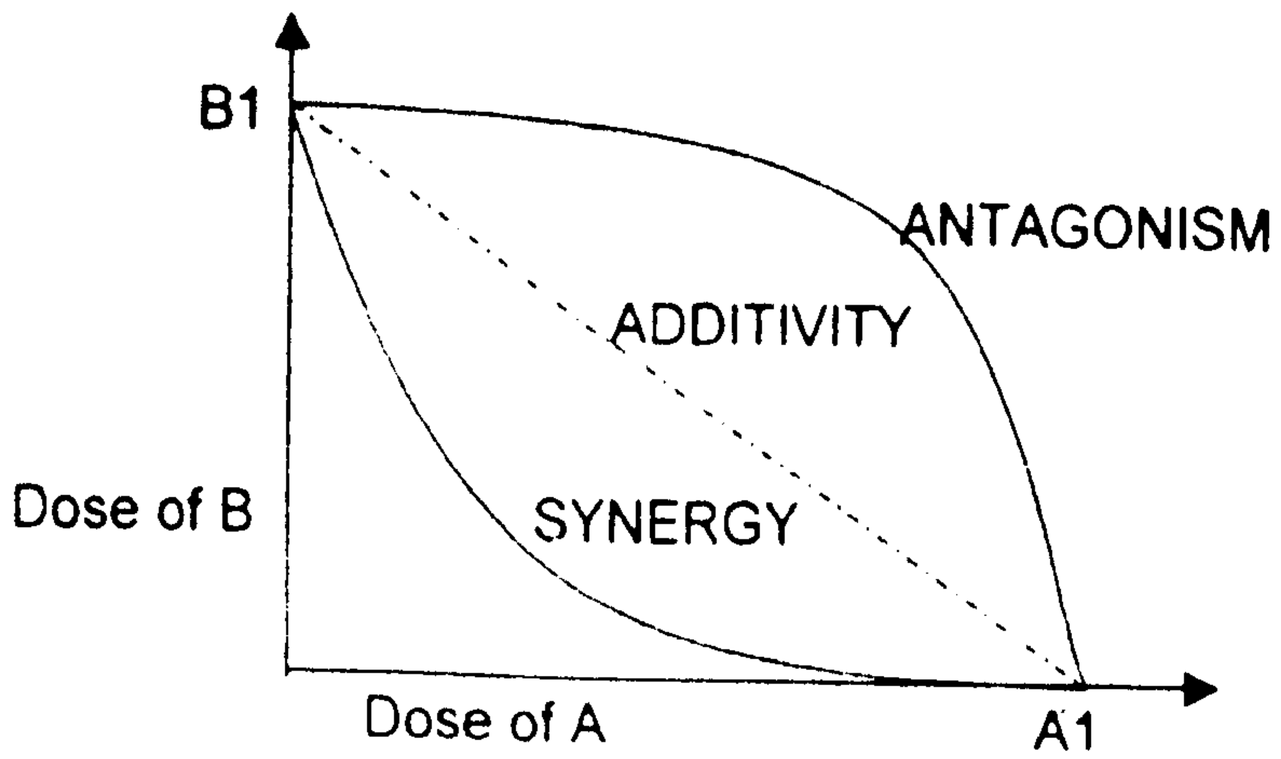


Figure 2.1 Isobologram illustrating additivity, synergy, and antagonism (Berenbaum 1989). A1 and B1 are the doses of the constituents A and B respectively, which produce an equal effect. The concave up isobole represents synergy. The concave down isobole represents antagonism.

The idea that the nature of an interaction might be decided by analysing the mechanism of action of the agents and on this basis calculating the expected effect of the combination, was the original approach to the measurement of synergy. This presupposes however that the mechanisms of action are fully understood and entirely depends therefore on the current state of knowledge. Berenbaum (1989) cites the following example. If two compounds have common receptor sites and an equation for their expected efficacy in combination is derived on this basis and if the combination is observed to be more effective than the equation predicts, then synergy is thought to have occurred. If however, it is subsequently found that the binding sites are in fact different, leading to the derivation of a different equation and the data fits this new equation, the two compounds will now be said to show zero interaction. Thus, as new knowledge about mechanisms of action have expanded, interactions defined in this way are no longer valid, so that an interaction initially said to be synergistic could subsequently be thought to be antagonistic although the observed data remained the same. Berenbaum, (1989) has suggested that observed effects of the agents and their combinations without reference to mechanisms may be a better approach to the assessment of synergy. Such an approach would be just as applicable to agents of which the modes of action are completely unknown as to those which are well understood and even to agents that are unidentified. He states that the requirements of a mechanism free model are analogous to those of a nonparametric statistical test such as Mann-Whitney or the Kolmogorov-Smirnov test that calculates the probability that two sets of values come from populations with the same distribution and to decide this question no information is required about what the values represent, nor about the real distribution of the populations from which the sets of values come. Many mechanism free models analogous to these requirements have been developed like the isobole method mentioned above, but to date none have been identified for reliably interpreting reactions between complex herbal mixtures. As a precursor to more complex investigations, statistical analysis using a nonparametric statistical test would at least indicate whether or not two or more plant extracts interact, simply on the basis of observed effects. For the preliminary assessment of any plant interaction the Mann-Whitney test is the preferred method of analysis in this study.

Three classes of interaction will be referred to in this study; zero interaction, in which the effect of a combination is the same as that expected from their effects alone; synergy, in which the effect is greater than expected; and antagonism, in which it is less.

2.2 SELECTION OF PLANTS

2.2.1 Introduction

The use of herbs to augment good health and to help in the treatment of some conditions associated with ageing such as circulatory disorders and joint disease are traditionally known to be effective. There is a growing need however to establish a scientific evidence base for the practise of herbal medicine. Conditions such as osteoarthritis that may respond poorly to orthodox treatment will possibly do better with appropriate herbal therapy (Long et al, 2001; Hoffman, 1993 p.11). Herbs commonly used by herbalists in their treatment of OA formed the basis of this study, with research into their reported efficacy focused on the establishment of a possible therapeutic link between the chemical constituents contained in these plants, and one of the pathological processes associated with OA, namely lipid peroxidation.

2.2.2 Osteoarthritis

Osteoarthritis is the most common degenerative disease affecting human articular cartilage, especially in the population over 65 years old (Heraud et al, 2000). Reactive oxygen species are implicated in both cartilage ageing and the pathogenesis of osteoarthritis (Tiku et al, 2000); see Chapter 1 of this thesis. Since micronutrient antioxidants provide defence against tissue injury, high dietary intake of these micronutrients it has been suggested could protect against osteoarthritis. Lane et al, (1999) found that high levels of vitamin D protected against both incident and progressive hip osteoarthritis. Herbal remedies have a long history of use in the treatment of 'Arthritis' generally. The word 'arthritis' means joint inflammation involving either inflammation of connective tissues or non-inflammatory degeneration of these tissues. Conclusions from a systemic review (Long et al, 2001) on herbal medicine used for the treatment of osteoarthritis and a pilot study (Bell et al, 1999) investigating herbal treatment outcomes present promising evidence for the effective use of some herbal preparations in the treatment of this condition. When considering the large number of people suffering from osteoarthritis, together with the known adverse

side effects associated with conventional drug treatments, he concluded that this area had been under researched (Long et al, 2001).

2.2.3 The Herbalists approach to 'Arthritis'

Western Herbal Medicine advocates a broad holistic approach to the treatment of 'arthritis' by selecting herbs with a wide range of actions, primarily anti-rheumatic, anti-inflammatory, circulatory stimulant effects, plus enhancement of elimination, particularly for the improvement of liver function (Mills, 1991, p.61; Hoffman, 1993 p.139-156). The term anti-rheumatic as used by Herbal practitioners refers to herbal remedies that have been traditionally observed to improve the patient's experience of rheumatic problems. It is a description of outcome rather than process that has anecdotally engendered herbal knowledge over many generations (Mills, 1991, p.206-209; Hoffman, 1993 p.139-156). The specific effect upon the disease or upon the musculo/skeletal tissue itself may not have been identified. Anti-inflammatory herbs ease symptoms overall by reducing inflammation; circulatory stimulants increase blood flow to muscles and joints and increase elimination through improved circulation; eliminative herbs, especially liver tonics, enhance the body's expulsion of toxic products of metabolism through the use of appropriate herbs. Supporting the liver to improve detoxification is considered an important focus of treatment in Western Herbal Medicine. Since many herbs fall into the above categories but may also have a wide range of other actions, the Herbal Practitioner's choice of one herb over another would be influenced by the botanical evidence-base in respect of both anecdotal and scientific evidence, the original preferences of the Practitioner's training Institution, and clinical experience. Osteoarthritis is a complex disorder and empirically it certainly seems that herbal remedies have qualities that lend themselves to the treatment of 'arthritis' in a very different way from that of conventional medicines. As science unravels the multifactorial aetiology and pathogenesis of chronic diseases like osteoarthritis we may discover that herbs traditionally used to treat arthritic conditions have actions as yet unidentified that are contributing to their reported efficacy.

2.2.4 A pilot study investigating outcomes of herbal treatment for osteoarthritis

A pilot study was undertaken by collaborators at Middlesex University and the Archway Clinic of Herbal Medicine to study patient outcomes following herbal treatment for osteoarthritis (Bell et al., 1999). Treatment was given from a choice of three herbal formulations generally based on the above approach to treatment [Table 2.1]. Preliminary results suggest that the herbal treatment administered over the three month period of the study was of benefit to the patients, some of whom had been long term sufferers of the condition. The herbs used in the prescriptions for this pilot study formed the basis of the herbs selected for this study.

Table 2.1 The list of herbs used by Herbal Practitioners in the pilot study by Bell et al (1999) for the treatment of osteoarthritis based on the following three presentations:- Metabolic/ Hormonal; Toxic/ Eliminative; Trauma/ Degenerative

Common name	Latin name	Plant part used
Celery	*Apium graveolens*	Seed
White willow	*Salix alba*	Bark
Liquorice	*Glycyrrhiza glabra*	Root
Ginkgo	*Ginkgo biloba*	Leaves
Dandelion	*Taraxacum officinale*	Root
Cramp bark	*Viburnum opulus*	Bark
Black cohosh	*Cimicifuga racemosa*	Root
Wild yam	*Dioscorea villosa*	Root and tuber
Nettle	*Urtica dioica*	Ariel parts
Meadowsweet	*Filipendula ulmaria*	Flowering tops and leaves
Horsetail	*Equisetum arvense*	Ariel parts
Hawthorn	*Crataegus* spp.	Leaves, flowers and berries
Devil's claw	*Harpagophytum procumbens*	Root
Angelica	*Angelica archangelica*	Root, leaves, stem, seeds
Withania	*Withania somnifera*	Leaves, root, berries
Ginger	*Zingiber officinale*	Rhizome

2.2.5 Method of plant selection for this study

2.2.5.1 Practitioner consultation for the selection of herbs

After consultation with clinic supervisors who were fully qualified and experienced Herbal Practitioners from a selection of specialist training clinics:- The Archway Clinic of Herbal Medicine, Westminster Poly clinic, The London clinic of Herbal Medicine and Dunboyne Herbs in Eire; a review of patients' case notes at the Archway clinic and a review of the existing literature, a list of 12 herbs most commonly used for the treatment of 'arthritis' was compiled [Table 2.2].

Since the pilot study by Bell et al (1999), was carried out at The Archway Clinic of Herbal Medicine, patient case notes at this clinic strongly reflected the herbal treatments given in this trial. A more balanced view of Practitioner's prescribing for arthritis was thus achieved by additionally consulting with Practitioners from other training institutions viz Westminster Poly clinic, The London clinic of Herbal Medicine and Dunboyne Herbs in Eire.

Key practitioners from all four training institutions were interviewed in regard to their general approach to the treatment of OA. All were specifically asked which single herb would be their primary choice in an herbal prescription for OA. They were then asked to indicate, which herbs from the list of those used in the pilot study by Bell et al (1999) they would include in this prescription plus any not on this list they would also routinely use.

2.2.5.2 Results of Practitioner consultation: final selection of herbs

As a result of all the data collated from the sources previously outlined, a list of twelve herbs most commonly used by Herbalists for the treatment of osteoarthritis was compiled as shown in Table 2.2.

Table 2.2 The 12 herbs identified as being those most commonly used by some Herbal Practitioners for the treatment of osteoarthritis. Herbs selected for study are marked with an asterisk (*).

Common name	Latin name	Plant part used
Devil's claw	*Harpagophytum procumbens*	Tuber
Boswellia	*Boswellia serrata*	Bark, gum resin
*Burdock	*Arctium lappa*	Root
*Celery	*Apium graveolens*	Seed
*Dandelion	*Taraxacum officinale*	Root
*Milkthistle	*Silybum marianum*	Seed
*Hawthorn	*Crataegus laevigata*	Leaves and flowers/ berry
*Marigold	*Calendula officinalis*	Flower
*White Willow	*Salix alba*	Bark
Black Cohosh	*Cimicifuga racemosa*	Root
Ginger	*Zingiber officinalis*	Rhizome
Prickly Ash	*Zanthoxylum clara-herculis*	Bark and berries

Seven of the twelve herbs shown in Table 2.2 were selected for study based on the criteria whereby the plant must be indigenous to Europe or the U.K. and that it could easily be grown and harvested from the Middlesex University herb garden. The seven herbs identified were Milkthistle (*Silybum marianum*), Marigold (*Calendula officinalis*), Dandelion (*Taraxacum officinale*), Celery (*Apium graveolens*), White Willow (*Salix alba*), Burdock (*Arctium lappa*) and Hawthorn (*Crataegus laevigata).*

Those herbs not selected were Devil's claw (*Harpagophytum procumbens*) a native plant of Southern Africa, Ginger (*Zingiber officinalis*) that grows in India, China and Mexico, Black Cohosh (*Cimicifuga racemosa*) and Prickly Ash *(Zanthoxylum clara-herculis*) that are native to North America and Boswellia *(Boswellia serrata*) which is a traditional Ayurvedic herb from India. Marigold (*Calendula officinalis*) is not a traditional remedy for 'arthritis' but since it was mentioned by several practitioners and there is a good evidence base for its anti-inflammatory action it was included in this study (Della Loggia, 1994; Akihisa, 1996). Although all of the selected herbs would have a role to play in the treatment of arthritis by a range of different actions, it is not known if they all possess the protective effects of antioxidant activity that could be postulated a part of the basis of their therapeutic effects.

2.3 PROFILES OF HERBS CHOSEN FOR STUDY

2.3.1 BURDOCK

Latin name:

Arctium lappa (Linné)

Family:

Asteraceae

Figure 2.2 Large heart-shaped foliage of *Arctium lappa*

Description

Arctium lappa is a biennial plant native to Britain and Europe which grows wild along roadsides, hedgerows and on waste ground (Fig. 2.2). The root, seeds and leaves are all used as herbal medicines but for the purpose of this study only the root, which is the part traditionally used in the treatment of arthritic conditions, will be considered. *Arctium lappa* root is composed of the fresh or dried root, which is harvested from plants in their first year of growth, when they are large and fleshy (British Herbal Pharmacopoeia, 1996).

Key constituents

Phenolic acids	For example, caffeic acid, chlorogenic acid (3-caffeoylquinic acid), isochlorogenic acid and other caffeolyquinnic acid derivatives) (Maruta et al, 1995; Bisset, 2001, p.100; Barnes et al, 2002, p.95).
Monocarboxcylic acids	For example, isovaleric acid, acetic acid, butyric acid, proprionic acid, tiglic acid) (Barnes et al, 2002, p.95).
Fatty acids	For example, lauric acid (dodecanoic acid), linoleic acid (9,12-octadecadienoic acid), linolenic acid (9,12,15-octadecadienoic acid), myristic acid (tetradecanoic acid), oleic acid (9-

	octadecanoic acid), palmitic acid (hexadecanoic acid), stearic acid (octadecanoic acid) (Barnes et al, 2002, p.95).
Polyacetylenes	Mainly trideca-1,11-dien-3,5,7,9-tetrayne and a number of sulphur-containing acetylene compounds (e.g. arctinone-a, arctinone-b, arctinol-a, arctinol-b, arctinal, arctic acid-b) (Washino, 1986a and 1986b; Bisset, 2001, p.100; Barnes et al, 2002, p.95).
Carbohydrates	Inulin (gamma-glucoside-fructose ester), mucilage, pectin (Tamayo et al, 2000; Bisset, 2001, p.100).
Flavonoids	Lignans (e.g. arctiin and its aglycone arctigenin) are thought to be present in the root (Bisset, 2001, p.100; Cho et al, 2004).
Bitter principals	Sesquiterpene lactones of the germacranolide type (e.g. arctiopicrin); glycosidal bitter (e.g. lappatin) mainly found in the leaf, but also reported for the root. (Bruneton, 1995, p.150-151; Barnes et al, 2002, p.95).
Other constituents	Sterols (e.g. sitosterol, stigmasterol) (Yochkova et al, 1989; Barnes, et al, 2002, p.95); essential oil containing more than 60 identified substances including aldehydes (e.g. benzaldehyde, phenylacetaldehyde), 2-alkyl-3-methoxypyrazines, aliphatic/ aromatic acids and sesquiterpenes (e.g. costic acid) (Washino et al, 1985; Bradley, 1992, p.48; Bisset, 2001, p.99); minerals (e.g. calcium, cobalt, copper, chromium, iron, potassium, selenium and zinc (Duke, 1992).

The term *Arctium lappa* is an umbrella term for several species e.g. *Lappa minus*, *Lappa majus*. The chemical composition of the root may vary between species (Barnes et al, 2002, p.95).

Current and traditional uses

Arctium lappa is known foremost in Western Herbal Medicine for its alterative or 'blood cleansing' properties. The term alterative is an expression used in herbal medicine to describe a concept, namely that certain herbs will gradually restore the proper function of the body by assisting with the elimination of waste materials that can

accumulate in the blood (Hoffman, 1990, p.143). No scientific mechanism has been established to describe this action. *Arctium lappa* is also known for its anti-inflammatory, aperient, diuretic, cholagogue and depurative actions. Although the evidence for use is mostly anecdotal, *Arctium lappa* is valued by herbalists as a treatment for arthritis, gout, skin diseases and other inflammatory conditions. *In vitro* and animal studies on *Arctium lappa* root and some of its isolated constituents have shown the following properties, antibacterial (Moskalenko, 1986; Healy et al, 1999; Holetz et al, 2002), antifungal (Holetz et al, 2002), antiviral (Eich et al, 1996; Collins et al, 1997), anti-inflammatory (Iwakami et al, 1992; Lin et al, 1996), antitumour (Koshimizu et al, 1988; Cho et al, 2004) and hepatoprotective which may in part be attributed to its antioxidant activity, which decreases the oxidative stress of hepatocytes, or to other unknown protective mechanisms (Lin et al, 2002).

2.3.2 CELERY

Latin name:

Apium graveolens (Linné)

Family:

Apiaceae/ Umbelliferae

Figure 2.3 Flowering top of *Apium graveolens*

Description:

Native to Great Britain and other European countries, *Apium graveolens* is often found growing wild in ditches and marshy areas not far from the sea (Fig. 2.3). The dried ripe fruits (seeds) are used medicinally, usually harvested from cultivated plants (Bradley, 1992, p.56).

Key constituents

Flavonoids	Flavones apigenin and glycoside apiin (apigenin 7-apiosylglucoside) and luteolin as glycosides (e.g. luteolin 7-apiosylglucoside); flavonol glycoside isoquercitrin (Harborne and Williams, 1972; Bradley, 1992, p. 56; Duke, 1992).
Phenolic acids	Caffeic and chlorogenic acids (Bradley, 1992, p.56).
Coumarins	For example, celerin, seselin, apigravin, umbelliferone, osthenol (Garg, 1979; Bradley, 1992, p.56).
Furanocoumarins	For example, bergapten, isopimpinellin, 8-hydroxy-5-methoxypsoralen; furanocoumarin glucosides (e.g. apiumoside, celereoside); dihydrofurocoumarins (e.g. rutaretin, apiumentin) (Garg, 1980; Bradley, 1992, p.56; Lombaert et al, 2001).
Volatile oils	Many components including terpenes/sesquiterpenes (e.g. limonene, α-selinene, β-selinene, nerolidiol, β-pinene, d-carvone, β-caryophyllene, α-eudesmol) (Duke, 1992; Rao et al, 2000).

The phenylpropene apiole (Harborne, 1984, p.52) and phthalide compounds (e.g. 3-n-butylphthalide, sedanolide) which determine the characteristic odour of celery (Zheng et al, 1994; Momin and Nair, 2002).

Other constituents

Fatty acids (e.g. lauric acid, linoleic acid, myristic acid, myristicic acid, oleic acid, palmitic acid, stearic acid); minerals (e.g. calcium, copper, iron, magnesium, zinc); ascorbic acid (Garg, 1979 Duke, 1992; Barnes et al, 2002, p.118); sesquiterpenoid glucosides (celerioside A-E), phthalide glycosides (celephtalide A-C) (Kitajima et al, 2003).

Current and traditional uses

Traditionally, *Apium graveolens* seed is known for its antirheumatic, anti-inflammatory, diuretic, carminative and hypotensive effects and is used in the treatment of rheumatic disorders, arthritis, gout and urinary tract infections. It is also used by Ayurvedic physicians for ailments of the liver and spleen. There is limited research, especially with human subjects, to support the effectiveness of its traditional uses, however, some *in vitro* and preliminary animal studies suggest anti-inflammatory (Atta and Alkofahi, 1998; Momin and Nair, 2002), antibacterial (Krishna and Banergee, 1999; Friedman et al, 2002), antifungal (Momin and Nair, 2001) and hepatoprotective (Singh and Handa, 1995; Ahmed et al, 2002) activity. Studies in laboratory mice show that Apium graveolens seed may help prevent the formation of cancerous tumours (Zheng et al, 1993; 1994; Sultana et al, 2005).

2.3.3 DANDELION

Latin name:

Taraxacum officinale (G.H. Weber ex F.G. Wigg)

Figure 2.4 Flower and foliage of *Taraxacum officinale*

Family:

Asteraceae

Description

Taraxacum officinale is a common perennial plant native to Western Europe (Fig.2.4). It grows wild in fields, along roadsides and on waste ground everywhere. Both the leaves and root are used medicinally but, for the purpose of this study, only the root will be considered since this is the part traditionally used in the treatment of arthritis. *Taraxacum officinale* root is composed of the dried root and rhizome which are harvested in the autumn when the roots are two years old (Chevallier, 1996, p.140; Blumenthal 2000, p.79).

Key constituents

Phenolic acids	For example, caffeic acid, chlorogenic acid, p-hydroxyphenylacetic acid) (Duke, 1992; Barnes et al, 2002, p.171).
Fatty acids	For example, linoleic acid, linolenic acid, palmitic acid, oleic acid) (Duke, 1992; Barnes et al, 2002, p.171).
Terpenoids	Sesquiterpene lactones of the eudesmanolide type (e.g. taraxacolide glucoside) and germacranolide type (e.g. taraxinic acid glucoside). Triterpenes (e.g. taraxol, taraxerol, β–amyrin, faradiol) (Blumenthal, 2000, p.81; Hagymasi et al, 2000).
Phytosterols	For example, sitosterol, stigmasterol, taraxasterol (Blumenthal, 2000, p.81; Hagymasi et al, 2000).

Other constituents Bitter glycoside, taraxacin; carbohydrates, inulin, pectin, mucilage; choline (Barnes et al, 2002, p.171; Blumenthal, 2000, p.81); beta-carotene (Duke, 1992); minerals (e.g. calcium, chromium, cobalt, iron, magnesium, potassium, selenium, zinc); sugars (e.g. glucose, fructose, sucrose) and vitamins A, B, C and D (Duke, 1992; Hagymasi et al, 2000).

Current and traditional uses

Taraxacum officinale is better known as a folk remedy than a conventional medicine. Its choleretic, diuretic, antirheumatic, anti-inflammatory, appetite-stimulating and laxative properties are well known anecdotally as a treatment for liver and gallbladder disorders, digestive complaints, arthritic and rheumatic disorders as well as eczema and other skin conditions. In folk medicine *Taraxacum officinale* is considered to be a 'blood purifier' and is used as a detoxifying remedy (Chevallier, 1996, p.140-1; Hagymasi et al, 2000).

2.3.4 HAWTHORN

Latin name:

Crataegus laevigata (Poiret) de Candolle [(syn. *Crataegus oxyacantha* (Linné)]; *Crataegus monogyna* (Jaquin emend. Lindman).

Family:

Rosaceae

Figure 2.5a Flowering tops of *Crataegus* spp.

Figure 2.5b Ripe berries (haws) of *Crataegus* spp.

Description:

Crataegus laevigata and C*rataegus monogyna* are spiny trees or shrubs native to Europe and Great Britain. The traditional use by farmers of planting Hawthorn to form into thorny, stock-proof hedges has contributed to the patchwork appearance of the English landscape. In herbal medicine, hawthorn refers to the flowers, leaves and berries called 'haws' from the two *Crataegus* species above (Figs. 2.5a and b). The flowers which bloom in May and June, are harvested immediately after opening; the leaves when they are completely developed. The berries are collected in September and October. Herbal preparations are usually made from the dried plant parts (Blumenthal, 2000, p.185-8). Both the flowering tops and berries are to be used in this study.

Key constituents

Flavonoids are considered the most important constituents and primarily responsible for the pharmacological activity of this plant (Upton, 1999b). The flavonoid profile and proportions of active constituents varies within each species. Although the constituents for the berries and leaf with flower are similar, the relative amounts of the individual flavonoids and procyanidins is different. The berries consist primarily of oligomeric and polymeric procyanidins with lower levels of flavonoids than the leaf with flower (Upton, 1999a and b). The main flavonoids in the berries are hyperoside and rutin with small amounts of quercetin derivatives e.g. isoquercetin (Shahat et al, 1996; Zhang et al, 2001). The primary flavonoids in the leaves are vitexin and vitexin-2"-O- rhamnoside with little or none present in the berries (Wagner, 1984, p.178; Upton, 1999b). The predominant flavonoid in the flowering tops is hyperoside (Upton, 1999b).

Flavonols

Flavonols present in the berries, leaves and flowering tops include the flavonols quercetin and glycosides (e.g. hyperoside (quercetin-3-D-galactoside), rutin (quercetin 3-rutinoside), isoquercitrin (quercetin-3-0-β–D-glucopyranoside), quercitrin (quercetin-3-0-rhamnoside), quercetin-3-O-rhamnogalactoside) and kaempferol and glycosides (e.g. 8-methoxykaempferol-3-O-glucoside, 8-methoxykaempferol-3-O-neohesperidoside, kaempferol-3-O-neohesperidoside) (Djumlija, 1994; Upton, 1999a and b; Zhang et al, 2001).

Flavones

Glycosides of apigenin and luteolin (e.g. vitexin (apigenin 8-C-glucoside), isovitexin (apigenin 6-C-glucoside), vitexin-2"-O-rhamnoside (leaves), acetylvitexin-2"-O-rhamnoside (leaves), orientin (luteolin 8-C-glucoside), luteolin-7-O-glucoside, luteolin-3'7-diglucoside) (Djumlija, 1994; Rehwald et al, 1994; Upton, 1999a).

Proanthocyanidins

Procyanidins (constituents (+)-catechin and (-)-epicatechin). The dimeric procyanidin B2 and the trimeric procyanidin C1 are the primary constituents of both the berries and leaf with flower. Tetramers (e.g. D1) and oligomers that primarily consist of (-)-epicatechin units are also present. Polymeric procyanidins are

	mainly present in the berries (Upton, 1999a and 1999b; Svedstrom et al, 2002a and 2002b).
Triterpene acids	For example, ursolic acid, crataegolic acid, oleanolic acid) (Djumlija, 1994; Zhang et al, 2001).
Phenolic acids	For example, caffeic acid, chlorogenic acid, cinnamic acid, protocatechuic acid) (Djumlija, 1994; Zhang et al, 2001).
Fatty acids	For example, lauric acid, linoleic acid, linolenic acid, palmitic acid) berries (Duke, 1992).
Other constituents	Amines (e.g. phenylethylamine, o–methoxyphenethylamine, tyramine) (Hobbs, 1990). These compounds are found mainly in the flowers. Leaves and fruit contain smaller amounts (Weiss, 1988, p.163). Sterols (e.g. beta-sitosterol) leaf with flower (Hobbs, 1990; Duke, 1992); minerals (e.g. calcium, cobalt, chromium, iron, magnesium, potassium, selenium, zinc) berries; vitamins (e.g. ascorbic acid, beta-carotene) (Duke, 1992; Djumlija, 1994);

Current and traditional uses

Crataegus spp. *(monogyna or laevigata)* leaves, tops and berries have traditionally been used for their cardiotonic, coronary and peripheral vasodilator, hypotensive, diuretic and antirrhythmic effects to treat conditions such as angina, arteriosclerosis, hypertension, cardiac failure and associated cardiovascular disorders. Both animal and human studies support the traditional use of these *Crataegus* spp. in treating various cardiovascular disorders (Djumlija, 1994; Miller, 1998a; Degenring et al, 2003). Based on the findings of a meta-analysis of randomized trials for the treatment of chronic heart failure (Pittler et al, 2003), Eaton and Kinkade, (2003) in The Journal of Family Practice concluded that *Crataegus* spp. could be recommended as an adjunctive therapy to improve the physical performance and ameliorate heart failure-related symptoms of patients with chronic heart failure. Research on individual constituents has shown that the therapeutic benefits of *Crataegus* spp. are only gained when a whole plant preparation is used (Upton, 1999a and 1999b).

2.3.5 MARIGOLD

Latin name:

Calendula officinalis (Linné)

Family:

Asteraceae

Figure 2.6 *Calendula officinalis* flower

Description

Calendula officinalis is an old garden favourite also known as pot marigold, native to Southern Europe (Fig. 2.6) and commonly cultivated in Britain. For medicinal purposes either the dried flower-heads or the dried ligulate flowers (ray florets) are used (Blumenthal, 2000, p.45).

Key constituents

Flavonoids	Different flavonol glycosides have been identified in *Calendula officinalis* including rutin, narcissin (isorhamnetin-3-rutinoside), isorhamnetin-3-glucoside, isorhamnetin-3-neohesperidoside, isorhamnetin rutinorhamioside and isoquercetrin, plus the flavonol aglycones isorhamnetin and quercetin (Wagner, 1984, p.176; Pietta et al, 1992; Bilia et al, 2001).
Phenolic acids	Caffeic acid, chlorogenic acid (Duke, 1992).
Terpenoids	Triterpene saponins, triterpene alcohols and their fatty acid esters (e.g.α -amyrin, β-amyrin, lupeol, helianol, calendulosides C-H) (Pizza, 1987; Akihisa et al, 1996; Hamburger et al, 2003); triterpenoid acids (e.g. oleanolic acid) (Duke 1992). The triterpenoid esters (e.g faradiol 3-O-laurate, palmitate amd myristate) are reported to significantly contribute to the anti-inflammatory effects of *Calendula officinalis* flowers (Della Loggia et al, 1994; Hamburger et al, 2003).

Phytosterols	For example, cholesterol, sitosterol, sigmasterol, taraxasterol (Duke, 1992; Barnes et al, 1996, p.103).
Other constituents	Carotenoid pigments (e.g. lutein) (Bako et al, 2002; Wei et al, 2003); a bitter, calendine (Willuhnand and Westhaus, 1987); immunostimulant polysaccharides and volatile oils (Barnes et al, 2002, p. ; Kaurinovic et al, 2003).

Current and traditional uses

Calendula officinalis has commonly been used in conventional as well as folk medicine for its anti-bacterial and antimycotic effects (Dumenil et al, 1980; Tarle and Dvorak, 1989); antiviral activity (De Tommasi et al, 1990; Kalvatchez et al, 1997), anti-inflammatory and antiphlogistic action (Della Loggia et al, 1994; Blumenthal, 2000, p.44; Hamburger et al, 2003); immunostimulant, antitumorous and anti-lipidemic effects (Lansky, 1993; Golding, 1994; Isaac, 1994).

Traditionally, *Calendula officinalis* is used externally as a lotion, ointment, oil or tincture for the treatment of diverse dermatological conditions such as wounds, ulcers eczema, burns, bruises and eruptions like impetigo. Infusions are used internally for indigestion and liver and gallbladder disorders (Kaurinovic et al, 2003).

2.3.6 MILKTHISTLE

Latin name:

Silybum marianum (L.) Gaertner [*Carduus marianus* (Linné)]

Family:

Asteraceae

Figure 2.7 Flowerhead of *Silybum marianum*

Description

A spiny annual or biennial plant, native to the Mediterranean but found growing throughout Europe (Fig. 2.7). The part used in Herbal Medicine is the small, hard-skinned fruit (achene) known as *Fructus silybi* that develops from the large purple flower head. Referred to commercially as *Silybum marianum* seeds, these fruits are used when they mature and are freed from the papus (a deciduous tuft of minutely barbed bristles that falls off the mature fruit) (Blumenthal, 2000, p.260).

Key Constituents

Flavonoids

Flavanone lignans collectively referred to as silymarin consisting of the flavanolignans silybin (silibinin), isosilybin, silydianin, silychristin (Wagner, 1984, p.190; Bilia et al, 2001; Campodonico et al, 2001).

Dihydroflavonols (e.g. dehydrokaempferol, dihydroquercetin (taxifolin); flavonols (e.g. quercetin, kaempferol); flavones, flavanones and their glycosides (e.g. apigenin, chrysoeriol (luteolin 3'-methyl ether), naringin (naringenin 7-0-neohesperidoside), eriodictyol (Wagner, 1984, p.190; Morazzoni and Bombardelli, 1995; Duke, 1992; Bisset, 2001, p.122; Barnes et al, 2002, p.341).

Lipids

For example, linoleic acid, oleic acid, palmitic acid) (Bisset, 2001, p.122; Barnes et al, 2002, p.341; El-Mallah et al, 2003).

Other constituents	Phytosterols (e.g. cholesterol, campesterol, stigmasterol, β sitosterol); mucilage (Bisset, 2001, p.122; Barnes et al, 2002, p.341; El-Mallah et al, 2003).

Current and traditional use

Historically, *Silybum marianum* has been used as a liver tonic. In Western Herbal Medicine it is the main remedy used to protect the liver and treat liver disorders such as hepatitis and jaundice, cirrhosis and alcoholic liver disease and problems associated with the gall bladder. Numerous biochemical and pharmacological studies carried out on *Silybum marianum* have provided support for its traditional use as a unique hepatoprotective agent (Saller et al, 2001; Fraschini et al, 2002). Clinical trials using formulations of the active constituent silymarin have shown positive hepatoprotective effects in the treatment of liver diseases of different actiology that include acute viral and chronic hepatitis, alcohol-related liver disease and poisoning by the highly toxic mushroom *Amanita phalloides* (Ferenci et al, 1989; Flora et al, 1998; Luper, 1998). Anti-inflammatory, anti-arthritic, antioxidant and anti-carcinogenic properties have also been documented (Morazzoni and Bombardelli, 1995; Gupta et al, 2000; Jiang et al, 2000; Kang et al, 2001; Davis-Searles et al, 2005).

2.3.7 WHITE WILLOW

Latin name:

Salix alba (Linné)

Family:

Salicaceae

Figure 2.8 Small branch of the tree *Salix alba*

Description

Salix alba is a large deciduous tree indigenous to Britain, central and southern Europe, with a stout deeply furrowed trunk and smooth silvery grey branches, found growing beside streams and waterways (Fig. 2.8). The part used as medicine is the dried bark of young 2-3 year old twigs and branches harvested during the early spring (Blumenthal, 2000, p.409).

Key constituents

Plant chemistry varies between the many S*alix* species (Upton, 1999c). The following chemical profile is representative of the main constituents present:-

Phenolic glycosides	Salicin and esters of salicin (e.g. salicortin, 2'0-acetyl salicortin, fragilin (2'-0-acetylsalicin), tremulacin). Salicyl alcohol (saligenin) aglycone of salicin (Wagner, 1984, p.282; Upton, 1999c, p.7).
Flavonoids	Flavanones (e.g. eriodictoyl-7-glucoside, naringenin-5-glucoside, naringenin-7-glucoside); chalcones (e.g. isosalipurposide) according to species (Upton, 1999c, p.7; Du et al, 2004).
Polyphenols	Flavan-3-ol monomers, mainly (+)-catechin accompanied by traces of (-)-epicatechin; dimeric and trimeric proanthocyanidins (Kolodziej, 1990; Bradley, 1992, p.224).

Other constituents	Acids (e.g. caffeic acid, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, salicylic acid, syringic acid, vanillic acid); minerals (e.g. calcium, chromium, cobalt, iron, magnesium, potassium, selenium, zinc) (Duke 1992; Bisset, 2001, p.438).

Current and traditional uses

The bark of *Salix alba* is an ancient remedy historically used for its anti-rheumatic, anti-inflammatory, antipyretic, analgesic and astringent properties for the symptomatic relief of arthritis and rheumatism, gout, fever and aches and pains of varying aetiology, particularly joint pain (Vane, 2000). Most of the research carried out on the bark of *Salix* spp. has isolated the salicylate compounds and shown these to have analgesic, anti-inflammatory and antipyretic activity that in principle support the herbal uses (Upton, 1999c). Since levels of salicylates in the bark may vary and can often be low, these findings are difficult to extrapolate to whole herbal extracts of *Salix alba* (Upton, 1999c).

Few studies have been carried out to evaluate the therapeutic efficacy of whole extracts of the bark of *Salix* spp. as opposed to isolated salicylates and their synthetic counterpart acetylsalicylic acid (aspirin). Two recent clinical studies investigating pain management with herbal anti-rheumatics as a replacement for conventional drugs, evaluated the effectiveness of *Salix* bark extracts for pain associated with osteoarthritis and lower back pain, with the common conclusion that it was a safe and effective remedy for the treatment of mild to moderate pain. In contrast to NSAIDS such as acetylsalicylic acid, no gastrointestinal side effects were observed at the pharmacologically active dose administered (Chrubasik et al, 2000; Schmid et al, 2000; Marz and Kemper, 2002). Using a similar *Salix* bark extract to that used in these trials a further *in vitro* study was carried out to investigate the mechanisms of its anti-inflammatory and analgesic actions (Fiebich and Chrubasik, 2004). The results indicated that the effectiveness of the extract i.e. inhibition of COX-2-mediated PGE (2) release, was through compounds contained in the extract other than salicin or salicylate. The authors suggest that the actions of *Salix alba* are only partly attributable to its salicylate content (Fiebich and Chrubasik, 2004).

2.3.8 Concluding comment

A small selection of herbs that generally reflect practitioner prescribing for OA has been chosen for this study. Reasons for their choice are based mainly on anecdotal evidence and clinical observation. Since free radicals may now be implicated in the pathogenesis of OA, many of the herbs traditionally used to treat arthritis, all attributed with different properties such as anti-inflammatory, pain relieving or improving elimination, may all share a common underlying property, antioxidant activity.

Based on the knowledge that many of the polyphenols present in plants, especially flavonoids, have antioxidant activity which has been fully recognised in both model systems and under *in vitro* conditions, the plants selected for study were extracted, separated and analysed for their polyphenolic content by a number of phytochemical methods as outlined in section one of the next chapter. Following phytochemical analysis, these extracts were then evaluated *in vitro* for any antioxidant propensity, including the ability to inhibit the pathological process of lipid peroxidation as described in section two.

CHAPTER 3: EXPERIMENTAL

3.1 METHODS OF PLANT ANALYSIS FOR IDENTIFICATION OF FLAVONOIDS

3.1.1 Selection, separation and extraction of plant material

General methods for flavonoid identification are outlined in this section, whilst those used for assessment of antioxidant activity are included in section 3.2.
The chemicals used were mainly purchased from Sigma-Aldrich Chemical Company, or Fisher Chemical Company, U.K.

3.1.2 Extraction of phenolic compounds from plant material

3.1.2.1 Plant material

Authenticated dried plant material provided by Proline Botanicals for the production of the crude plant extracts and extracts prepared as tinctures and commercial tinctures (liquid extracts) purchased from Rutland Biodynamics are described in Table 3.1. Dried herbs were all stored in sealed plastic bags at normal room temperature. Tinctures (liquid extracts) were supplied in brown glass bottles and stored at normal room temperature (approximately 20°C).

3.1.2.2 Preparation and extraction methodology

Solvent extraction was carried out by two different methods, Soxhlet extraction and cold maceration. Extracts produced by Soxhlet extraction are referred to as crude plant extracts. Extracts referred to as commercial tincture extracts (liquid extracts) and 'extracts prepared as tinctures' were produced by cold maceration. In the text, where the term fresh tincture extract is used, this refers to the 'extract prepared as tincture' which was freshly prepared by the author, using the same plant material as the crude plant extract in order to provide a better comparison of extraction methods.

Table 3.1 Plant material: dried herbs and commercially produced tinctures (liquid extracts) used in this study.

Latin name	Common name	Plant part used	Dried herbs (Proline Botanicals) Batch No. and country of origin	Tinctures (liquid extracts) (Rutland Biodynamics) Batch No. and country of origin	Tinctures (liquid extracts) final % alcohol content [1.]
Silybum marianum	Milkthistle	Seed	B4433/ France	030822-2/ UK	25%
Calendula officinalis	Marigold	Flower heads	B5157/ Egypt	010925/-1 UK	45%
Apium graveolens	Celery	Seed	B2044/ India	020813-1/ UK	60%
Salix alba	White willow	Cut bark	B4758/ Bulgaria	021211-1/ UK	25%
Taraxacum officinale	Dandelion	Cut root	B5063/ France	030723-1/ UK	25%
Crataegus laevigata	Hawthorn	Berries	B2934/ Bulgaria	011109-2/ UK	25%
Crataegus laevigata	Hawthorn	Leaves and flowers	B5392/ Bulgaria	010601-6/ UK	25%
Arctium lappa	Burdock	Cut root	B4522/ Hungary	020627-3/ UK	25%

[1.] The final % alcohol content shown for the commercial tinctures is the alcohol content at the point of sale and not the alcohol/water solvent ratio used in the initial extraction process which for most plants is different and shown in Table 3.2.

3.1.2.2.1 Crude plant extracts

Extraction was based on the methods of Markham (1982, p.16), Harborne (1984, p.5) and Waterman and Mole (1994, sec. 4.2.3). Dried plant material (30g) was ground to a fine power for solvent extraction carried out by continuous Soxhlet extraction for one hour at a temperature of 60°C. Flavonoid constituents are unaffected by heating at this temperature (Jodrell Laboratory, 2004).

To separate the extract from plant material filtration was carried out using a filter funnel and filter paper (Whatman No.1). The extract was filtered until clear and then concentrated to a final volume of about 1 ml under vacuum using a rotary evaporator. The residue was weighed and re-dissolved in the original solvent to give a stock solution of 100mg per 1 ml, which was stored at a temperature of -18°C. The ratio of 1g dried plant material to 5ml solvent as recommended by Wagner (1984, p.291), was used for all extractions in the proportion 30g powdered plant material to 150ml solvent. (30g of powdered plant material was the maximum volume that could be used in the 'thimble' used for Soxhlet extraction, and increase in solvent volume above 150ml failed to increase the final yield).

The above procedure was used for extraction of all dried plant material. Different solvents and solvent mixtures were experimented with as outlined below. The final extracts were assayed and the most appropriate adopted for use.

3.1.2.2.2 Experimental procedure for the extraction of dried plant material

Following the method of Markham, (1982, p.16) the crude extract obtained from Soxhlet extraction using the recommended methanol:water (1:1) solvent ratio was filtered and the methanol removed by rotary evaporation. The resultant aqueous extract was then further extracted in a separating funnel with chloroform, to remove low polarity contaminants such as chlorophylls and fats. This chloroform extract was then evaporated under reduced pressure as above (3.1.2.2.1). To simplify the method and avoid any further use of chloroform because of its hazardous nature, single solvent soxhlet extractions were carried out using 100% ethanol and 100% methanol, which were filtered and evaporated to dryness as outlined in (3.1.2.2.1) without further extraction. Although TLC analysis (3.1.3.1) of the two single solvent extractions

showed similar results, HPLC analysis (3.1.6) showed that neither the ethanol nor methanol alone was extracting the full range of flavonoids. This was indicated by the absence of the flavonol quercetin aglycone and quercetin methyl ether (Grayer, 2003) which were present in the authentic silymarin standard [Fig 3.1; t_R 17.47 and t_R 20.80], but not in the ethanolic or methanolic crude plant extracts from *Silybum marianum* [Figs. 3.2 and 3.3 visualised at 335 nm]. In accordance with a recommendation from the research team at the Jodrell Laboratory, Kew (2003) and review of the literature (Zhang, 2001), a soxhlet extraction of *Silybum marianum* was carried out with 80% aqueous ethanol, using the method outlined in (3.1.2.2.1). HPLC analysis of this extract showed the presence of the quercetin aglycone and quercetin methyl ether at t_R 17.39 and t_R 20.71 (Grayer, 2003) [Fig 3.4 visualised at 335 nm], suggesting that some flavonoid compounds may only be water soluble and that the composition of the solvent should therefore be a mixture of alcohol and water. All further experimental work was therefore carried out using the crude plant extracts from Soxhlet extraction with 80% ethanol.

The procedure most suitable for flavonoid extraction depends on the type of flavonoids to be extracted and by whether the extraction is for qualitative or quantitative purposes. At the Jodrell laboratory, Kew, methanol (80:20) or ethanol (80:20) are traditionally used for extraction of flavonoid glycosides (Jodrell Laboratory, 2003). Ethanol is considered to be the less toxic of the two solvents and is also the solvent of choice for the production of herbal tinctures (Mills, 1991, p.369). The method outlined in 3.1.2.2.1 is for qualitative flavonoid analysis. Quantitative yields of the constituent flavonoid glycosides require several sequential extractions of the original plant material (Markham and Bloor, 1998; Jodrell Laboratory, 2003).

3.1.2.2.3 'Extracts prepared as tinctures'

Extracts were prepared in the laboratory as follows from the same dried plant material as the crude plant extracts (Table 3.1) and by the same method of cold maceration as that used in the production of the commercial tinctures (liquid extracts), to provide better comparability between the two types of extract i.e. crude plant and tincture extracts, produced by two different methods of extraction. The solvent ratios were the same as those used for commercial manufacture (Table 3.2).

Dried plant material (1g) was pulverised and macerated in an ethanol and water menstruum (100ml) in a covered airtight conical flask and agitated daily for three days. The ethanol-water solvent ratio used for the extraction of each plant is shown in Table 3.2 [A]. The extract obtained was clarified by vacuum filtration using a Buchner flask and filter paper (Whatman No.1). The filtrate was then concentrated under reduced pressure to a final volume of about 1ml. Each plant extract was reconstituted with aqueous ethanol (5ml) to the same final ethanol content (%) as that of the commercially produced tinctures (liquid extracts) as shown in Table 3.2 [B]. Each extract (1 ml) was then filtered into an HPLC vial and stored at a temperature of 4°C ready for HPLC DAD analysis.

Table 3.2 Alcohol-water ratios used in the production of the commercially produced tinctures (liquid extracts) from Rutland Biodynamics outlined in Table 3.1.

Plant	Ethanol-water solvent ratios used in the extraction process of the commercial tincture (liquid extracts) [A]	Final percent (%) ethanol content of the commercially produced tinctures (liquid extracts) [B]
Silybum marianum seed	25%	25%
Calendula officinalis flowers	90%	45%
Apium graveolens seed	90%	60%
Salix alba bark	50%	25%
Taraxacum officinale root	45%	25%
Crataegus laevigata berries	45%	25%
Crataegus laevigata leaves and flowers	45%	25%
Arctium lappa root	65%	25%

3.1.2.2.4 Tinctures (liquid extracts)

Using a rotary evaporator, the commercially produced tinctures (liquid extracts) (30ml), listed in Table 3.1, were prepared for study by concentrating to a final volume of about 1ml. Each reduced extract was then weighed and reconstituted with aqueous ethanol to the same final ethanol content (%) as the original product (Table 3.2 [B]), to give a stock solution of 100mg per 1ml, which was then stored at a temperature of -18°C.

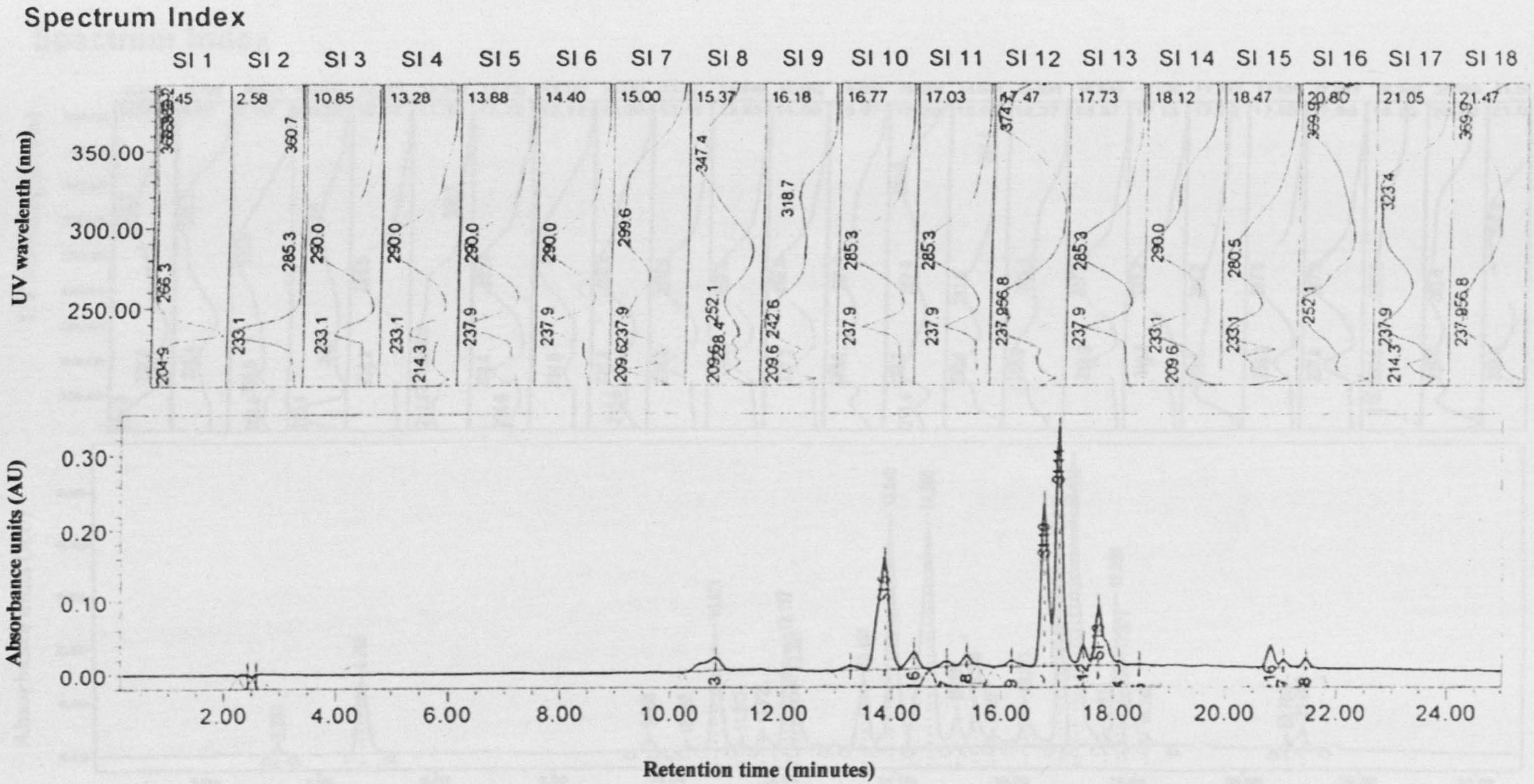


Figure 3.1. HPLC analysis and related on-line UV spectra of the reference compound silymarin (1mg/ml) recorded at 335nm showing the presence of the flavonol quercetin aglycone (t_R 17.47) and quercetin methyl ether (t_R 20.80). Separation on 5µm Li Chrospher 100RP-18e capillary column (250 x 4mm i.d.); gradient elution with 2% Aq. acetic acid and methanol : acetic acid : water (18:1:1); flow rate, 1ml/min.

Spectrum Index

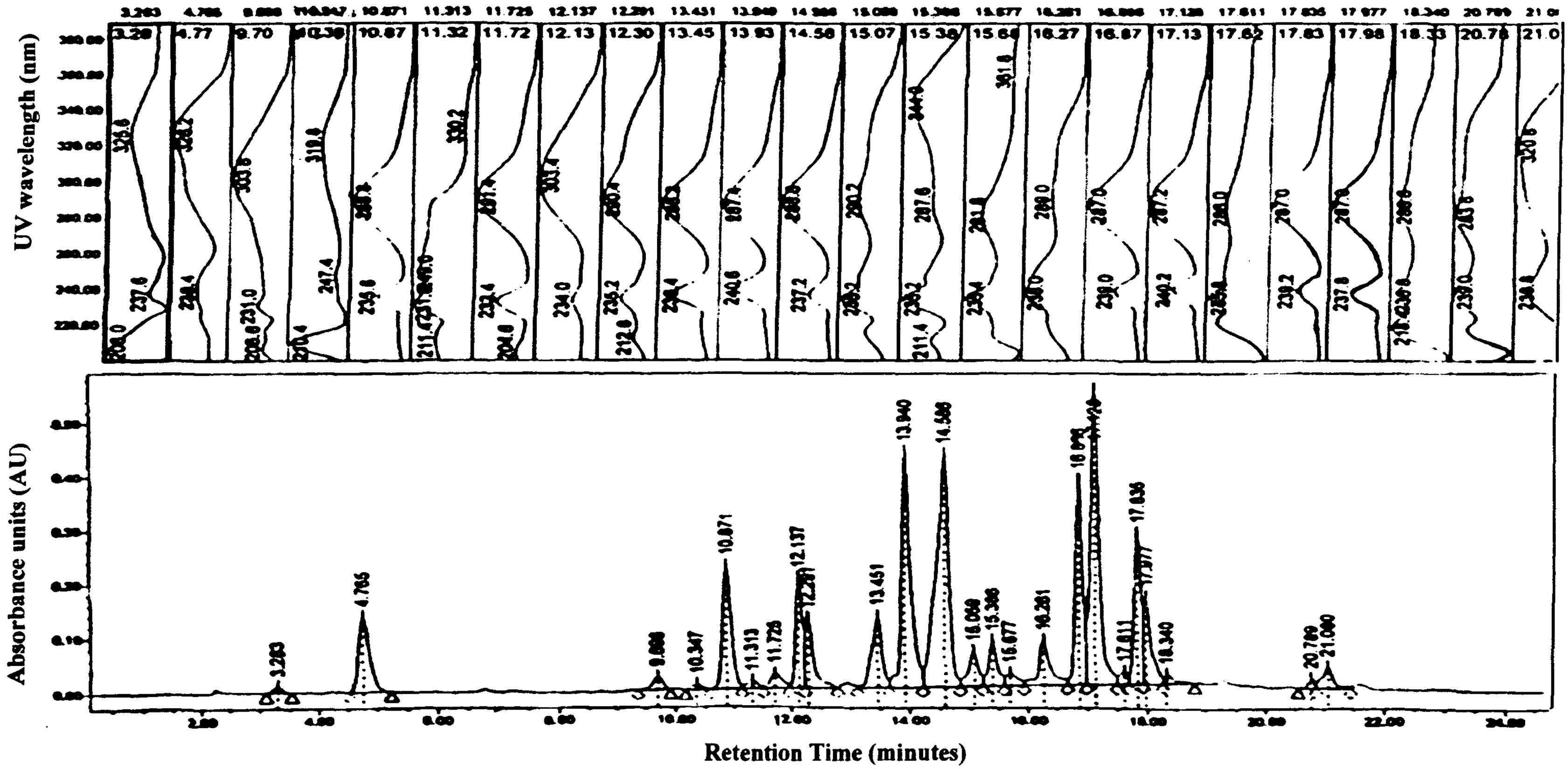


Figure 3.2. HPLC analysis and related on-line UV spectra of an ethanolic extract of *Silybum marianum* (Milkthistle) recorded at 335nm. Separation on 5µm Li Chrospher 100RP-18e capillary column (250 x 4mm i.d.); gradient elution with 2% Aq. acetic acid and methanol : acetic acid : water (18:1:1); flow rate, 1ml/min.

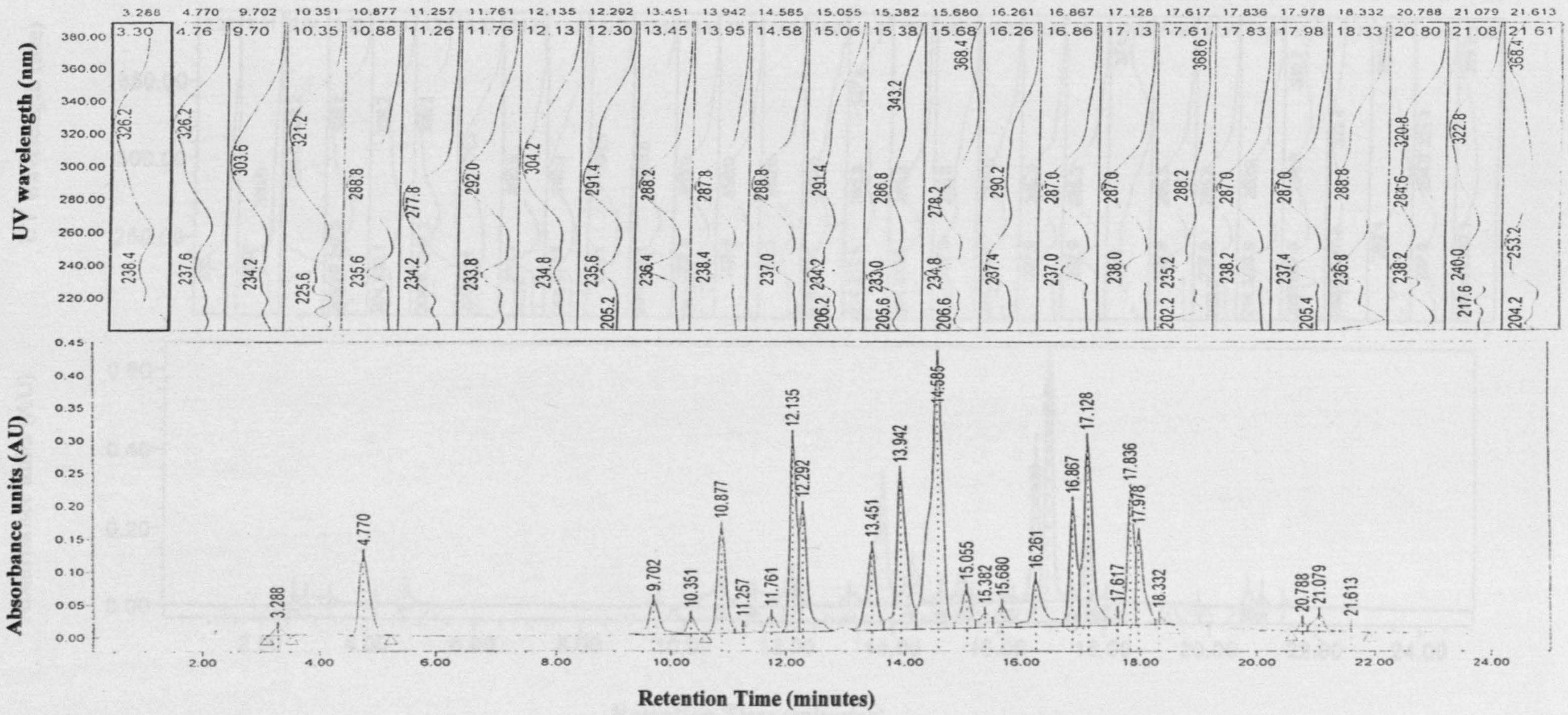


Figure 3.3. HPLC analysis and related on-line UV spectra of a methanolic extract of *Silybum marianum* (Milk thistle) recorded at 335nm. Separation on 5µm Li Chrospher 100RP-18e capillary column (250 x 4mm i.d.); gradient elution with 2% Aq. acetic acid and methanol : acetic acid : water (18:1:1); flow rate, 1ml/min.

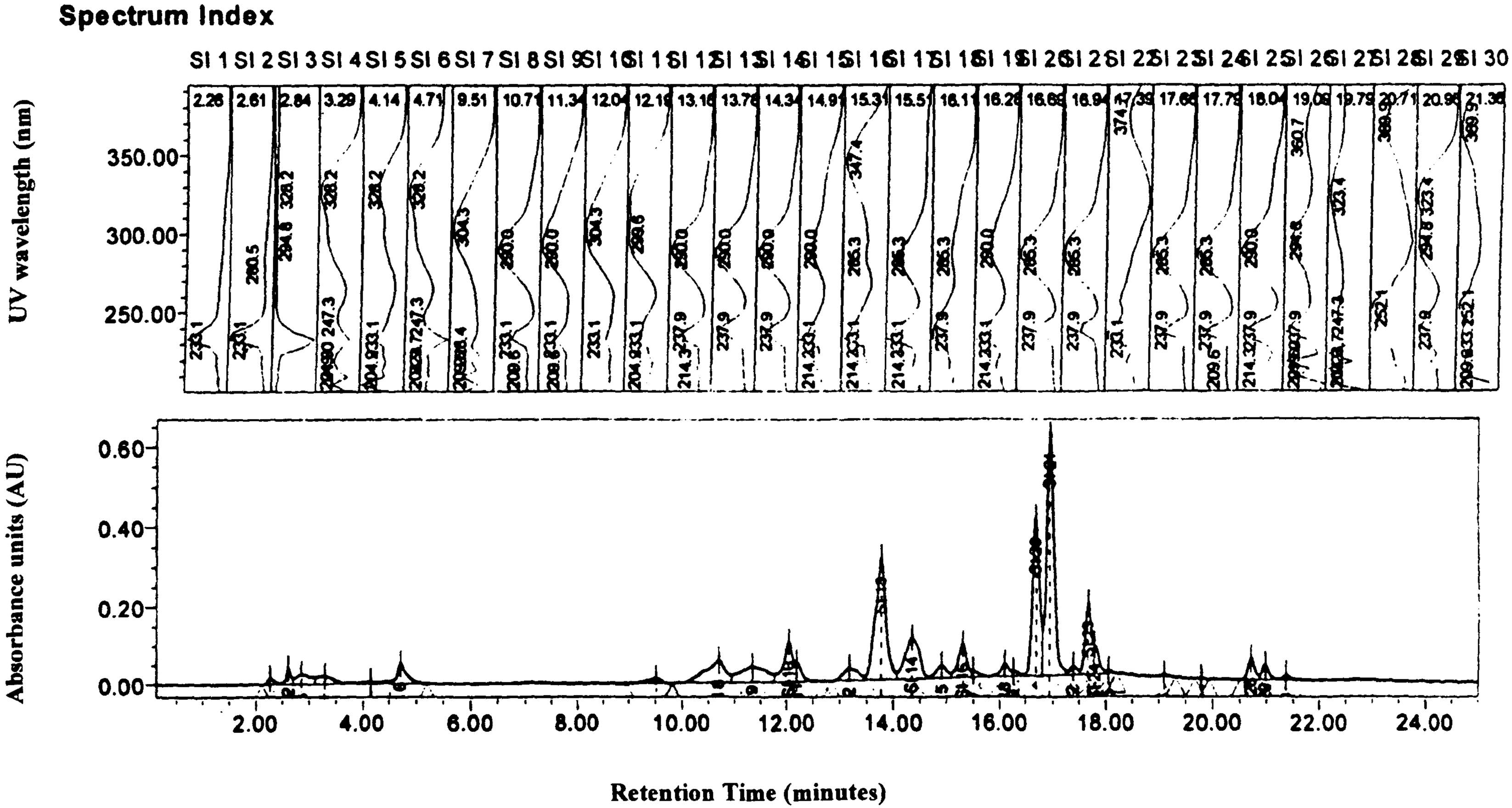


Figure 3.4. HPLC analysis and related on-line UV spectra of *Silybum marianum* (Milk thistle) crude plant extract (80% EtOH) recorded at 335nm showing the presence of the flavonol quercetin aglycone (t_R 17.39) and quercetin methyl ether (t_R 20.71). Separation on 5µm Li Chrospher 100RP-18e capillary column (250 x 4mm i.d.); gradient elution with 2% Aq. acetic acid and methanol : acetic acid : water (18:1:1); flow rate, 1ml/min.

3.1.3 Separation and identification of compounds from the extracts

3.1.3.1 Thin Layer Chromatography (TLC)

Thin layer chromatography provides a quick and simple means of separation and preliminary detection of plant compound from small samples.

3.1.3.1.1 Materials and methods

3.1.3.1.2 Standard compounds

Caffeic acid (99%); rutin (95%); quercetin (98%); taxifolin (85%); chlorogenic acid (95%); hyperoside and silymarin purchased from Sigma-Aldrich Company Ltd. Where no percentages are quoted they were not specified by the manufacturer.

3.1.3.1.3 Detection reagents

Reagent A	Natural products reagent (NP/PEG) The plate is sprayed with 1% methanolic diphenylboric acid-β-ethylamino ester (NP) followed by 5% ethanolic polyethyleneglycol 4000 (PEG)
Reagent B	The plate is sprayed with a mixture of 1 volume sulphuric acid and 9 volumes of ethanol.
Reagent C	The plate is sprayed with 0.3g vanillin in 85 ml of methanol with 3 ml sulphuric acid added.

3.1.3.1.4 Method

TLC analysis was carried out on Macherey-Nagel TLC plates coated with fluorescent Kieselgel 60 F_{254} eluting with the appropriate solvent system as indicated in Table 3.3 according to the methods described by Stahl (1974, p.3-23) and Wagner (1984, p. 163-5). The compounds were detected by direct visualisation under shortwave UV light, developing the plates in spray reagent (see Table 3.3) and heating on a hot plate. The identified compounds were characterized by their R_f values.

The development distances on the chromatogram are expressed by the R_f value that is constant for a particular compound. TLC of each crude plant extract (3.1.2.2.1) was carried out using key reference compounds prepared as 0.1% solutions, as indicated by the literature (Wagner, 1984; Bisset, 2001). Where the specified reference compound was unavailable documented R_f values were used to aid interpretation (Wagner, 1984; Bisset, 2001).

3.1.3.2 Thin layer chromatography (TLC) for the identification of salicylic acid derivatives in *Salix alba* (Willow bark)

Willow bark contains numerous phenolic compounds that are hydrolysed to salicin (Upton, 1999c, p.8). Hydrolyzed plant material was therefore used to detect the presence of salicin and its derivatives.

3.1.3.2.1 Materials and method

TLC was carried out according to the method given in the American Herbal Pharmacopoeia (Upton, 1999c, p.8)). A solution of plant extract (sample A) was prepared by heating a mixture of powdered Willow bark (1g) and methanol (20ml) on a water bath at a temperature of 50°C for 10 minutes. The mixture was allowed to cool and then filtered using a filter funnel and filter paper (Whatman No.1).

Alkaline hydrolysis was performed by adding 1ml of a solution of anhydrous sodium carbonate (50g/L solution) to an aliquot of the prepared plant extract (5ml) and heating in a 60°C water bath for 10 minutes. This solution (sample B) was cooled and filtered as above.

TLC of samples A and B was carried out as outlined under 3.1.3.1.4 using the appropriate solvent system listed in Table 3.3. Documented R_f values for salicin were used to aid interpretation (Wagner, 1984, p.282; Upton, 1999c, p.8).

Table 3.3 Chromatography solvent systems and detection reagents for TLC analysis of selected plants (Wagner, 1984; British Pharmacopoeia, 1996; Bisset, 2001).

Plant	Chromatography Solvent System	Detection
Silybum marianum (Milk thistle seed)	chloroform:acetone: anhydrous formic acid (75:16.5:8.5)	Reagent A (3.1.3.1.3)
Apium graveolens (Celery seed)	ethyl acetate:anhydrous formic acid: glacial acetic acid:water (100:11:11:27)	Reagent A (3.1.3.1.3)
Arctium lappa (Burdock root)	ethyl acetate:anhydrous formic acid: glacial acetic acid:water (100:11:11:26)	Reagent A (3.1.3.1.3)
Calendula officinalis (Marigold flower)	ethyl acetate:anhydrous formic acid: glacial acetic acid:water (100:11:11: 26)	Reagent A (3.1.3.1.3)
Salix alba (White willow bark)	ethyl acetate:methanol:water (100:13.5:10)	Reagent B (3.1.3.1.3)
Taraxacum officinalis (Dandelion root)	chloroform:ethanol (95:5)	Reagent C (3.1.3.1.3)
Crataegus laevigata (Hawthorn berries Leaf and flower)	ethyl acetate:formic acid:glacial acetic acid:ethylmethyl ketone: water (50:7:3:30:10)	Reagent A (3.1.3.1.3)

3.1.4 Column chromatography

3.1.4.1 Materials and method

Commercially prepared polyethylene tubes packed with 8g silica containing a fluorescence indicator were supplied by Fisher Chemicals and used for the isolation of compounds identified by TLC.

The crude plant extract (3.1.2.2.1) was dissolved in the selected solvent mixture (Table 3.3) and applied to the top of the column. The column was allowed to develop and then directly examined under UV light to visualize the separated compounds and the desired

portions cut out and extracted using the same selected solvent mixture. The resulting eluent was analysed using TLC as outlined in 3.1.3.1

3.1.5 Assays for the detection or quantification of specific flavonoids

3.1.5.1 Detection of plant proanthocyanidins

Proanthocyanins or flavolans are mainly associated with woody plants and are rarely found in herbaceous angiosperms (Walker, (1975, p.37). Three prepared extracts outlined under 3.1.2.2 of the plants identified by the literature to contain proanthocyanidins (*Salix alba* and *Crataegus* spp. berries plus leaves and flowers), were analysed for the presence of proanthocyanidins.

3.1.5.1.1 Materials and method

The standard method for acid hydrolysis used was adapted from Harborne, (1984, sec.283) and Walker, (1975, p.52). An aliquot of each plant extract (1ml) was placed in a boiling tube with an equal volume of 2M HCl (1ml) and the colour of each extract recorded. These were then heated on a 100°C water bath for 30 minutes. The tubes were removed from the water bath, allowed to cool and any colour changes recorded. A red colour indicates a positive result.

To further extract the anthocyanidins, ethyl acetate (1ml) was added to each tube which was shaken for 30 seconds. The top layer (ethyl acetate) was pipetted off and placed on labelled watch-glasses to dry in the fume cupboard. The bottom layers were discarded. The ethyl acetate layers were re-dissolved in 1ml of 80% aqueous methanol then filtered into crimp top HPLC vials which were stored in the dark at a temperature of 4°C ready for analysis. The separated anthocyanidins may be tentatively identified from their colours but these only serve as a rough guide (Walker, 1975, p.54; Harborne, 1984, p.62-64).

3.1.5.2 Quantitative analysis by Spectroscopy

The following two spectrophotometric assays quantitatively determine the concentration of the two principal flavonoids contained in the berries and in the flowers and leaves of *Crataegus* spp (Hawthorn).

3.1.5.2.1 Spectrophotometric measurement of procyanidin content in *Crataegus* spp. (Hawthorn berries)

3.1.5.2.1.1 Materials and method

Acid hydrolysis of a flavonoid glycoside leads to the separation of the aglycone and the sugar moieties thereby enabling independent analysis of the separated portions (Markham and Bloor, 1998). The assay was carried out according to the method given in the American Herbal Pharmacopoeia, (Upton, 1999a, p.11). A mixture of 2.5g powdered hawthorn berries and 30 ml aqueous methanol (70% v/v) were refluxed for 30 minutes and then filtered. After 15ml hydrochloric acid and 10ml water were added to the filtrate the residue was then washed with 10 ml aqueous methanol and added to the acidified filtrate. The filtrate was then refluxed for a further 80 minutes and re-filtered. The remaining residue was washed with aqueous methanol and the resulting solution added to the filtrate. The final volume was adjusted to 250 ml with aqueous methanol. Using a rotary evaporator, 50 ml of this solution was concentrated to a volume of about 3 ml and transferred to a separating funnel. The flask was rinsed with 15 ml water which was also transferred to the separating funnel and the combined solutions shaken with 45 ml butanol. The organic layer was diluted to a final volume of 100 ml with butanol and the absorbance of this solution measured at 545nm.

Total procyanidin available from hawthorn berries was calculated as percentage cyanidin chloride using the following formula cited in The American Pharmacopoeia, (Upton, 1999c, p.11):-

Total procyanidin available as percentage of mass = A x 500/ 75 x *m*

A = absorbance of the test solution at 545 nm

The specific absorbance of cyanidin chloride is 75

m = mass of substance to be analysed in grams

3.1.5.2.2 Spectrophotometric measurement of hyperoside in *Crataegus* spp. (Hawthorn leaf and flower)

3.1.5.2.2.1 Materials and method

The assay was carried out according to the method given in the American Herbal Pharmacopoeia (Upton, 1999b, p.14). A stock solution was prepared by heating a mixture of 40 ml aqueous ethanol (60%v/v) and 0.4g powered hawthorn leaves and flowers on a 60°C water bath for 10 minutes that was allowed to cool, and then filtered using a filter funnel and filter paper (Whatman No.1) into a 100 ml volumetric flask. The extraction was then repeated as above by adding 40 ml aqueous ethanol to the filter paper and plant residue. The two filtrates combined were adjusted with aqueous ethanol to a final volume of 100 ml.

To prepare the test solution, 5 ml of the above stock solution was evaporated to dryness and 8 ml methanol-acetic acid reagent (10 volumes methanol: 100 volumes glacial acetic acid) added to the dried extract. This solution was then transferred into a 25 ml volumetric flask and 10ml of boric acid-oxalic acid reagent (2.5g boric acid and 2g oxalic acid made up to 100 ml with anhydrous formic acid) added and the final volume adjusted to 25 ml with anhydrous acetic acid.

The blank solution was prepared as above replacing the boric acid-oxalic acid reagent with 10 ml anhydrous formic acid. Absorbance of both solutions was measured at 410nm after standing for 30 minutes at room temperature.

Concentration of flavonoids available in hawthorn leaf and flower was calculated as percentage hyperoside using the following formula cited in The American Pharmacopoeia, (Upton, 1999b, p.14):-

% available hyperoside = A x D/M x 405

A = absorbance of the test solution minus the blank at 410nm

D = dilution factor = 100/5 x 25

M = mass of powdered material in grams

405 = E (1%) value of hyperoside at 410nm

3.1.6 Analysis of flavonoids and phenolic compounds by High Performance Liquid Chromatography (HPLC)

3.1.6.1 Materials and methods

3.1.6.1.1 Standard compounds

Caffeic acid (99%); chlorogenic acid (95%); p-coumaric acid; ferulic acid; rutin (95%); quercetin (98%); taxifolin (85%) and silymarin prepared as 1mg/ml and 0.1mg/ml solutions in 80% ethanol or methanol, filtered into HPLC vials. Where no percentages are quoted they were not specified by the manufacturer.

3.1.6.1.2 Equipment

HPLC analysis was carried out using a Waters 600 HPLC pump with a 600E system controller and a Waters 717 plus autosampler coupled to a Waters 996 Photodiode array detector. The HPLC was controlled by a PC workstation (Elonex) running Millennium 3.11 software (Waters Ltd.) Compounds were separated on a 250mm x 4mm i.d., 5µm

particle, LiChrospher 100 RP-18e capillary column, (Merck) maintained at 25°C. Helium was used for de-gassing solvents.

3.1.6.1.3 Method

Since retention times can vary between methods a standard method is used by many workers in the field (Grayer et al., 2000; Merken and Beecher, 2000). To separate the flavonoids present in the samples the mobile phase utilised a gradient solvent system prepared from methanol and water and containing acetic acid in order to avoid peak tailing (Table 3.4) (Grayer, 2000; Hostettmann and Hostetttmann, 1981); the flow rate was 1ml/min. Solvent A (100% methanol) was used to wash the column. A delay of 13 minutes between each run was used to re-equilibrate the column. The UV-visible spectra were recorded on-line during the HPLC analysis in the range 210-400 nm for flavonoids and phenolic acids, 210-600 nm for acid hydrolysed anthocyanidins. The characteristic of each peak was ascertained at 254 nm and 335 nm for flavonoids and phenolic acids and 535 nm for anthocyanidins. Crude plant extracts (3.1.2.2.1) and tincture extracts (3.1.2.2.5) were diluted to 10mg/ml and filtered into HPLC vials prior to analysis. The volume of sample solution injected was 30µl. (Markham and Bloor 1998).

Table 3.4 HPLC conditions for the analysis of flavonoids in plant extracts

Time (min)	Solvent A	Solvent C	Solvent D	Flow (ml/min)
0	0	75	25	1.0
20	0	0	100	1.0
24	0	0	100	1.0
25	0	75	25	1.0

Solvent A = Methanol
Solvent C = 2% Aq. Acetic acid
Solvent D = Methanol: water: acetic acid 18:1:1

3.1.6.1.4 Identification procedures

The UV-visible spectra of phenolic compounds provide a valuable aid to their identification since the wavelengths of the absorption maxima are often characteristic of a particular class of compound. Flavonoids are easily detectable using UV detectors

since they possess a chromphore which allows them to absorb UV light (Waterman and Mole, 1994, p.79-80). Flavonoids and hydroxycinnamic acids present in the extracts were identified where possible by retention times and by comparing the UV spectra of the peaks with those of the available standards. Where authentic samples were unavailable general identification of flavonoid types was made by interpretation of the general flavonoid spectrum which typically consists of two absorption maxima in the ranges 240-285nm (band 11) and 300-550 (band 1), (Markham, 1982, p.37; Jodrell Laboratory, 2004). Band 1 absorption can be used as a guide to the type of flavonoid present. Flavones tend to absorb between 310 – 350nm, whilst flavonols absorb between 330 – 385nm. Flavanones tend to have the major band 11 maximum in the 270 – 295nm region. They also have a small shoulder in the 300 – 330nm region. Anthocyanins have a principal maxima at 475-550nm (Markham, 1982, p.39; Markham and Bloor, 1998, p.25). Caffeic acid and its derivatives have characteristic absorbance bands at 243nm and 326nm with a distinct shoulder at 300nm (Markham, 1982, p.50-51). Documented ranges of absorption maxima for different flavonoid types were used to aid interpretation (Mabry, 1970; Markham 1982; Pietta, 1998).

3.1.7 Analysis of plant compounds by Gas Chromatography/ Mass Spectroscopy (GC/MS)

This technique, although not the chromatographic technique of choice for the identification of flavonoid compounds, was used to identify any volatile components in the crude plant extracts. Flavonoids have a molecular weight which is generally too high to volatilize and they may be best chromatographed as their trimethylsiloxy derivatives (Evans, 1996). After using a BPX5 column (SGE) which produced a poorly separated chromatogram, an attempt was made with a BPX20 column, which produced a very acceptable separation for all the plant extracts tested.

3.1.7.1 Equipment

GC analysis was carried out on a Fisons/Carlo Erba GC interfaced to a MassLab MD800 mass spectrometer. A medium polarity column (SGE BPX20) was used with a retention gap to provide protection for the column.

3.1.7.2 Materials and method

Helium at 8 pounds per square inch was used as a carrier gas; the oven programme was set at 50°C for 2 minutes then increased by 10°C per minute up to 250°C where it was held for 7 minutes. A volume of 1µl of the crude plant extract diluted to approximately 1 mg per ml was injected onto the column via a splitless vapour rising injector at 270°C.

3.1.7.3 Identification procedures

The volatile components of the extract separate out at different times according to their affinity for the column coating. Although analytes show a characteristic retention time, only tentative identification of plant constituents could be made, because most of the compounds appeared to be absent from the system's database and reference compounds would have been required for positive identification. A characteristic fragment profile or mass spectrum was produced for each compound. The mass/charge ratio against relative abundance of the resultant ions for each GC peak gives a characteristic profile for each compound. Mass spectra for significant GC peaks were qualitatively analysed using the National Institute for Science and Technology database to allow identification of the type of compound present.

3.2. EVALUATION OF PLANT EXTRACTS AS ANTIOXIDANTS

3.2.1 LIPID PEROXIDE ASSAY

3.2.1.1 Introduction: the membrane structure

The scope of this project was influenced by the existing knowledge that the plasma membrane forms the outer surface of a cell and protects it from harmful substances in its immediate environment. The basic structure of all membranes is a phospholipid bilayer containing both free-floating peripheral proteins and integral proteins embedded in the bilayer. The membrane lipids are distinct and separate from each other yet are tightly packed in order to form a flexible cell membrane. This unique structure allows the lipids to move laterally freely and rapidly, a process referred to as membrane fluidity (1.1.6.1). Proteins account for about 50% of the membrane mass, but there are around 50 lipid molecules for every protein molecule (Kingsland, 2000).

Biological membranes are one of the major targets of reactive oxygen species, the attack of which affects membrane functions by inducing lipid peroxidation. The primary reaction site for lipid peroxidation is the polyunsaturated fatty acid moities, oleic, linoleic and linolenic acids. The rate of oxidation of these fatty acids increases with the degree of unsaturation (Mihaljevic et al, 1996). Monounsaturated and saturated fatty acids are much less reactive and are not usually affected.

3.2.1.2 Peroxidation of unsaturated lipids

To expand on the process of lipid peroxidation outlined in chapter 1: (section 1.3.4), poly unsaturated fatty acids can become peroxidised according to the following scheme (Abuja, 2001).

The initiator (free radical $R^{\cdot}$) abstracts a hydrogen atom from a polyunsaturated fatty acid side chain (LH) leaving a carbon-centred radical ($L^{\cdot}$).

$$LH + R^{\cdot} \rightarrow L^{\cdot} + RH$$

The carbon-centred radical ($L^{\cdot}$) combines with molecular oxygen yielding a highly reactive lipid peroxyl radical ($LOO^{\cdot}$)

$$L^{\cdot} + 0_2 \rightarrow LOO^{\cdot}$$

The lipid peroxyl radical formed is capable of abstracting a hydrogen from an adjacent fatty acid to give a lipid hydroperoxide and a new carbon-centred radical ($L^{\cdot}$) and thus propagating the chain reaction of lipid peroxidation. A single initiation event can result in conversion of hundreds of fatty acid side chains into LOOH. The length of the propagation chain reaction depends on many factors including the oxygen concentration, composition of the fatty acid, and the presence within the membrane of chain breaking antioxidants.

$$LH + LOO^{\cdot} \rightarrow L^{\cdot} + LOOH$$

A termination reaction limits the extent of lipid peroxidation by generating non-radical products, i.e. less reactive compounds with no free electrons.

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow \text{ non-radical products.}$$

3.2.1.3 Autoxidation of linoleic acid

Based on the scheme outlined for the lipid peroxidation of unsaturated lipids, Figure 3.5 below shows the oxidation of a typical unsaturated fatty acid, linoleic acid.

Figure 3.5 Mechanism of linoleic acid peroxidation. Adapted from Vaya and Avriam (2001).

Lipid peroxidation, an irreversible reaction, may be considered a result of a pathological process. The presence of lipid peroxides in certain tissues has been demonstrated in association with several pathological states (Chapter 1). Due to its biological significance therefore, assays to assess lipid peroxidation are the most appropriate to determine the antioxidant activities of the crude plant extracts and herbal preparations in this study.

3.2.2 EXPERIMENTAL PROCEDURES

3.2.2.1 Principles of the lipid peroxide assay using the ferric thiocyanate method for the measurement of peroxides.

The lipid peroxide assay is based on the use of linoleic acid that can undergo lipid peroxidation which in turn can be interrupted by antioxidants. Linoleic acid is widely reported in the literature as an oxidising substrate and although easily oxidised by air, requiring storage under an inert gas such as nitrogen, it is convenient and effective. Primary antioxidants such as flavonoids can halt chain reactions by donating an electron to the peroxyl radical of the linoleic acid and thus breaking the propagation chain. Assessment of antioxidant activity is based on the ability of the crude plant extract/herbal preparation to retard oxidation of the linoleic acid when exposed to oxidative stress, i.e. exposure to oxygen and heat (incubation).

The products of lipid peroxidation can be measured by colorimetric methods. A convenient spectrophotometric method to measure lipid hydroperoxides is based on the oxidation of ferrous to ferric ion and subsequent complexation of the latter with thiocyanate. This method for the detection of peroxides was initially developed by the food industry to detect the presence of peroxides in the fat of such foods as butter and milk, to study the oxidative deterioration of these products (Loftus Hills and Thiel, 1945). This method, in conjunction with the lipid peroxide assay, has now been adopted by a number of workers in the field to asses the antioxidant potential of crude plant extracts and phytochemicals against lipid peroxidation. The colour reagent thiocyanate permits the direct estimation of the amount of ferric iron formed and the sensitivity of the test is particularly high in a solvent of low dielectric strength such as ethanol-water mixture with high ethanol content. The ferric thiocyanate response is fast and, since only small amounts of sample are needed, the method is suitable for multiple sample analysis (Mihaljevic et al, 1996). The sensitivity of the method enables 170pmol LOOH/ml in the analytical solution to be measured (Mihaljevic et al, 1996). The presence of antioxidants in the reaction solution does not interfere with formation of the ferric thiocyanate complex (Mihaljevic et al, 1996).

3.2.2.2 Ferric thiocyanate assay principle

Peroxides will convert Fe^{2+} ion to Fe^{3+} ion at acidic pH. Fe^{3+} ion will then form a coloured adduct with ammonium thiocyanate ($(Fe(SCN)_6)^{-3}$) which is observed at a wavelength of 500nm.

The oxidation of ferrous to ferric is a consequence of the one-electron reduction of LOOH, followed by monolytic cleavage of LOOH, giving rise to lipid alkoxy radicals, $LO^{\cdot}$ (Mihaljevic et al, 1996; Kolthoff and Medalia, 1951):

$$LOOH + Fe^{2+} \rightarrow LO^{\cdot} + OH^{-} + Fe^{3+}$$

The alkoxyl radical is very reactive, and capable of further reacting with another ferrous ion:

$$LO^{\cdot} + Fe^{2+} + H^{+} \rightarrow LOH + Fe^{3+}$$

3.2.2.3 Validity of the ferric thiocyanate assay for lipid hydroperoxides

The preparation and concentration of reagents have previously been established (Mihaljevic et al, 1996; Glavind and Hartmann, 1955; Loftus Hills and Thiel, 1946).

A 30% solution of ammonium thiocyanate has been established as the optimum concentration. Reducing the concentration noticeably reduces the colour intensity and an increase above 30% does not result in an increase in the colour intensity (Loftus Hills and Thiel, 1946).

The optimum concentration of the ferrous chloride solution is established at 20mM. More concentrated ferrous solutions give higher blank values reducing the accurate quantification of lipid hydroperoxides (Loftus Hills and Thiel, 1946).

Maximal colour intensity of the ferric thiocyanate complex formed has been found to be dependent on the hydrogen ion concentration (Loftus Hills, & Thiel, 1946; Kolthoff et al, 1951). Methods described in the literature use ferrous chloride dissolved in 3.5% hydrochloric acid. Ferric and ferrous chlorides are both prone to hydrolysis and the

hydrochloric acid is added to prevent this. By experimentation, it was established that the colour intensity of the ferrous thiocyanate assay in 75% ethanol was improved if the ferrous chloride reagent was made up using a 3.5% hydrochloric acid solution based on the actual hydrochloric acid content of the acid (35.45%). Experimentation established that this concentration of $FeCl_2$ did not produce, at full oxidation of the ferrous ion by hydrogen peroxide, an absorbance beyond the range of the spectrophotometer after the thiocyanate was added. Lower concentrations were naturally less sensitive. Since ferrous chloride is prone to rapid oxidation, the use of ferrous sulphate acidified with sulphuric acid was substituted in the assay. This resulted in the production of a precipitate in the test solution and some cloudiness. It was therefore decided to revert to the use of ferrous chloride. The acid inhibits the formation of hydroxides (i.e. ferric hydroxide) which may add to the absorbance by causing turbidity.

$$FeCl_3 + 3H_2O \rightleftharpoons Fe(OH)_3 + 3HCl$$

$Fe(OH)_3$ could form a precipitate without sufficient hydrochloric acid.

3.2.2.3.1 Kinetics of the development response

The complete response to micromolar concentrations of LOOH can be achieved within 5 minutes irrespective of the structure of LOOH (Mihaljevic et al, 1996). Previous studies in which this technique is used to measure the antioxidant activity of plant material report recording results at 3 minutes after the addition of the ferrous chloride solution.

3.2.2.3.2 Absorption spectrum

To establish optimum wavelength for the measurement of the red ferric thiocyanate complex, an absorbance curve was plotted using hydrogen peroxide as the oxidising agent. The ferric thiocyanate complex absorbs in the green part of the spectrum. Readings were therefore taken every 20nm between 400nm and 600nm. Results are shown in Fig. 3.6. Absorption maximum was found to be 500nm.

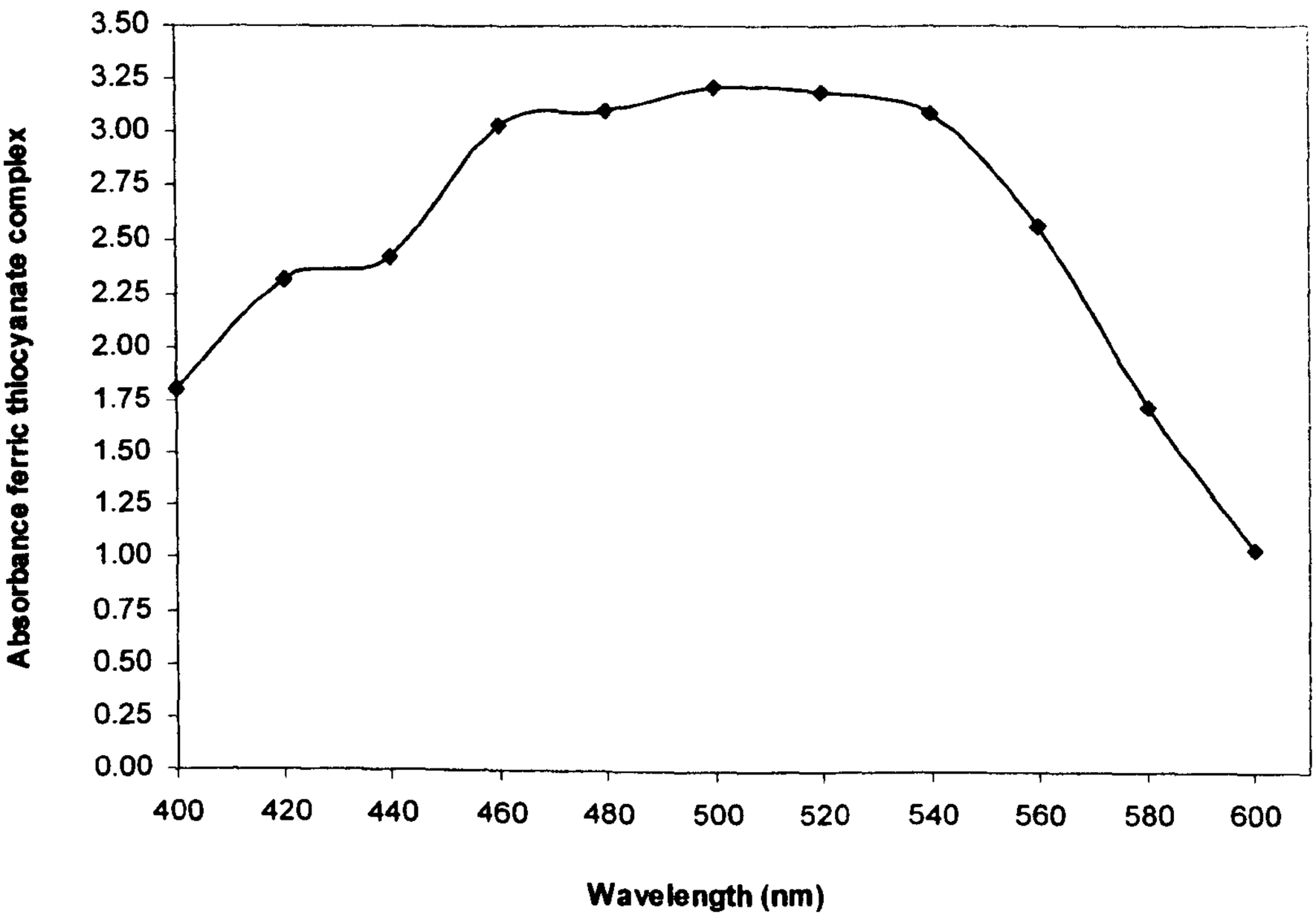
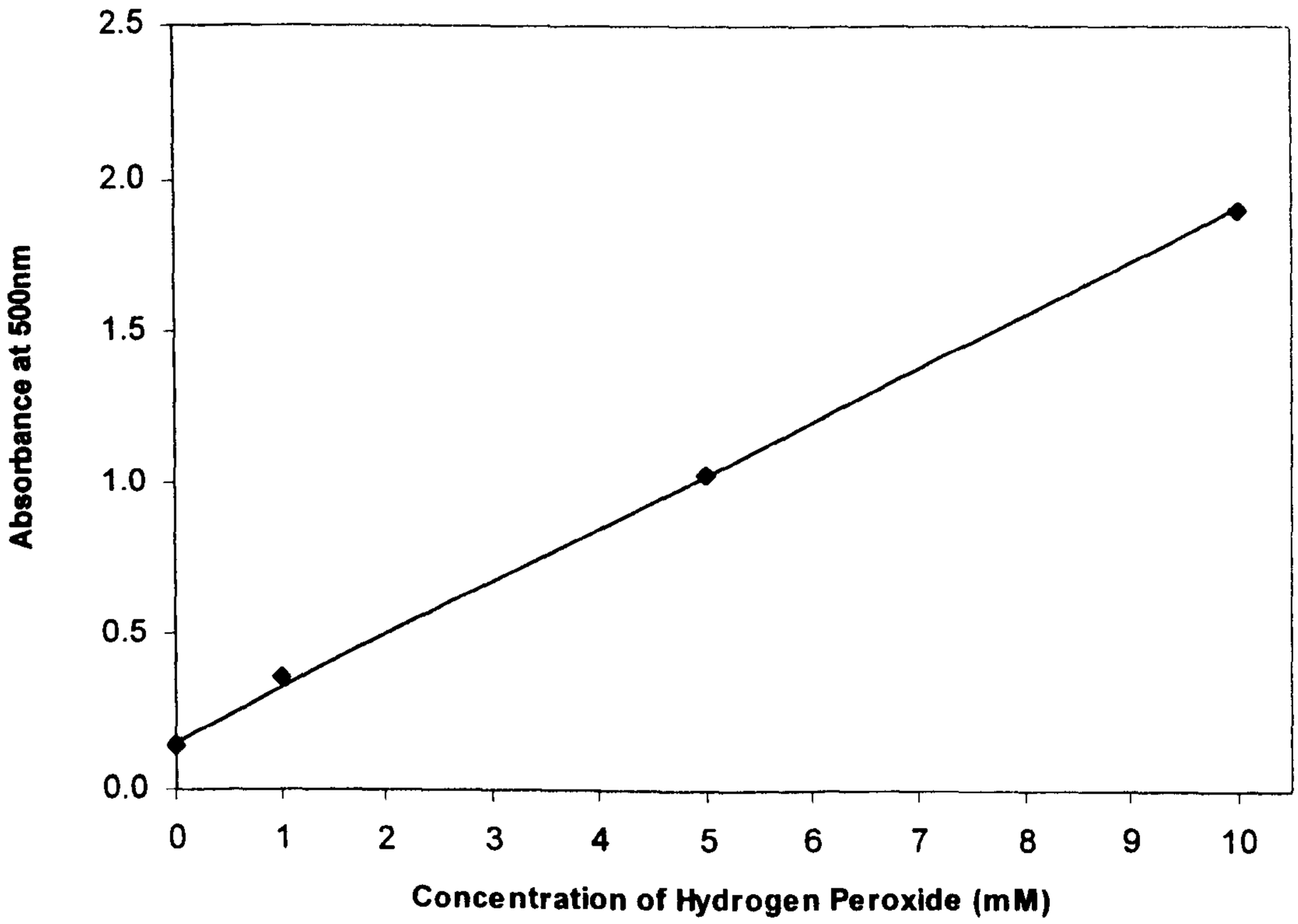


Figure 3.6. Absorption spectrum of ferric thiocyanate in 75% ethanol, using hydrogen peroxide as the oxidizing agent.

3.2.2.3.3 Stoichiometry

The stoichiometry of the reaction of the hydroperoxy compound hydrogen peroxide with ferrous ions has previously been established as a 1:2 stoichiometry (Mihaljevic et al, 1996). The ferrous thiocyanate assay was performed as outlined in section 3.2.2.4 using different concentrations of hydrogen peroxide as the sample and a standard curve constructed to confirm the linearity of the response. Absorbance at 500nm was measured at the following concentrations of hydrogen peroxide, 1mM, 5mM, 10mM, and 1M as these covered the expected experimental range. [Fig. 3.7] The absorbance reading for 1M was outside the expected range and too high to register an accurate result. The 1:2 stoichiometry relates to hydrogen peroxide only (Mihaljevic et al, 1996).



Slope: 0.175774
Intercept: 0.152772
Correlation: 0.99971

Figure 3.7. Absorbance of ferric thiocyanate complex in 75% ethanol at 500nm generated by hydrogen peroxide at concentrations of: 1mM; 5mM; 10mM as determined by the ferric thiocyanate assay method.

3.2.2.3.4 Sources of error

In the presence of transition metal ions, Fenton's reaction, resulting in the production of additional Fe^{3+} ions, would lead to an overestimation of LOOH.

Autoxidation of Fe^{2+} ions by atmospheric O_2 may produce a small concentration of superoxide ion which could be subsequently involved in the production of additional Fe^{3+} ions which could also lead to an overestimation of LOOH.

3.2.2.4 Ferric thiocyanate assay procedure

3.2.2.4.1 Materials

Iron (II) chloride tetrahydrate and ammonium thiocyanate 97.5% ACS reagent were purchased from Sigma-Aldrich and hydrochloric acid (35.4%) and analytical grade ethanol from Fisher Chemical Co.

3.2.2.4.2 Preparation of reagents

Ammonium thiocyanate solution was made by dissolving ammonium thiocyanate (6g) in distilled water using a 20 ml volumetric flask and adjusting the volume with distilled water to 20 ml.

Ferrous chloride (0.08g) was dissolved in 3.5% hydrochloric acid solution in a 20 ml volumetric flask. Small aliquots of the solution were then stored under liquid nitrogen. To maintain a low blank reading, several zinc pellets were added to the solution during use. The zinc reacted with the hydrochloric acid reducing all Fe^{3+} to Fe^{2+} (hydrogen ion takes an electron from Fe^{3+} reducing it back to Fe^{2+}). It was observed that ferrous chloride solutions prepared with water used immediately from the laboratory two-stage still gave lower blank readings. This water was found to contain only traces of oxygen, which was determined by the use of a dissolved oxygen meter that showed the level of oxygen to be appreciably less than 5% at ambient temperature (20°C). Ferrous (Fe^{2+})

compounds are very readily oxidised to ferric (Fe^{3+}) and this reaction appeared to be minimised by the low level of dissolved oxygen present in this water.

Reagents were freshly prepared each time.

3.2.2.4.3 Apparatus

To ensure reproducibility, the same micropipettes were used each time and disposable plastic 30ml universal screw cap vials and cuvettes were used to perform the assay. A Spectronic 20 Genesys spectrophotometer with a wavelength range of 325 – 1100 nm was used to measure the absorbance of the ferric thiocyanate complex.

3.2.2.4.4 Procedure

To 10 ml of a 75% ethanol solution, 200µl 30% ammonium thiocyanate, 200 µl sample solution and 200 µl ferrous chloride (20mM) in 3.5% hydrochloric acid were added sequentially and stirred immediately after the addition of the ferrous chloride. After three minutes the absorbance was read at 500nm.

For each batch of tests, a blank containing no test solution measured against solvent (75% ethanol) was included to confirm the quality of the reagent and as an indication of the absence of ferric ions. High blank readings prevent accurate quantification of lipid hydroperoxides in samples containing lipids (Richards and Feng, 2000). Under the laboratory conditions used, the background absorbance reading varied between 0.007 – 0.02. If the sample (crude plant extract) was coloured, the absorbance was measured against a different sample blank which had the same composition, except for the omission of the reagent solutions. The absorbance at 500nm was measured against this blank and the reagent blank subsequently subtracted.

3.2.2.5 Lipid peroxide assay (linoleic acid system)

3.2.2.5.1 Materials

The Linoleic acid 97% used was purchased from Lancaster Synthetics Ltd. (Batch No. FA003793). Phosphate buffer (pH7) and analytical grade ethanol were obtained from

Fisher Chemical Company and n-Propyl gallate and caffeic acid were purchased from Sigma-Aldrich.

3.2.2.5.2 Apparatus

Oxidising substrate was incubated in 30 ml glass universal screw cap vials, double rinsed with distilled water prior to use. A standard temperature controlled microbiological incubator was used for incubation.

3.2.2.5.3 Optimization of the method

A reaction mixture containing varying proportions of 99.8% ethanol, distilled water, phosphate buffer (pH7) and a 2.5% linoleic acid solution was tested following the methods of Kikuzaki and Nakatani (1993); Osawa and Namiki (1981); Duh (1998); Ono et al, 1999); Kim et al, (2000) and Habash (2000). The amounts of 2.5% linoleic acid solution used in the assay mixture differed from author to author ranging from 130µl in a total volume of 25 ml (Duh, 1998) to 4 ml in a total volume of 20 ml (Habash, 2000) giving variable and inconsistent results. The following method was adapted from the literature and used to test oxidising substrates containing 2.5% linoleic acid solution in a range of concentrations for rate and hydroperoxide formation.

3.2.2.5.4 Procedure

A mixture of 2.5% linoleic acid in 99.8% ethanol in varying amounts made up to 8 ml with 99.8% ethanol, 8 ml phosphate buffer (pH7), and 4 ml water were placed in screw top bottles and put in a microbiological incubator at 37°C. A total of 6 concentrations were tested:- 100µl; 250µl; 500µl; 1 ml; 2 ml and 4 ml. A blank sample containing the above mixture with no 2.5% linoleic acid solution was included. Commencing on the third day of incubation, absorbance values of each solution were measured by the ferric thiocyanate method at 500nm and then measured every 24 hours up to and including the 11^{th} day.

3.2.2.5.5 Effect of time and concentration on the rate of oxidation and hydroperoxide formation

Figure 3.8 shows results over an eleven day period for the 6 concentrations tested. A volume of 4 ml of 2.5% linoleic acid in 99.8% ethanol in a total volume of 20 ml, was found to produce the highest absorbance readings. Absorbance readings for this sample on days 6, 7, 10 and 11 were outside the accurate measurement range of the instrument, although an indication of absorbance can be provided as shown in Fig. 3.8. Albeit these results may not be precisely accurate, it seems reasonable to assume that they are relative to each other and a trend of increasing absorbance is apparent. These results confirm that incubating the assay solution over a 5 day period as recommended in the literature (Ono et al, 1999) generates hydroperoxides to the maximum level that can be accurately measured at the established wavelength of 500nm.

A concentration curve for the 6 samples on day 5 was plotted and the results shown in Figure 3.9. Because of the general trend of the results, an exponential curve gave the line of best fit. It has previously been shown that peroxide values reach a maximum and then start to decline (Vaya and Avriam, 2001). This was confirmed with samples containing 2ml and 4ml 2.5% linoleic acid in 99.8% ethanol in a total volume of 20ml, both showing a slightly reduced absorbance after the 10^{th} day [Fig.3.8]. The blank sample (assay solution without linoleic acid) registered no increase in absorbance above the background blank.

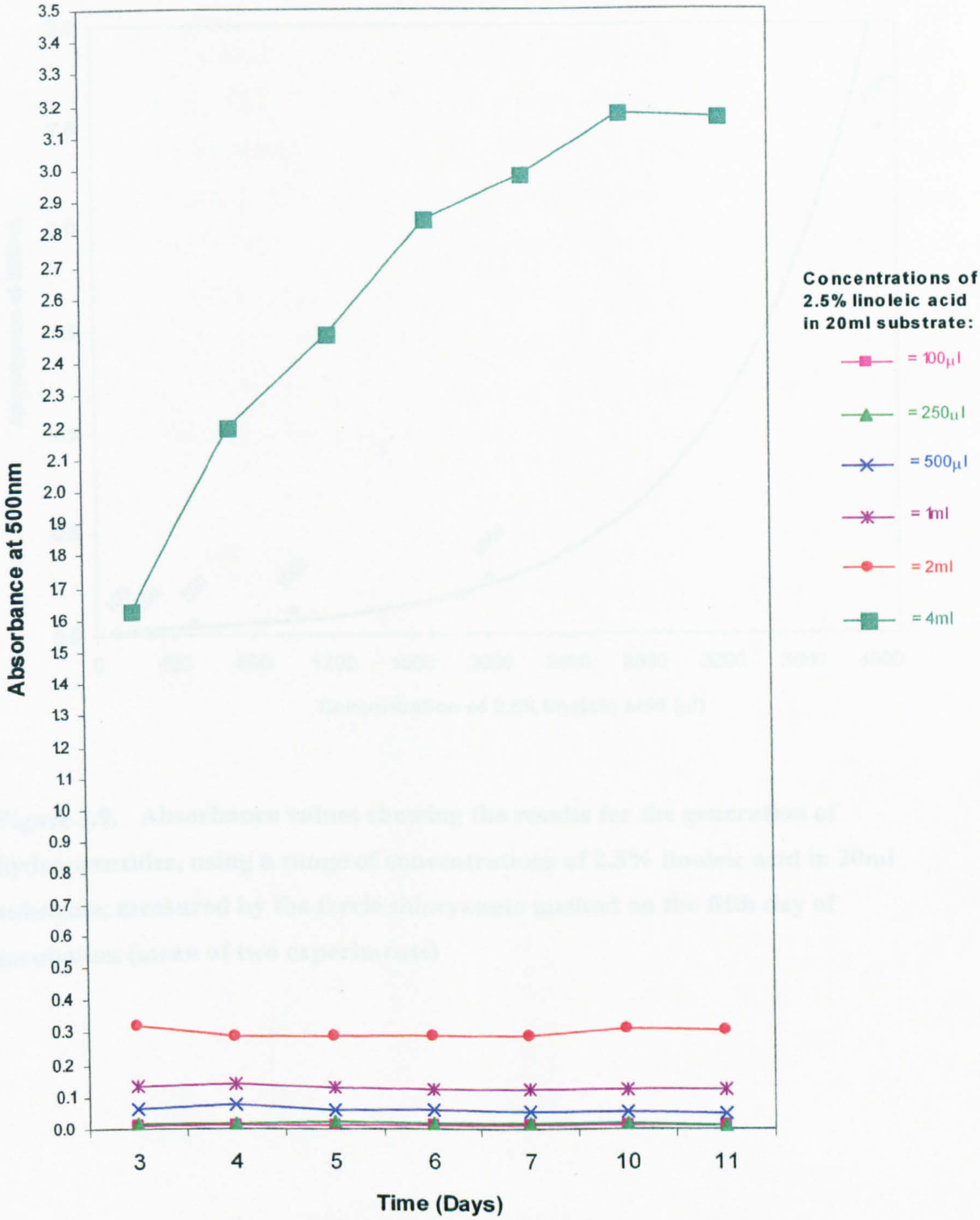


Figure 3.8. Absorbance at 500 nm for different amounts of 2.5% linoleic acid in 20 ml substrate measured by the ferric thiocyanate method over an eleven day period, to quantify the rate of oxidation and hydroperoxide formation (mean of two experiments).

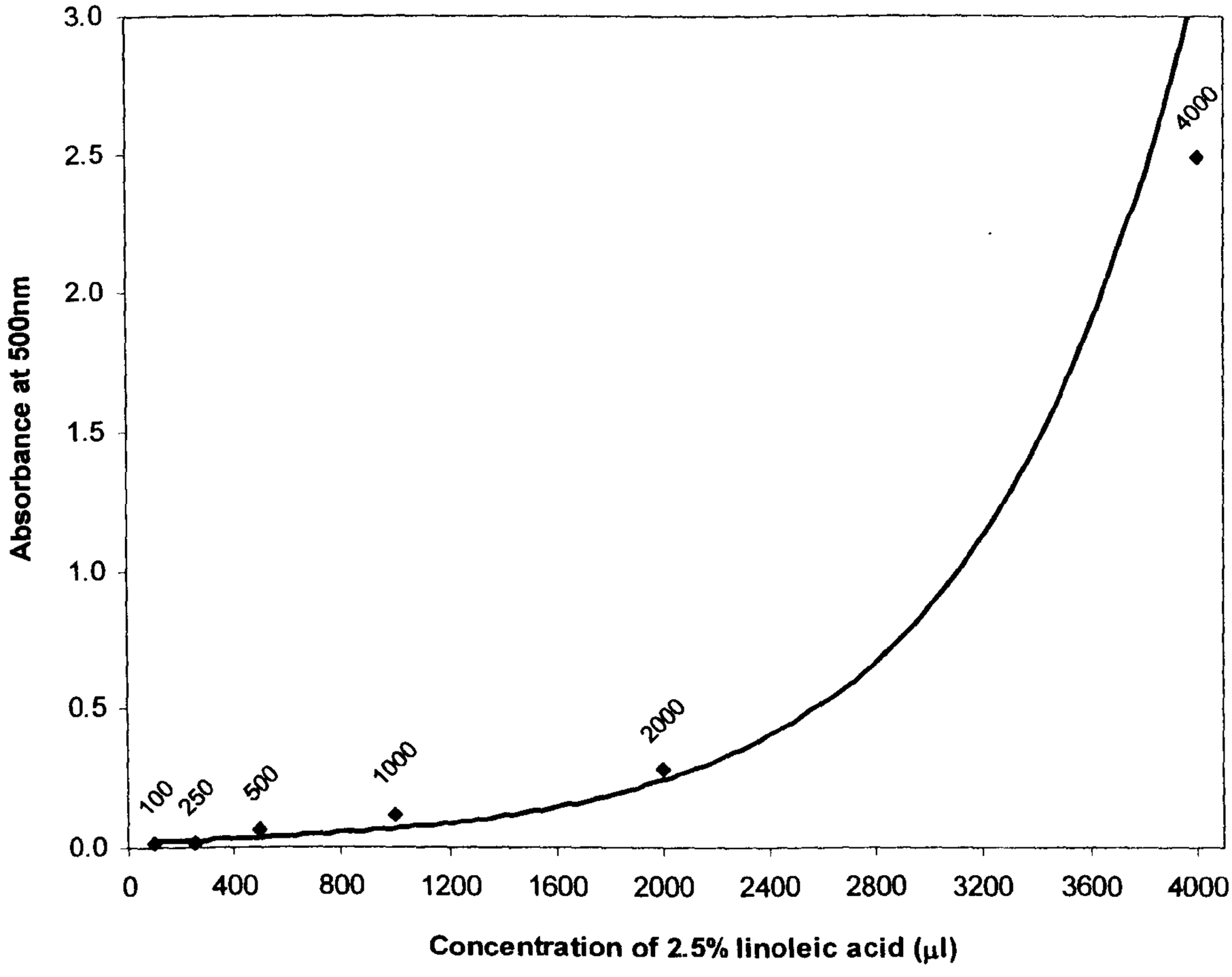


Figure 3.9. Absorbance values showing the results for the generation of hydroperoxides, using a range of concentrations of 2.5% linoleic acid in 20ml substrate, measured by the ferric thiocyanate method on the fifth day of incubation (mean of two experiments)

3.2.2.5.6 Effect of incubation temperature on hydroperoxide formation

The assay mixture found to produce the highest absorbance as described in 3.2.2.6.1 (4ml 2.5% linoleic acid in 99.8% ethanol in a total volume of 20ml) was incubated at the following temperatures:- 4°C; 25°C; 37°C; 60°C and 85°C. Samples were measured by the ferric thiocyanate method at 500nm on the 5th day of incubation. Results are shown in Figure 3.10. Maximum generation of lipid hydroperoxides was achieved by incubation at 37°C (body temperature). At 85°C no hydroperoxides were generated and a red precipitate was observed, indicating that the linoleic acid may have denatured. Results confirm that 37°C (body temperature) as recommended in the literature, is the most effective incubation temperature, providing the highest absorbance readings.

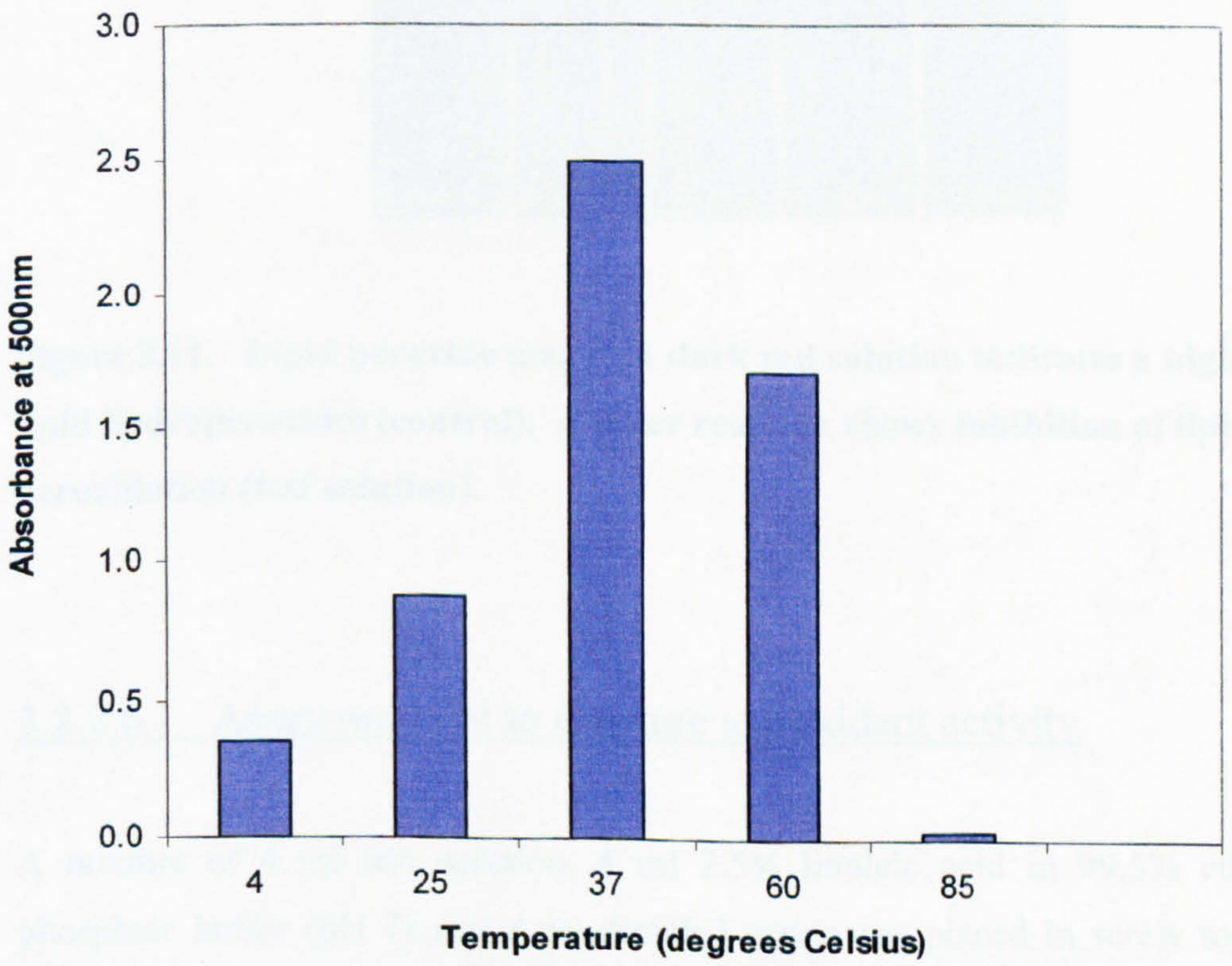


Figure 3.10. Effect of incubation temperature on the lipid peroxide assay, measured by the ferric thiocyanate method, on the fifth day of incubation (mean of two experiments).

3.2.2.6 Assessment of antioxidant activity

The measurement of antioxidant activity is based on the ability of the test substance to donate an electron (or hydrogen ion) to the lipid peroxide radical ($LOO^{\cdot}$), halting propagation (chain breaking mechanism). Using the ferric thiocyanate test, antioxidant activity is reflected in a reduction in the absorbance of the sample (oxidising substrate with the test solution added), against the absorbance of the control [Fig. 3.11]. The test solution was prepared by diluting the stock solution (plant extract) to the desired concentration in 4 ml of 99.5% ethanol.

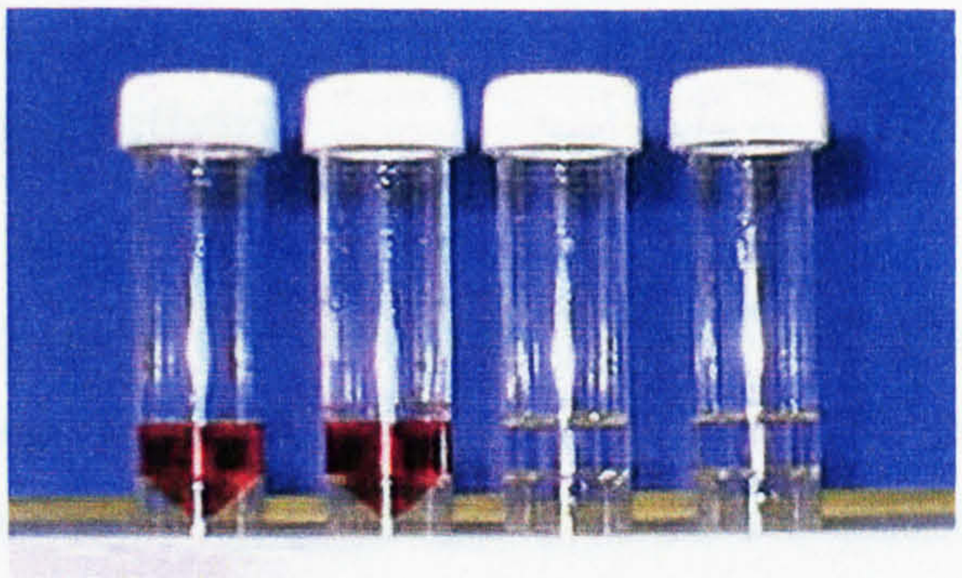


Figure 3.11. Lipid peroxide assay. A dark red solution indicates a high level of lipid hydroperoxides (control). A paler reaction shows inhibition of lipid peroxidation (test solution).

3.2.2.6.1 Assay protocol to measure antioxidant activity

A mixture of 4 ml test solution, 4 ml 2.5% linoleic acid in 99.5% ethanol, 8 ml phosphate buffer (pH 7) and 4 ml distilled water was placed in screw top bottle and incubated at 37°C in a microbiological incubator for 5 days. On the fifth day of incubation, the absorbance of the reaction mixture (sample) was measured by the ferric thiocyanate method at 500nm as described above. For each crude plant/tincture extract different concentrations were tested.

The synthetic antioxidant n-propyl gallate, a known chain-breaking lipid antioxidant (Halliwell et al. 1992) and caffeic acid, a natural antioxidant phenolic compound found in plants, were both selected to compare their antioxidant activity with that of the test

substances (Pharmaceutical Soc., 1979). To determine an appropriate concentration a literature search and brief programme of experimentation was conducted and 50μM was deemed to be the most suitable for use in this assay

3.2.2.6.2 Calculation

Antioxidant activity was expressed using the formula cited in Duh (1998).

100 – [(absorbance of sample/ absorbance of control) x 100]

(Absorbance readings are minus the blank).

3.2.2.6.3 Pro-oxidant activity

The antioxidant activity of *Silybum marianum* has previously been demonstrated in the inhibition of lipid peroxidation in rat hepatocytes (Farghali, 2000). This plant was therefore selected to use in the development of the lipid peroxide assay. During early development of this assay, results showed that the concentration of 2.5% linoleic acid in the reaction mixture compared with the concentration of the crude plant extract affected the antioxidant activity. Pro-oxidant results against the control were recorded for *Silybum marianum* at a final concentration of 0.02% w/v in a reaction mixture containing 250μl 2.5% linoleic acid solution, compared with 62.7% inhibition in an assay containing 4 ml 2.5% linoleic acid solution at the same concentration. Furthermore, use of a 1% solution of the silymarin authentic standard produced pro-oxidant results when added to a reaction mixture containing 250μl 2.5% linoleic acid solution at a final concentration of 0.01% w/v.

Since the ability of polyphenols to react with metal ions rendering them pro-oxidant has been shown in some studies (Cao et al, 1997), to gain further insight, ICP analysis of all the selected plant material was undertaken to ascertain the presence of transition metal ions capable of promoting the Fenton reaction.

3.2.2.7 Experimental design for evaluating the synergistic, additive and antagonistic interactions of pairs of crude plant extracts for antioxidant activity in the linoleic acid system.

Synergy is defined as "the phenomenon whereby the effect of two substances acting together is greater than the sum of their individual effects" (Bailey, 1999). Measurement of synergy is a complex process and there is yet to be a universally accepted formula for its determination (Boik, 2001, p. 147). Either additive or synergistic interactions are sufficient to increase the potency of most natural compounds but there is no accepted definition of either process to distinguish between the two.

The object of this test is to establish if the antioxidant activity of two herbs tested in combination is greater than the sum of both herbs tested singly.

3.2.2.7.1 Procedure

The eight crude plant extracts were tested in pairs (28 combinations), following the procedure outlined in 3.2.2.6.1. These tests were run in triplicate and the mean percentage inhibition for single extracts versus pairs of extracts analysed for antioxidant activity using the Mann Whitney test. The pairs of extracts were all tested at a final concentration of 0.01 mg/ml, i.e. each crude plant extract was used at a concentration of 0.005 mg/ml.

3.2.3 INDUCTIVELY COUPLED PLASMA (ICP) ANALYSIS FOR SELECTED METAL IONS

There is established evidence for the occurrence of the Fenton and Haber Weiss reactions *in vitro* involving transition metal ions that may catalyse a pro-oxidant reaction with flavonoids (1.1.2.5). In view of the pro-oxidant effects observed for *Silybum marianum* during the development of the Lipid Peroxide assay, the possibility that the Fenton or a similar reaction catalysed by the presence of transition metal ions was occurring was explored. ICP analysis was used to detect the presence of selected metal ions in each of the eight plant parts to be tested *in vitro* for antioxidant activity.

Metal ions such as zinc and selenium are involved in protecting the body against oxidative stress. Selenium is an integral component of the enzyme glutathione peroxidase (Sunde and Hoeksra cited in Lentner, 1981, p239). Zinc combined with copper is found in the cytoplasmic form of the enzyme superoxide dismutase whereas zinc and magnesium occur in the mitochondrial enzyme. Plants containing important trace elements such as these may contribute to the plants *in vivo* antioxidant activity.

3.2.3.1 Equipment

ICP analysis was carried out on a Perkin Elmer Plasma 40 ICP with an AES (atomic emission spectroscopy) detector. Table 3.5 lists the metals used as standards and their characteristic wavelengths.

3.2.3.2 Materials and method

ICP analysis was carried out according to the method recommended by Professor Mike Revitt (verbal communication), Middlesex University. A weighed sample (20g) of dried plant material was ground to a fine powder and left for 24 hours to digest in a glass beaker using sufficient hydrochloric acid (4M) to cover the plant material. This extract, which had reduced to dryness, was then re-dissolved in 60% hydrochloric acid, filtered through Whatman filter No. 1 and made up to (250 ml) with double distilled water. The resulting solution was analysed by ICP at the appropriate wavelength as

shown in Table 3.5 to determine the presence of the listed metal ions. The use of hydrochloric acid instead of the more usual nitric acid was to dissolve only the easily dissolvable ionic components of the plant matrix and not the organically bound metals. A mixed aqueous standard solution (1ppm) containing the metals listed in Table 3.5 was prepared from 100ppm standard solutions and calibration curves plotted to quantify the levels of each metal ion present in the prepared plant material. The amount of each metal shown on the machine output copy is the amount present in the weighed plant extract. Integration was set at three times sequentially for each individual metal and each plant extract. A one point calibration was used because of the excellent linearity of the instrument. A high coefficient of variation, i.e. over-range readings, is normally a result of the ICP working at its limit of detection (LOD). The limit of detection can be calculated from the standard deviation obtained from analysis of the blank sample at a specified wavelength [Table 3.5] using the following equation:- Limit of detection (LOD: ppm) = standard deviation (s.d. : ppm) x 3.

Table 3.5 Metals used as standards in ICP analysis of the selected plants and their detection wavelengths.

Metal	Wavelength (nm)
Nickel (Ni)	221.647
Iron (Fe)	238.204
Copper (Cu)	324.754
Cobalt (Co)	238.892
Chromium (Cr)	205.552
Selenium (Se)	196.090
Magnesium (Mg)	279.553
Zinc (Zn)	213.856

3.2.3.3 Experimental procedure for ICP analysis of the selected plants

To explore the pro-oxidant activity of *Silybum marianum* during the development of the lipid peroxide assay, ICP analysis was carried out to investigate if this plant contained iron or copper documented in the literature to catalyse free radical reactions with flavonoids (Cao et al, 1997; Sugihara et al, 1999). ICP analysis was initially carried out on the diluted crude plant extract but this solution was incompatible with the instrument. Following the above method (3.2.3.2) a nitric acid digestion of *Silybum marianum* dried plant material was performed and ICP analysis showed the presence of both iron and

copper. Since nitric acid completely breaks down the plant material releasing all forms of the metal, an acid digestion of *Silybum marianum* dried plant material was carried out using the less caustic hydrochloric acid (3.2.3.2) to dissolve only the easily dissolvable ionic components of the plant matrix. ICP analysis again showed the presence of iron and copper. Intensity counts however, which are directly related to the concentration of the target metals, were higher for iron and copper from the nitric acid digestion than that from the hydrochloric acid digestion, suggesting more metals were recovered using nitric acid. ICP on all the selected plant material was therefore carried out using hydrochloric acid digestion.

3.2.4 DPPH· SCAVENGING ACTIVITY

3.2.4.1 Introduction

DPPH· or α,α–diphenyl–β–picrylhydrazyl (Fig. 3.12) is a stable free radical capable of abstracting the labile hydrogen atom of chemical compounds (Ratty, 1988). This assay, which measures the scavenging effect on DPPH, is a simple but effective method for investigating the potential free radical scavenging activity (FRSA) of crude plant extracts.

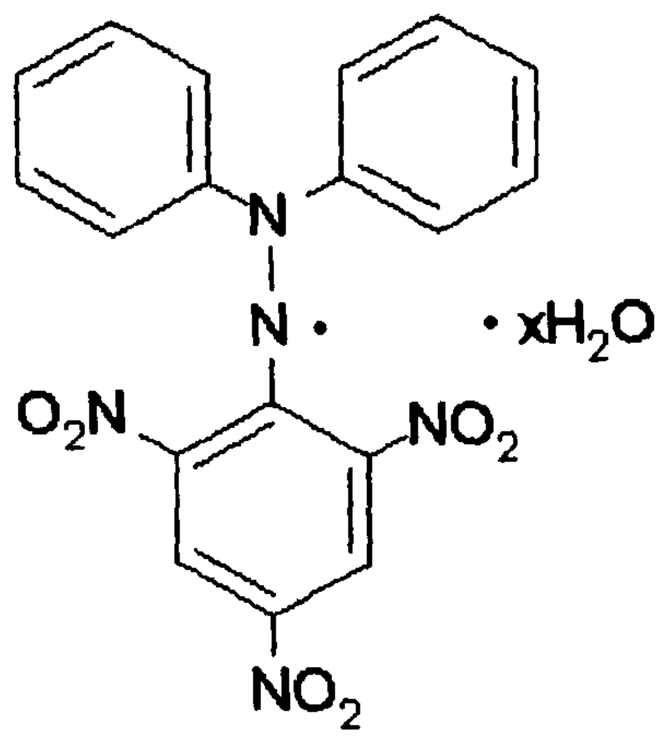


Figure 3.12 The structure of α,α-diphenyl-β-picrylhydrazyl free radical (DPPH)

Ethanolic solution of a known concentration of DPPH· /EtOH produces a deep violet colour, which shows maximum absorbance at 517 nm. To evaluate antioxidant activity, test compounds (TC-H) are added to the DPPH· solution which may reduce the DPPH· radical to the yellow-coloured diphenypicrylhydrazine resulting in a decrease in optical absorbency. Decolourisation is stoichiometric with respect to the number of electrons taken up (Blois 1958).

$(DPPH)^{\cdot} + TC\text{-}H \rightarrow DPPH\text{-}H + TC^{\cdot}$ (Blois, 1958)

The antioxidant compounds that can scavenge the DPPH· radical are expected to depress lipid peroxidation (Aniya et al, 1999).

3.2.4.2 Materials

DPPH, 1M Tris HCl buffer (pH 7.4), propyl gallate and caffeic acid were purchased from Sigma-Aldrich. Analytical grade ethanol was obtained from Fisher Chemical Company.

3.2.4.3 Experimental procedure

Methodology was adapted from the methods of Pieroni et al (2002) and Aniya et al (1999). The stable free radical DPPH· was dissolved in ethanol to give a 0.1mM solution and the test solution was prepared by diluting the stock solution (plant extract) with ethanol to the desired concentration. For each test solution different concentrations were tested. The optical absorbency of DPPH is unaffected by pH except for highly alkaline solutions (Blois, 1958). A Tris HCl buffer (pH 7.4) was used in the reaction mixture to stabilise the pH of the test solutions.

The reaction mixture added sequentially, consisted of 1 ml 0.1mM DPPH, 1 ml Tris HCl buffer (pH 7.4) diluted to 0.05M and 1 ml test solution or ethanol/water at the same ethanol concentration as the test sample (control), mixed in a cuvette and shaken. The decrease in DPPH absorption was measured at 517 nm using a spectrophotometer exactly 30 seconds after adding the test solution and every 60 seconds for 30 minutes. Readings taken at 30 seconds showed the immediate reducing ability of the test solutions. At 2 minutes absorbance readings had stabilized. Subsequent readings showed only minimal change after 10 minutes. All results were therefore based on how much each test solution reduced the DPPH radical after 10 minutes. To measure the absorbance of the plant extract alone as a blank, DPPH was omitted from the reaction mixture and replaced by ethanol. All tests were run in triplicate and the mean values obtained used to calculate the "Free Radical Scavenging Activity" (FRSA) of the test solution. Propyl gallate and caffeic acid were used at a concentration of 100μm as positive controls (Pieroni et al, 2002; Yan et al, 1998).

3.2.4.4 Calculation

To calculate the DPPH radical scavenging activity (FRSA), the absorbance of the blank (crude plant extract alone) was subtracted from the absorbance of the reaction mixture.

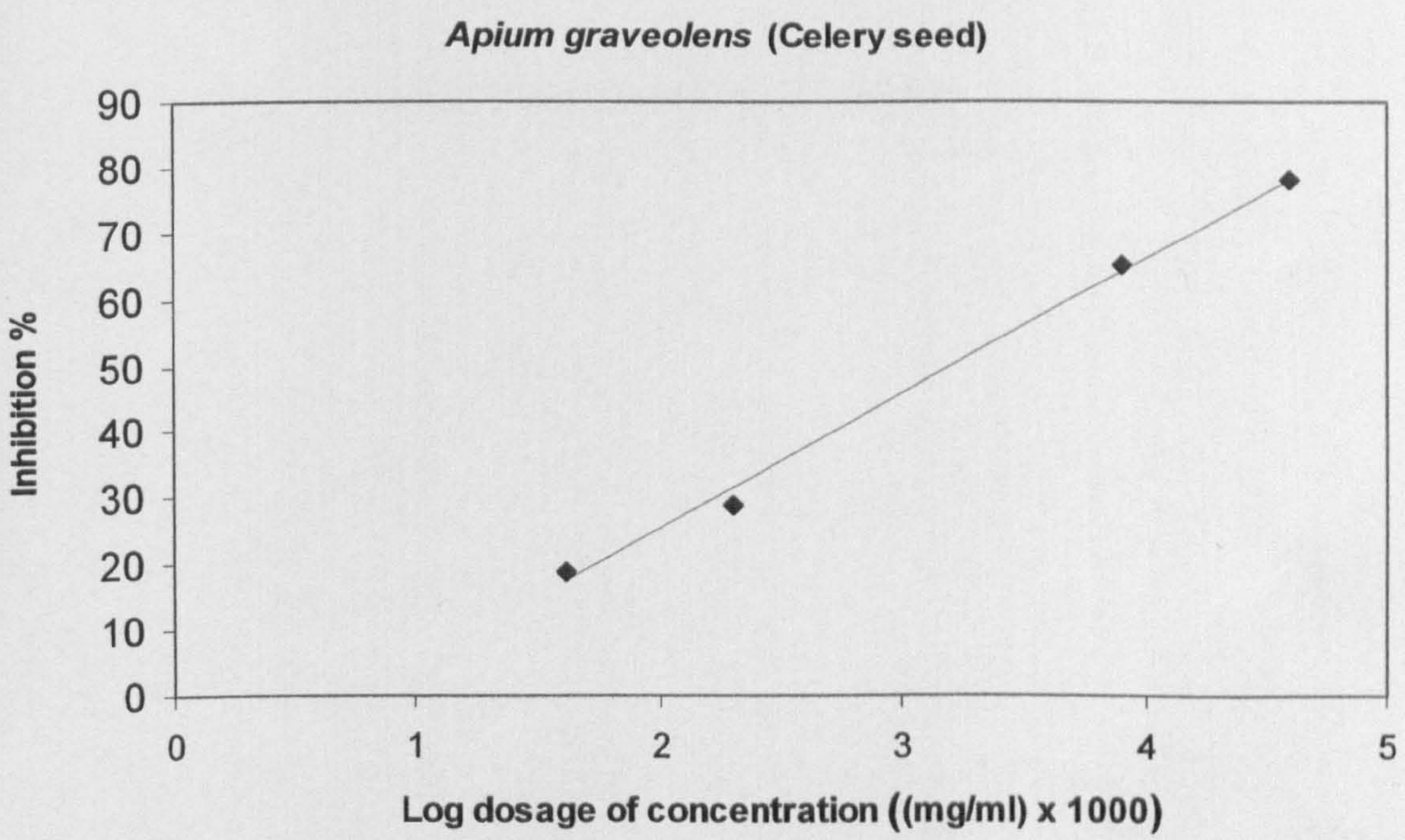
The resultant figure was then subtracted from the absorbance of the control and expressed as the percent decrease in the absorbance of the reaction mixture compared with that of the control. The antioxidant activity of each test solution was also expressed as an IC_{50} value (concentration in mg/ml or μM/ml required to reduce the $DPPH^{\cdot}$ radical by 50%), which was determined from the log dose-inhibition curve. By experimentation it was shown that this method, widely used in the literature, should be utilised, due to the non-linearity of the crude plant/tincture extracts. The co-efficient of correlation relating to the logarithmic curve was superior to that produced by the linear graph.

3.2.4.5 Determination of the IC_{50} value

The FRSA of each test compound was expressed as an IC_{50} value, determined graphically. This was obtained by calculating the correlation co-efficient from the log concentration of the test substances by linear regression analysis, i.e. FRSA of the test substance as calculated above was plotted for the concentration expressed as a logarithm. The IC_{50} was calculated by using the following equation:-

IC_{50} = y50 – intercept / slope = log of IC_{50} (expressed as concentration in mg/ml or μM/ml)

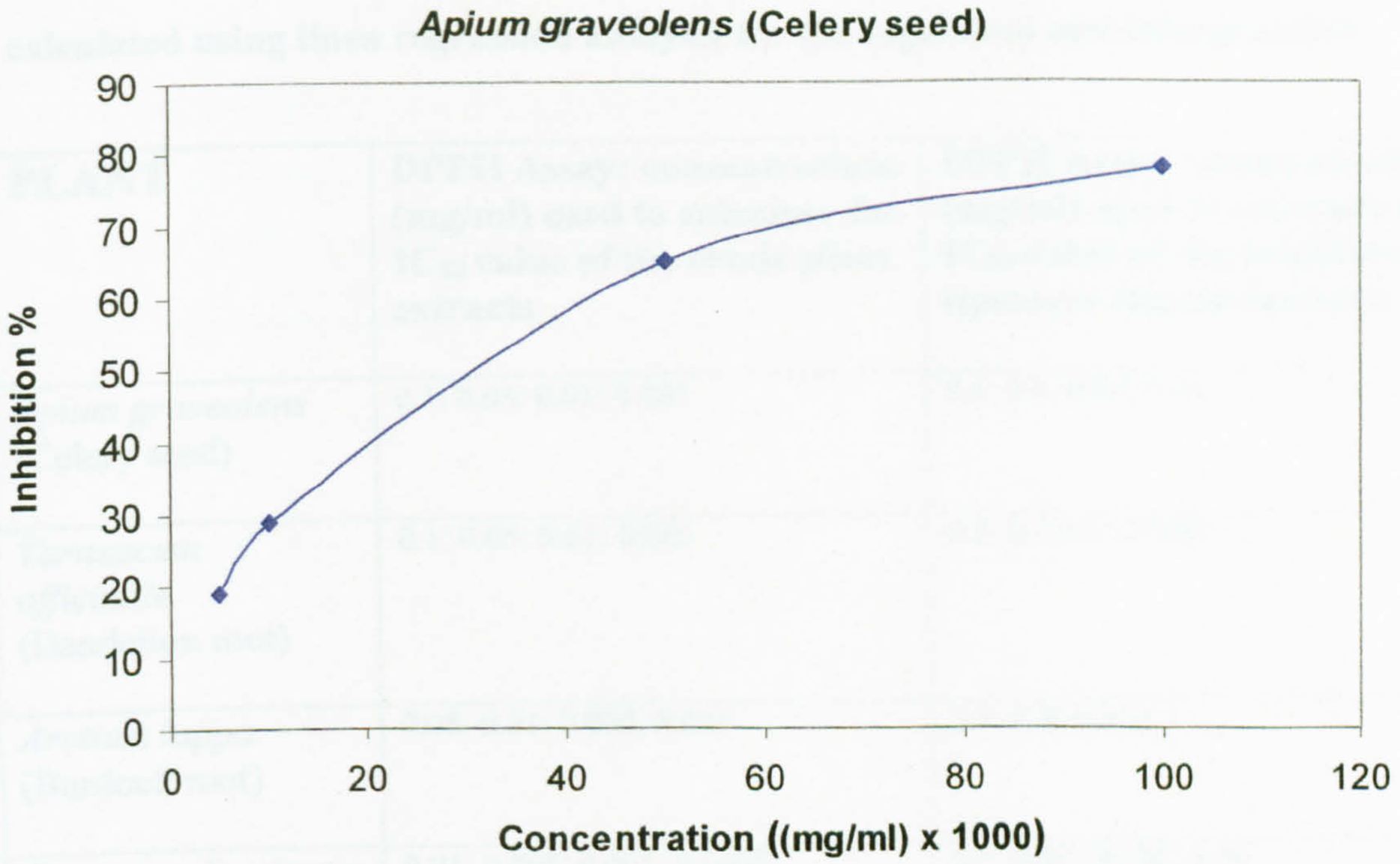
In figure 3.13 on the following page, the log dose-inhibition curve is shown for the crude plant extract of *Apium graveolens*. Using the equation above the IC_{50} value is found to be 0.024983 (0.025) mg/ml.



Correlation	0.998025
Slope	20.29347
Intercept	-15.30799
IC 50 (log)	3.218177
IC 50	0.024983

Figure 3.13 The log dose-inhibition curve of *Apium graveolens* crude plant extract corresponding to percent inhibition of the DPPH· radical (FRSA)

Because a linear response is not always obtained from work involving plant extracts the above method (3.2.4.5) is used by many authors in the field. Concentration against inhibition for *Apium graveolens* crude plant extract created a curve [Fig. 3.14] whereas the log dose against inhibition produced a straighter line [Fig. 3.13] and better coefficient of correlation. The IC_{50} concentrations calculated from these graphs, were 0.024983 (0.025) mg/ml [Fig 3.13] and 0.044931 (0.045) mg/ml [Fig. 3.14]. Tests based on these results established 0.024mg/ml as the exact IC_{50} confirming the use of linear regression analysis based on the log dose of the test substances in this study.



Correlation	0.953477
Slope	0.611171
Intercept	22.53921
IC 50	0.044931

Figure 3.14 The linear dose-inhibition curve for *Apium graveolens* crude plant extract corresponding to percent inhibition of the DPPH· radical (FRSA)

The concentrations (mg/ml) of crude plant extract and commercial tincture extract used to calculate the IC_{50} value of each of the extracts from all the selected plants are shown in Table 3.6 on the following page. The IC_{50} value of each extract was calculated using linear regression analysis for the logarithm and interpolation as outlined for *Apium graveolens* (celery seed), Figure 3.13.

Table 3.6 Concentrations (mg/ml) used to calculate the IC_{50} value i.e. the concentration in mg/ml required to reduce the DPPH· radical by 50%, which was calculated using linea regression analysis for the logarithm and interpolation.

PLANT	DPPH Assay: concentrations (mg/ml) used to calculate the IC_{50} value of the crude plant extracts	DPPH Assay : concentrations (mg/ml) used to calculate the IC_{50} value of the commercial tinctures (liquid extracts)
Apium graveolens (Celery seed)	0.1: 0.05: 0.01: 0.005	0.5: 0.1: 0.05: 0.01
Taraxacum officinale (Dandelion root)	0.1: 0.05: 0.01: 0.005	0.5: 0.25: 0.1: 0.05
Arctium lappa (Burdock root)	0.05: 0.01: 0.005; 0.001	2.5: 1.0: 0.5; 0.1
Crataegus laevigata (Hawthorn leaves and flowers)	0.01: 0.005: 0.001: 0.0005	0.1: 0.05: 0.025: 0.01
Crataegus laevigata (Hawthorn berry)	0.1: 0.05: 0.01: 0.005	0.05: 0.025: 0.01: 0.005
Silybum marianum (Milkthistle seed)	0.1: 0.05: 0.01: 0.005	0.5: 0.25: 0.1: 0.05
Salix alba (White Willow bark)	0.01: 0.005: 0.001: 0.0005	0.025: 0.01: 0.005: 0.001
Calendula officinalis (flower)	0.5: 0.1: 0.05: 0.01	0.1: 0.01: 0.005: 0.001

3.2.4.6 Experimental design for evaluating the synergistic, additive or antagonistic interaction of the crude plant extracts for antioxidant activity in the DPPH assay

In order to evaluate any synergistic, additive, or antagonistic interactions in the DPPH assay, IC_{50} values for the crude plant extracts were all tested to establish exact percentage inhibition (IC_{50} is only an estimation).

By experimentation, it was shown that by using half the IC_{50} concentration to reduce the DPPH radical by 25%, variable results were achieved, i.e. they did not display linearity. The log dose-inhibition curve was shown to be unsuitable for calculating IC_{25} concentrations. Exact percentage inhibition (FRSA) of the crude plant extracts at half the IC_{50} values was established and the crude plant extracts were tested in pairs, by using half the IC_{50} value for each herb. The FRSA of the combination of two individual herbs was compared with the combined FRSA's of the same herbs tested singly.

CHAPTER 4: RESULTS and DISCUSSION

4.1 METHODS OF PLANT ANALYSIS FOR IDENTIFICATION OF FLAVONOIDS

The flavonoid content of the plant samples used in this study has been investigated using various chromatographic techniques. The results for each plant are presented individually. A typical set of chromatograms is shown for *Silybum marianum* (Milk thistle). In HPLC analysis, only compounds showing a distinct peak are reported.

4.1.1 Extraction

Extraction of the compounds from the dried plant material listed in Table 3.1 by two different solvents is shown in Table 4.1.

Table 4.1 Yield and description of the crude plant extracts obtained from the selected plant parts extracted with 100% MeOH and 80% EtOH from 30g dried plant material

Plant	Yield (g) 100% methanol	Extract	Yield (g) 80% ethanol	Extract
Silybum marianum	3.5g	Deep russet brown separating into a solid brown layer and yellow oil.	2g	Dry brown extract
Arctium lappa	4g	Deep brown	4g	Dry deep brown
Crataegus laevigata (berries)	6.5g	Brown	6g	Solid maroonish-brown extract
Crataegus laevigata (leaves and flowers)	0.5g	Dark green	3g	Dark green syrupy liquid
Apium graveolens	0.5g	Bright green	3g	Green and syrupy
Taraxacum officinale	4.5g	Dark brown	5g	Dry golden brown
Salix alba	2g	Dark woody brown	1g	Solid woody brown
Calendula officinalis	4.5g	Sticky bright orange- brown	4g	Bright orange-brown and sticky

The results indicated that the extract yields for most plants were similar, but the nature of the extracts differed in that the 80% ethanol solvent generally produced a drier extract.

4.1.2 Chromatographic analysis

4.1.2.1 *Silybum marianum* (Milkthistle)

4.1.2.1.1 Thin Layer Chromatography

TLC analysis of the crude plant extract (sec. 3.1.2.2.1) carried out using the reference compounds silymarin and taxifolin gave R_f values which were consistent with those reported in the literature (Wagner 1984, p.190). Three spots were observed for the extract, each corresponding to the appropriate reference compound, Fig. 4.1. Silymarin was resolved into two yellow spots identified as silybin (R_f 0.6) and silychristin (R_f 0.35). Taxifolin appeared as a pinky orange spot at R_f 0.4. Silydianin appeared as a pale yellow fluorescent zone between taxifolin and silybin (Wagner, 1984, p.190).

4.1.2.1.2 Column Chromatography

Silymarin has been shown to consist of a number of related flavonoid compounds including silybin, silydianin, silychristin, isosilybin and dehydrosilybin (Bilia et al, 2001; Campodonico et al, 2001). Column chromatography of the crude plant extract of *Silybum marianum* was used to ascertain if this new technique achieved a better separation of the silymarin compounds than TLC. Using a newly developed commercially prepared polyethylene column gave no better separation and TLC analysis of the extracted fractions were the same as reported above. Glass column chromatography was slow and unsuccessful in separating the components. As a result of this no further analysis was carried out using this technique.

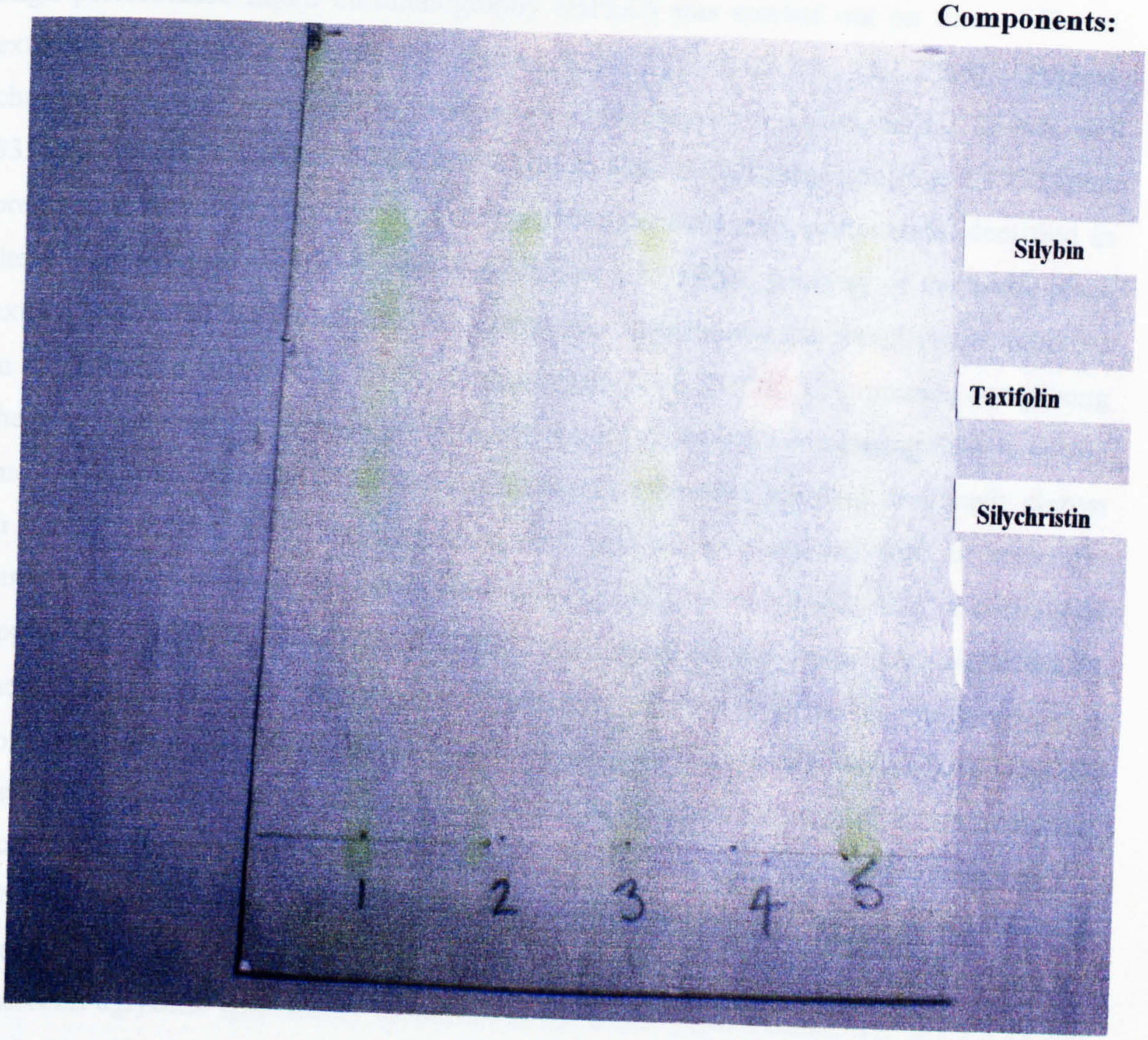


Figure 4.1. TLC chromatogram of *Silybum marianum* (Milkthistle) (Solvent system A/ Detection reagent A, pages 89 and 91).

1. Methanol extraction
2. Ethanol extraction
3. Reference compound silymarin
4. Reference compound taxifolin
5. All samples 1-4

4.1.2.1.3 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was carried out on three different extracts of *Silybum marianum* (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4). Typical chromatograms of an extract viewed at two characteristic wavelengths i.e. 254nm and 335nm (Jodrell laboratory, 2004) are shown in Fig. 4.2 (254nm) and Fig. 3.4 (335nm) previously shown in Chapter 3. The flavonoid/phenolic acid compounds identified in these three extracts are listed in Tables 4.2 and 4.3. HPLC analysis of the crude plant extract confirmed the presence of the flavonoids, in particular the flavolignans, reported in the literature (Bilia et al, 2001; Campodonico et al, 2001). Compounds comprising the silymarin complex were identified in the three extracts by comparing their t_R values and UV spectra with the corresponding authentic silymarin standard previously shown in Chapter 3 [Fig. 3.1]. The peak at t_R 10.7 min when compared with an authentic sample gave a spectrum typical of taxifolin [Fig. 4.2/ 3.4 and Table 4.2]. Compounds appearing at wavelength maxima of 285/290 nm in all three extracts were identified by their UV spectra as flavanones (Jodrell laboratory, 2004). Neither the tincture extract nor the extract prepared as tincture however contained compounds that equated with the authentic silymarin standard, possibly due to a slight shift in retention times, resulting from the difference in alcohol concentration of the two types of injected samples, i.e. 80% MeOH (silymarin) and 25% EtOH (tincture extracts). A flavonol glycoside was identified by its UV spectrum in all three extracts (t_R 15.31min) and the flavonol quercetin aglycone (previously identified in the crude plant extract; sec. 3.1.2.2.2) was again identified in this extract (t_R 17.39 min), but could not be identified in the two tincture extracts. Quercetin methyl ether plus its aglycone (t_R 20.71min and 21.36min) were also only identified in the crude plant extract (Grayer, 2004). Hydroxycinnamic acids in all three extracts i.e. caffeic acid derivatives, *p*-coumaric acid and ferulic acid were identified by comparing the UV spectra of the peaks with authentic standards.

As illustrated in Table 4.2 the differences in the phytochemical profiles of the two types of extracts, crude plant extract (sec. 3.1.2.2.1) and tincture extract (sec. 3.1.2.2.4) are quite significant. Although an extraction as tincture (sec. 3.1.2.2.3) prepared in the laboratory using the same plant material as the crude plant extracts [Table 3.1] recovered more flavonoid compounds than the commercially produced tincture, none of these compounds appeared to belong to the silymarin complex. Thus it would appear that both the composition of the solvent mixture and method of extraction had a significant effect on the flavonoid profile of the two types of extract.

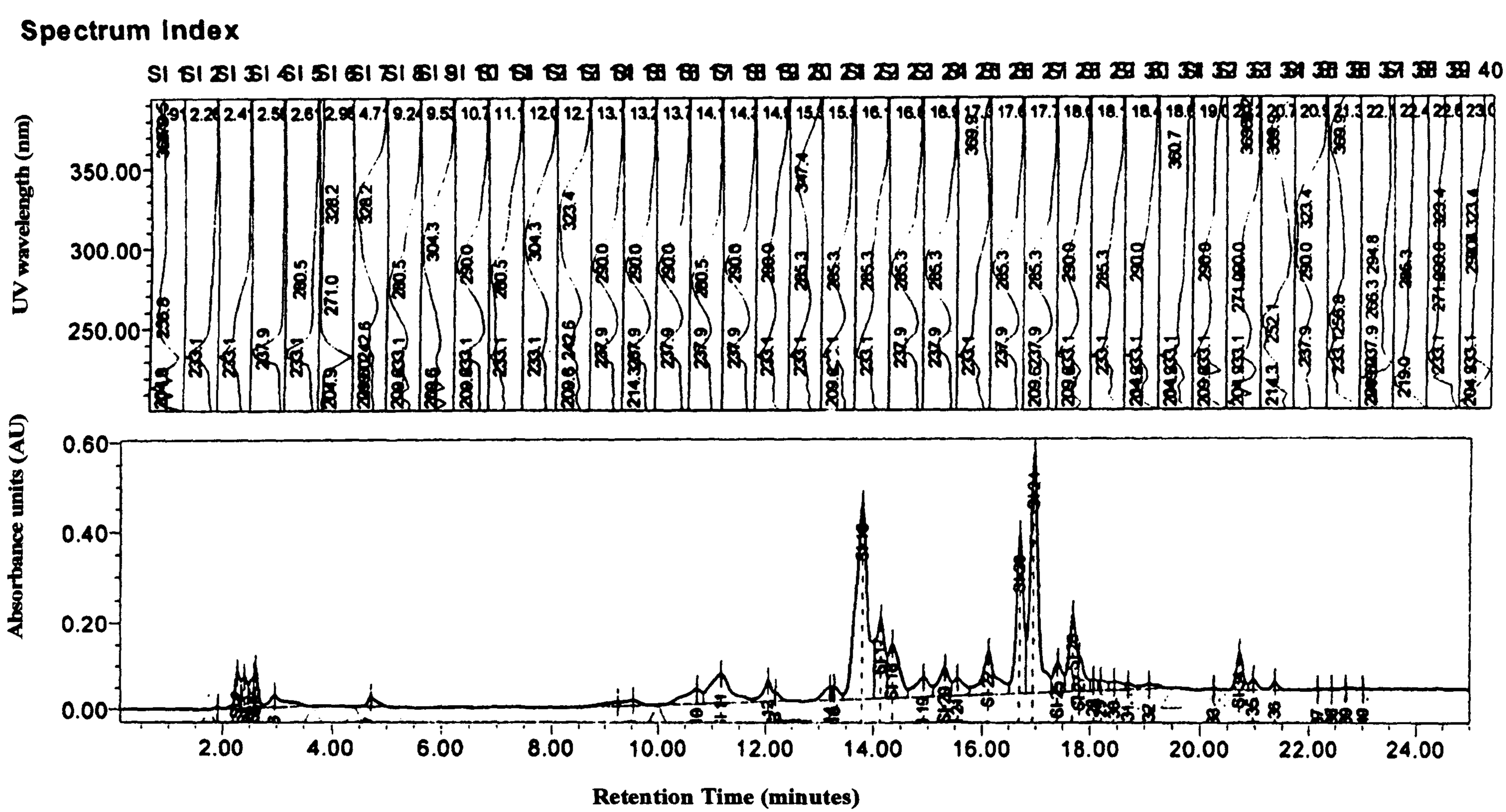


Figure 4.2. HPLC analysis and related on-line UV spectra of an 80% ethanol extract of *Silybum marianum* (Milk thistle) recorded at 254nm. Separation on 5µm Li Chrospher 100RP-18e capillary column (250 x 4mm i.d.); gradient elution with 2% Aq. acetic acid and methanol : acetic acid : water (18:1:1); flow rate, 1ml/min.

Table 4.2. Results of HPLC-DAD analysis of *Silybum marianum* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and crude plant extract used in the testing of antioxidant activity.

Compounds detected at 254nm and 335nm

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (25/25%EtOH)		CRUDE PLANT EXTRACT (80% EtOH)
	Retention Time (Minutes)	
	3.29	Caffeic acid derivative
	4.71	Chlorogenic acid
	10.71	Dihydroflavonol (taxifolin)*
p-coumaric acid derivative	10.78	
	11.34	Dihydroflavonol (silymarin)*
Ferulic acid	11.48	
	12. 11	Caffeic acid derivative
	13.18	Dihydroflavonol (silymarin)*
	13.78	Dihydroflavonol(silymarin)*
	14.1	Flavanone
	14.34	Dihydroflavonol (silymarin)*
Flavanone	14.40	
	14.91	Dihydroflavonol (silymarin)*
Flavonol glycoside	15.31	Flavonol glycoside*
	15.51	Dihydroflavonol (silymarin)*
	16.11	Dihydroflavonol (silymarin)*
	16.28	Flavanone
	16.69	Dihydroflavonol (silymarin)*
	16.94	Dihydroflavonol (silymarin)*
	17.39	Quercetin aglycone**
	17.66	Dihydroflavonol (silymarin)*
	17.79	Flavanone
	18.04	Flavanone
	20.71	Quercetin methyl ether**
	21.36	Quercetin methyl ether aglycone**

* Compounds identified by direct comparison with the authentic Silymarin standard.
** Compounds identified by their retention times and on-line UV spectra in collaboration with experienced staff at the Jodrell laboratory, Kew (Grayer, 2004).

Table 4.3. Results of HPLC-DAD analysis of *Silybum marianum* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and extract prepared as tincture. Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (25/25% EtOH)		EXTRACT PREPARED AS TINCTURE (25/25% EtOH)
	Retention Time (Minutes)	
	5.51	Caffeic acid derivative
	5.80	Caffeic acid derivative
	8.41	Caffeic acid derivative
	8.83	*p*-coumaric acid derivative
	8.90	*p*-coumaric acid derivative
p-coumaric acid	10.78	
Ferulic acid	11.48	
	11.6	Flavanone
	11.7	*p*- coumaric acid
	12.6	Ferulic acid
	12.9	Caffeic acid derivative
	13.8	Flavanone
Flavanone	14.1	Flavanone
	14.8	Flavanone
	15.5	Flavanol glycoside
	16.6	*p*-coumaric acid derivative
	16.9	Flavanone
	17.8	Flavanone
	18.8	*p*-coumaric acid derivative
p-coumaric acid derivative	19.1	*p*-coumaric acid derivative

4.1.2.1.4 Gas Chromatography/Mass Spectrometry (GC/MS)

GC analysis of the authentic reference sample of silymarin was unable to detect any compounds, indicating the non-volatile nature of this flavonoid complex. Results of GC/MS analysis of the crude plant extract (sec., 3.1.2.2.1) and tincture extract (sec., 3.1.2.2.4) are shown in Fig 4.3 and Table 4.4. Using the chemical database, tentative analysis of the two extracts showed the presence of several fatty acids and components consistent with the literature (Bisset, 2001, p.122; El Mallah et al, 2003):- lauric acid ethyl ester (t_R 20.7 min), glycerol (t_R 21.4 min, tincture extract) a basic component of nearly all complex lipids (Bailey, 1999), lauric acid (t_R 22.6 min, tincture extract), ethyl pentadecanoate (t_R 22.8 min, tincture extract), palmitic acid (t_R 27.3 min), stearic acid

(t_R 31.1 min, crude plant extract), palmitoleic acid (t_R 32.0 min, crude plant extract) and arachidic acid (t_R 37.16 min, crude plant extract). The following compounds were also tentatively identified using the database:-

- Acetic acid (t_R 11.5 min, crude plant extract), an essential primary metabolite particularly as acetyl- CoA (Evans, 1996)
- A heterocyclic 4H pyran-4-one compound (t_R 21.0 min, crude plant extract), 4H pyran-4-one derivatives are widely distributed in nature.
- A furan compound possibly 5-(hydroxymethyl) furfural at t_R 23.2 min (crude plant extract)
- 7 dodecanol, an unsaturated long chain aliphatic alcohol at t_R 33.5 min.
- A high peak at t_R 23.3 min (tincture extract) whose identity could not be established from the database but was identified by the mass spectrum to most likely be an unsaturated alcohol, probably C18 with 2 double bonds.

Acquisition of pure samples of all the above compounds to verify retention time equality would be necessary for positive identification. Since no flavonoid compounds were detected in any of the plant samples, no further results for GC/MS are reported. This method was used as an exploratory exercise to ascertain the nature of some of the other constituents present. Results for all the selected plants were similar to those reported for *Silybum marianum*.

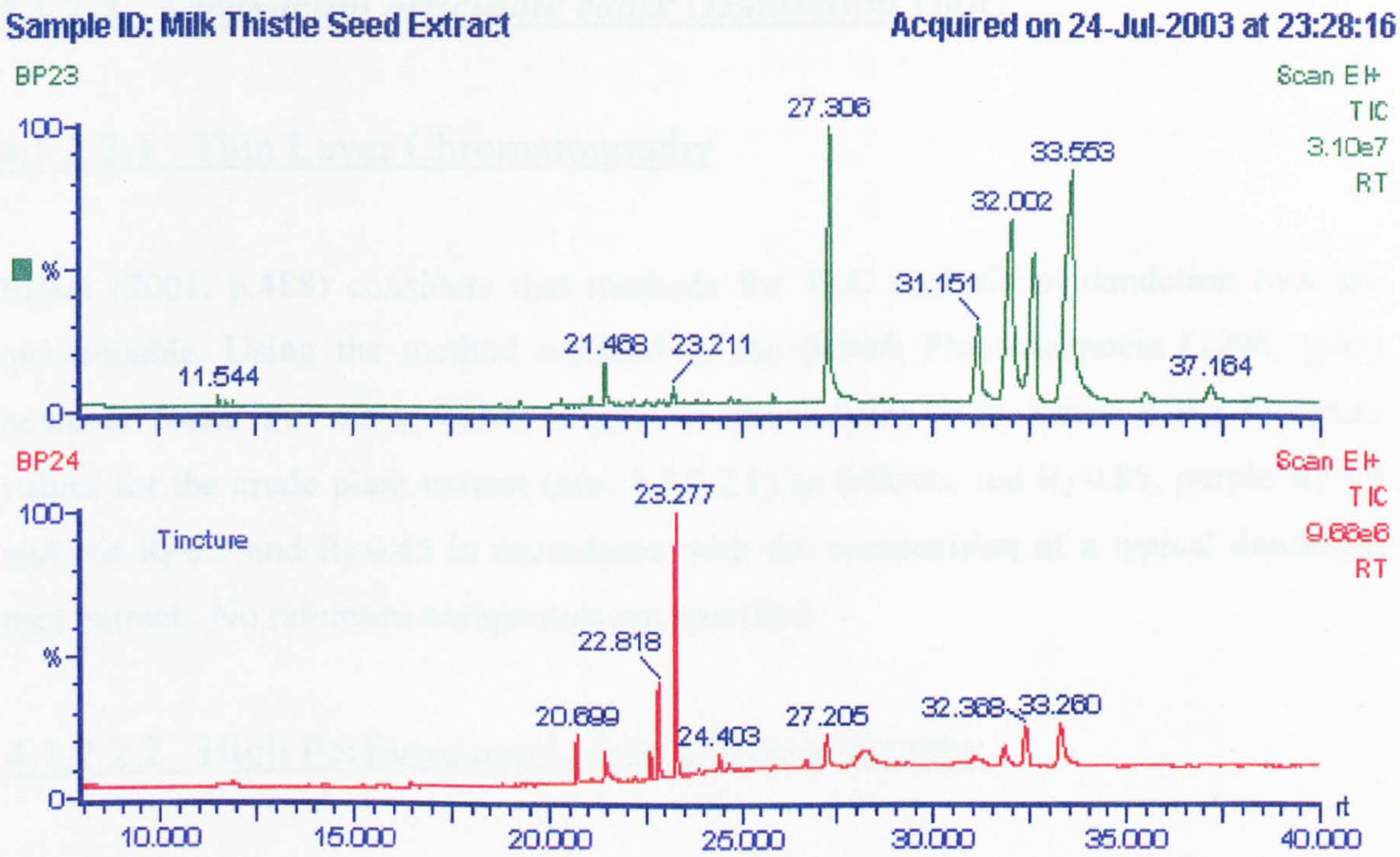


Figure 4.3. GC analysis of the separations of *Silybum marianum* showing 1) crude plant extract (BP23) and 2) commercial tincture extract (BP24).

Table 4.4. Results of GC-MS analysis of *Silybum marianum* showing the volatile components of the commercial tincture extract and crude plant extract used in the testing of antioxidant activity.

TINCTURE EXTRACT (Liquid extract 25/25% EtOH)		CRUDE PLANT EXTRACT (80% EtOH)
	Retention Time (Minutes)	
	11.5	Acetic acid
Dodeconoic (lauric) acid ethyl ester	20.7	Dodeconoic (lauric) acid ethyl ester
	21.0	2 methyl-3,5-dihydroxy (4H) pyran-4-one
Glycerol	21.4	Glycerol
Dodecanoic (lauric) acid	22.6	
Pentadecanoic acid, ethyl ester (ethyl pentadeconoate)	22.8	
	23.2	5 (hydroxymethyl) furfural
Unsaturated aliphatic alcohol	23.3	
Hexadecanoic (palmitic) acid	27.3	Hexadecanoic (palmitic) acid
	31.1	Octadecanoic (stearic) acid
	32.0	Palmitoleic acid
7 dodecanol	33.3	7 dodecanol
	37.16	Eicosanoic (arachidic) acid

4.1.2.2 *Taraxacum officinale radix* (Dandelion root)

4.1.2.2.1 Thin Layer Chromatography

Bisset (2001, p.488) considers that methods for TLC analysis of dandelion root are questionable. Using the method outlined in the British Pharmacopoeia (1996, p.69) however found the major bands relative to the solvent front matched the literature values for the crude plant extract (sec. 3.1.2.2.1) as follows, red R_f 0.85, purple R_f 0.6 and red R_f 0.5 and R_f 0.45 in accordance with the composition of a typical dandelion root extract. No reference compounds are specified.

4.1.2.2.2 High Performance Liquid Chromatography

High performance liquid chromatography was carried out on three different extracts of *Taraxacum officinale* radix (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4). The phenolic compounds identified in these three extracts are listed in Tables 4.5 and 4.6. HPLC analysis of the crude plant extract confirmed the presence of caffeic acid derivatives the main phenolic compounds reported in the literature (Barnes et al, 2002, p.171). Identity of these compounds was established by confirming their UV spectra with those of the corresponding standards. The peak at t_R 4.71 min, when compared with a known sample, was indicative of chlorogenic acid. No phenolic compounds were identified in the commercial tincture extract. This was in contrast to the extract prepared as tincture [Table 4.6] which presented a similar profile to the crude plant extract [Table 4.5], although fewer caffeic acid compounds were identified in the extract prepared as tincture. It is possible that the difference in alcohol concentration of the two different injected samples, i.e. 80% EtOH (crude plant extract) and 25% EtOH (tincture extracts), caused a slight shift in retention times of the two types of extract, resulting in their apparent disparity.

Since the two methods of extraction (sec. 3.1.2.2.1; sec. 3.1.2.2.3) produced similar phytochemical profiles, the absence of phenolic compounds from the commercial tincture extract (sec. 3.1.2.2.4) could be postulated to be attributable to storage conditions, sample preparation or methods of harvesting and drying (sec. 2.1.5; 2.3.3).

Table 4.5. Results of HPLC – DAD analysis of *Taraxacum officinale* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and crude plant extract used in the testing of antioxidant activity. Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (45/25% EtOH)		CRUDE PLANT EXTRACT (80% EtOH)
	Retention time (minutes)	
No flavonoid compounds or phenolic acids could be detected in this sample	3.31	Caffeic acid derivative
	4.71	Chlorogenic acid
	4.85	Caffeic acid derivative
	6.56	Caffeic acid derivative
	8.85	Caffeic acid derivative
	9.60	Caffeic acid derivative
	11.88	Caffeic acid derivative
	12.18	Caffeic acid derivative
	12.51	Caffeic acid derivative
	12.85	Caffeic acid derivative
	13.55	Caffeic acid derivative
	13.70	Caffeic acid derivative
	13.93	Caffeic acid derivative

Table 4.6. Results of HPLC – DAD analysis of *Taraxacum officinale* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and extract prepared as tincture. Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (45/25% EtOH)		EXTRACT PREPARED AS TINCTURE (45/25% EtOH)
	Retention time (minutes)	
No flavonoid compounds or phenolic acids could be detected in this sample	4.60	Caffeic acid derivative
	6.90	Caffeic acid derivative
	7.65	Caffeic acid derivative
	8.43	Caffeic acid derivative
	11.27	Caffeic acid derivative
	11.67	Caffeic acid derivative
	11.95	Caffeic acid derivative
	13.48	Caffeic acid derivative
	16.73	Caffeic acid derivative

4.1.2.3 *Arctium lappa radix* (Burdock root)

4.1.2.3.1 Thin layer Chromatography

Standard methods given for TLC analysis of *Arctium lappa* provide only a fingerprint chromatogram for authentication of the plant material (British Herbal Pharmacopoeia, 1996, p.49; Bisset, 2001, p.101). Using the method outlined in section 3.1.3.1, TLC analysis of the crude plant extract (sec. 3.1.2.2.1) found the following major bands relative to rutin (R_f 0.32), matching the literature values as follows:- a whitish blue band (Rx 2.1) and two pale blue bands (Rx 1.74 and Rx 1.4). A narrow intense blue band indicated by the literature at Rx 2.35 could not be identified (British Herbal Pharmacopoeia 1996, p.49). Chlorogenic acid at R_f 0.45 (Rx 1.4) was identified by comparison with the standard R_f value cited for chlorogenic acid in this solvent system (Wagner, 1984, p.176).

4.1.2.3.2 High Performance Liquid Chromatography

HPLC was carried out on three different extracts of *Arctium lappa* root (secs. 3.2.2.1; 3.1.2.2.3; 3.1.2.2.4). The polyphenolic compounds identified in these extracts are listed in Tables 4.7 and 4.8. The presence in the crude plant extract of the principal components of *Arctium lappa*, namely the caffeylquinic acids composed of polyphenols such as chlorogenic acid, caffeic acid and derivatives reported in the literature (Bisset, 2001, p.100; Maruta et al, 1995) was confirmed by HPLC analysis. The identity of these compounds was established by comparing their UV spectra with those of the corresponding standards. No phenolic compounds were identified in the tincture extract, while the extract prepared as tincture [Table 4.8] presented a similar profile to the crude plant extract [Table 4.7]. Although chromatographic results of the extract prepared as tincture and crude plant extract were broadly similar, it is possible that the comparative differences in retention times may be due to the difference in alcohol concentration of the two types of injected samples, i.e. 80% EtOH (crude plant extract) and 25% EtOH (tincture extracts). The peak at t_R 17.63 min [Table 4.7] and 17.5 min [Table 4.8] with a major absorption peak (band 11) at 275.8nm gave a UV spectrum typical of a flavanone in both the crude plant extract and tincture extraction.

Since the two methods of extraction (sec. 3.1.2.2.1; sec. 3.1.2.2.3) produced similar phytochemical profiles, the absence of phenolic compounds from the commercial tincture extract (sec. 3.1.2.2.4) could be attributed to storage conditions, sample preparation, harvesting or drying as discussed previously (sec. 2.1.5; 2.3.1).

Table 4.7. Results of HPLC-DAD analysis of *Arctium lappa* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and crude plant extract used in the testing of antioxidant activity.

Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (65/25% EtOH)		CRUDE PLANT EXTRACT (80% EtOH)
	Retention time (Minutes)	
No flavonoids or phenolic acid compounds could be detected in this sample.	3.28	Caffeic acid derivative
	4.17	Caffeic acid derivative
	4.70	Chlorogenic acid
	7.10	Caffeic acid derivative
	10.45	Caffeic acid derivative
	11.70	Caffeic acid derivative
	12.27	Caffeic acid derivative
	12.48	Caffeic acid derivative
	12.80	Caffeic acid derivative
	13.32	Caffeic acid derivative
	13.65	Caffeic acid derivative
	13.92	Caffeic acid derivative
	14.23	Caffeic acid derivative
	14.52	Caffeic acid derivative
	14.63	Caffeic acid derivative
	15.02	Caffeic acid derivative
	15.38	Caffeic acid derivative
	17.63	Flavanone

Table 4.8. Results of HPLC-DAD analysis of *Arctium lappa* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and extract prepared as tincture

Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (65/25% EtOH)		EXTRACT PREPARED AS TINCTURE (65/25% EtOH)
	Retention time (Minutes)	
No flavonoids or phenolic acid compounds could be detected in this sample.	4.61	Chlorogenic acid
	6.91	Caffeic acid derivative
	10.11	Caffeic acid derivative
	11.0	Caffeic acid derivative
	11.2	Caffeic acid derivative
	11.8	Caffeic acid derivative
	12.0	Caffeic acid derivative
	12.2	Caffeic acid derivative
	12.4	Caffeic acid derivative
	12.6	Caffeic acid derivative
	13.1	Caffeic acid derivative
	13.4	Caffeic acid derivative
	13.7	Caffeic acid derivative
	14.0	Caffeic acid derivative
	14.4	Caffeic acid derivative
	14.8	Caffeic acid derivative
	16.7	Caffeic acid derivative
	17.5	Flavanone

4.1.2.4 *Calendula officinalis* (Marigold)

4.1.2.4.1 Thin layer Chromatography

TLC analysis of the crude plant extract (sec. 3.1.2.2.1) carried out using the reference compounds rutin and chlorogenic acid gave some of the R_f values consistent with the literature (Wagner, 1984, p.176). The chromatogram showed strong orange and yellow-green fluorescent zones between R_f 0.2 and R_f 0.4. Rutin at R_f 0.3 as identified by the reference sample was an orange zone which lay between two yellow-green fluorescent

zones identified as narcissin (isorhamnetin 3-O-rutinoside) above rutin and isorhamnetin rutinorhamnoside below it (Wagner, 1984, p.176).

4.1.2.4.2 High Performance Liquid Chromatography

HPLC was carried out on three different extracts of *Calendula officinalis* (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4). The phenolic compounds identified in these extracts are listed in Tables 4.9 and 4.10. All the extracts contained a number of flavonols, some of which were identified as quercetin and isorhamnetin as reported in the literature (Bilia et al, 2001; Barnes et al, 2002, p.103). Flavonol compounds were generally identified by their characteristic UV spectra. Specific flavonols like quercetin were identified by comparing their t_R values and UV spectra with the corresponding authentic samples, combined with advice from experienced staff at Jodrell laboratory, Kew. Hydroxycinnamic acids in all three extracts, i.e. caffeic acid derivatives, chlorogenic acid and *p*-coumaric acid were identified by comparing the UV spectra of the peaks with pure samples.

Chromatograms for the crude plant extract and extract prepared as tincture were broadly similar and comparable to those available in the literature (Pietta et al, 1992; Bilia et al, 2001). Although a number of flavonols were identified in the commercial tincture extract [Fig. 4.10], this chromatogram was significantly different. Not only were more flavonol compounds recovered from the crude plant extract [Fig. 4.9] but two clearly-defined peaks at t_R 14.78 min and t_R 15.50 min in the crude plant extract, identified as isorhamnetin glycosides, were missing from the commercial tincture extract. Since flavonoid compounds are mainly water soluble (Harborne, 1984, p.55) the use of 90% aqueous ethanol for the initial extraction process of the commercial tincture [Table 3.2] may have contributed to a reduced efficiency of extraction.

Table 4.9. Results of HPLC-DAD analysis of *Calendula officinalis* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and crude plant extract used in the testing of antioxidant activity.
Compounds detected at 254 nm and 335 nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (90/45% EtOH)		CRUDE PLANT EXTRACT (80% EtOH)
	Retention time (Minutes)	
	4.73	Chlorogenic acid
Caffeic acid derivative	6.73	
Caffeic acid derivative	7.21	
	10.83	Flavonol (quercetin glycoside)*
	11.35	Flavonol (quercetin glycoside)*
	11.70	Flavonol (quercetin glycoside)*
Flavonol (quercetin glycoside)	12.26	Flavonol (quercetin glycoside)*
Flavonol (quercetin glycoside)	12.4	
Flavonol (quercetin glycoside)	12.71	Flavonol (quercetin glycoside)*
	12.98	Flavonol (quercetin glycoside)*
	13.25	Flavonol (quercetin glycoside)*
Flavonol (quercetin glycoside)	13.4	
	13.50	Flavonol (acylated)*
	13.70	Caffeic acid derivative
	13.81	Flavonol glycoside
Flavonol glycoside	14.5	
	14.78	Flavonol (isorhamnetin glycoside)*
	15.40	Flavonol (isorhamnetin glycoside)*
Flavonol glycoside	15.6	
Flavonol glycoside	16.1	
Flavonol glycoside	16.8	
Flavonol aglycone	18.6	
	18.8	*p*-coumaric acid derivative
p-coumaric acid derivative	19.0	
Quercetin aglycone	19.4	Quercetin aglycone*
	19.6	Flavonol aglycone

*Compounds identified by their retention times and on-line UV spectra in collaboration with staff at the Jodrell laboratory, Kew (Grayer, 2004).

Table 4.10. Results of HPLC-DAD analysis of *Calendula officinalis* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and extract prepared as tincture.

Compounds detected at 254 nm and 335 nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (90/45% EtOH)		EXTRACT PREPARED AS TINCTURE (90/45% EtOH)
	Retention time (Minutes)	
	4.3	Caffeic acid derivative
	4.6	Caffeic acid derivative (probably chlorogenic acid)
Caffeic acid derivative	6.73	
Caffeic acid derivative	7.21	
	10.7	Flavonol (quercetin glycoside)
	11.10	Flavonol (quercetin glycoside)
	11.75	Flavonol (quercetin glycoside)
Flavonol (quercetin glycoside)	12.26	Flavonol (quercetin glycoside)
Flavonol (quercetin glycoside)	12.4	Flavonol (quercetin glycoside)
	12.6	Flavonol (quercetin glycoside)
Flavonol (quercetin glycoside)	12.71	
	13.2	Flavonol (quercetin glycoside)
Flavonol (quercetin glycoside)	13.4	
	13.7	Caffeic acid derivative
	13.8	Flavonol glycoside
Flavonol glycoside	14.5	
	14.6	Caffeic acid derivative
	15.3	Flavonol glycoside
Flavonol glycoside	15.6	
Flavonol glycoside	16.1	
Flavonol glycoside	16.8	
	17.6	Flavonol aglycone
p-coumaric acid derivative	19.0	
Quercetin aglycone	19.6	

4.1.2.5 *Apium graveolens* (celery seed)

4.1.2.5.1 Thin Layer Chromatography

Standard methods given for TLC analysis of *Apium graveolens* provide only a fingerprint chromatogram for authentication of the plant material (British Herbal Pharmacopoeia, 1996, p.56; Bisset, 2001, p.82). TLC analysis of the crude plant extract (sec., 3.1.2.2.1) using the method outlined in section 3.1.3.1. showed major bands relative to rutin (R_f 0.31) that matched the literature values as follows, a pale orange band (1.7) and strong wide orange band (1.05) indicative of *Apii fructus* (British Herbal Pharmacopoeia, 1996, p. 56).

4.1.2.5.2 High Performance Liquid Chromatography

HPLC analysis carried out on three different extracts of *Apium graveolens* (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4) confirmed the presence of flavones and phenolic acids reported in the literature (Bradley, 1992, p.56). These compounds are listed in Tables 4.11 and 4.12. Luteolin glycosides, luteolin aglycone and a flavone aglycone recognised as either chrysoerial or diosmentin were identified by their t_R values and UV spectra combined with advice from staff at Jodrell laboratory, Kew (Grayer, 2004). Hydroxycinnamic acids, i.e. caffeic acid derivatives *p*-coumaric acid and ferulic acid were identified by comparing the UV spectra of the peaks with authentic standards. The phenylpropene compound apiole (t_R 20.8 min), a component of the essential oil that particularly occurs in the fruits of the apiaceae (Harborne, 1989, p.52), was tentatively identified in the crude plant extract and in the extract prepared as tincture (Grayer, 2004).

Chromatograms for all three extracts showed a broadly similar pattern in relation to the aforementioned compounds. Three clearly defined peaks at t_R 16.0 min – 16.9 min (detected at 254 nm) that could not be identified, were also observed on the chromatogram of the commercial tincture extract.

Table 4.11. Results of HPLC-DAD analysis of *Apium graveolens* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and crude plant extract used in the testing of antioxidant activity.

Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (90/60% EtOH)		CRUDE PLANT EXTRACT (80% EtOH)
	Retention time (Minutes)	
p-coumaric acid derivative	7.7	*p*-coumaric acid derivative
	10.0	*p*-coumaric acid derivative
p-coumaric acid derivative	10.8	
Flavone (luteolin glycoside)	12.0	Flavone (luteolin glycoside)*
Flavone (luteolin glycoside)	12.4	Flavone (luteolin glycoside) *
Flavone (luteolin glycoside)	12.6	
	13.0	Flavone (luteolin glycoside) *
	13.3	Flavone (luteolin glycoside) *
Flavone (luteolin glycoside)	13.9	Flavone (luteolin glycoside) *
Flavone (luteolin glycoside)	14.3	Flavone (luteolin glycoside) *
	15.5	Flavone (luteolin glycoside) *
	17.1	Caffeic acid derivative
Luteolin aglycone	17.4	Luteolin aglycone*
	17.8	Caffeic acid derivative
	18.8	*p*-coumaric acid derivative
Flavone (possibly the same as the crude plant extract)	19.0	Chrysoeriol (luteolin 3'methyl ether or diosmetin (luteolin 4' methyl ether) *
Caffeic acid derivative	20.0	
	20.8	Apiole*
	24.4	Caffeic acid derivative

*Compounds identified by their retention times and on-line UV spectra combined with advice from staff at Jodrell laboratory, Kew (Grayer, 2004).

Table 4.12. Results of HPLC-DAD analysis of *Apium graveolens* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and extract prepared as tincture

Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (90/60% EtOH)		EXTRACT PREPARED AS TINCTURE (90/45% EtOH)
	Retention time (Minutes)	
	7.3	*p*-coumaric acid derivative
p-coumaric acid derivative	7.7	
	9.7	*p*-coumaric acid derivative
	10.0	*p*-coumaric acid derivative
p-coumaric acid derivative	10.8	
	11.8	Ferulic acid
Flavone (luteolin glycoside)	12.0	
	12.2	Flavone (luteolin glycoside)
Flavone (luteolin glycoside)	12.4	
Flavone (luteolin glycoside)	12.6	
	12.8	Flavone (luteolin glycoside)
Flavone (luteolin glycoside)	13.9	Flavone (luteolin glycoside)
Flavone (luteolin glycoside)	14.3	Flavone (luteolin glycoside)
	15.4	Flavone (luteolin glycoside)
	17.0	Caffeic acid derivative
Luteolin aglycone	17.4	Luteolin aglycone
	17.6	Caffeic acid derivative
	18.8	Flavone (possibly the same as the crude plant extract)
Flavone (possibly the same as the crude plant extract)	19.0	
	19.9	*p*-coumaric acid derivative
Caffeic acid derivative	20.0	
	20.6	Apiole
	24.4	Caffeic acid derivative

4.1.2.6 Salix alba (White willow)

4.1.2.6.1 Thin Layer Chromatography: Thin Layer Chromatography for the identification of salicylic acid derivatives in Willow bark

TLC analysis of the crude plant extract produced a chromatogram consisting of two distinct zones of red. Red zones observed between R_f 0.4 – R_f 0.6 were identified by the literature as the phenol glycosides that are characteristic of *Salix* spp. (Wagner 1984, p.282). Salicin is documented as the main compound. The red zones observed above R_f 0.6 (R_f 0.6- R_f 0.8) are reported to be due to catechin, *p*-coumaric acid, saligenin and salicylic acid (yellow-brown) (Wagner, 1984, p.282). It was not possible to detect any colours other than deep red. No reference compounds were used.

Since the numerous phenolic compounds contained in Willow bark may be hydrolysed to salicin, alkaline hydrolysis was carried out further to identify the presence of salicylic acids in the raw plant material [Table 3.1]. TLC after alkaline hydrolysis showed only a single band of red at R_f 0.45 in accordance with the literature (Upton, 1999c, p.8). Salicin is reported to have an R_f value of 0.44 (Upton, 1999c, p.8) and it would seem reasonable to suppose that this compound was salicin.

4.1.2.6.2 High Performance Liquid Chromatography (HPLC)

HPLC analysis carried out on three different extracts of *Salix alba* (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4) confirmed the presence of flavonoids, namely flavanones and phenolic acids, *p*-coumaric and caffeic acid derivatives as reported in the literature (Bisset 2001, p.438; Upton, 1999c, p.7). These compounds are listed in Tables 4.13 and 4.14. Flavanones in all three extracts were generally identified by their characteristic UV spectra. Without authentic samples however, this information was insufficient for individual compound analysis. Hydroxycinnamic acids, i.e. *p*-coumaric acid and caffeic acid derivatives, were identified by comparing the UV spectra of the peaks with authentic standards. A flavonol glycoside (t_R 14.5 min) and flavonol aglycone (t_R 17.55 min) were identified by their general UV spectra in the crude plant extract only.
Chromatograms for the crude plant extract and extract prepared as tincture were broadly similar [Tables 4.13/14]. Variations in the type and amount of compounds recovered from each extract may be attributable to the different methods and solvent ratios used in

the initial extraction process e.g. absence of flavonol compounds from both the tincture extracts. Although the commercial tincture extract contained similar compounds to the other two extracts [Tables 4.13/14], fewer compounds were detected and the chromatographic profile was notably different. These differences could be explained by insufficient sample preparation, i.e. prior to extraction, the bark should ideally be pulverized or shredded to increase the surface area exposed to the solvent; poor harvesting of raw material or inherent differences in the original source species (sec. 2.3.7).

Table 4.13. Results of HPLC-DAD analysis of *Salix alba* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and crude plant extract used in the testing of antioxidant activity.
Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (50/25% EtOH)		CRUDE PLANT EXTRACT (80% EtOH)
	Retention time (Minutes)	
	6.06	Flavanone
	6.4	Flavanone
	10.2	Flavanone
	10.6	Flavanone
Flavanone	10.9	Flavanone
	11.2	Flavanone
	12.2	Caffeic acid derivative
	14.17	Flavanone
Flavanone	14.22	
	14.5	Flavonol glycoside
	15.0	*p*-coumaric acid derivative
	15.3	*p*-coumaric acid derivative
Flavanone	15.44	Flavanone
	15.6	Caffeic acid derivative
	16.4	*p*-coumaric acid derivative
p-coumaric acid derivative	16.53	
Caffeic acid derivative	16.95	
p-coumaric acid derivative	19.13	
	17.55	Flavonol aglycone
	21.70	Caffeic acid derivative

Table 4.14. Results of HPLC-DAD analysis of *Salix alba* showing the flavonoid/phenolic acid profiles of the commercial tincture extract and extract prepared as tincture.

Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (50/25% EtOH)		EXTRACT PREPARED AS TINCTURE (50/25% EtOH)
	Retention time (Minutes)	
	6.06	Flavanone
	6.9	Caffeic acid derivative
	9.97	Flavanone
	10.4	Flavanone
	10.6	Flavanone
Flavanone	10.9	Flavanone
	11.2	Flavanone
	12.1	Caffeic acid derivative
	13.1	Flavanone
	13.6	Flavanone
	13.9	Flavanone
Flavanone	14.22	
	14.8	Caffeic acid derivative
	15.2	Flavanone
Flavanone	15.44	
	16.3	*p*-coumaric acid derivative
	16.5	Flavanone
p-coumaric acid derivative	16.53	
	16.8	Flavanone
Caffeic acid derivative	16.95	
	17.5	Flavanone
	18.9	*p*-coumaric acid derivative
p-coumaric acid derivative	19.13	

4.1.2.6.3 Detection of plant proanthocyanidins

Anthocyanidins (anthocyanin aglycones) are formed in acid hydrolysed plant tissue from colourless polymeric tannins and proanthocyanidins (Pigman et al, 1953; Harborne, 1984, p.88). Three extracts of *Salix* spp. (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4) were analysed by acid hydrolysis for the presence of proanthocyanidins. Results are listed in Table 4.15. All three samples developed different shades of magenta identified

by colour as cyanidin (Walker, 1975, p.54; Harborne, 1984, P.64) confirming the presence of proanthocyanidins as reported in the literature (Kolodziej, 1990; Upton, 1999c, p.7). HPLC-DAD analysis of the three extracts detected cyanidin in only the crude plant extract that had turned a dark, dense magenta. UV-Vis spectra were recorded and visually scanned in the range 210-600 nm and chromatograms viewed at 280nm, and 535nm. Although all the samples developed a red colour, the depth of colour varied and the levels of cyanidin appeared to have been too low in both the commercial tincture extract and the extract prepared as tincture to register on the chromatogram. This may be attributable to the following factors.

Since the same plant material was used for both the crude plant extract (sec. 3.1.2.2.1) and extract as tincture (sec. 3.1.2.2.3), the preparation of the extracts by two different methods may have contributed to the apparently different levels of anthocyanin aglycones formed by acid hydrolysis in the two extracts.

Due to the unstable nature of the acid hydrolysed anthocyanin aglycones, the colour of the samples awaiting analysis may have faded slightly indicating changes to the structure of the anthocyanin that may have affected the resultant chromatograms (Harborne, 1984, p.63). The colour and stability of an anthocyanin in solution is highly dependent on the pH. They are most stable and most highly coloured at low pH values and gradually lose colour as the pH is increased. Exposure to light results in loss of colour (Walker, 1975, p.51; Goiffon et al, 1991).

Table 4.15. Results of acid hydrolysis to detect the presence of proanthocyanidins in *Salix alba* bark, *Crataegus laevigata* berries and *Crataegus laevigata* leaves and flowers expressed as the production of anthocyanidins.

HPLC-DAD Compounds detected at 280 nm and 535 nm.

Plant/type of extract	Colour of extract	Colour of extract after acid hydrolysis	Visible colour analysis	Anthocyanidin identified by HPLC-DAD analysis
***Salix alba* (bark)**				
Crude plant extract (80% EtOH)	brown	Very dense dark (magenta) red	Cyanidin	Cyanidin
Commercial tincture extract (liquid extract) (50/25% EtOH)	Pale amber brown	Magenta red	Cyanidin (traces)	None detected
Plant material (bark) extracted as tincture (50/25% EtOH)	Pale amber brown	Magenta red	Cyanidin (traces)	None detected
***Crataegus laevigata* (berries)**				
Crude plant extract (80% EtOH)	Pale yellow	Pinky red	Cyanidin (traces)	None detected
Commercial tincture extract (liquid extract) (45/25% EtOH)	Amber	Pale pink	Cyanidin (traces)	None detected
Plant material (berries) extracted as tincture (45/25% EtOH)	Pale yellow	Pinky red	Cyanidin (traces)	None detected
***Crataegus laevigata* (leaves and flowers)**				
Crude plant extract (80% EtOH)	Pale yellow	Deep scarlet red	Pelargonidin	Anthocyanidin aglycone (unidentified)
Commercial tincture extract (liquid extract) (45/25% EtOH)	Orange	Pinkish	Pelargonidin (traces)	None detected
Plant material (leaves and flowers) extracted as tincture (45/25% EtOH)	Pale yellow	Weak red	Pelargonidin (traces)	None detected

4.1.2.7 *Crataegus laevigata* (Hawthorn berries)

4.1.2.7.1 Thin Layer Chromatography

TLC analysis of the crude plant extract (sec., 3.1.2.2.1) carried out using the recommended reference compounds vitexin, hyperoside, rutin, caffeic acid and chlorogenic acid produced an unresolved chromatogram that gave some of the R_f values consistent with those reported in the literature (Wagner, 1984, p.178; Upton, 1999a, p.10-12). Three weak bands were observed for the extract. Two pale blue bands were identified as caffeic acid (R_f 0.9) and chlorogenic acid (R_f 0.45) and a pale yellow band at R_f 0.55 corresponded to hyperoside.

4.1.2.7.2 High Performance Liquid Chromatography

HPLC analysis was carried out on three extracts of *Crataegi fructus* (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4). The flavonoids/phenolic acids identified in these extracts are listed in Tables 4.16 and 4.17. HPLC analysis of the crude plant extract confirmed the presence of flavonoids and hydroxycinnamic acids reported in the literature (Djumlija, 1994). Hydroxycinnamic acids, i.e. caffeic acid derivatives, chlorogenic acid and *p*-coumaric acid, were identified by comparing the UV spectra of the peaks with authentic standards. *Crataegi fructus* is documented to have a low flavonoid content (Wagner, 1984, p.178). However several compounds generally identified as flavonols and flavones were identified by their characteristic UV spectra [Table 4.16]. Some of these compounds were further identified as a quercetin glycoside (t_R 12.79 min) and quercetin aglycone (t_R 16.74 min) by comparing their t_R values and UV spectra with the corresponding authentic standards, combined with advice from experienced staff at Jodrell laboratory, Kew (Grayer, 2004). Both the commercial tincture extract and extract prepared as tincture contained several hydroxycinnamic acids as outlined above for the crude plant extract [Table 4.17]. Only one flavonol compound was identified in both tincture extracts corresponding with the quercetin glycoside (t_R 12.79 min) in the crude plant extract. No flavones were identified in the tincture extracts.

Chromatograms of the crude plant extract and extract prepared as tincture were broadly similar with a higher proportion of hydroxycinnamic acids identified in the tincture extract and more flavonoids in the crude plant extract [Tables 4.16/17]. Since both

extracts originate from the same source material, these differences could be attributed to the different methods and solvent ratios used for the initial extraction of the plant material. The chromatographic profile of the commercial tincture extract in comparison to the previous two extracts was notably dissimilar, especially in relation to the extract prepared as tincture. In addition to differences in extraction method, the source material for the commercial tincture extract may have originated from another species of *Crataegus*. The composition of the flavonoids present in the berries has been reported to differ with each species (Upton, 1999a, p.10).

Table 4.16. Results of HPLC – DAD analysis of *Crataegi fructus* showing the flavononid/ phenolic acid profiles of the commercial tincture extract and crude plant extract used in the testing of antioxidant activity.
Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (45/25% EtOH)		CRUDE PLANT EXTRACT (80% EtOH)
	Retention time (Minutes)	
	4.82	Caffeic acid derivative (probably chlorogenic acid)
	4.87	Caffeic acid derivative
Caffeic acid derivative	6.61	
Caffeic acid derivative	7.05	
	9.22	*p*-coumaric acid derivative
p-coumaric acid derivative	10.78	
Flavonol (quercetin glycoside)	12.79	Flavonol (quercetin glycoside)
	14.56	Flavonol glycoside
	15.31	Flavone
	15.92	Flavone
	16.74	Flavonol (quercetin aglycone)
Caffeic acid derivative	16.85	
p- coumaric acid derivative	19.03	

Table 4.17. Results of HPLC – DAD analysis of *Crataegi fructus* showing the flavononid/ phenolic acid profiles of the commercial tincture extract and extract prepared as tincture.

Compounds detected at 254nm and 335nm.

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (45/25% EtOH)		EXTRACT PREPARED AS TINCTURE (45/25% EtOH)
	Retention time (Minutes)	
	4.63	Caffeic acid derivative (probably chlorogenic acid)
Caffeic acid derivative	6.61	
	6.9	Caffeic acid derivative
Caffeic acid derivative	7.05	
p-coumaric acid derivative	10.78	
	12.0	Caffeic acid derivative
	12.2	Caffeic acid derivative
Flavonol (quercetin glycoside)	12.79	Flavonol (quercetin glycoside)
	14.8	Caffeic acid derivative
Caffeic acid derivative	16.85	
p-coumaric acid derivative	19.03	

4.1.2.7.3 Detection of plant proanthocyanidins

Three extracts of *Crataegi fructus* (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4) were analysed for the presence of anthocyanidins following acid hydrolysis. Results are given in Table 4.15. All three samples developed different depths of a weak pinky red identified by colour as cyanidin, confirming the presence of proanthocyanins reported in the literature (Bahorun et al, 1994; Upton, 1999a, p.9). However no anthocyanin aglycones could be identified by HPLC-DAD analysis in any of the three extracts. UV-Vis spectra were recorded and visually scanned in the range 210-600nm and chromatograms viewed at 280nm and 535nm. Since the colour developed in all three extracts was weak, the levels of cyanidin aglycones formed by acid hydrolysis appear to have been too low to register as a peak on the chromatograms. Solutions of anthocyanins are unstable and may fade prior to analysis – (sec., 4.1.2.6.3).

4.1.2.7.4 Quantification of procyanidin content in *Crataegus laevigata* berries

Since oligomeric proanthocyanidins are documented as one of the major constituents of *Crataegi fructus,* a spectrometric assay to quantify as a percentage the concentration of these compounds in the raw plant material [Table 3.1] was carried out using the method outlined in section 3.1.5.2.1. The presence of cyanidin chloride expressed as an absorbance reading at 545 nm was used as the basis of the calculation (Upton, 1999a, p.11).

Formula as outlined in section 3.5.2.1.1:- A x 500/ 75 x *m*

0.118 x 500/ 75 x 2.5 = 0.31%

Total procyanidin concentration calculated as percentage cyanidin chloride in a 2.5g sample of dried *Crataegus laevigata* berries = 0.31% The procyanidin content of *Crataegus* spp. are mainly unknown, however the procyanidin concentration in the fruits is dependent on the degree of maturity of the fruits, the concentration being highest in immature fruits (Svedstromet et al, 2002).

4.1.2.8 *Crataegus laevigata* (Hawthorn leaves and flowers)

4.1.2.8.1 Thin Layer Chromatography

TLC of the crude plant extract (sec., 3.1.2.2.1) was carried out according to the method outlined in section 3.1.3.1 using the reference compounds rutin, hyperoside and vitexin. The chromatogram showed three distinct bands, each corresponding to the appropriate reference compound that gave R_f values consistent with the literature (Upton, 1999b, p.12-13):- a weak orange band at R_f 0.35 in the region of rutin, a strong orange band corresponding with hyperoside at R_f 0.52 and yellowish green band of vitexin at R_f 0.65. Rutin at R_f 0.35 is reported to co-elute with vitexin-2"-O-rhamnoside (Wagner, 1984, p.178; Upton, 1999b, p.12-13).

4.1.2.8.2 High Performance Liquid Chromatography

HPLC analysis carried out on three different extracts of *Crataegus* spp. leaves and flowers (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4) confirmed the presence of some flavonoids as reported in the literature (Upton, 1999b, p.10-11). Flavones and flavonols are the main flavonoids of *Crataegus* spp. leaves and flowers. Identified compounds are listed in Tables 4.18 and 4.19. Flavone compounds vitexin (apigenin 8-C glucoside) and apigenin C-glycoside were identified in all three extracts by their t_R values and UV on-line spectra combined with advice from experienced staff at Jodrell laboratory, Kew (Greyer, 2004). Flavonol compounds present included kaempferol and quercetin, also identified as above. Quercetin glycosides were present in all three extracts. Kaempferol glycosides (t_R 13.39 min) could be identified only in the crude plant extract and the extract prepared as tincture and a quercetin aglycone (t_R 16.76 min) solely in the crude plant extract [Tables 4.18/19]. Hydroxycinnamic acids, caffeic acid derivatives, chlorogenic acid and *p*-coumaric acid were identified by comparing the UV spectra of the peaks with authentic samples of these compounds.

The chromatographic results, particularly for the crude plant extract and the extract prepared as tincture, were broadly similar. It is possible however that the difference in alcohol content of the two different injected samples, i.e. 80% EtOH (crude plant extract) and 25% EtOH (tincture extracts), has caused a slight shift in retention times as observed for the same compound of vitexin which registered at t_R 11.59 for the crude plant extract and t_R 11.4 for the extract prepared as tincture. The chromatographic differences observed for the commercial tincture may be related to the source material which, like the berries, may have originated from another species of *Crataegus*. Such is the variation in flavonoids present in the leaves and flowers of the *Crataegus* spp. that the compounds can often be used to separate the different species (Bisset, 2001, p.162).

Table 4.18. Results of HPLC-DAD analysis of *Crataegus* spp. leaves and flowers showing the flavonoid/phenolic acid profiles of the commercial tincture extract and crude plant extract used in the testing of antioxidant activity.
Compounds detected at 254nm and 335nm

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (45/25% EtOH)		CRUDE PLANT EXTRACT (80% EtOH)
	Retention Time (Minutes)	
	3.27	Caffeic acid derivative
Caffeic acid derivative	3.36	
	3.89	Caffeic acid derivative
	4.16	Caffeic acid derivative
Caffeic acid derivative (probably chlorogenic acid)	4.69	Caffeic acid derivative (probably chlorogenic acid)*
Caffeic acid derivative	6.61	
Caffeic acid derivative	7.09	
	7.39	*p*-coumaric acid derivative
p-coumaric acid derivative	9.91	
	10.22	Flavone
	10.52	Flavone
p-coumaric acid derivative	10.86	
Flavone (vitexin – apigenin 8-C-glucoside)	11.59	Flavone (vitexin – apigenin 8-C-glucoside)*
	12.12	Caffeic acid derivative
Flavone	12.63	
	12.81	Flavonol (quercetin glycoside)*
Flavonol (quercetin glycoside)	12.83	
	13.39	Flavonol (kaempferol glycoside)*
Flavone (apigenin C-glycoside)	14.74	Flavone (apigenin C-glycoside)*
	15.71	*p*-coumaric acid derivative
	16.76	Flavonol (quercetin aglycone)*
Caffeic acid derivative	16.88	
p-coumaric acid derivative	19.04	

*Compounds identified by their retention times and on-line UV spectra combined with advice from staff at Jodrell laboratory, Kew (Grayer, 2004).

Table 4.19. Results of HPLC-DAD analysis of *Crataegus* spp. leaves and flowers showing the flavonoid/phenolic acid profiles of the commercial tincture extract and extract prepared as tincture

Compounds detected at 254nm and 335nm

COMMERCIAL TINCTURE EXTRACT (Liquid extract) (45/25% EtOH)		EXTRACT PREPARED AS TINCTURE (45/25% EtOH)
	Retention Time (Minutes)	
Caffeic acid derivative	3.36	
	4.6	Caffeic acid derivative
Caffeic acid derivative (probably chlorogenic acid)	4.69	
Caffeic acid derivative	6.61	
	7.1	*p*-coumaric acid derivative
Caffeic acid derivative	7.09	
p-coumaric acid derivative	9.91	
	10.0	Flavone
	10.3	Flavone
p-coumaric acid derivative	10.86	
	11.4	Flavone (vitexin – apigenin 8-C-glucoside)
Flavone (vitexin – apigenin 8-C-glucoside)	11.59	
	11.9	Caffeic acid derivative
	12.3	Flavone
Flavone	12.63	
	12.81	Flavonol (quercetin glycoside)
Flavonol (quercetin glycoside)	12.83	
	13.3	Flavonol (kaempferol glycoside)
	14.6	Flavone (apigenin C-glycoside)
Flavone (apigenin C-glycoside)	14.74	
	16.7	Caffeic acid derivative
Caffeic acid derivative	16.88	
p-coumaric acid derivative	19.04	
	21.5	*p*-coumaric acid derivative

4.1.2.8.3 Quantification of flavonoid concentration in *Crataegus laevigata* leaves and flowers

Crataegus leaves and flowers contain a wide array of flavonoids that are considered to contribute strongly to its pharmacological activity (Upton, 1999b, p.10). Since the concentration of these compounds varies according to species, time of harvesting and different plant parts, a spectrophotometric assay to determine the flavonoid content of the raw plant material (Table 3.1.) was carried out using the method outlined in section 3.1.5.2.2. Percentage calculation of total flavonoids present is based on the concentration of hyperoside expressed as an absorbance reading at 410 nm.

Formula as outlined in section 3.1.5.5.2:- A x D/ M x 405

0.163 x 500/ 0.400 x 405 = 0.5%

The total flavonoid concentration calculated as hyperoside in a 0.400g sample of dried *Crataegus laevigata* leaves and flowers was therefore calculated as 0.5% and this is consistent with levels reported in the literature (Upton, 1999b, p.10).

4.1.2.8.4 Detection of plant proanthocyanidins

Three samples of *Crataegus* spp. (secs. 3.1.2.2.1; 3.1.2.2.3; 3.1.2.2.4) were analysed for the presence of anthocyanidins following acid hydrolysis. Results are listed in Table 4.15. All three samples developed different depths of scarlet confirming the presence of proanthocyanidins as reported in the literature (Upton, 1999b, p.11). HPLC- DAD analysis of the three extracts showed the presence of an anthocyanin aglycone in the crude plant extract only that did not give a spectrum typical of cyanidin (Jodrell laboratory, 2004). UV-Vis spectra were recorded and visually scanned in the range 210nm – 600nm and chromatograms viewed at 280nm and 535nm. The resultant chromatograms were poor due to the levels of anthocyanin aglycone seemingly being too low to produce a spectrum from which a positive identification could be made. Tentative identification by colour indicated that the compound may be the orange red anthocyanin aglycone pelargonidin (Walker, 1975, p.54; Harborne, 1984, p.64). Although all the samples developed a red colour, the depth of colour varied and the levels of anthocyanin aglycones appeared to have been too low in the two tincture

extracts to register on the chromatogram. The same reasons as outlined in sec. 4.1.2.6.3 may apply.

4.1.3 Discussion

The reported findings of the qualitative analysis of all the crude plant extracts confirmed the presence of phenolic compounds, including flavonoids and phenolic acids, identified by the UV spectra from High Performance Liquid Chromatography and the use of authentic samples utilizing Thin Layer Chromatography. Since a crude plant extract is not the form in which herbs are generally administered as medicine, a set of commercial tinctures in the form of liquid extracts was also evaluated by HPLC for their flavonoid content in order to compare the antioxidant activity of the two types of extract, with the purpose of investigating whether they exhibit similar levels of antioxidant activity. Due to the different extraction procedure and varying strengths of alcohol and water being used for the production of the commercial tinctures, replica tincture extractions were carried out using the same source material as used for the crude plant extract, to ensure a more objective comparison of the two different types of extract (i.e. crude plant extract and tincture extract) and to establish if the method of tincture extraction produced similar constituent profiles when using different source material.

In general, more flavonoid compounds and hydroxycinnamic acids were extracted by using 80% ethanol than by other ethanol concentrations. Freshly prepared tincture extracts were found to contain more compounds than commercially produced extracts that had been stored for some time and flavonol aglycones could only be identified in extractions made with either 80% or 90% ethanol. Although a colour change after acid hydrolysis indicated the presence of proanthocyanidins in all the extracts of *Salix alba* and *Crataegus lavigata* (leaves, flowers and berries), the only anthocyanidin that could be identified by HPLC was cyanidin in the crude plant extract of *Salix alba*. Since detectable levels of proanthocyanidins are generally reported for both *Salix alba* and *Crataegus lavigata* (leaves, flowers and berries) (Kolodziej, 1990; Bahorun et al, 1994), the unstable nature of the acid hydrolysed anthocyanidins or method of extraction, particularly tincture extraction, may have been responsible for the lack of results from HPLC analysis (Walker, 1975, p.54; Goiffon et al, 1991). According to Harborne (1984, p.87) extraction of the full range of proanthocyanidins can rarely be achieved

and analysis of anthocyanidin solutions is dependent on the structural type of the original proanthocyanidins.

Flavonoid profiles and the hydroxycinnamic acid content of crude plant extracts were found to be generally dissimilar to those of commercially prepared tinctures, except for those prepared from *Apium graveolens* and *Crataegus* spp. leaves and flowers,. The fresh tincture extracts produced from the same plant material as the crude plant extract also, in most cases, produced HPLC chromatograms that were different to their commercial tincture counterparts, but broadly similar to the crude plant extracts, with the exception of *Crataegus* spp. fruit and *Silybum marianum*. The differences observed between all three types of extract appeared to be controlled by two factors, firstly, use of different source material and secondly, the strength of alcohol and water used for the extraction process. As outlined in the results, qualitative analysis of the content of both the commercial and replica tincture extracts 25% *v/v Silybum marianum* showed negligible amounts of flavanolignans present in each of these extracts compared to the 80% ethanol crude plant extract. These findings would appear to support results reported by Bilia et al, (2001) whose HPLC analysis of a 40% *v/v Silybum marianum* tincture showed a low flavanolignan content (88.6mg/100ml tincture measured as silymarin), compared with a 60% *v/v* tincture with a content which was much higher (178mg/100ml tincture). This illustrates the crucial effect that the solvent ratio can have on the final extract. Variations relating to extracts produced from raw material obtained from different sources could be associated with factors such as geography, climate and harvesting procedures as outlined in 2.1.5. This does not explain however the absence of any phenolic compounds, namely caffeic acid derivatives from both *Arctium lappa* (Burdock) and *Taraxacum officinale* (Dandelion) commercial tincture extracts. Phenolic acids such as these are heat-sensitive and susceptible to oxidation under certain pH conditions (Batovska et al, 2005). Plant phenols are also very prone to enzyme oxidation and due to the action of enzymes like glycosidases, esterases, or oxidases present in all plants, phenolic material may be lost or degraded in the production or storage of liquid herbal products (Stefan and Chantal, 2005). If plant material is to be dried before extraction, this should be carried out as quickly as possible after collection, without using high temperatures, to avoid the occurrence of chemical changes (Harborne, 1984, p.4). Due to the complexity of a plant extract, the evaluation of the chemical stability of a finished product during the storage period is very challenging and current information which is mainly focused on the stability of an isolated

compound and its decomposition products does not always accurately reflect the chemical stability of the compound in an extract (Stefan and Chantal, 2005). Tinctures are traditionally stored at normal room temperature in brown glass to minimise degradation by sunlight. Storage of the crude plant extracts at -18°C may have contributed to their continuing efficacy. Since HPLC analysis of the 'extracts prepared as tinctures' of *Arctium lappa* and *Taraxacum officinale* was carried out on freshly prepared samples and both showed the presence of a range of caffeic acid derivatives, manufacturing procedures other than the method of extraction would seem to be implicated. Failure to follow harvesting guidelines as outlined in sections 2.3.1 and 2.3.3 may result in an inferior product. Insufficient sample preparation may affect the efficacy of the extraction process. While some plant material can be efficiently extracted without extensive preparation, others, like roots, will require some pre-treatment for efficient extraction to occur. Plant material such as this should ideally be pulverized or shredded to increase the surface area exposed to the solvent. Chen et al, (2004) investigating the influence of different treatments, i.e. methods of preparation on the free radical scavenging activity of Burdock root, found that the phenolic components, caffeic acid and chlorogenic acid, exist mainly in the skin of the root and that peeling the root and discarding the skin greatly decreased the concentration of these components and subsequent free radical scavenging activity.

Caffeic acid derivatives detected by HPLC in the 'extract prepared as tincture' of *Arctium lappa* using 65% ethanol, although registering at slightly different retention times, were equal in number to these compounds detected in the crude plant extract. By comparison, using 45% ethanol, fewer caffeic acid derivatives were detected in the 'extract prepared as tincture' of *Taraxacum officinale* than the crude plant extract. This emphasizes yet again the importance of the solvent ratio. It is still not clear, however, what impact the effect of the different extracts, i.e. those extracted using different alcohol and water mixtures, would have on the efficacy of the extracts *in vivo,* which would require testing for bioequivalence in a suitable pharmacological model.

Although various pharmacopoeias like the European Pharmacopoeia (1997) contain general monographs about tinctures, in which their modes of preparation and the amounts of the herbal drug in the tinctures are specified, the individual herbal monographs do not specify the amount of alcohol required to facilitate extraction of the phytocomplex. This results in commercially manufactured tinctures that contain

different amounts of alcohol and varying phytochemical profiles. Since the commercial tinctures evaluated are representative of those widely used by Herbalists in the U.K. (Table 3.1) they were tested with the crude plant extracts for their antioxidant propensity.

Analysis of the authentic sample of silymarin by Gas Chromatography did not register any of the flavonoid compounds that constitute the silymarin complex, thereby confirming the need to derivatise these compounds when using Gas Chromatography for flavonoid analysis (Markham and Bloor, 1998, p.30). It is quite possible however that the large molecular weight of these compounds will mean that derivatisation will fail to make the compounds sufficiently mobile. The compounds identified by Gas Chromatography were generally similar in both types of extract. A number of compounds like fatty acids, furan compounds, monocarboxylic acids and simple phenols were common to most of the tested extracts, but no flavonoid compounds were detected. For this reason only a representative set of results namely *Silybum marianum* were reported. Notably, recent studies suggest that in addition to the polyphenols, other compounds like fatty acids (e.g. palmitic acid) and some volatile heterocyclic furan compounds that have exhibited antioxidant activity *in vitro* may contribute to the antioxidant activity of some plants (Fuster, 2000; Zhang, 2003). This supports the view of some authors that the activity of certain plant extracts is not only due to the compounds established as being active but also to the contribution from a number of other components (Gordon and An, 1995; Houghton, 2000).

Although all the plants analysed were shown to contain a range of polyphenolic compounds, each plant had its own unique profile of flavonoid compounds and phenolic acids. *Taraxacum officinale* and *Arctium lappa* were shown to contain only caffeic acid derivatives. *Calendula officinalis* contained a number of flavonols, *Apium graveolens* flavones and *Crataegus laevigata* (leaves and flowers) contained a mixture of both, all in combination with caffeic acid derivatives. *Silybum marianum* was the only plant to contain the dihydroflavonol silymarin and proanthocyanidins (condensed tannins) were only detected in *Salix alba* and *Crataegus laevigata* (leaves and flowers/berries). Although, in general, flavonoid compounds all possess the same basic properties (section 1.2.7.4), variations in structure and different combinations of these compounds are likely to influence their activity and antioxidant potential, along with the presence of

other nonflavonoidic compounds such as volatile oils and other plant metabolites not analysed in this study (section 1.2.7.5).

As already outlined, one of the underlying principles of Western Herbal Medicine is that the therapeutic value of a plant rests not only on its pharmacologically active compounds, but on the complex mix of many different compounds. Numerous studies now show that isolation of the pharmacologically active compounds may produce less activity than the whole plant extracts (Cott and Misra, cited in Gilbert and Alves 2003; Houghton, 2000). Many of the flavonoid compounds and hydroxycinnamic acids identified in the selected plant extracts have been shown to possess antioxidant activity largely in *in vitro* systems and it is postulated that these compounds may also exert antioxidant effects in the whole plant extract. Generally it is considered that the activity of medicinal plants containing flavonoids depends not on the flavonoid fraction alone, but also on other components which may either directly contribute or play an adjuvant role in strengthening the potency of the flavonoid compounds (Pietta, 1998). The reported results support the use of whole plant extracts in this study.

4.2 EVALUATION OF PLANT EXTRACTS AS ANTIOXIDANTS

4.2.1 LIPID PEROXIDE ASSAY

4.2.1.1 Results of the antioxidant action of the crude plant extracts on linoleic acid oxidation

Results of the antioxidant activity of the crude plant extracts (sec. 3.1.2.2.1) on linoleic acid oxidation are shown in Figs. 4.4 – 4.11. Results are expressed as percentage inhibition of the experimental control (oxidizing substrate without antioxidant). All the crude plant extracts were tested at the following concentrations (expressed as mg/ml):- 0.001; 0.0025; 0.005; 0.01; 0.05; 0.1; 0.2; 0.5; 1mg/ml and 5mg/ml. All eight crude plant extracts exhibited antioxidant activity for most of these concentrations. Results generally showed that the antioxidant activity of the crude plant extracts increased with increasing concentration up to 1mg/ml but with antioxidant activity remaining fairly constant in the range from 0.005mg/ml to 0.1mg/ml. Experimental results for 0.0025mg/ml and 0.001mg/ml were variable. At 0.0025mg/ml and 0.001mg/ml *Calendula officinalis* exhibited consistent pro-oxidant activity. At 0.001mg/ml pro-oxidant activity was also recorded for *Taraxacum officinale*. At this concentration however, results for *Apium graveolens, Silybum marianum,* and *Arctium lappa* were changeable with both pro-oxidant and antioxidant activity recorded, albeit by a very narrow margin. For example, results from four experiments for *Silybum marianum* ranged from pro-oxidant activity (results x 2) to antioxidant activity (15%) and a result (0%) where neither antioxidant nor pro-oxidant activity were observed. At 0.001mg/ml the only extracts to exhibit antioxidant activity were those shown by phytochemical analysis to contain proanthocyanidins which were *Salix alba* and *Crataegus laevigata* leaves, flowers and berries. Since antioxidant activity was consistently observed for all the extracts tested at concentrations 0.005mg/ml and above, results for 0.001 and 0.0025 mg/ml suggest that antioxidant activity is dose dependent and that a threshold level exists for each extract at which a change in behaviour from antioxidant to pro-oxidant is observed.

The strongest antioxidant activity was exhibited by *Crataegus laevigata* leaves and flowers [92%, Fig. 4.5] and *Arctium lappa* root [91%, Fig. 4.10] at a final concentration of 1mg/ml. Maximum antioxidant activity was also observed at this concentration for *Crataegus laevigata* berry [89%, Fig. 4.9]; *Taraxacum officinalis* root [87%, Fig. 4.7]; *Salix alba* [87%, Fig. 4.11] and *Apium graveolens* [80%, Fig 4.4]. A decrease in antioxidant activity at 5mg/ml was observed for all the crude plant extracts except *Silybum marianum* [90%, Fig 4.6] and *Calendula officinalis* [86%, Fig. 4.8] which exhibited the highest level of antioxidant activity at this concentration. Seemingly anomalous results were observed for *Calendula officinalis* and *Crataegus laevigata* berry which both exhibited over 70% antioxidant activity at 0.2mg/ml, 1mg/ml and 5mg/ml, but showed a significant decrease in activity at 0.5mg/ml [Figs. 4.8 and 4.9]. *Salix alba* also registered a similar pattern, with a decrease in antioxidant activity at 0.2mg/ml [Fig.4.11].

Silybum marianum was the only extract to show a consistent increase in antioxidant activity with increasing concentration [Fig. 4.6]. Since the flavanolignans comprising the silymarin complex were identified as the main flavonoids present in our crude plant extract (sec., 2.3.6) and it is this isolated complex that has mainly been used in biochemical and pharmacological studies (Morazoni and Bombanelli, 1995), the authentic silymarin compound was evaluated for the inhibition of linoleic acid peroxidation at 0.001mg/ml; 0.5mg/ml and 1mg/ml as a comparison with the crude plant extract. The commercial silymarin complex was pro-oxidant at 0.001mg/ml. Results achieved for 0.5mg/ml (30%) and 1mg/ml (62%) however, were considerably lower than those registered for the crude plant extract at the same concentrations i.e. 0.5mg/ml (79.5%) and 1mg/ml (85%), suggesting a possible loss of activity when the silymarin complex was used in isolation.

Propyl gallate and caffeic acid at a concentration of 50μM were used as positive controls to provide a frame of reference against which to assess the extracts' antioxidant activity. Both compounds at this concentration were approximately equivalent to 0.01mg/ml of extract. Propyl gallate and caffeic acid both inhibited linoleic acid peroxidation by 50% (n = the mean of three experiments) showing that when tested at an equivalent concentration, most of the crude plant extracts were at least as effective as the two reference compounds used as antioxidants.

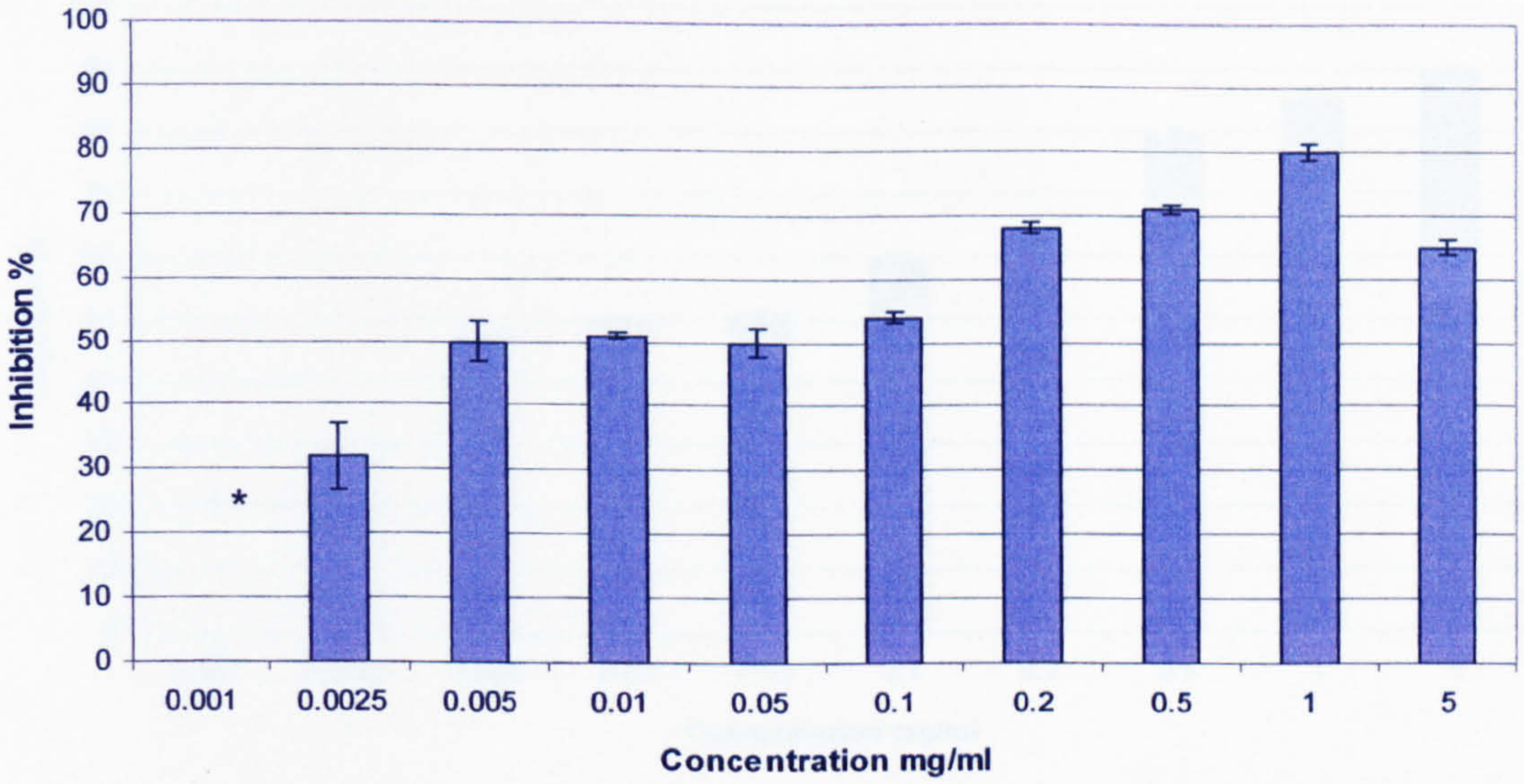


Figure 4.4. Evaluation of the inhibition of linoleic acid peroxidation by a range of concentrations of *Apium graveolens* (seed) crude plant extract (80%EtOH) in a lipid peroxide assay to measure antioxidant activity. Results are given as a percentage of the control (oxidizing substrate without antioxidant). Each column shows the mean ± SD of triplicate determinations except 0.001mg/ml which represent data from four experiments. Average coefficient of variation for concentrations (0.0025-5 mg/ml) = 4%

*** (0.001mg/ml) Experimental results were inconsistent and varied from pro-oxidant activity to 17% inhibition.**

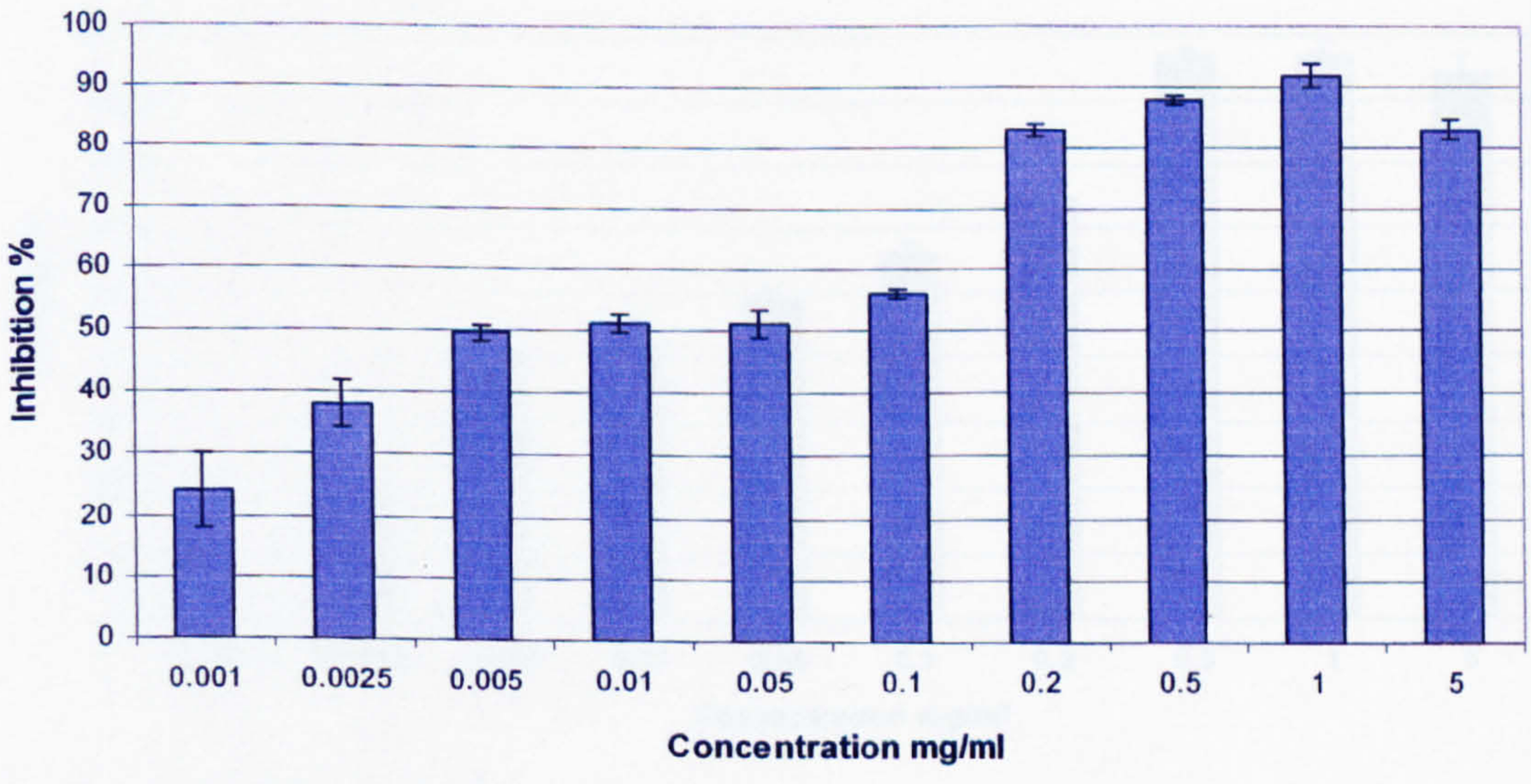


Figure 4.5. Evaluation of the inhibition of linoleic acid peroxidation by a range of concentrations of *Crataegus laevigata* (leaves and flowers) crude plant extract (80% EtOH) in a lipid peroxide assay to measure antioxidant activity. Results are given as a percentage of the control (oxidizing substrate without antioxidant). Each column shows the mean ± SD of triplicate determinations except 0.001mg/ml which shows the mean of four experiments.

Average coefficient of variation for concentrations (0.0025-5 mg/ml) = 2%

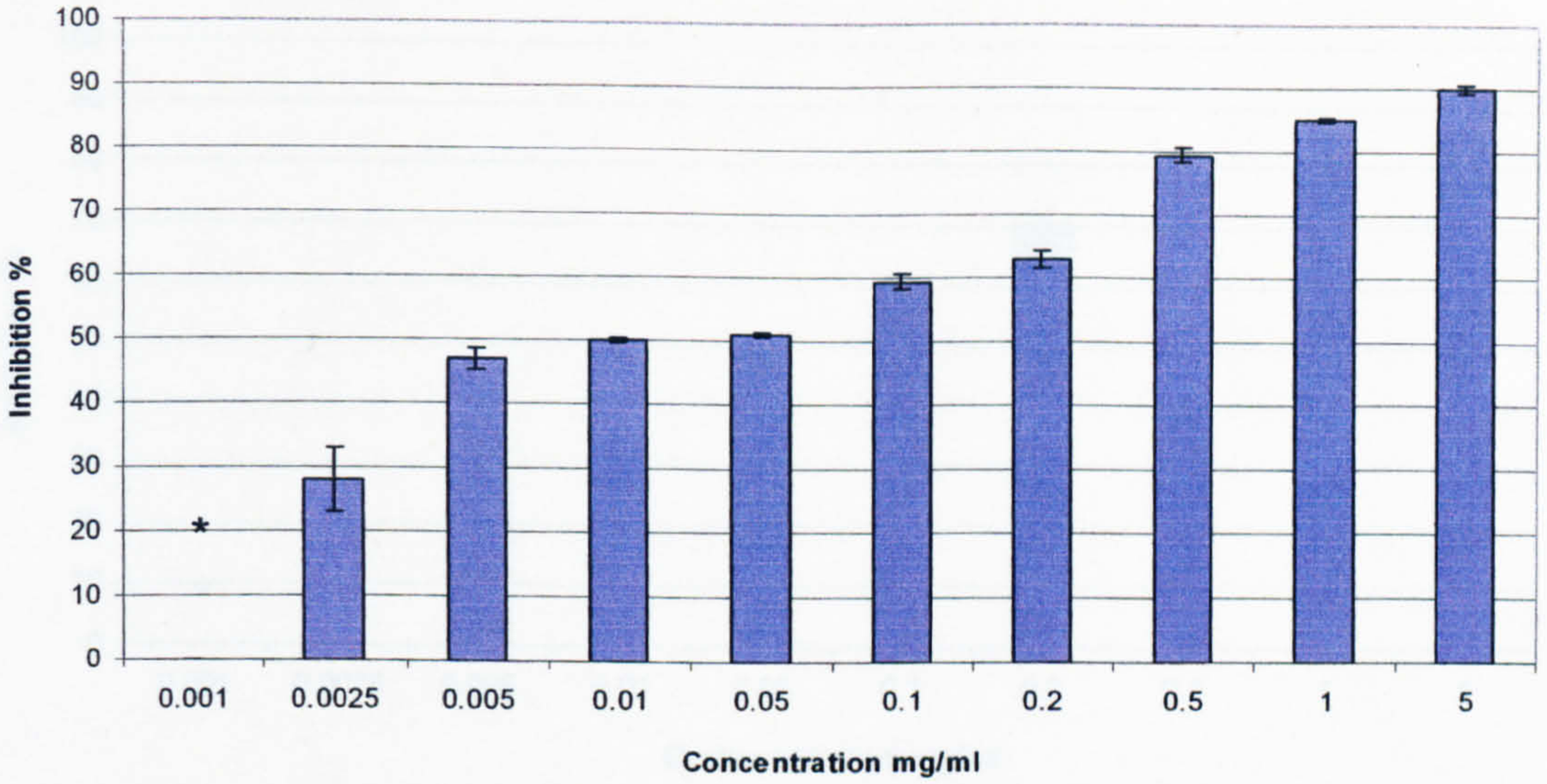


Figure 4.6. Evaluation of the inhibition of linoleic acid peroxidation by a range of concentrations of *Silybum marianum* (seed) crude plant extract (80%EtOH) in a lipid peroxide assay to measure antioxidant aactivity. Results are given as a percentage of the control (oxidizing substrate without antioxidant). Each column shows the mean ± SD of triplicate determinations except 0.001mg/ml which represents the data from four experiments. Average coefficient of variation for concentrations (0.0025-5 mg/ml) = 3%

*** (0.001mg/ml) Experimental results were inconsistent and varied from just pro-oxidant to 15% inhibition.**

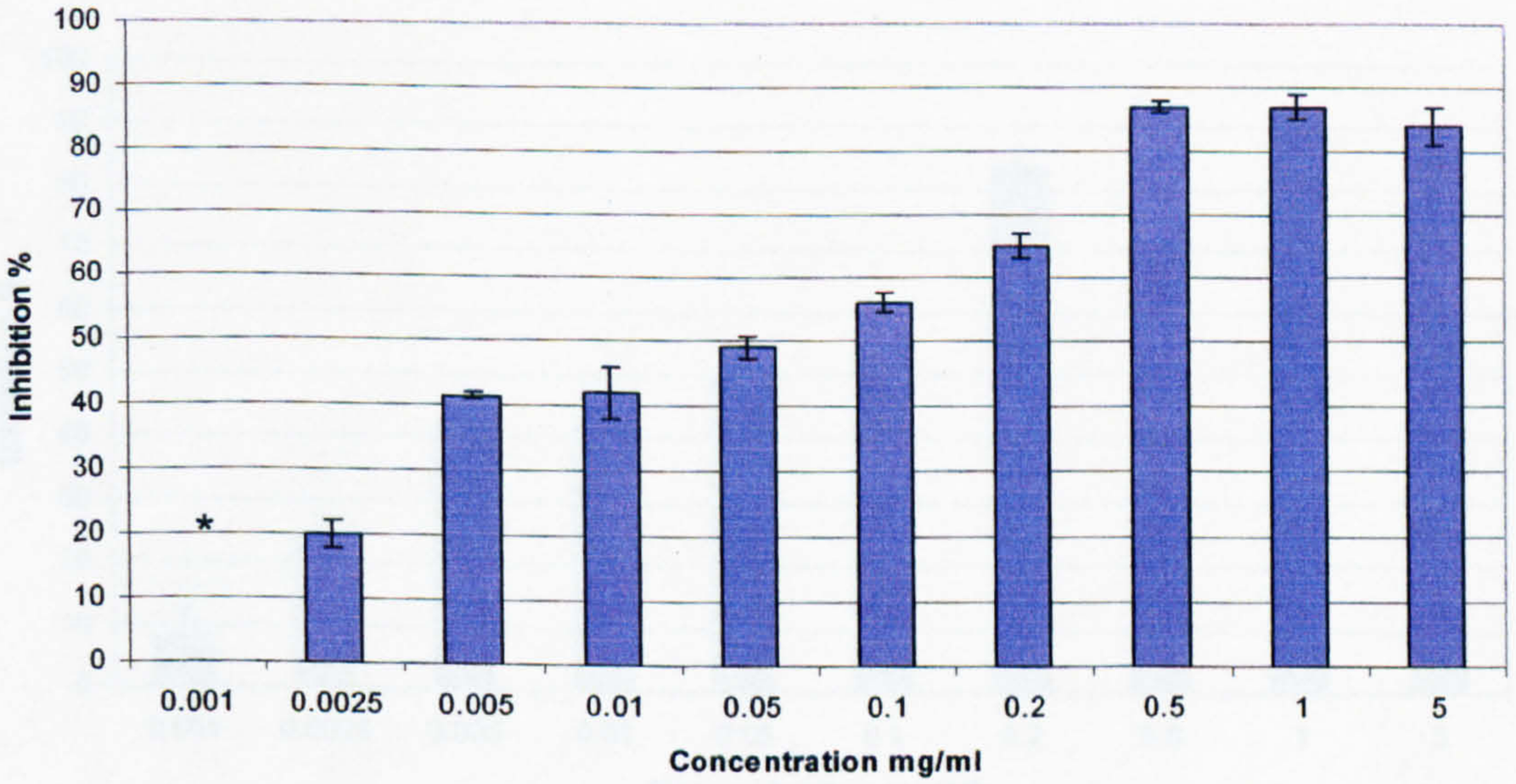


Figure 4.7. Evaluation of the inhibition of linoleic acid peroxidation by a range of concentrations of *Taraxacum officinale* (root) crude plant extract (80% EtOH) in a lipid peroxide assay to measure antioxidant activity. Results are given as a percentage of the control (oxidizing substrate without antioxidant). Each column shows the mean ± SD of triplicate determinations except 0.001mg/ml which represents the data from four experiments. Average coefficient of variation for concentrations (0.0025-5 mg/ml) = 4%

*** (0.001mg/ml) Experimental results were consistently pro-oxidant at this concentration.**

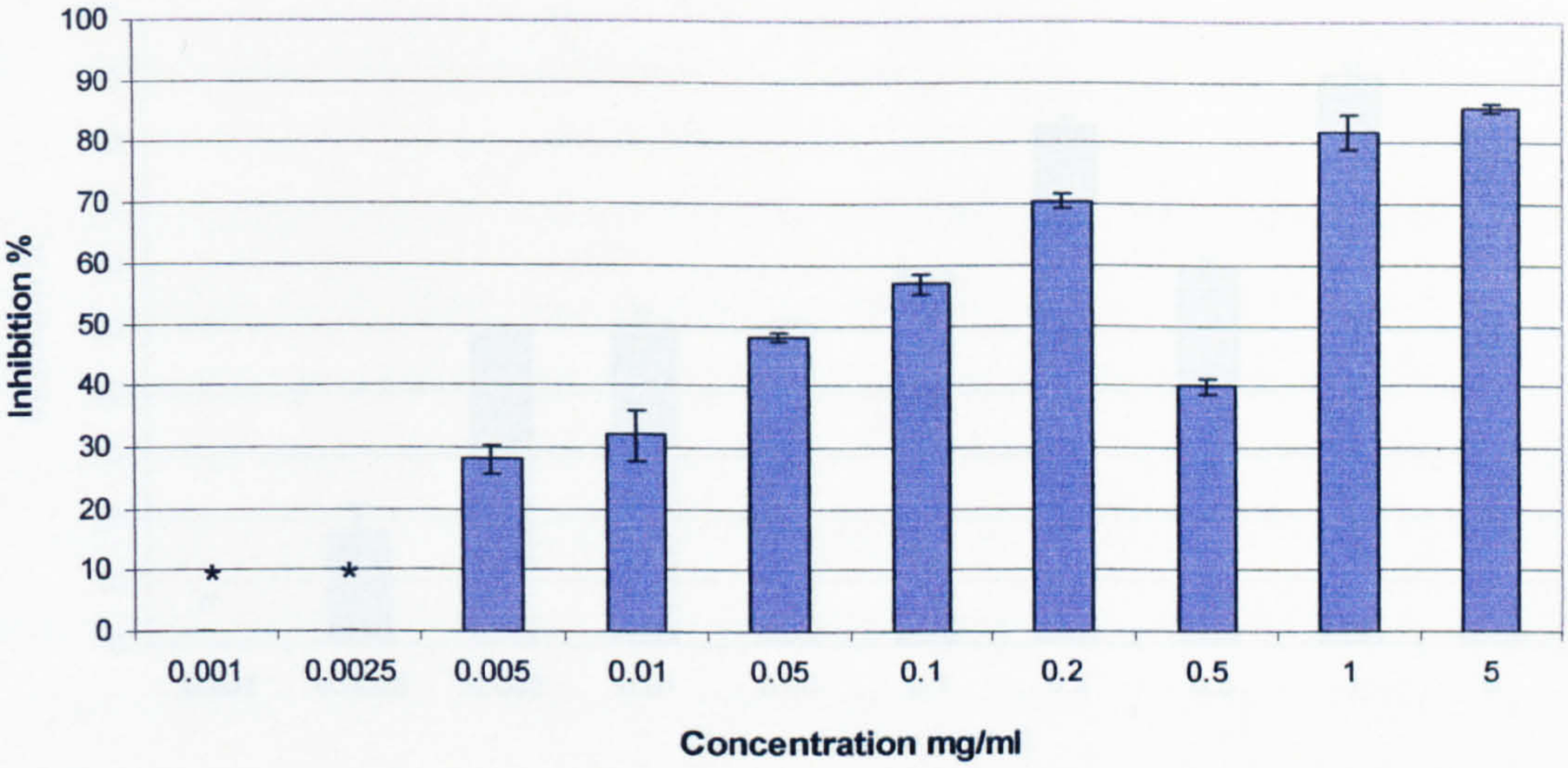


Figure 4.8. Evaluation of the inhibition of linoleic acid peroxidation by a range of concentrations of *Calendula officinalis* (flower) crude plant extract (80% EtOH) in a lipid peroxide assay to measure antioxidant activity. Results are given as a percentage of the control (oxidizing substrate without antioxidant). Each column shows the mean ± SD of triplicate determinations except 0.001mg/ml which represents the data from four experiments. Average coefficient of variation for concentrations (0.005-5 mg/ml) = 4.5%

*** Experimental results suggested consistent pro-oxidant activity at both 0.001mg/ml and 0.0025mg/ml.**

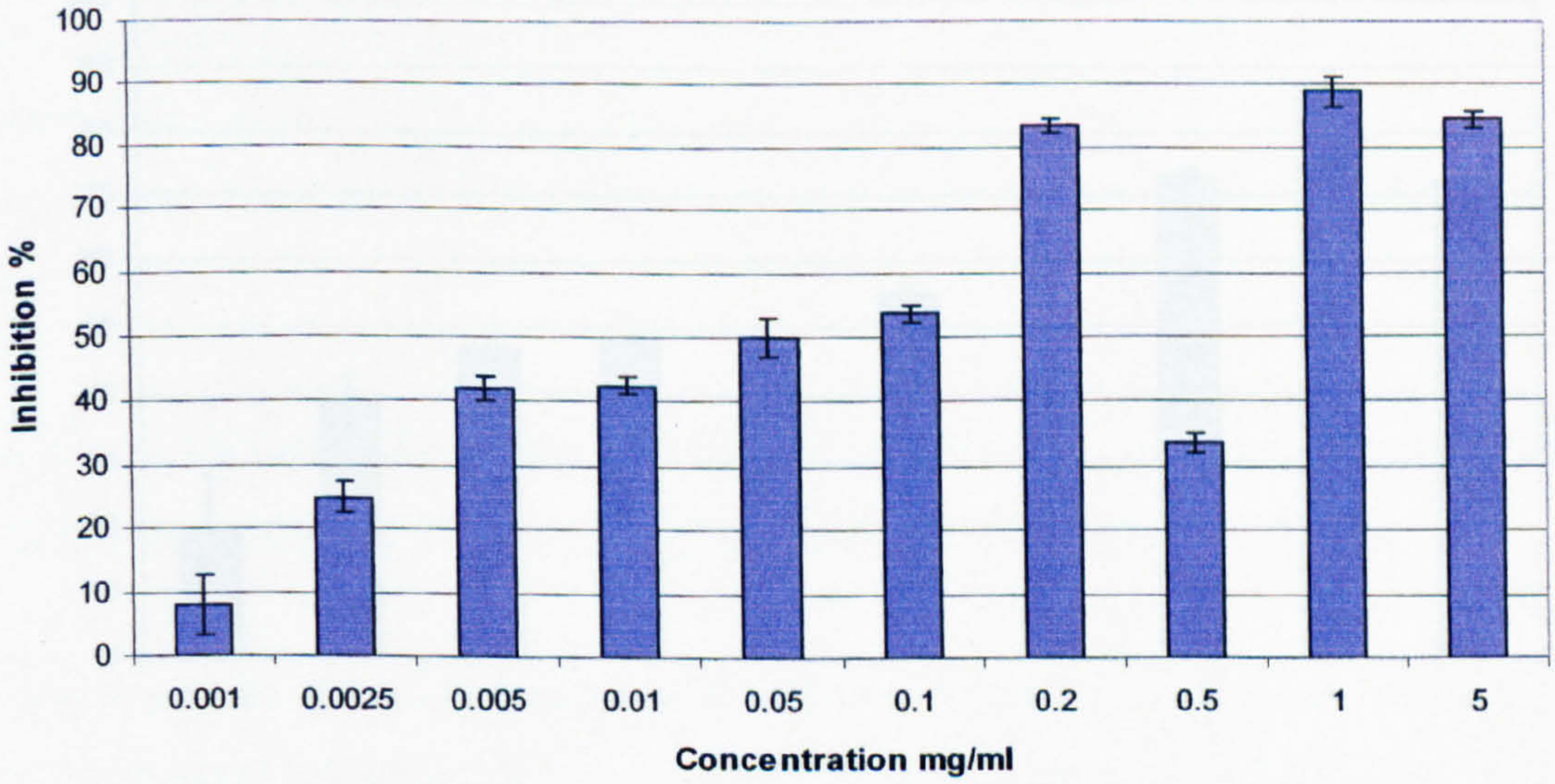


Figure 4.9. Evaluation of the inhibition of linoleic acid peroxidation by a range of concentrations of *Crataegus laevigata* (berry) crude plant extract (80% EtOH) in a lipid peroxide assay to measure antioxidant activity. Results are given as a percentage of the control (oxidizing substrate without antioxidant). Each column shows the mean ± SD of triplicate determinations except 0.001mg/ml which shows the mean of four experiments. Average coefficient of variation for concentrations (0.0025-5 mg/ml) = 4%

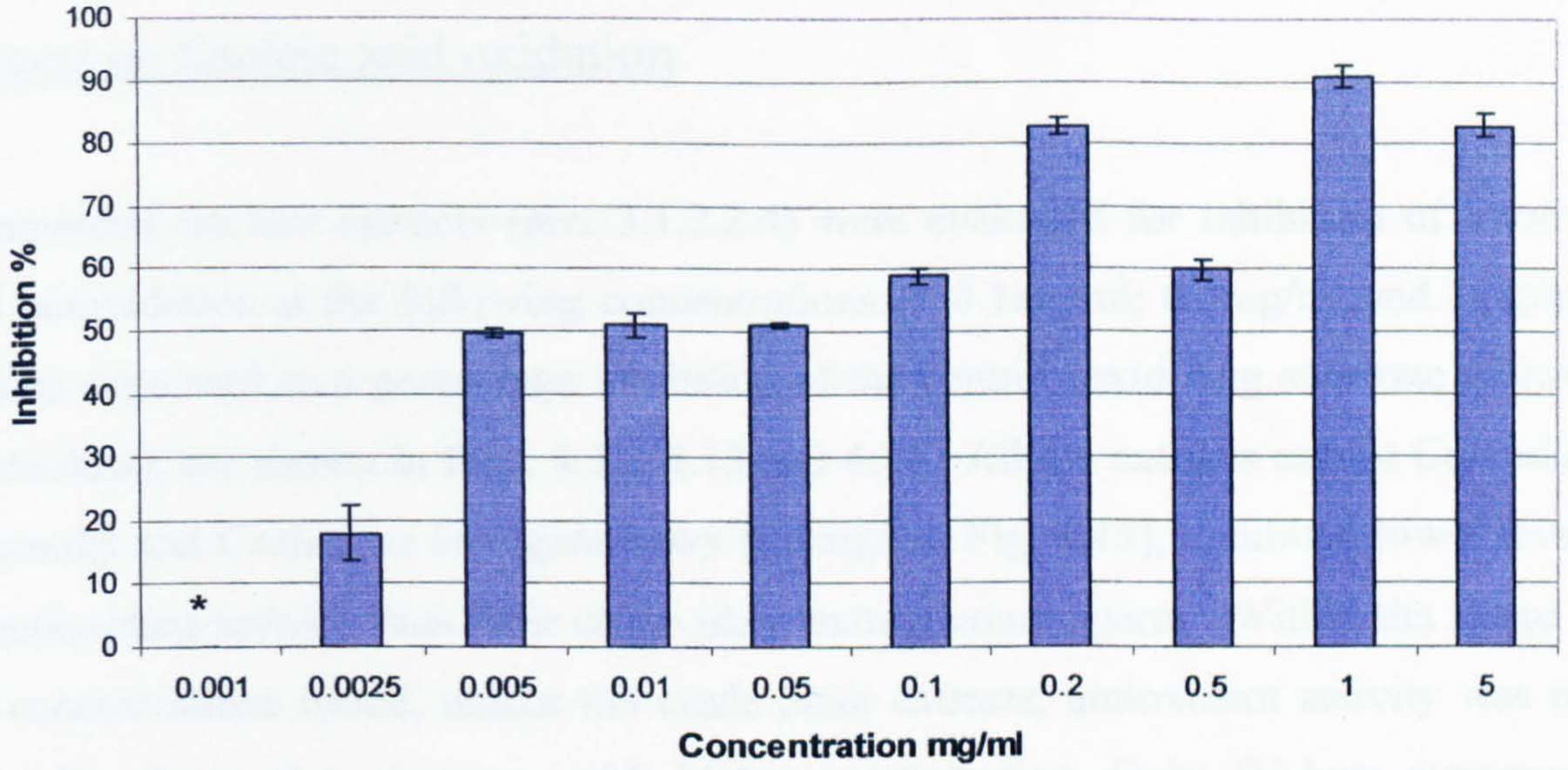


Figure 4.10. Evaluation of the inhibition of linoleic acid peroxidation by a range of concentrations of *Arctium lappa* (root) crude plant extract (80% EtOH) in a lipid peroxide assay to measure antioxidant activity. Results are given as a percentage of the control (oxidizing substrate without antioxidant). Each column shows the mean ± SD of triplicate determinations except 0.001mg/ml which represents the data from four experiments. Average coefficient of variation for concentrations (0.0025-5 mg/ml) = 4%

*** (0.001mg/ml) Experimental results were inconsistent and activity varied from pro-oxidant to inhibition (10%).**

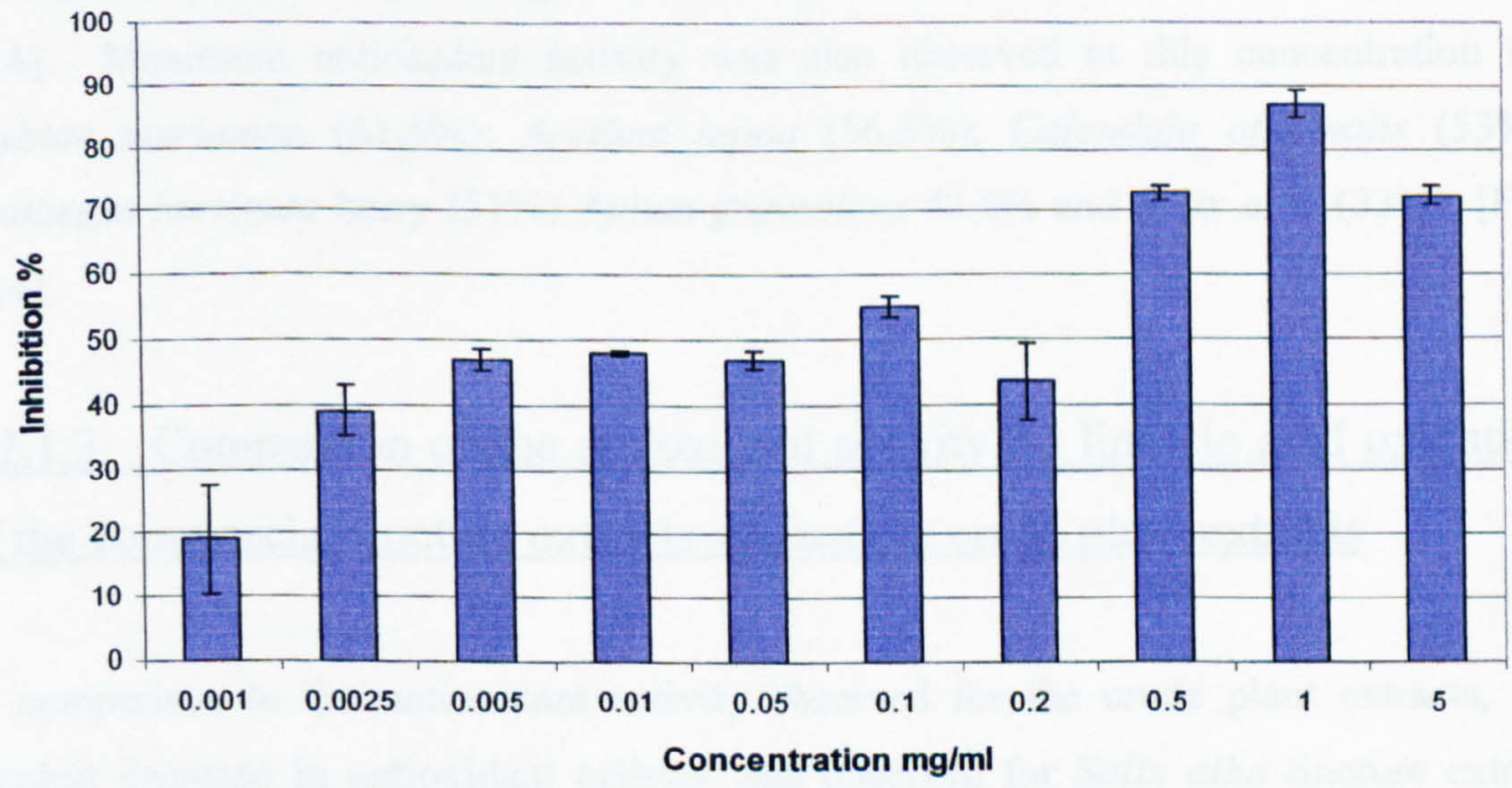


Figure 4.11. Evaluation of the inhibition of linoleic acid peroxidation by a range of concentrations of *Salix alba* (bark) crude plant extract (80% EtOH) in a lipid peroxide assay to measure antioxidant activity. Results are given as a percentage of the control (oxidizing substrate without antioxidant). Each column shows the mean ± SD of triplicate determinations except 0.001mg/ml which shows the mean of four experiments. Average coefficient of variation for concentrations (0.0025-5 mg/ml) = 4.5%

4.2.1.2 Results of the antioxidant action of the commercial tincture extracts on linoleic acid oxidation

Commercial tincture extracts (sec. 3.1.2.2.4) were evaluated for inhibition of linoleic acid peroxidation at the following concentrations:- 0.1mg/ml; 0.5mg/ml and 1mg/ml. Results expressed as a percentage inhibition of the control (oxidizing substrate without antioxidant), are shown in Figs. 4.12; 4.13 and 4.14. All the extracts except *Calendula officinalis* and *Crataegus laevigata* berry [0.5mg/ml, Fig. 4.13], exhibited lower levels of antioxidant activity than their crude plant extract counterparts. Within the range of the concentrations tested, unlike the crude plant extracts, antioxidant activity was not generally observed to increase with higher concentration. Only *Silybum marianum*, *Taraxacum officinale*, *Crataegus laevigata* (leaves and flowers) and *Arctium lappa* displayed a consistent increase in antioxidant activity with higher concentration [Figs. 4.12 - 4.14]. The antioxidant activity of *Salix alba* and *Apium graveolens* was almost the same for all three concentrations [Figs. 4.12 – 4.14], also, similar results were observed for *Calendula officinalis* at 0.1mg/ml [Fig. 4.12] and 0.5mg/ml [Fig. 4.13] and *Crataegus laevigata* berry at 0.5mg/m [Fig. 4.13] and 1mg/ml [Fig. 4.14]. The strongest antioxidant activity was exhibited by both *Crataegus laevigata* leaves and flowers (65%) and *Taraxacum officinalis* (64%) at a final concentration of 1mg/ml [Fig. 4.14]. Maximum antioxidant activity was also observed at this concentration for *Silybum marianum* (61.5%); *Arctium lappa* (56.5%); *Calendula officinalis* (53%); *Crataegus laevigata* berry (51%) *Apium graveolens* 43.5% and *Salix alba* (33%), [Fig. 4.14].

4.2.1.3 Comparison of the antioxidant activity on linoleic acid oxidation of the commercial tincture extracts against the crude plant extracts

In comparison to the antioxidant activity observed for the crude plant extracts, the greatest decrease in antioxidant activity was observed for *Salix alba* tincture extract (final concentration 1mg/ml). This exhibited only 33% inhibition, 54% less than the 87% inhibition recorded for its counterpart crude plant extract, [Fig. 4.14]. Compared to the crude plant extracts, a significant reduction in antioxidant activity was also observed for the following tincture extracts:- a decrease of 44% and 35% for *Arctium lappa* at concentrations of 0.1mg/ml [Fig. 4.12] and 1mg/ml [Fig. 4.14] respectively;

40% for *Taraxacum officinalis* [0.5mg/ml – Fig. 4.13]; 38% for *Crataegus laevigata* leaves and flowers [0.5mg/ml – Fig. 4.13]; 37.5% for *Crataegus laevigata* berry [1mg/ml – Fig. 4.14]; 30% and 36.5% for *Apium graveolens* at concentrations of 0.5mg/ml [Fig. 4.13] and 1mg/ml [Fig. 4.14] respectively and 31.5% and 34% for *Silybum marianum* at concentrations of 0.1mg/ml [Fig. 4.12] and 0.5mg/ml respectively [Fig. 4.13]. Excluding the preceding data, the mean average decrease in antioxidant activity of the remaining tincture extracts compared to the crude plant extracts over the three concentrations tested was approximately 20%. From this data it is apparent that to achieve a pharmacological effect equal to that of the crude plant extract a much higher dose of tincture may need to be administered.

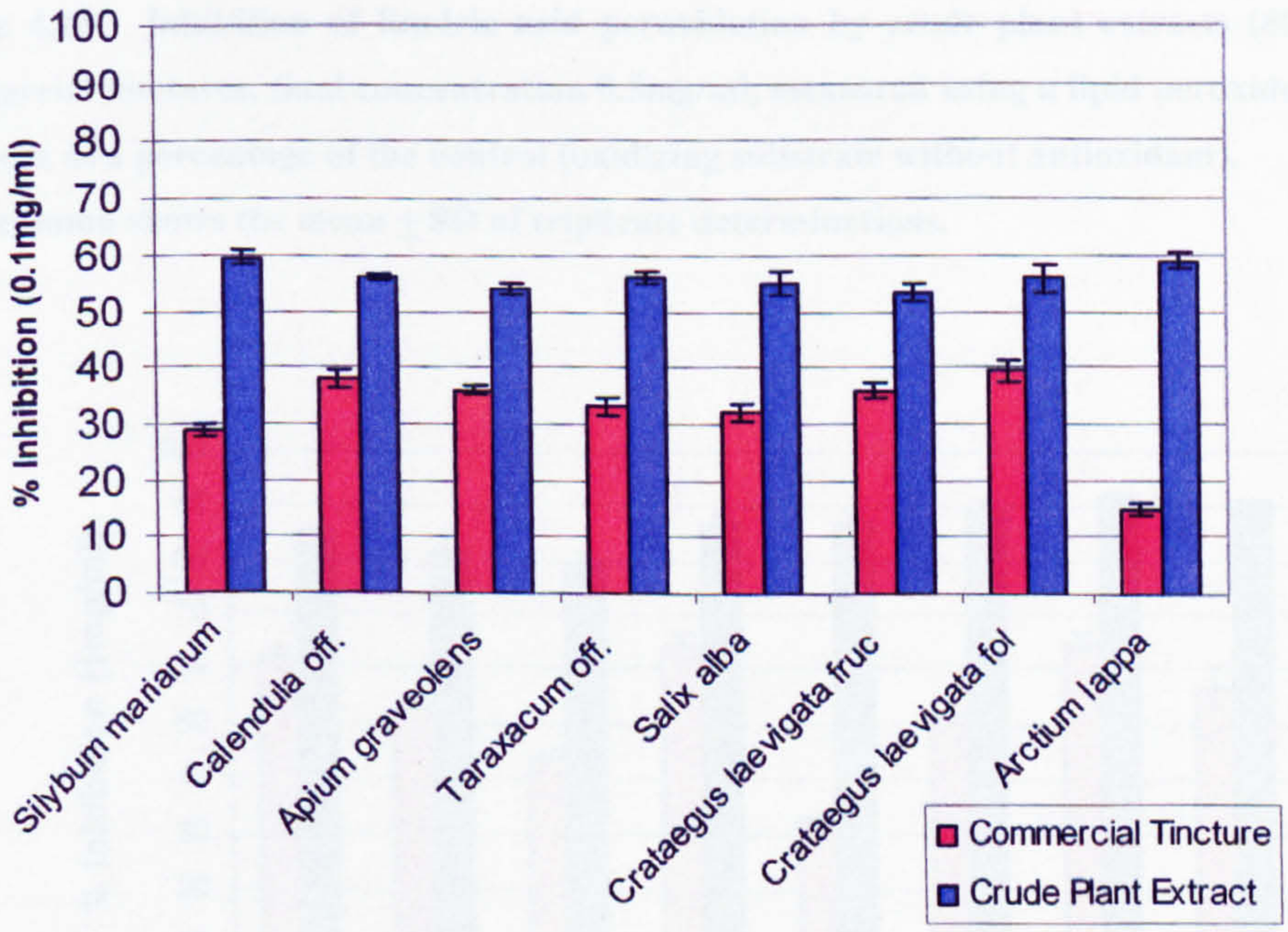


Figure 4.12 Inhibition of linoleic acid peroxidation by crude plant extracts (80% EtOH) and commercial tinctures, final concentration 0.1mg/ml, measured using a lipid peroxide assay. Results are given as a percentage of the control (oxidizing substrate without antioxidant). Each column shows the mean ± SD of triplicate determinations.

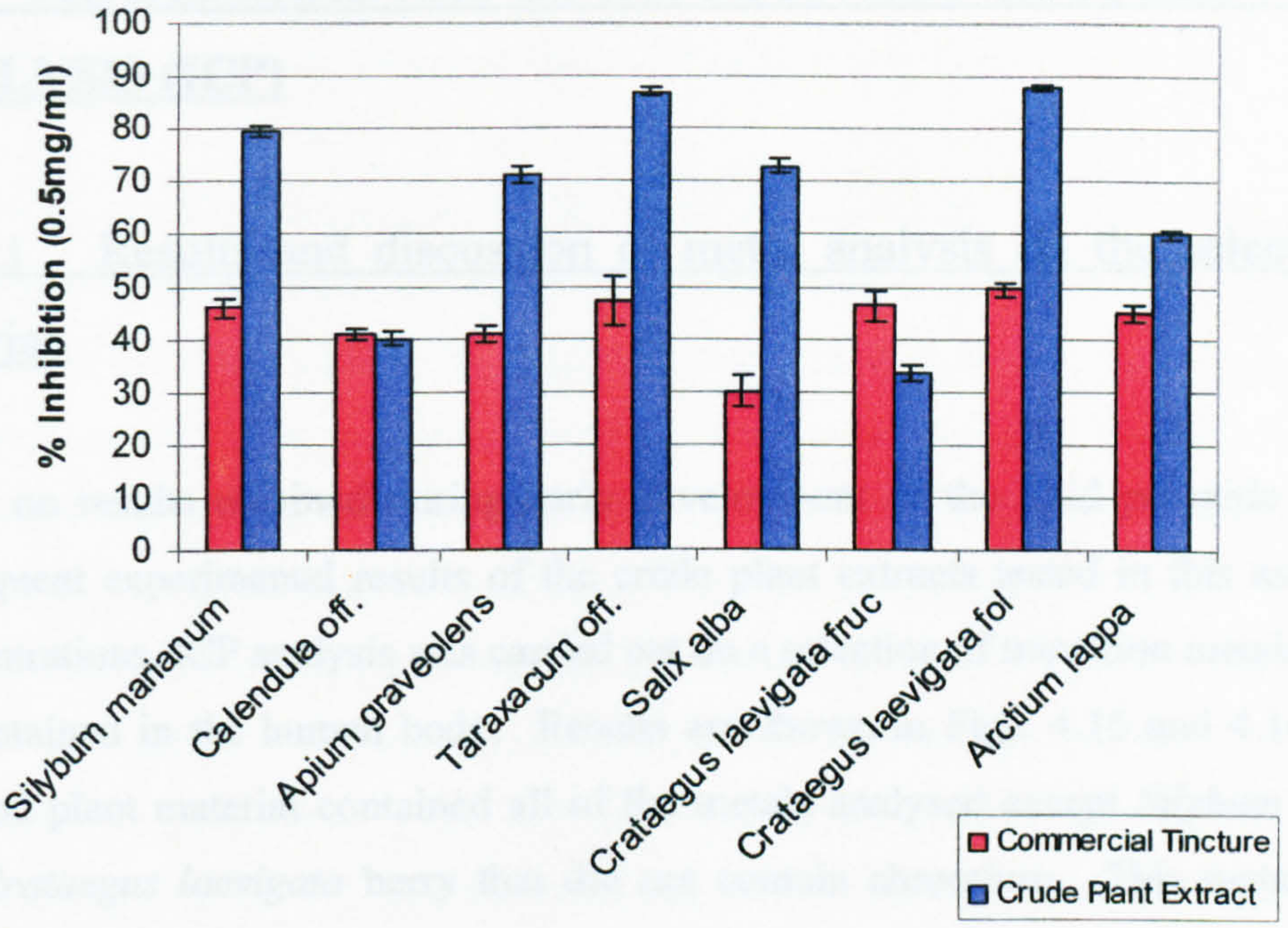


Figure 4.13 Inhibition of linoleic acid peroxidation by crude plant extracts (80% EtOH) and commercial tinctures, final concentration 0.5mg/ml, measured using a lipid peroxide assay. Results are given as a percentage of the control (oxidizing substrate without antioxidant).
Each column shows the mean ± SD of triplicate determinations.

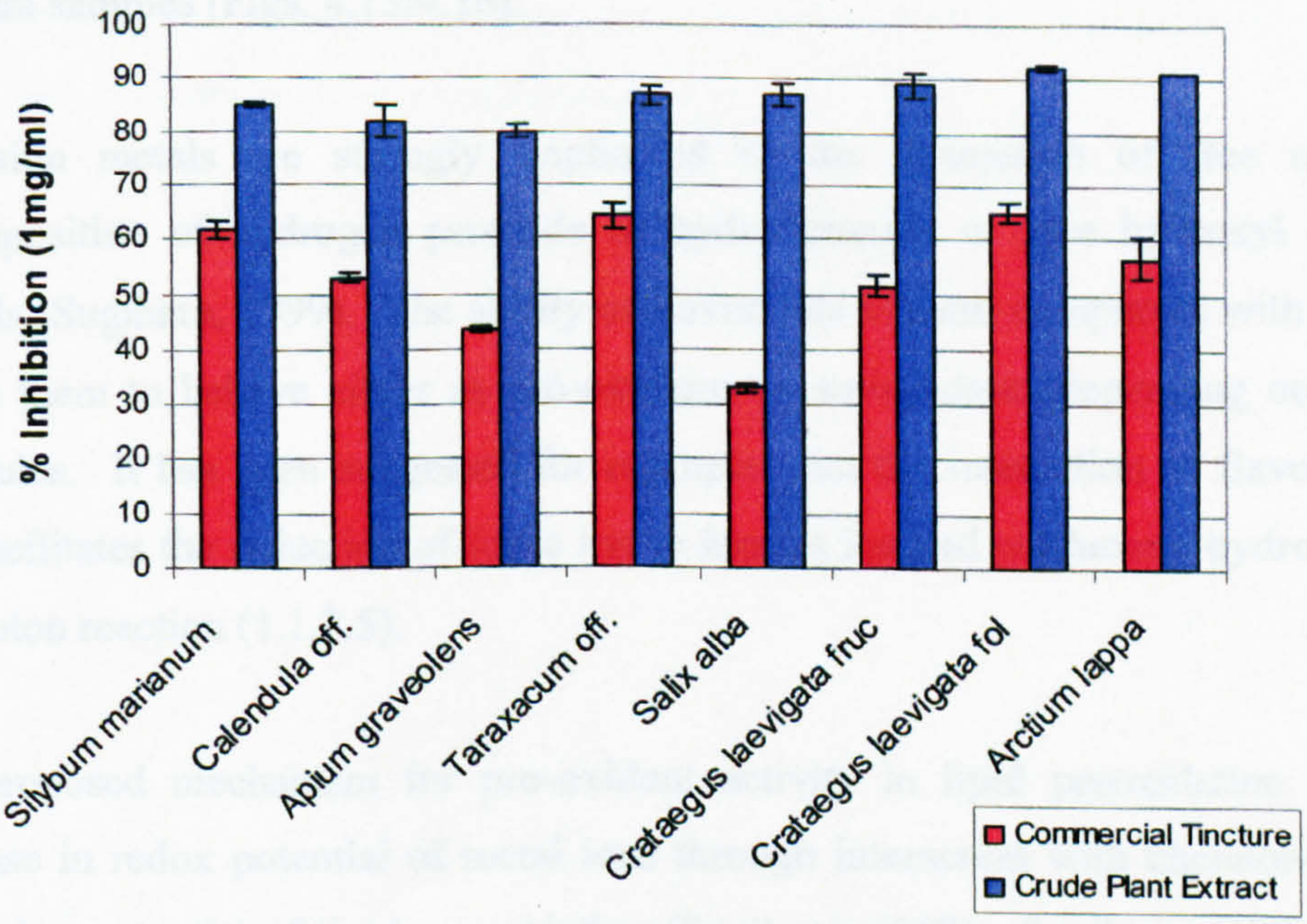


Figure 4.14 Inhibition of linoleic acid peroxidation by crude plant extracts (80% EtOH) and commercial tinctures, final concentration 1mg/ml, measured using a lipid peroxide assay. Results are given as a percentage of the control (oxidizing substrate without antioxidant).
Each column shows the mean ± SD of triplicate determinations.

4.2.2 METAL ANALYSIS BY INDUCTIVELY COUPLED PLASMA ANALYSIS (ICP)

4.2.2.1 Results and discussion of metal analysis on the selected plant material

Based on results obtained during early development of the lipid peroxide assay and subsequent experimental results of the crude plant extracts tested in this assay at low concentrations, ICP analysis was carried out on a selection of transition metals known to be contained in the human body. Results are shown in Figs. 4.15 and 4.16. All the selected plant material contained all of the metals analysed except *Silybum marianum* and *Crataegus laevigata* berry that did not contain chromium. This metal was also detected in all the other plant samples, at much lower levels (less than 6μg/g), than all the other metals [Fig. 4.15]. Iron, copper and cobalt all previously shown in studies to catalyse pro-oxidant reactions (Cao, 1997) were present in all the samples in varying amounts [Fig. 4.16]. Magnesium, zinc and selenium, essential co-factors of some enzymes with antioxidant function (sections 1.1.3 and 3.2.3) that are known to contribute to our endogenous antioxidant defence mechanisms, were also present in all the plant samples [Figs. 4.15/4.16].

Transition metals are strongly implicated in the generation of free radicals by decomposition of hydrogen peroxide or hydroperoxide to give hydroxyl or alkoxyl radicals (Sugihara, 1999). The ability of flavonoids to form complexes with metal ions causes them to behave either as pro-oxidants or antioxidants depending on the target molecules. It has been suggested for example, that the interaction of flavonoids with iron facilitates the reduction of ferric ion to ferrous ion and produces a hydroxyl radical by Fenton reaction (1.1.2.5).

The proposed mechanism for pro-oxidant activity in lipid peroxidation is that the decrease in redox potential of metal ions through interaction with chelators incurs the catalyzing potential of lipid peroxidation (Sugihara, 1999). Sugihara, (1999) observed that certain flavones and flavonols changed from antioxidant to pro-oxidant activity when iron concentrations increased in linolenic acid loaded hepatocytes. When the flavonoid concentration increased however, antioxidant effects at high iron

concentrations were maintained suggesting that chelation alters the redox potential of the metal complexes, depending on the molar ratio of the flavonoid to metal ion.

Because the exact composition of the extracts, which not only contains one but a multiplicity of compounds, and the profile of metal ion concentrations are unknown, no conclusions can be reached in regard to any metal-catalysed pro-oxidant activity exhibited in the lipid peroxide assay at low concentrations. Some studies have established that certain flavonoids *in vitro* can exert pro-oxidant or antioxidant effects depending on the concentration (Cos, 2000; 2001) and that only trace amounts of transition metals are needed to catalyse the Fenton reaction (Halliwell, 1987). A pro-oxidant mechanism involving transition metal ions and flavonoids should therefore be considered a possibility. An electron paramagnetic resonance (EPR) study of flavonoids by Cos (2001), demonstrated that the pro-oxidant activity of quercetin at a concentration of 500μM was decreased significantly when rutin was added to the quercetin at the same concentration. This illustrates the difficulties in analysis that exist in the comparison of studies using single compounds against multi-compound crude plant extracts present.

It is appreciated that there are differences in the extraction regimes used for ICP and the remainder of the experimental programme. The difficulties alcoholic extracts presented for ICP however made this approach necessary.

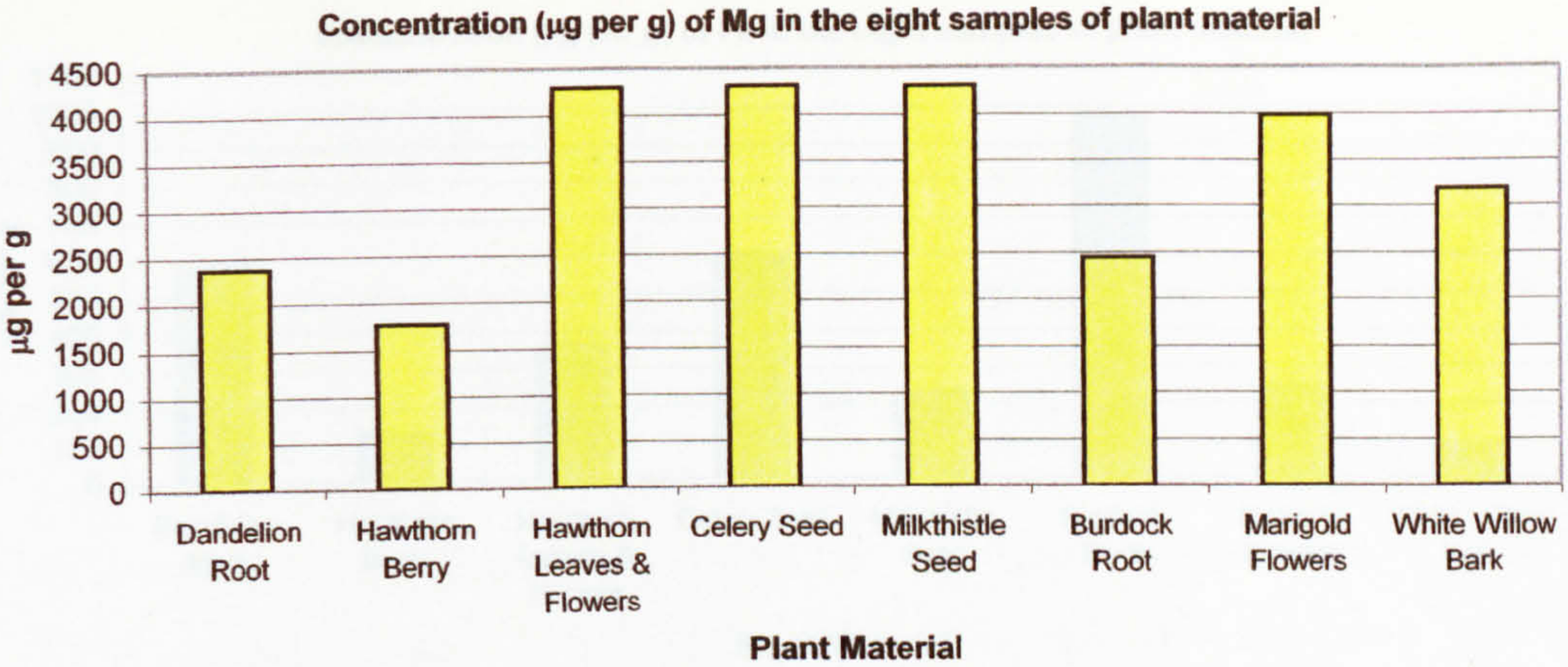


Figure. 4.15. Results of ICP metal analysis showing the concentration (µg / g) of Mg, Cr, Ni and Zn in the selected plant samples measured against 1ppm metal standards. (Differences in scale)

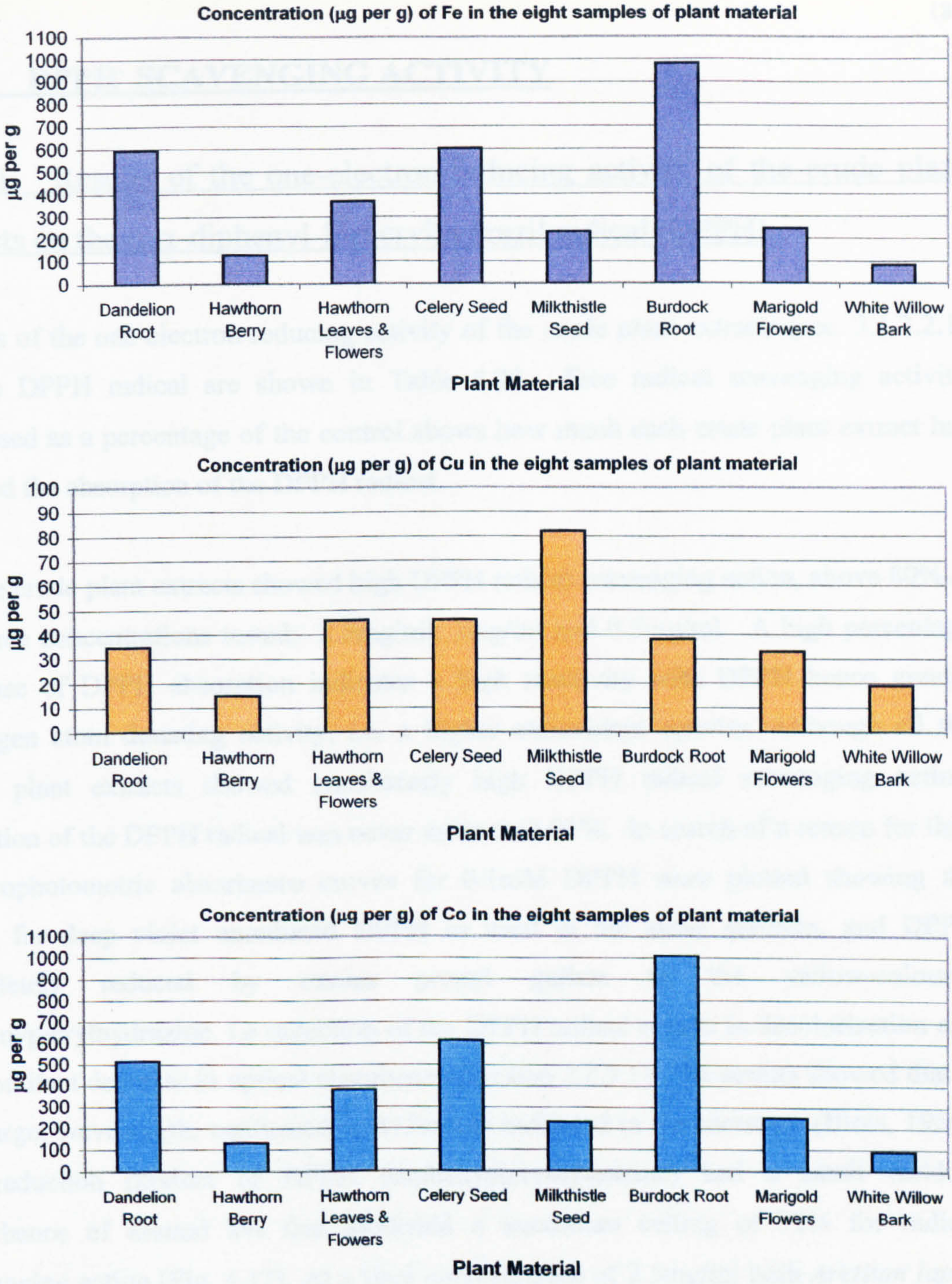


Figure 4.16. Results of ICP metal analysis showing the concentration (µg / g) of Fe, Cu, Co and Se in the selected plant samples measured against 1ppm metal standards. (Differences in scale)

4.2.3 DPPH· SCAVENGING ACTIVITY

4.2.3.1 Results of the one-electron reducing activity of the crude plant extracts on the α,α-diphenyl-β-picrylhydrazil radical (DPPH)

Results of the one-electron reducing activity of the crude plant extracts (sec. 3.1.2.2.1) on the DPPH radical are shown in Table 4.20. Free radical scavenging activity expressed as a percentage of the control shows how much each crude plant extract has reduced the absorption of the DPPH radical.

All the crude plant extracts showed high DPPH radical scavenging action, above 80% at the three concentrations tested:- 2.5mg/ml; 1mg/ml and 0.5mg/ml. A high percentage decrease of DPPH absorption indicates a high reactivity with DPPH hence greater hydrogen atom donating activity, i.e. a higher antioxidant activity. Although all the crude plant extracts showed consistently high DPPH radical scavenging action, reduction of the DPPH radical was never more than 91%. In search of a reason for this, spectrophotometric absorbance curves for 0.1mM DPPH were plotted showing the curve for deep violet unreduced DPPH as used in the assay solution, and DPPH completely reduced by excess propyl gallate to the yellow-coloured diphenypicrylhydrazine, i.e reduction of the DPPH radical results in decolorization and concomitant decrease in optical absorbency (section 2.2.3.1). The results showed that at the target wavelength, confirmed as 517nm as indicated in the literature (Blois, 1958), the reduction product of DPPH (diphenypicrylhydrazine) had a small residual absorbance of around 8% that produced a maximum ceiling of 92% for radical scavenging action [Fig. 4.17]. At a final concentration of 2.5mg/ml both *Arctium lappa* root and *Crataegus laevigata* leaves and flowers reached this ceiling.

Extracts were also tested at a concentration of 25 mg/ml but at this concentration the reaction mixture for some plant extracts was too turbid to take meaningful absorbance readings.

All crude plant extracts at the three concentrations tested showed a higher or comparable DPPH radical scavenging action to the reference compounds propyl gallate, a known antioxidant used for preserving oils and fats, and caffeic acid, a natural antioxidant compound found in plants (Table 4.20). Strong antioxidant activity on the

DPPH radical by propyl gallate and caffeic acid have been previously demonstrated by Yan et al, (1998).

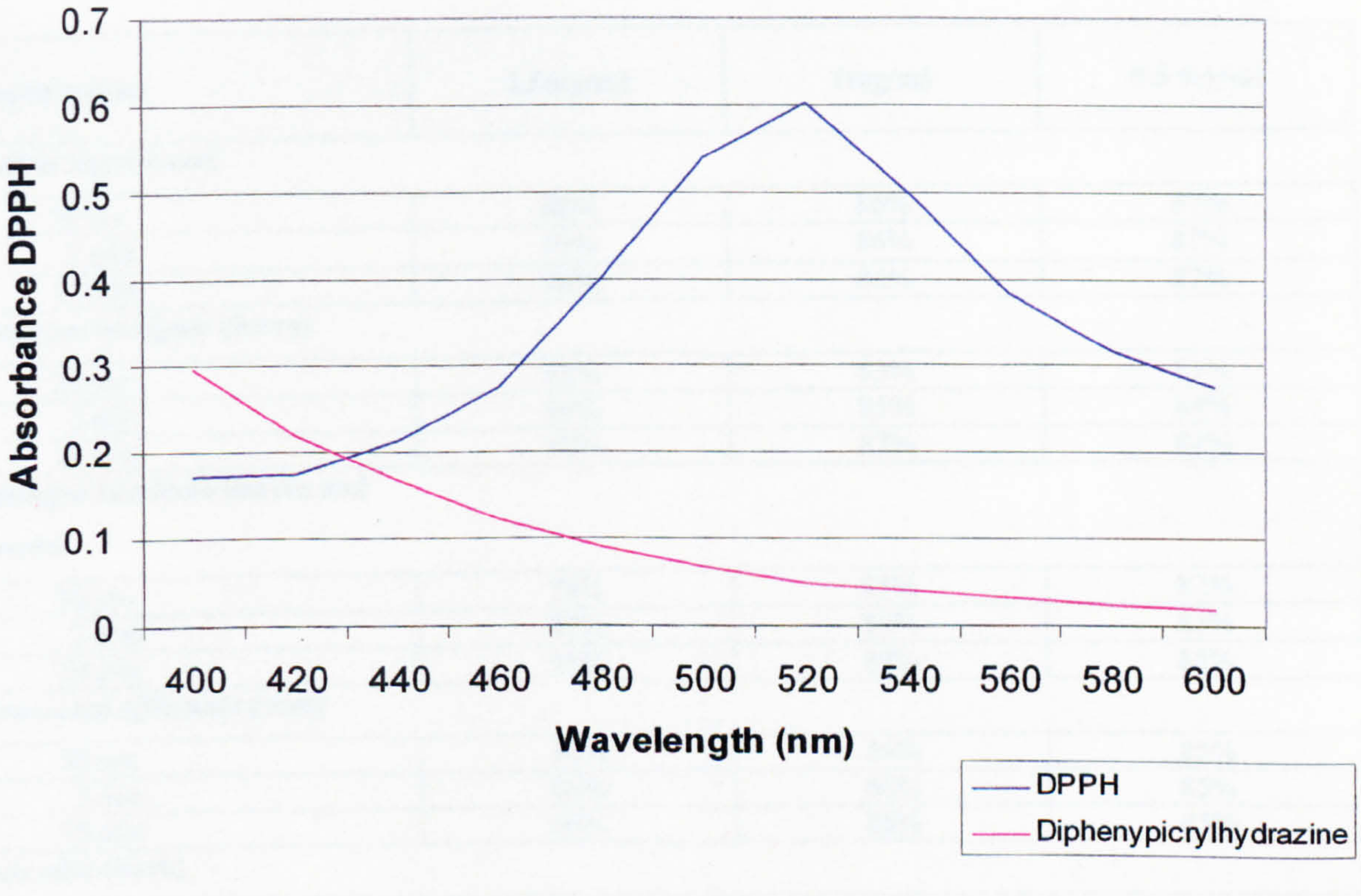


Figure 4.17. Spectrophotometric absorbance curves of a 0.1mM ethanolic solution of DPPH, unreduced (deep purple) and completely reduced by antioxidants to diphenypicrylhydrazine (pale yellow), showing that at a wavelength of 517nm, the confirmed absorption maximum (Blois, 1958), the reduction product diphenypicrylhydrazine had a residual percentage of approximately 8% that produced a ceiling of 92% for DPPH radical scavenging action.

Table 4.20 Evaluation of the Free Radical Scavenging Activity (FRSA) of the selected crude plant extracts on the 1,1-diphenyl-2-picrylhydrazil free radical (DPPH). Reduction of the DPPH radical was measured by reading absorbance at 517 nm. A high percentage decrease of the DPPH radical indicates high reactivity with DPPH.
Each concentration shows the mean of duplicate determinations.

Concentration	2.5mg/ml	1mg/ml	0.5 mg/ml
***Arctium lappa* (root)**			
30 sec	88%	86%	87%
2 min.	89%	86%	87%
10 min.	90%	86%	87%
***Crataegus laevigata* (berry)**			
30 sec	83%	83%	83%
2 min	86%	85%	84%
10 min	88%	87%	84%
***Crataegus laevigata* (leaves and flowers)**			
30 sec	79%	85%	82%
2 min	87%	85%	85%
10 min	91%	89%	85%
***Taraxacum officinale* (root)**			
30 sec	86%	86%	85%
2 min	86%	86%	85%
10 min	86%	86%	85%
***Salix alba* (bark)**			
30 sec	81%	85%	86%
2 min	84%	86%	86%
10 min	87%	87%	86%
***Apium graveolens* (seed)**			
30 sec	82%	85%	83%
2 min	84%	86%	83%
10 min	85%	87%	83%
***Calendula officinalis* (flower)**			
30 sec	81%	87%	84%
2 min	82%	87%	86%
10 min	84%	87%	86%
***Silybum marianum* (seed)**			
30 sec	69%	73%	78%
2 min	72%	76%	79%
10 min	79%	80%	81%
Propyl gallate 100μM final concentration:- 84%			
Caffeic acid 100μM final concentration:- 86%			

4.2.3.2 DPPH radical-scavenging activities of crude plant extracts (80% EtOH) expressed as an IC_{50} value

Since the DPPH assay was used as an initial screening process to test the propensity of the crude plant extracts to any possible antioxidant activity, it was impossible to predict such high results for all the crude plant extracts that gave almost the same results for all the concentrations initially tested. The antioxidant activity of each extract was therefore expressed in terms of IC_{50} value (concentration in mg/ml or μM required to inhibit DPPH radical formation by 50%) that would facilitate a more accurate comparison of antioxidant activity. Results are shown in Table 4.21. The most potent DPPH radical scavengers were *Salix alba* (bark) and *Crataegus laevigata* (leaves and flowers) which showed IC_{50} values of 0.003mg/ml and 0.006mg/ml. *Arctium lappa* root also showed strong scavenging effects with an IC_{50} value of 0.017mg/ml. After these plants the order of potency was *Apium graveolens* (seed), *Crataegus laevigata* (berry) and *Silybum marianum* (seed) with IC_{50} values of 0.025mg/ml and *Taraxacum officinale* (root) with an IC_{50} value of 0.054mg/ml. *Calendula officinalis* (flower), although still an effective DPPH scavenger, had the highest IC_{50} value of 0.102mg/ml.

4.2.3.3 DPPH radical-scavenging activities of commercial tincture extracts expressed as an IC_{50} value

DPPH scavenging activities of commercial tincture extracts (3.1.2.2.4) are shown in Table 4.21. Most of the commercial tincture extracts, except *Calendula officinalis* (flower), in comparison to their crude plant extract counterparts showed reduced DPPH radical scavenging activity that resulted in higher IC_{50} values [Table 4.21; Fig. 4.18]. The most potent DPPH radical scavengers were *Calendula officinalis* (flowers) and *Salix alba* (bark) with IC_{50} values of 0.005mg/ml and 0.007mg/ml. *Crataegus laevigata* (berry), *Crataegus laevigata* (leaves and flowers) and *Apium graveolens* (seed) were less potent with IC_{50} values of 0.025, 0.035 and 0.05mg/ml respectively. Thereafter the order of potency was *Silybum marianum* (seed), *Taraxacum officinale* (root) and *Arctium lappa* (root) with IC_{50} values of 0.17, 0.23 and 0.81mg/ml. To achieve 50% inhibition of the DPPH radical, considerably higher concentrations of tincture extract than crude plant extract were required for *Silybum marianum* (seed), *Taraxacum*

officinale (root) and *Arctium lappa* (root) reflecting the significant differences in phytochemical profiles of the two types of extract reported in sections 4.1.2.1.3; 4.1.2.2.2 and 4.1.2.3.2.

Table 4.21 DPPH radical scavenging activity of the crude plant extracts and commercial tincture extracts expressed as the IC_{50} value (mg/ml) graphically determined by linear regression analysis.

PLANT AND PART USED	CRUDE PLANT EXTRACT (80% EtOH) IC_{50} value (mg/ml)	COMMERCIAL TINCTURE EXTRACT IC_{50} value (mg/ml)
Apium graveolens (seed)	0.025	0.05
Taraxacum officinale (root)	0.054	0.23
Arctium lappa (root)	0.017	0.81
Crataegus laevigata (leaves and flowers)	0.006	0.035
Crataegus laevigata (berry)	0.025	0.025
Silybum marianum (seed)	0.025	0.17
Salix alba (bark)	0.003	0.007
Calendula officinalis (flower)	0.102	0.005
Propyl gallate	0.00032 mg/ml	
Caffeic acid	0.00061 mg/ml	

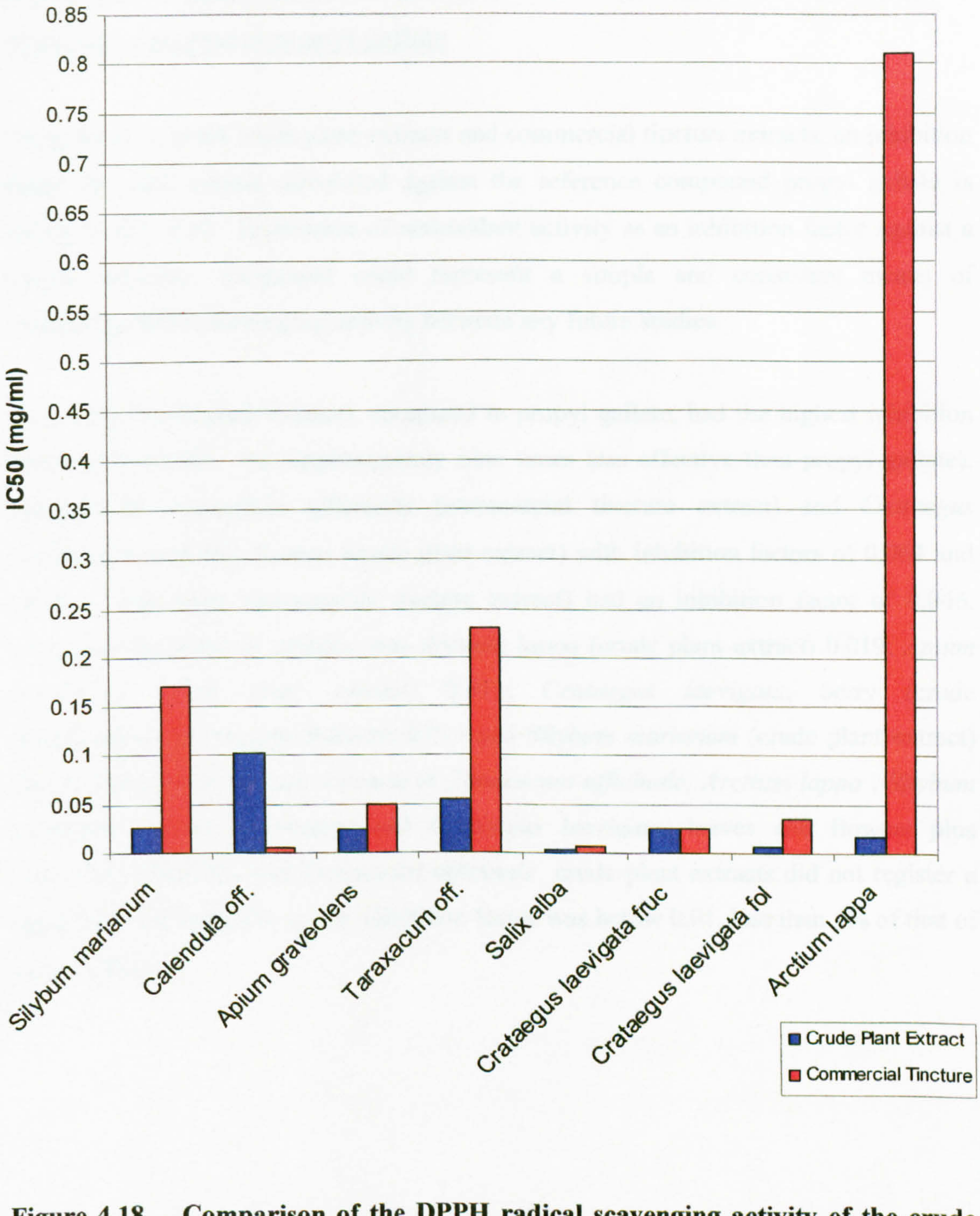


Figure 4.18. Comparison of the DPPH radical scavenging activity of the crude plant extracts and commercial tincture extracts expressed as the IC_{50} value (mg/ml).

4.2.3.4 DPPH radical-scavenging activities of the crude plant extracts and commercial tincture extracts expressed as an inhibition factor against the reference compound propyl gallate

Using the IC_{50} of the crude plant extracts and commercial tincture extracts, an inhibition factor for each extract calculated against the reference compound propyl gallate is shown in Fig. 4.19. Expression of antioxidant activity as an inhibition factor against a known reference compound could represent a simple and consistent means of comparing DPPH scavenging activity between any future studies.

Salix alba (crude plant extract), compared to propyl gallate, had the highest inhibition factor at 0.107 (i.e. was approximately nine times less effective than propyl gallate), followed by *Calendula officinalis* (commercial tincture extract) and *Crataegus laevigata*, leaves and flowers (crude plant extract) with inhibition factors of 0.064 and 0.053. *Salix alba* (commercial tincture extract) had an inhibition factor of 0.046. Thereafter the order of potency was *Arctium lappa* (crude plant extract) 0.019; *Apium graveolens* (crude plant extract) 0.013; *Crataegus laevigata*, berry (crude plant/commercial tincture extracts) 0.013 and *Silybum marianum* (crude plant extract) 0.013. Commercial tincture extracts of *Taraxacum officinale, Arctium lappa* , *Silybum marianum, Apium graveolens* and *Crataegus laevigata,* leaves and flowers plus *Calendula officinalis* and *Taraxacum officinale* crude plant extracts did not register a significant inhibition factor, i.e. inhibition factor was below 0.01, less than 1% of that of propyl gallate.

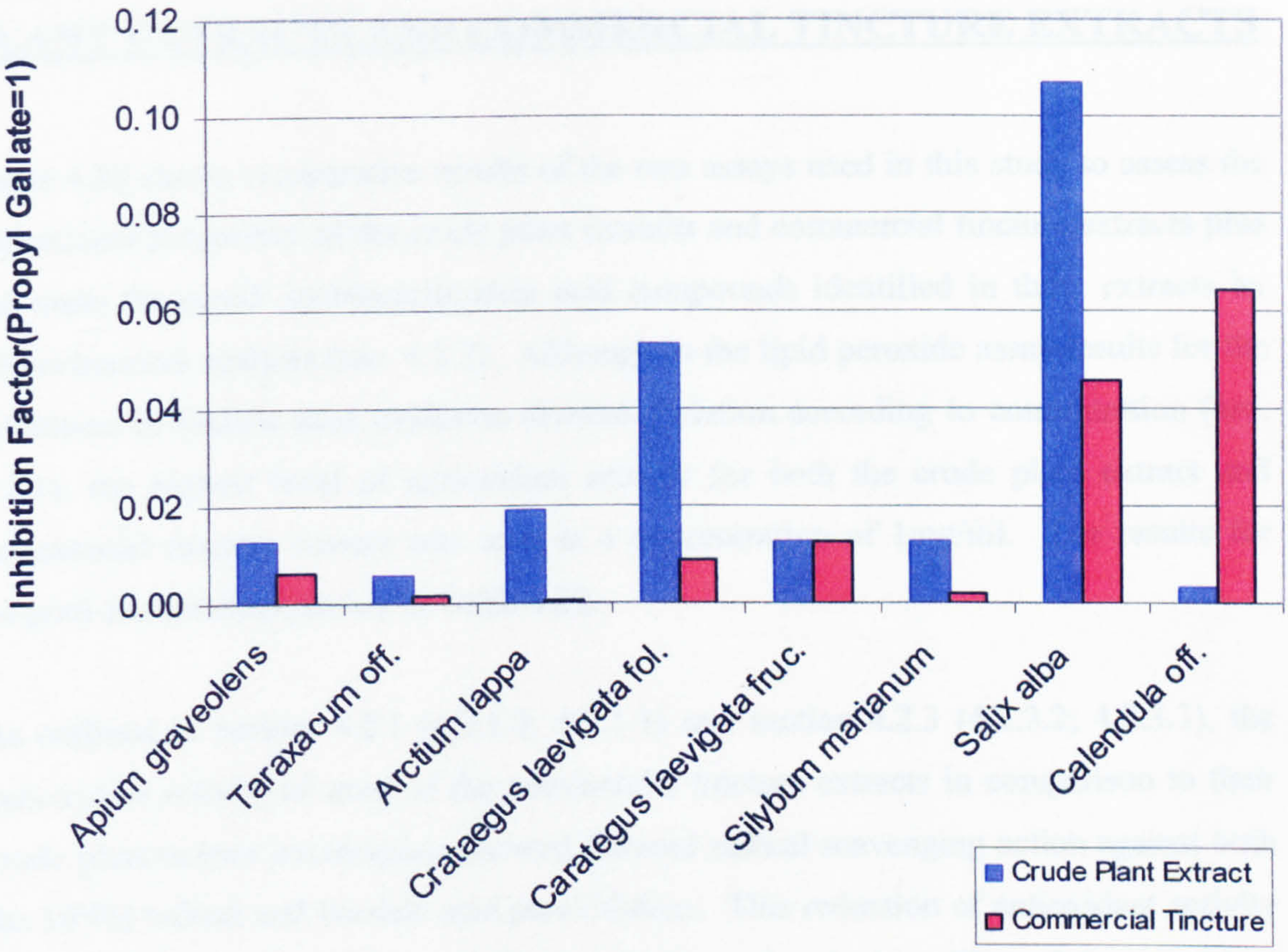


Fig. 4.19. DPPH radical-scavenging activities of the crude plant extracts and commercial tincture extracts expressed as an inhibition factor against the reference compound propyl gallate. Inhibition factor calculated as (IC_{50} extract /IC_{50} propyl gallate)

4.2.3.5 Experimental results for evaluating the synergistic, additive or antagonistic interaction of the crude plant extracts for antioxidant activity in the DPPH assay

Experimentation with combinations of crude plant extracts indicated that there are no synergistic effects to be observed between any of the extracts tested in pairs. In all cases the results using this assay, i.e. electron scavenging, were purely additive. Even triple combinations showed no inclination to synergy. Furthermore, no mutually inhibitory activity was noted.

4.2.4 SYNOPSIS OF RESULTS: COMPARISON OF CRUDE PLANT EXTRACTS AND COMMERCIAL TINCTURE EXTRACTS

Table 4.22 shows comparative results of the two assays used in this study to assess the antioxidant propensity of the crude plant extracts and commercial tincture extracts plus the main flavonoid/ hydroxycinnamic acid compounds identified in these extracts by phytochemical analysis (sec. 4.1.2). Although in the lipid peroxide assay results for the inhibition of linoleic acid oxidation showed variation according to concentration (sec. 4.2.1), the highest level of antioxidant activity for both the crude plant extract and commercial tincture extract was seen at a concentration of 1mg/ml. The results for 1mg/ml are therefore shown in Table 4.22.

As outlined in section 4.2.1 (4.2.1.2; 4.2.1.3) and section 4.2.3 (4.2.3.2; 4.2.3.3), the antioxidant activity of most of the commercial tincture extracts in comparison to their crude plant extract counterparts showed reduced radical scavenging action against both the DPPH radical and linoleic acid peroxidation. This reduction of antioxidant activity was reflected by a significant difference in phytochemical profiles of the two plant extracts particularly *Silybum marianum*, *Taraxacum officinale* and *Arctium lappa*. A general reduction in the number of flavonoid/hydroxycinamic compounds identified in the commercial tincture extracts, compared to the crude plant extracts also appeared to influence the level of radical scavenging activity. For example, the apparently higher levels of proanthocyanidins detected in *Salix alba* crude plant extract compared to the commercial tincture extract (Table 4.15) may have contributed to the superior radical scavenging activity of this crude plant extract.

Table 4.22 Comparative results of phytochemical analysis and assays for the evaluation of plant extracts as antioxidants. Where plant compounds are marked with an asterisk* this indicates that a significantly reduced number of these compounds were present in the commercial tincture extract compared to the crude plant extract.

Plant	Crude plant extract: types of flavonoid/ hydroxycinnamic acid compounds detected by HPLC and acid hydrolysis	Commercial tincture extract: types of flavonoid/ hydroxycinnamic acid compounds detected by HPLC and acid hydrolysis	Crude plant extract: IC_{50} value (mg/ml) DPPH assay	Commercial tincture extract: IC_{50} value (mg/ml) DPPH assay	Crude plant extract: % inhibition (1mg/ml) linoleic acid peroxidation	Commercial tincture extract: % inhibition (1mg/ml) linoleic acid peroxidation
Apium graveolens **(seed)**	Flavones; Caffeic acid derivatives	*Flavones; *Caffeic acid derivatives	0.025	0.05	80%	43.5%
Taraxacum officinale **(root)**	Caffeic acid derivatives	No flavonoid compounds or phenolic acids detected	0.054	0.23	87%	64%
Arctium lappa **(root)**	Caffeic acid derivatives	No flavonoid compounds or phenolic acids detected	0.017	0.81	91%	56.5%
Crataegus laevigata **(leaves & flowers)**	Flavonols; Flavones; Caffeic acid derivatives; Proanthocyanidins	*Flavonols; Flavones; Caffeic acid derivatives; *Proanthocyanidins	0.006	0.035	92%	65%
Crataegus laevigata **(berry)**	Flavonols; flavones; caffeic acid derivatives; proanthocyanidins	Flavonols; caffeic acid derivatives; *Proanthocyanidins	0.025	0.025	89%	51%
Silybum marianum **(seed)**	Dihydroflavonols (silymarin complex); Flavonols; Flavanones; Caffeic acid derivatives	No dihydroflavonols (silymarin complex) detected *Flavonols; Flavanones.	0.025	0.17	85%	61.5%
Salix alba **(bark)**	Favanones; Flavonols; Caffeic acid derivatives; Proanthocyanidins	*Favanones; *Caffeic acid derivatives; *Proanthocyanidins	0.003	0.007	87%	33%
Calendula officinalis **(flower)**	Flavonols; Caffeic acid derivatives	*Flavonols; Caffeic acid derivatives	0.102	0.005	82%	53%

4.2.5 DISCUSSION

The antioxidant action of the eight selected plant parts in the form of crude plant and tincture extracts were evaluated *in vitro*. All the extracts exhibited varying levels of antioxidant activity on both DPPH radical and linoleic acid peroxidation. These plants, selected on the basis of successful treatment outcomes (Bell et al, 1999), were postulated to have antioxidant activity.

The results showed that the method of extraction and composition of the solvent mixture influenced the activity of the resulting extracts. The crude plant extracts generally exhibited the strongest antioxidant activity, in a dose-dependant manner (Figs. 4.4 – 4.11).

The electron-donating properties of flavonoids and many phenolic acids have been repeatedly emphasized as the basis of their antioxidant action (Sugihara et al, 1999; Rice-Evans, 2001). The scavenging effects of different amounts of crude plant extracts and tincture extracts on the free radical DPPH increased with increasing amounts of extract. The activity of the extracts could therefore be attributed to their hydrogen donating ability. Duh (1998) states that "antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable radicals which do not initiate nor propagate further oxidation of lipids". As DPPH is known to abstract the labile hydrogen atom of chemical compounds (Ratty et al., 1988) the antioxidant that can scavenge the DPPH radical is expected to depress lipid peroxidation (Aniya et al, 1999). In this study, DPPH scavenging activity was found in all the extracts tested and it was confirmed that these extracts would also inhibit lipid peroxidation. Thus, the action of the plant extracts on DPPH would suggest that they acted as free radical inhibitors and primary antioxidants, which is likely to be the main reason for the inhibition of linoleic acid peroxidation in the lipid peroxide assay.

All the plant extracts examined were able to inhibit linoleic acid peroxidation with some relationship between concentration and scavenging ability. Inhibitory effects on lipid peroxidation may be considered as the principal effect established for flavonoids (Bors et al, 1998, p.119). The reported antioxidant action of flavonoids on lipid peroxidation is based on scavenging the principal propagating intermediates, the peroxyl and alkoxyl

radicals, by hydrogen donation, halting the radical chain (Bors et al, 1994). It is suggested therefore, that the plant extracts which have been shown to contain a variety of flavonoid compounds and polyphenols could be acting as electron donors, reacting with free radicals to convert them to less reactive compounds like flavonoid aroxylradicals, which would terminate a radical chain reaction (Bors et al, 1994). Although the polyphenols and flavonoids may not be the only active compounds, their antioxidant properties may, in part, explain the mechanism of antioxidant activity in the extracts tested.

The results for the free radical scavenging activity of the crude plant extracts on the DPPH radical at the concentrations initially tested, i.e. 2.5mg/ml, 1mg/ml and 0.5mg/ml, were comparable with, or higher than, the reference compound propyl gallate (Table 4.20). The IC_{50} value for propyl gallate however, was considerably lower than the IC_{50} values of all the crude plant extracts (Table 4.21). The antioxidant activity of the crude plant extracts on linoleic acid peroxidation compared to propyl gallate showed that when tested at a similar concentration, generally they were equally effective (section 4.2.1.1). The differences in the antioxidant activity of propyl gallate in these assays may be explained by the different antioxidant mechanisms being investigated. As already outlined above, DPPH scavenging involves only a single hydrogen-donating mechanism whereas inhibition of linoleic acid oxidation involves a complex sequence of oxidation and reduction processes. Since DPPH is a synthetic radical it is generally only used for primary screening to test the propensity of a crude plant extract to antioxidant activity. It is however, a simple, rapid screening method sensitive enough to detect natural and synthetic antioxidants at low concentrations.

The antioxidant effect of flavonoids has been reported to be dependent on the structure of the flavonoid. Flavonoids are antioxidants by virtue of the number and arrangement of their phenolic hydroxyl groups attached to ring structures (Rice-Evans, 2001). Their ability to act as antioxidants by donating an electron to an oxidant critically depends on the reduction potential of their radicals and their availability at the site of the pathological oxidative process (Rice-Evans, 2001). Since each individual extract contains a mixture of different polyphenol and flavonoid compounds, it is not possible to relate antioxidant activity to a specific compound or structural characteristic. It has been observed however, that the three crude plant extracts *Salix alba* (bark) and *Crataegus laevigata* (leaves and flowers/berries) which were the only plant parts to

contain proanthocyanins, still exhibited antioxidant activity in the lipid peroxide assay at 0.001mg/ml, the concentration at which all the other crude plant extracts changed from antioxidant to pro-oxidant activity. One possible explanation is that the flavonoid aroxyl (semiquinone) radicals formed after initial scavenging of an oxidizing radical behave differently depending on the type of flavonoid. All the flavonoid aroxyl (semiquinone) radicals decay by second-order kinetics, i.e. bi-molecular disproportionation, forming quinones (quinone methides) and the parent hydroxy compound (Bors et al, 2001). The quinones and quinone methides of flavonols for example, can undergo futile redox recycling with the formation of reactive oxygen species making them potentially pro-oxidant, whereas the quinones of proanthocyanins preferentially react via phenolic coupling reactions to form dimers and oligmers, each retaining its original number of reactive hydroxy groups, thereby enhancing their antioxidant capacity (Metodiewa et al., 1999; Bors et al, 2001). *Crataegus laevigata* (leaves and flowers), the only crude plant extract to contain a wide range of flavones, flavonols, hydroxycinnamic acids and detectable levels of proanthocyanins (Tables 4.15 and 4.18), displayed the highest level of antioxidant activity (92%) against linoleic acid oxidation.

Tinctures, widely used as herbal medicines, were evaluated against the crude plant extracts to ascertain if they exhibited similar levels of antioxidant activity. HPLC analysis generally detected fewer polyphenols and flavonoid compounds in the tincture extracts (section 4.1.2), which was reflected in reduced levels of antioxidant activity against the crude plant extracts when tested at the same dosage on both the free radical DPPH and linoleic acid oxidation. Since tinctures are among the most widely employed herbal medicinal products, a review of the extraction method, composition of the solvent and resultant levels of the active constituents are urgently required to optimize extraction of the active constituents.

Lipid peroxide assays are often used to establish the antioxidant potential of plant extracts and individual compounds. Although it is not possible to elucidate exact mechanisms of action, the observed antioxidant activity on lipid peroxidation is the result of all oxidation and reduction processes which might occur *in vivo* (Van Acker et al, 1998). Comparison of results between authors is generally difficult, due to differences in method and experimental conditions. The results obtained for *Arctium lappa* (root) in the linoleic acid system (90%), is comparable however, with those

reported by Duh (1998) using a similar method of analysis. Apart from the direct scavenging activity of compounds other factors such as interactions with transition metals and uptake into membranes may play an important role in antioxidant activity.

Previous studies have established that besides antioxidant activity, flavonoids can also act as pro-oxidants *in vitro* depending on conditions that include concentration of the antioxidant, the presence of other antioxidants and the presence of transition metals (Boik, 2001, p.189;255). There are two main mechanisms responsible for the pro-oxidant activity of flavonoids (Cos, 2001). The first is their ability to participate in reactions catalysed by transition metal ions such as the Fenton reaction, which produces the highly reactive hydroxyl radical. The second is their polyphenolic structure which makes them susceptible to auto-oxidation reactions, resulting in the production of reactive oxygen species (Bors et al, 1998). According to Halliwell and Chirico (1993) most biological studies of lipid peroxidation involve transition metal ions, added to, or contaminating, the reaction mixtures. As outlined in section 4.2.2.1 the decomposition of lipid hydroperoxides by transition metals such as iron and copper to give hydroxyl or alkoxyl radicals is also strongly implicated in the generation of free radicals. Lipid hydroperoxides are thought to be reductively cleaved by metal ions in a low valence state to highly reactive alkoxyl radicals, which then remove hydrogen ions from lipids to form new lipid alkyl radicals. For the reduction of the metal ions back from the high valence state to low valency, reducing compounds are required and flavonoids may have the capacity to reduce metal ions directly through autoxidation (Sugihara et al., 1999).

At the lowest concentrations tested, 0.001mg/ml and 0.0025mg/ml, some of the crude plant extracts were observed to change from antioxidant to pro-oxidant activity. ICP analysis confirmed the presence in all the selected plant parts of transition metals capable of participating in metal catalysed reactions that can accelerate production of reactive oxygen species. Although the pro-oxidant activity of antioxidants *in vitro* is well documented, most work has been carried out using individual flavonoid standards without considering the presence of other components occurring in natural matrices (Boik, 2001, p.189). No conclusions in regard to mechanisms of pro-oxidant activity in the crude plant extracts can be reached therefore, especially since the composition of each extract as shown by phytochemical analysis is so different.

Caution is needed when extrapolating from the *in vitro* results to the *in vivo* situation, especially since factors such as the bioavailability of flavonoids is still unclear. Furthermore, the antioxidant assays used in this study represent only two antioxidant mechanisms and it may be important to investigate how these plant extracts perform in other antioxidant systems. It has been shown for example that antioxidants that protect lipid peroxidation against free radical damage may actually accelerate damage to other molecules such as carbohydrates, under certain conditions (Burits and Bucar, 2000). Human-body chemistry is complex and it does not currently appear possible to simulate *in vitro* studies accurately *in vivo*. Data on biological markers such as blood levels of flavonoids and their metabolites are not widely available, thus making it difficult to determine the individual or the combined role of the flavonoids and other antioxidants (Pietta, 2000). There is however strong evidence that flavonoid-containing foods like fruit and vegetables and some medicinal plants have a protective effect against many of the degenerative diseases of ageing where lipid peroxidation is strongly implicated in the pathogenisis (Pietta, 2000). The results of these assays, which have demonstrated antioxidant activity of the eight plant parts traditionally used in the treatment of osteoarthritis, indicate that their radical scavenging actions may contribute to their therapeutic effects and that their use in this way may be useful and reasonable.

4.3 EVALUATION OF PLANT SYNERGY

4.3.1 Experimental results for evaluating the synergistic, additive or antagonistic interactions of pairs of crude plant extracts for antioxidant activity in the linoleic acid system

Combining herbs into a formula is common practice throughout traditional medicine. Practitioners generally assume that when several plant extracts are combined, synergy ensues and that mixtures of different plant extracts will support and enhance each other. The term synergy in this context refers to the enhanced effect of a mixture of two or more plant extracts, which is greater than that which would be expected by a consideration of the effects of the individual components. Although there appears to be some evidence in support of herbal synergy, because it is so difficult to prove, there is a scarcity of reports in the scientific literature (Williamson, 2001).

All of the crude plant extracts (sec. 3.1.2.2.1) assessed for antioxidant activity against linoleic acid peroxidation were tested in pairs in this assay, to observe if certain two-herb combinations would increase antioxidant activity above the levels already achieved for the extracts when assessed singly. 28 pairs of extracts were tested at a final total concentration of extract in the mixture of 0.01mg/ml (i.e. 0.005 mg/ml for each plant extract). Since there are few documented studies on plant synergy and results for the individual crude plant extracts showed no linear relationship to dose, a preliminary study at this concentration was deemed appropriate for the following reasons:-

1) Results for inhibition of linoleic acid oxidation at 0.01mg/ml (final concentration) and 0.005mg/ml (dose at which each crude plant extract was used in the pair) were almost the same [Tables 4.4 – 4.11].
2) Results for the individual extracts tested at 0.01mg/ml that ranged from 32% to 51% were at a level that would allow for a reasonable percent increase or decrease in antioxidant activity to be observed [Tables 4.4 – 4.11].

There are a number of methods which have been proposed to demonstrate synergistic interactions between agents (Berenbaum, 1989), none of which have successfully addressed the comparison of two complex plant extracts each comprising many

constituents (Wagner, 2004). This work was therefore carried out as an exploratory study, using statistical analysis to explore the possibility that an interaction between two extracts may have taken place as a precursor to more exhaustive studies. The comparative results for each pair of extracts, showing both their individual and combined antioxidant activity against linoleic acid peroxidation, as measured using the method given in sections 3.2.2.6.1 are illustrated by box plot graphs Figs. 4.20(a-e).

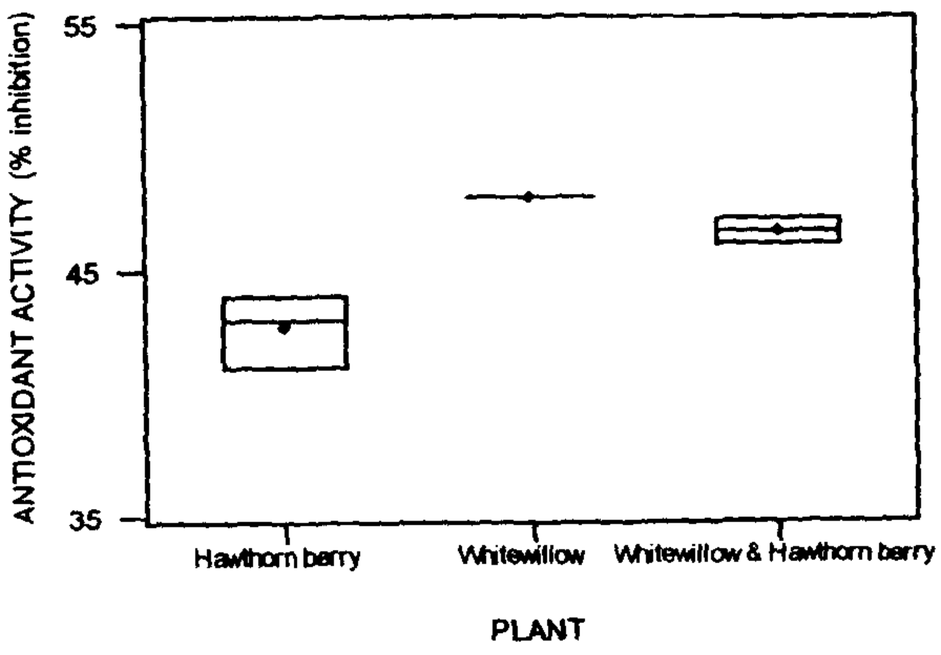
4.3.2 Statistical analysis

The Mann Whitney test, a distribution free nonparametric test suitable for comparing the difference between the medians of two independent groups of sampled data, was used statistically to analyse the results, i.e. the percent antioxidant activity of each single extract compared to the combined antioxidant activity of the pair. Results of the statistical analysis (Mann Whitney < 0.1) for each pair of crude plant extracts are shown in Table 4.23. The results for most pairs of extracts were not significant, indicating zero interaction. Increased activity, i.e. more than expected, indicating synergism, was displayed by the combinations *Silybum marianum* (Milkthistle) and *Arctium lappa* (Burdock) ($p = 0.03$), Fig. 4.20(e) graph B; *Crataegus laevigata fruc.* (Hawthorn fruit) and *Arctium lappa* (Burdock) ($p = 0.03$), Fig. 4.20(e) graph C; *Calendula officinalis* (Marigold) and *Arctium lappa* (Burdock) ($p = 0.07$), Fig. 4.20(c) graph B; *Salix alba* (White willow) and *Crataegus laevigata fol.* (Hawthorn leaves and flowers) ($p = 0.08$), Fig. 4.20(b) graph B; *Crataegus laevigata fruc.* (Hawthorn berry) and *Taraxacum officinale* (Dandelion) ($p = 0.1$), Fig. 4.20(d) graph C.

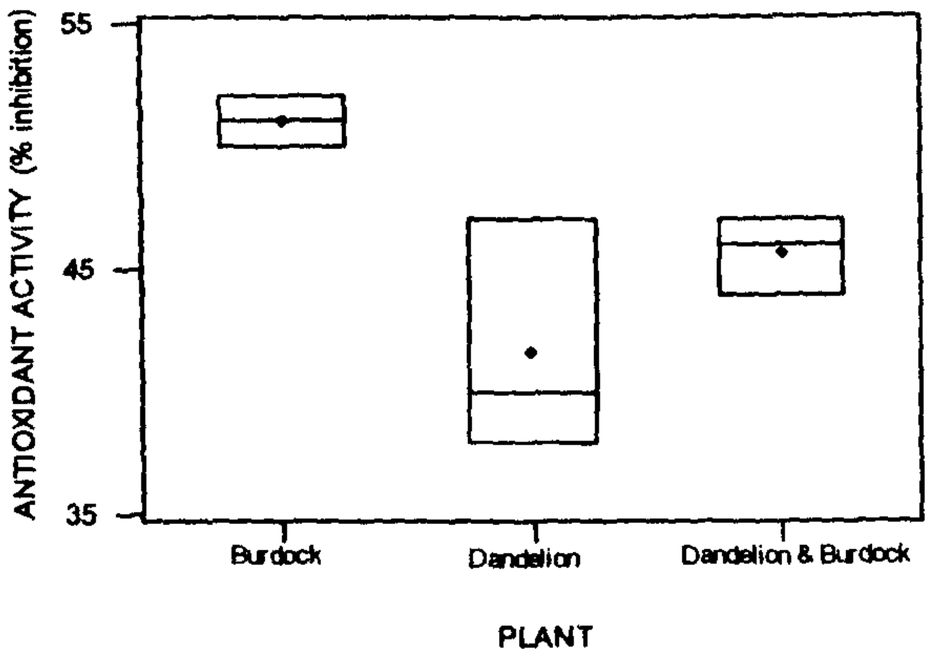
A decrease in activity, i.e. less than expected, indicating antagonism was shown by *Calendula officinalis* (Marigold) and *Silybum marianum* (Milkthistle) ($p = 0.03$), Fig. 4.20(e) graph D; *Crataegus laevigata fol.* (Hawthorn leaves and flowers) and *Silybum marianum* (Milkthistle) ($p = 0.03$), Fig.4.20(b) graph A and *Calendula officinalis* (Marigold) and *Taraxacum officinale* (Dandelion) ($p = 0.1$), Fig. 4.20(c) graph A.

Table 4.23. Results of statistical analysis (Mann Whitney test < 0.1) for the synergistic or antagonistic interactions of pairs of crude plant extracts, final concentration 0.01mg/ml, assessed for antioxidant activity using a lipid peroxide assay. X = no statistical significance. The crude plant extracts from each statistically significant combination, i.e. displaying synergy (increased activity) or antagonism (decreased activity) are shown in corresponding colours.

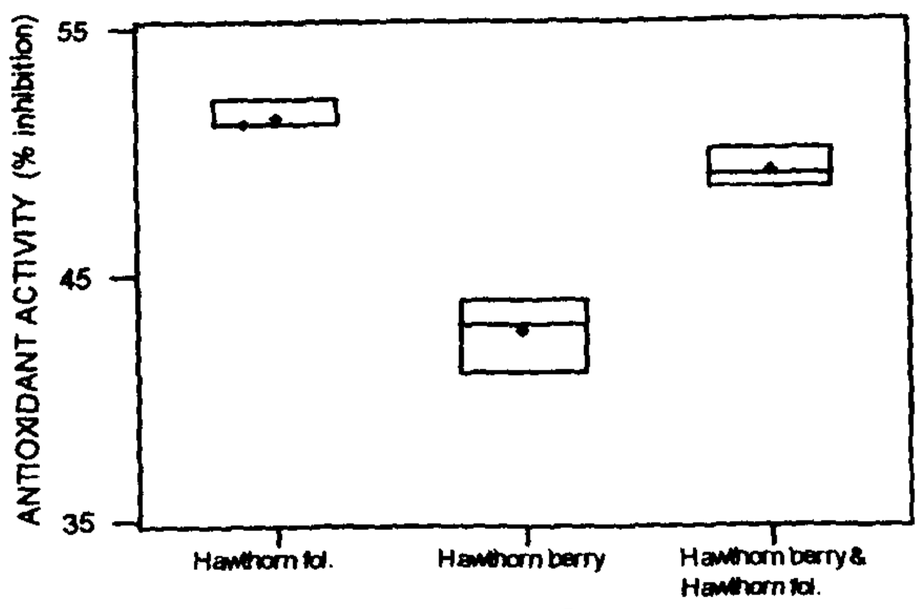
	***Silybum marianum* (Milkthistle)**	***Arctium lappa* (Burdock)**	***Taraxacum officinale* (Dandelion)**	***Salix alba* (White willow)**	***Calendula officinalis* (Marigold)**	***Apium graveolens* (Celery)**	***Crataegus laevigata* (Hawthorn berry)**	***Crataegus laevigata* (Hawthorn leaves and flowers)**
***Silybum marianum* (Milkthistle)**		Synergy p = 0.03	X	X	Antagonism p = 0.03	X	X	Antagonism p = 0.03
***Arctium lappa* (Burdock)**	Synergy p = 0.03		X	X	Synergy p = 0.07	X	Synergy p = 0.03	X
***Taraxacum officinale* (Dandelion)**	X	X		X	Antagonism p = 0.1	X	Synergy p = 0.1	X
***Salix alba* (Whitewillow)**	X	X	X		X	X	X	Synergy p = 0.08
***Calendula officinalis* (Marigold)**	Antagonism p = 0.03	Synergy p = 0.07	Antagonism p = 0.1	X		X	X	X
***Apium graveolens* (Celery)**	X	X	X	X	X		X	X
***Crataegus laevigata* (Hawthorn berry)**	X	Synergy p = 0.03	Synergy p = 0.1	X	X	X		X
***Crataegus laevigata* Hawthorn leaves and flowers)**	Antagonism p = 0.03	X	X	Synergy p = 0.08	X	X	X	



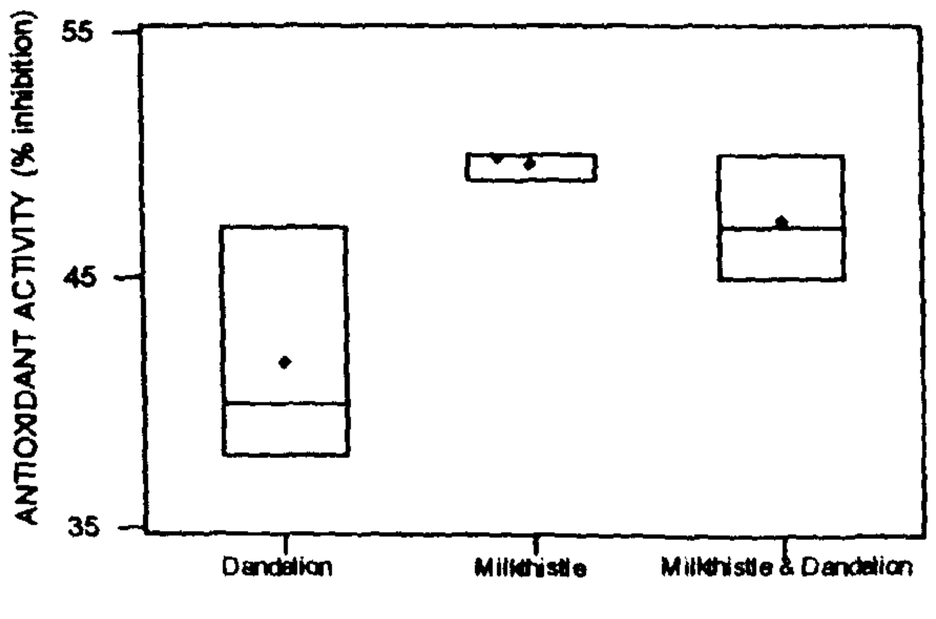
A. *Crataegus laevigata* fruc. (Hawthorn berry) and *Salix alba* (White willow)



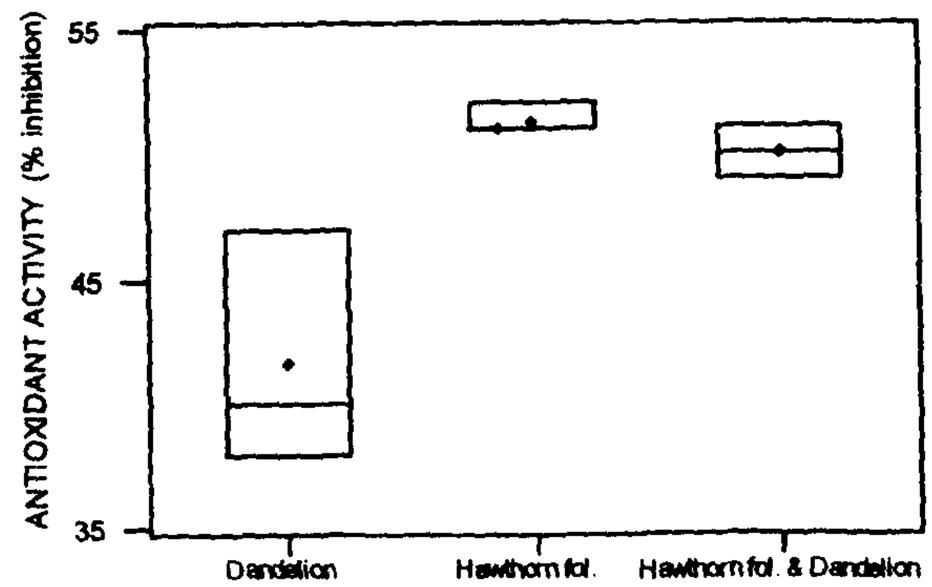
B. *Arctium lappa* (Burdock) and *Taraxacum officinale* (Dandelion)



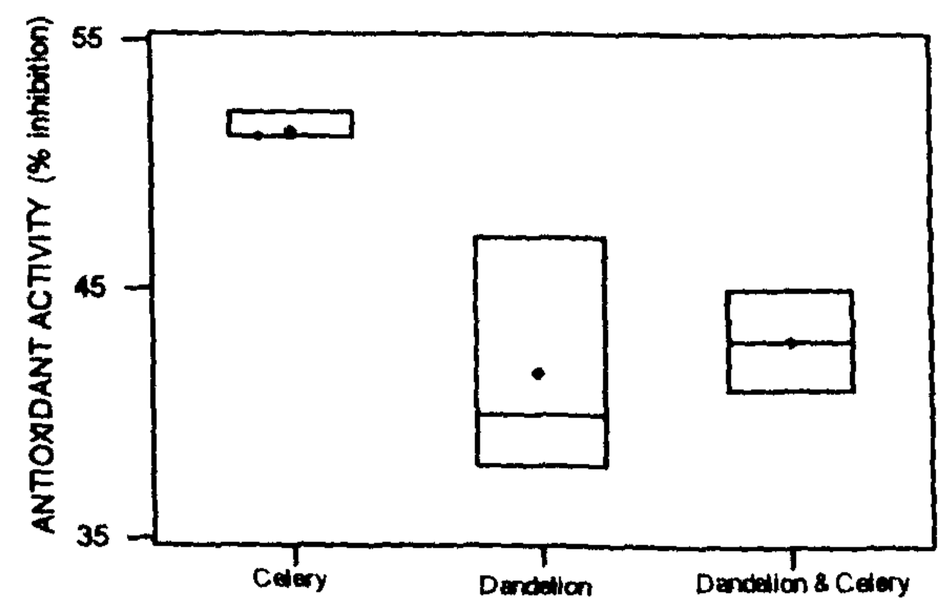
C. *Crataegus laevigata* (Hawthorn fol.) and *Crataegus laevigata* fruc. (Hawthorn berry)



D. *Taraxacum officinale* (Dandelion) and *Silybum marianum* (Milkthistle)

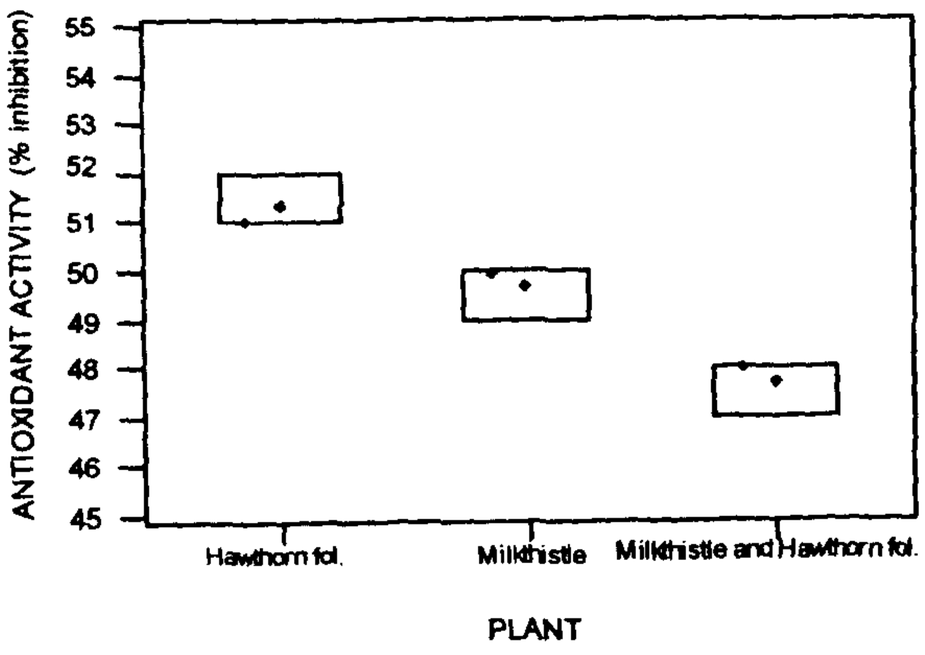


E. *Taraxacum officinale* (Dandelion) and *Crataegus laevigata* (Hawthorn fol.)

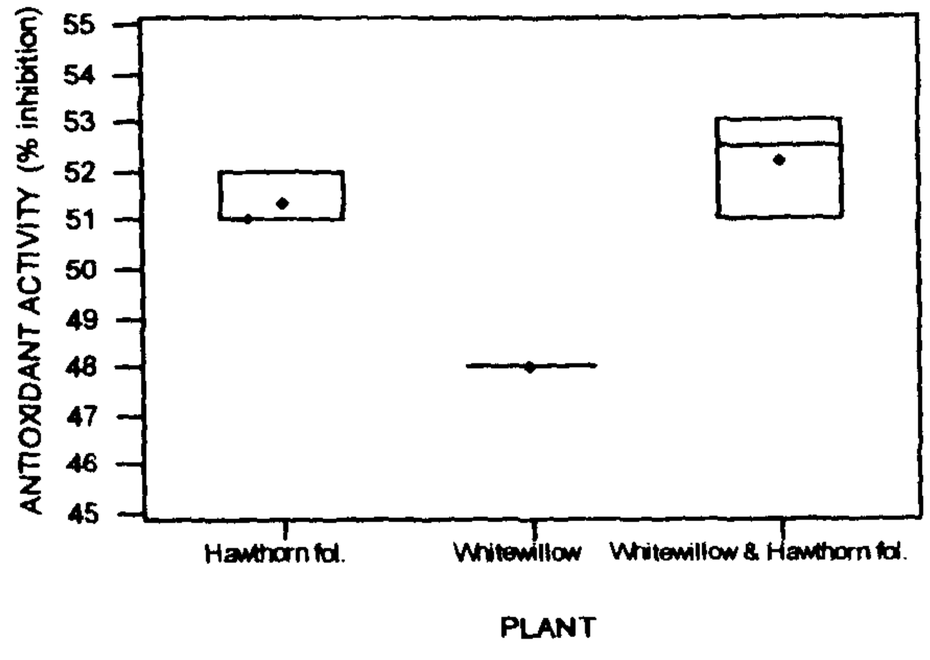


F. *Apium graveolens* (Celery) and *Taraxacum officinale* (Dandelion)

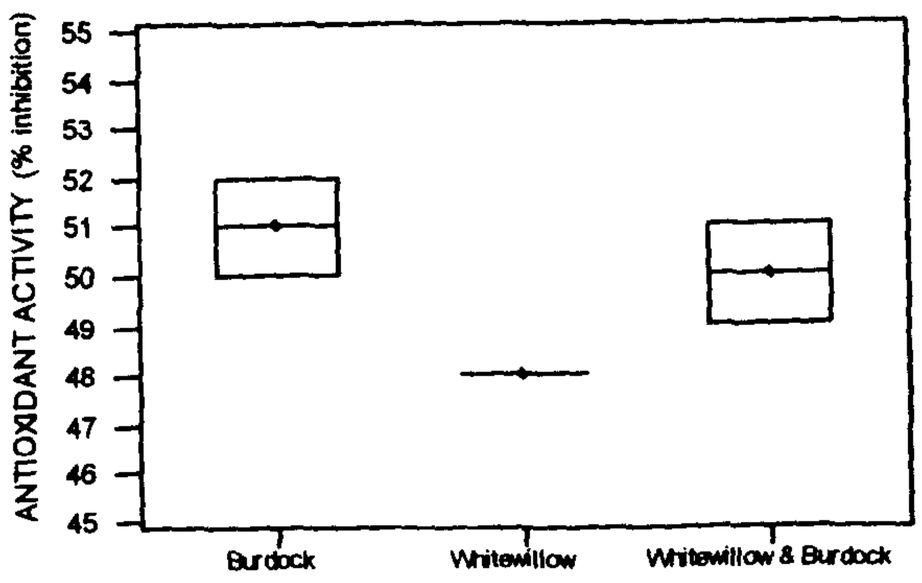
Figure 4.20(a) Boxplot graphs illustrating the % inhibition of linoleic acid peroxidation by crude plant extracts (final concentration 0.01mg/ml) of single herbs versus pairs of herbs. The median is shown by a line drawn across the box except where data is skewed and the median is either equal to, or too close to the upper/lower quartile to be visible on the graph in which case it is marked by a red dot. A black dot denotes the mean. No significant differences were found in any of these results.



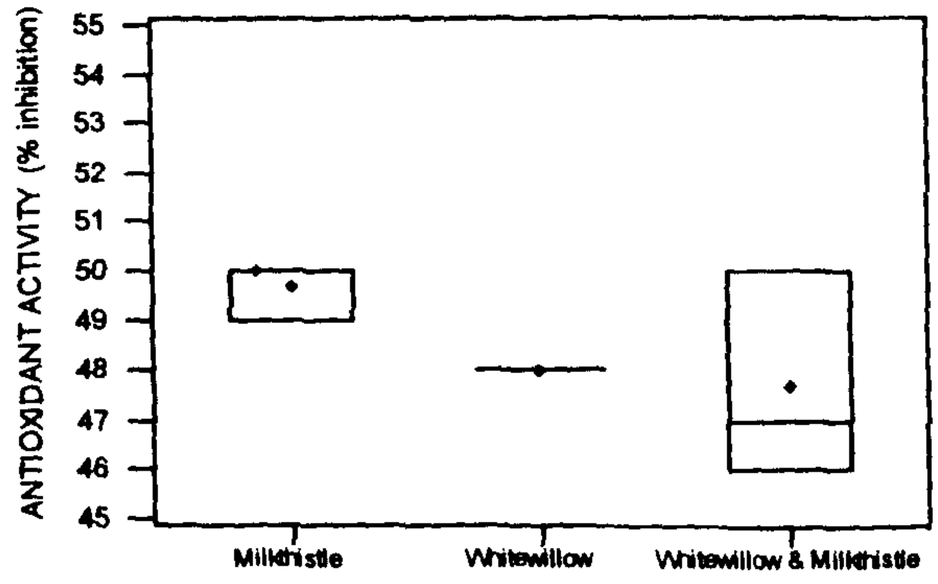
A. *Crataegus laevigata* (Hawthorn fol.) and *Silybum marianum* (Milkthistle) [Inhibition]



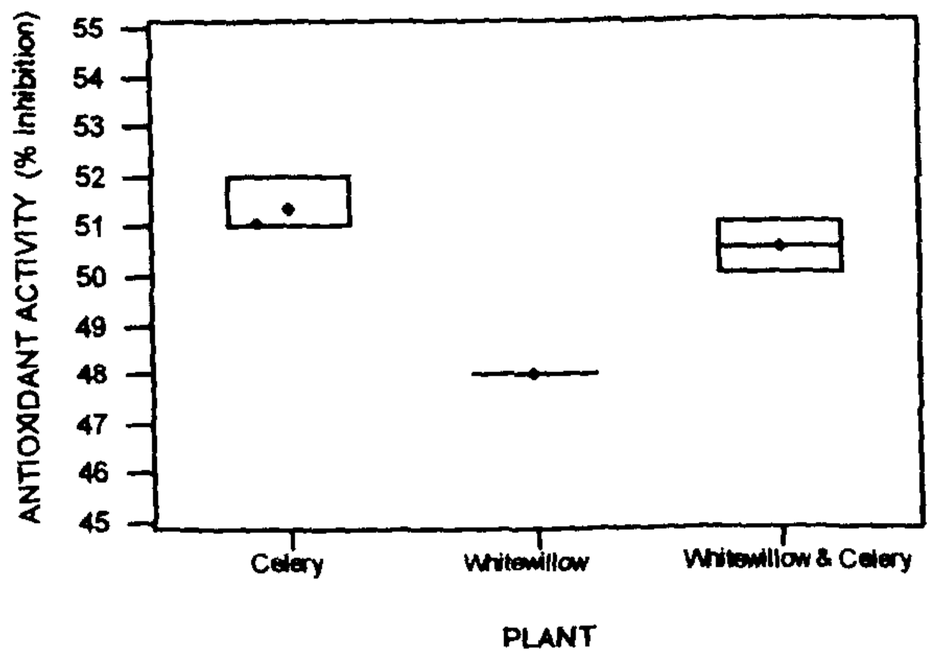
B. *Crataegus laevigata* (Hawthorn fol.) and *Salix alba* (White willow) [Synergy]



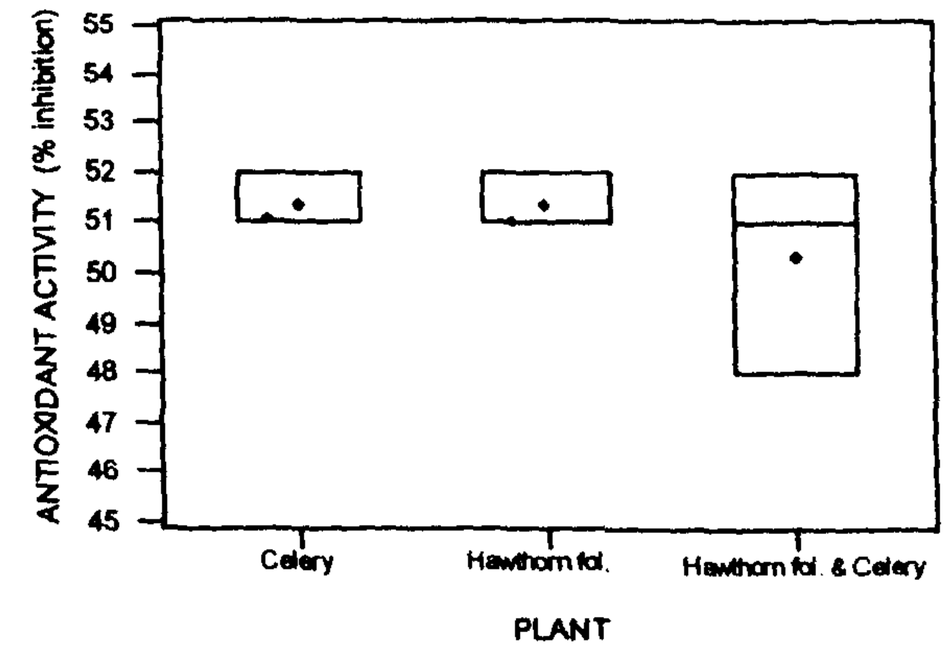
C. *Arctium lappa* (Burdock) and *Salix alba* (White willow)



D. *Silybum marianum* (Milkthistle) and *Salix alba* (White willow)

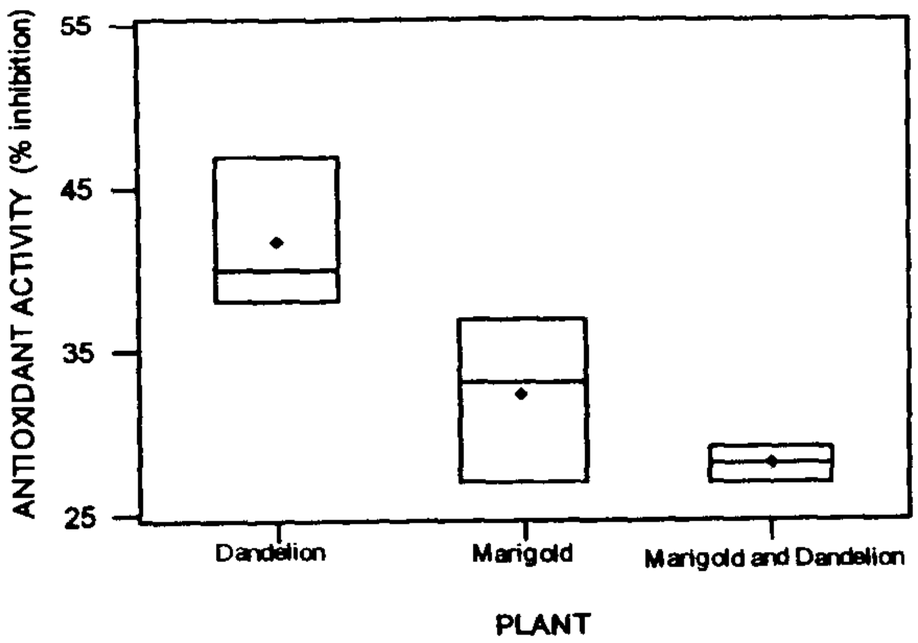


E. *Apium graveolens* (Celery) and *Salix alba* (White willow)

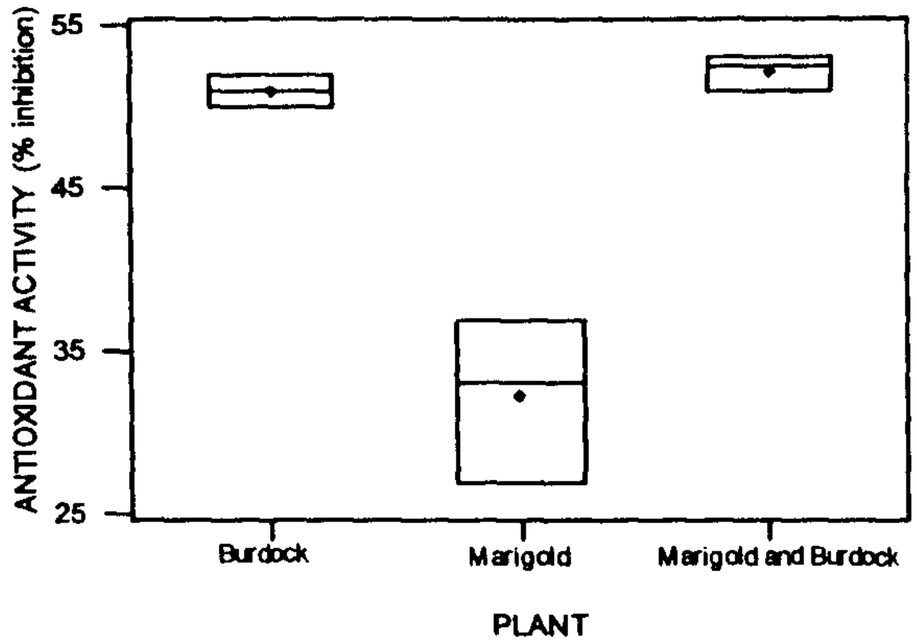


F. *Apium graveolens* (Celery) and *Crataegus laevigata* (Hawthorn fol.)

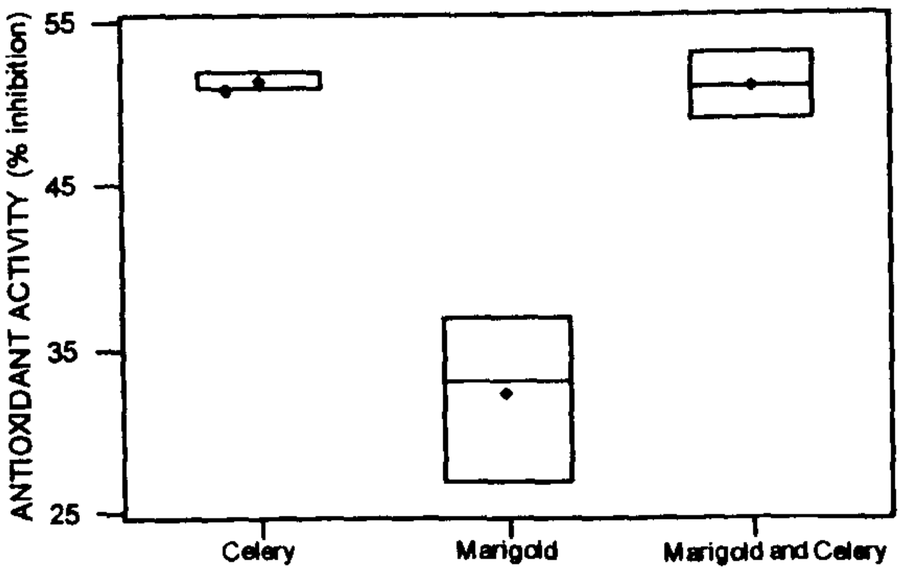
Figure 4.20(b) Boxplot graphs illustrating the % inhibition of linoleic acid peroxidation by crude plant extracts (final concentration 0.01mg/ml) of single herbs versus pairs of herbs. The median is shown by a line drawn across the box except where data is skewed and the median is either equal to, or too close to the upper/lower quartile to be visible on the graph in which case it is marked by a red dot. A black dot denotes the mean. Graph (A) decreased activity (inhibition, MW p = 0.03); Graph (B) increased activity (synergy, MW p = 0.08)



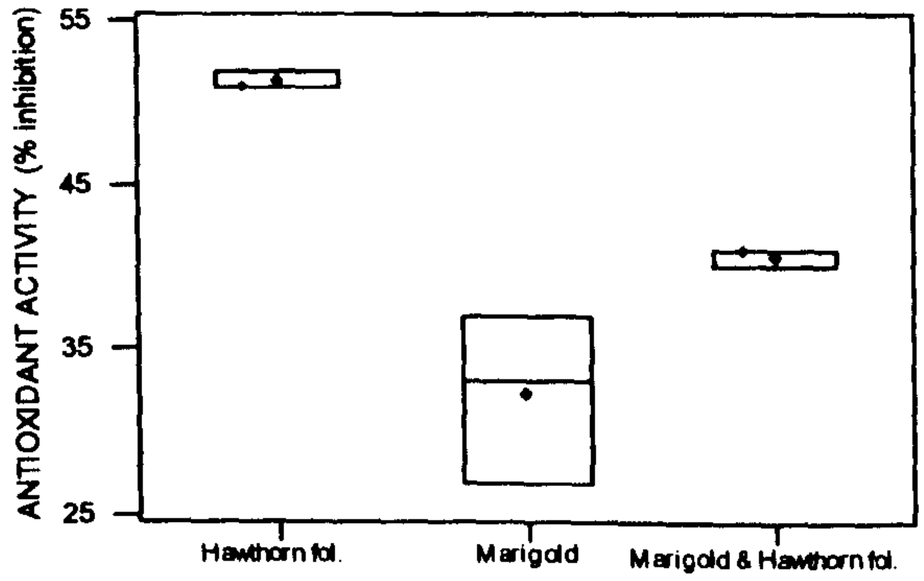
A. *Taraxacum officinale* (Dandelion) and *Calendula officinalis* (Marigold) [Inhibition]



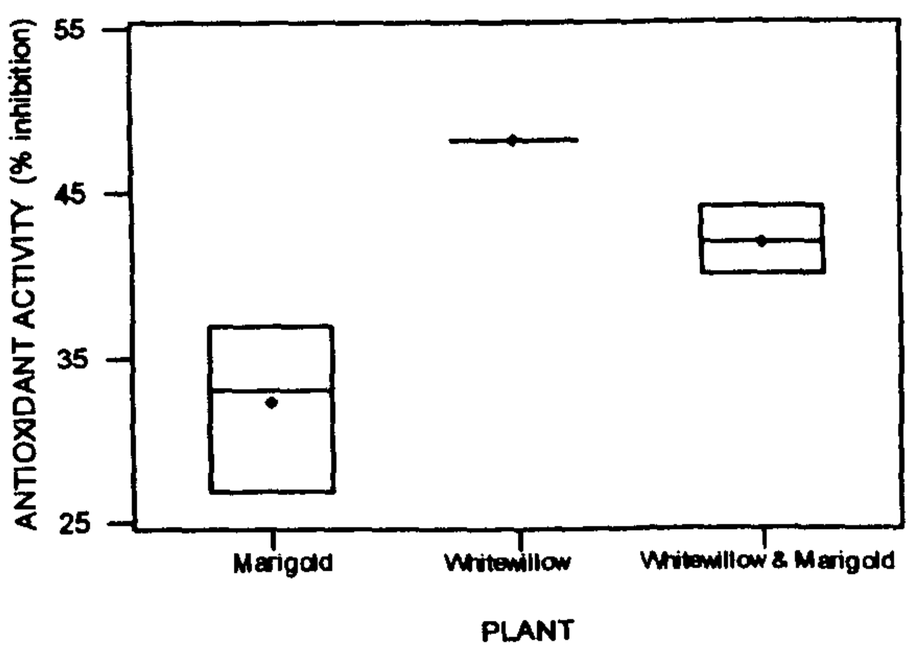
B. *Arctium lappa* (Burdock) and *Calendula officinalis* (Marigold) [Synergy]



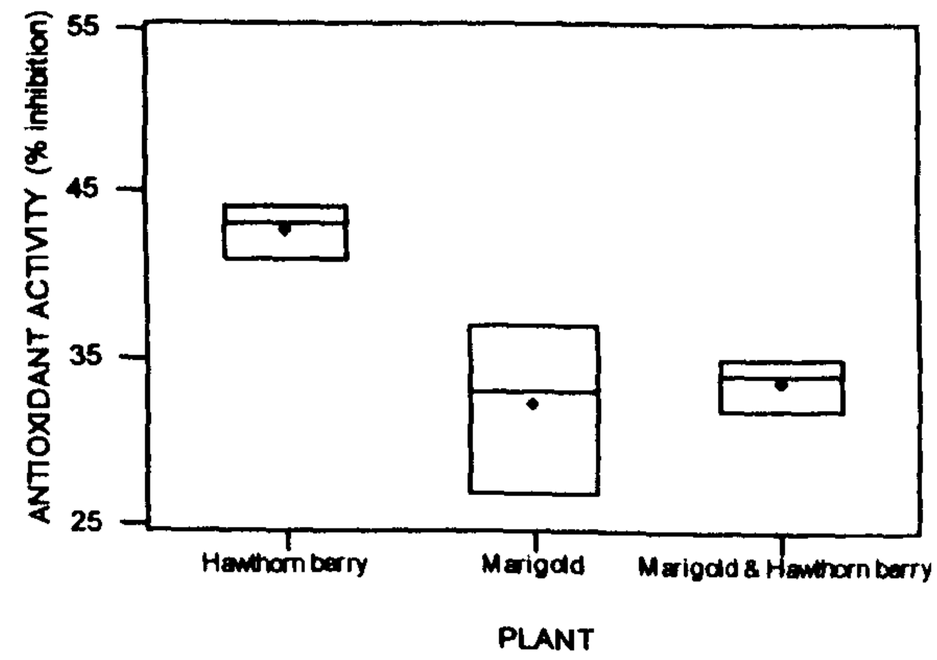
C. *Apium graveolens* (Celery) and *Calendula officinalis* (Marigold)



D. *Crataegus laevigata* (Hawthorn fol.) and *Calendula officinalis* (Marigold)

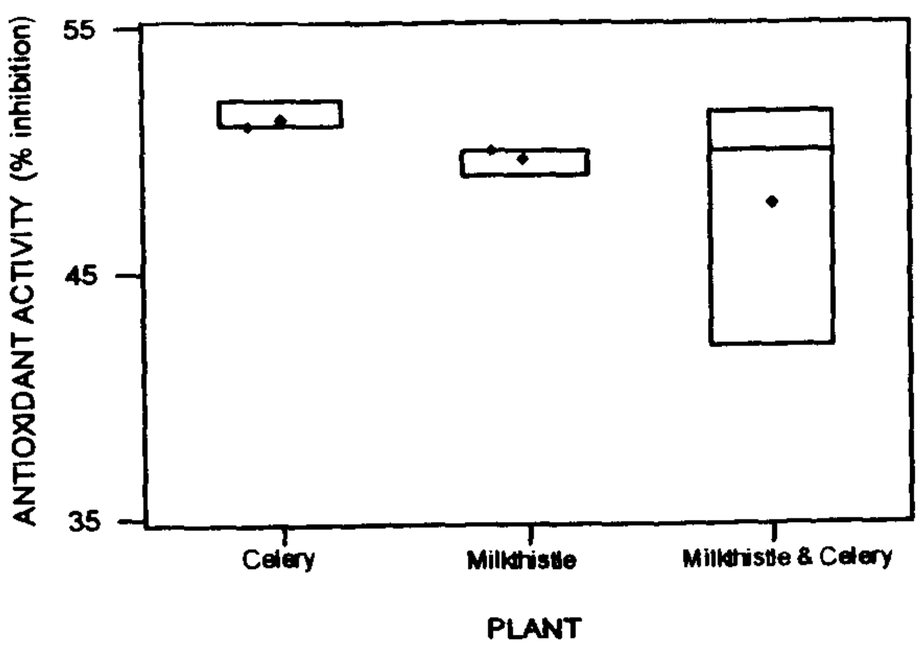


E. *Calendula officinalis* (Marigold) and *Salix alba* (White willow)

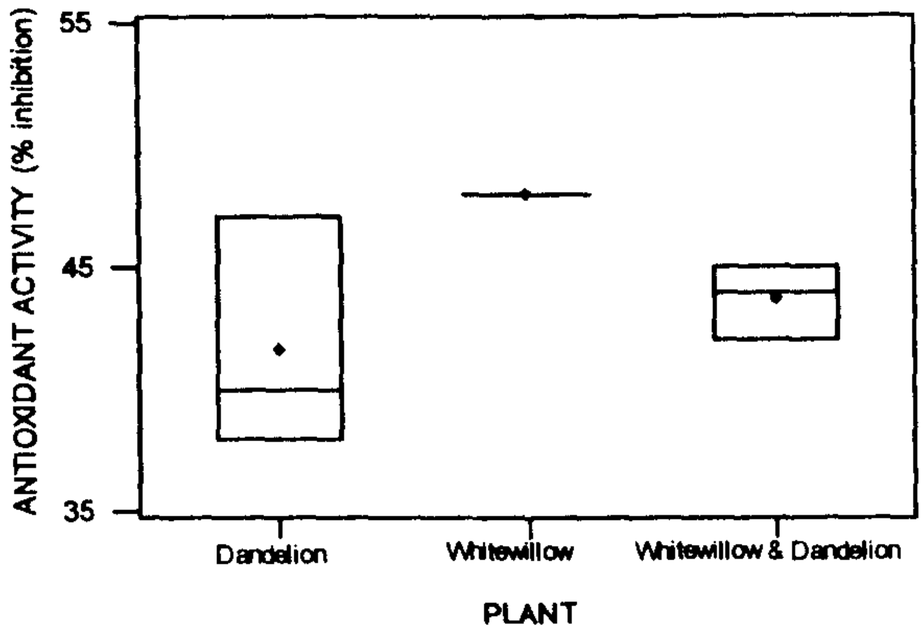


F. *Crataegus laevigata* (Hawthorn berry) and *Calendula officinalis* (Marigold)

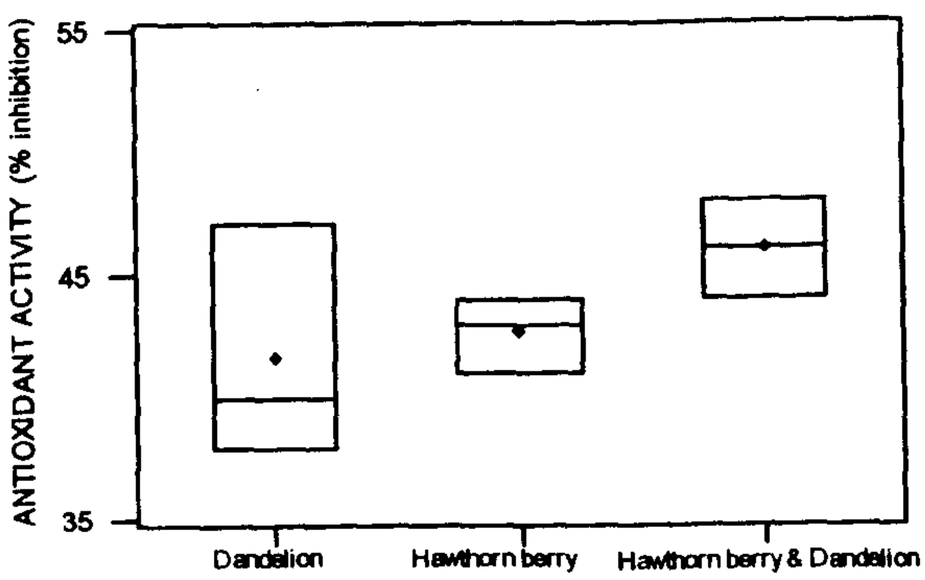
Figure 4.20(c) Boxplot graphs illustrating the % inhibition of linoleic peroxidation by crude plant extracts (final concentration 0.01mg/ml) of single herbs versus pairs of herbs. The median is shown by a line drawn across the box except where data is skewed and the median is either equal to, or too close to the upper/lower quartile to be visible on the graph in which case it is marked by a red dot. A black dot denotes the mean. Graph (A) decreased activity (inhibition, MW p = 0.1); Graph (B) increased activity (synergy, MW p = 0.07).



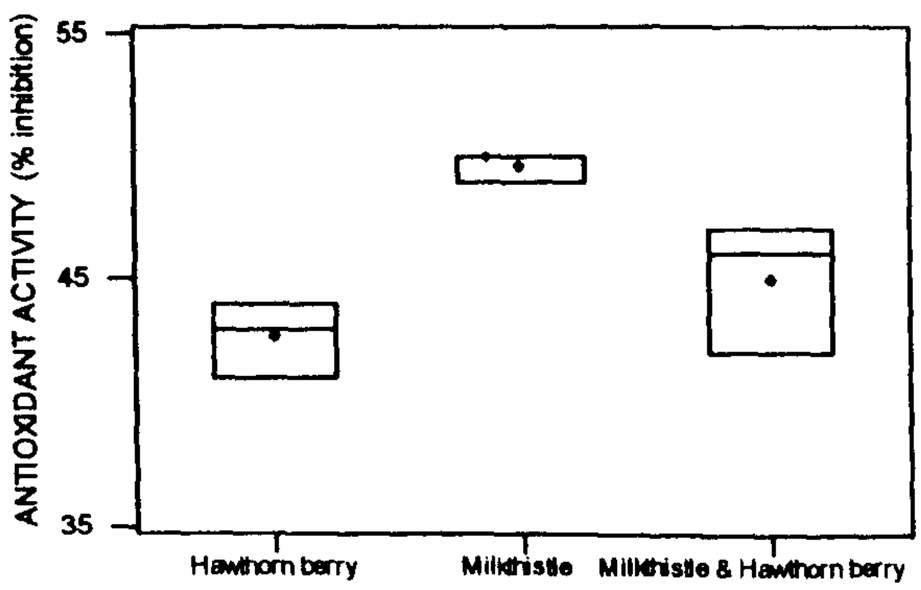
A. *Apium graveolens* (Celery) and *Silybum marianum* (Milkthistle)



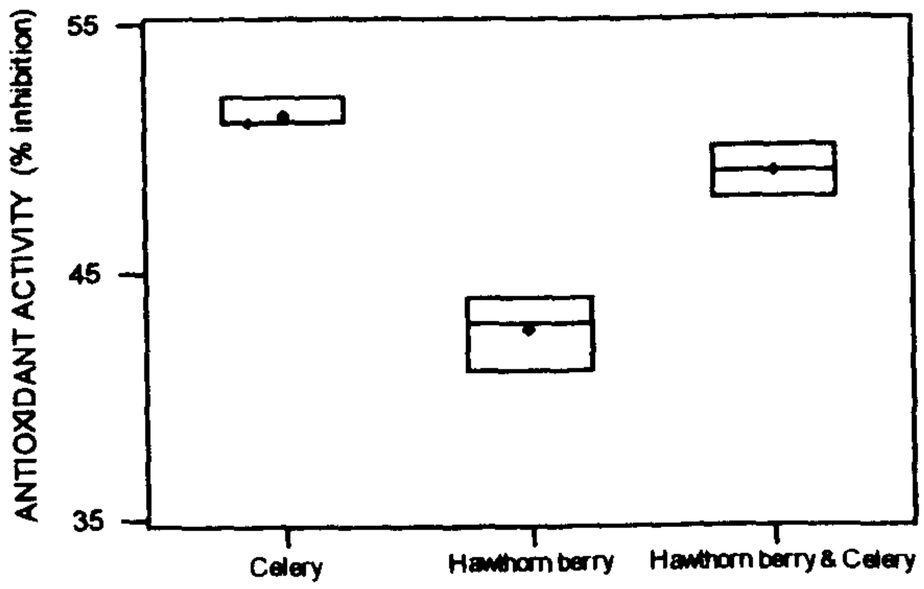
B. *Taraxacum officinale* (Dandelion) and *Salix alba* (White willow)



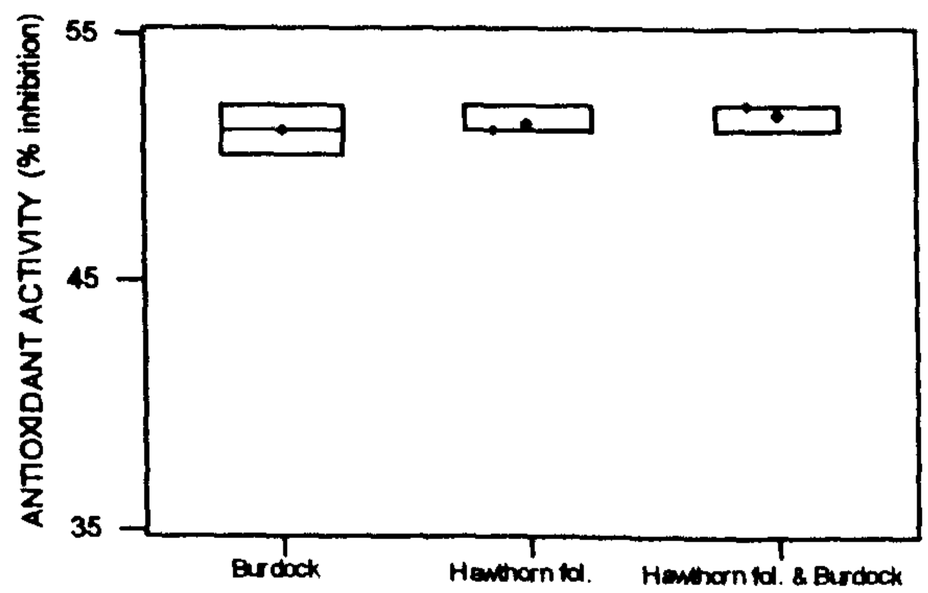
C. *Crataegus laevigata* fruc. (Hawthorn berry) and *Taraxacum officinale* (Dandelion) [Synergy]



D. *Crataegus laevigata* fruc. (Hawthorn berry) and *Silybum marianum* (Milkthistle)

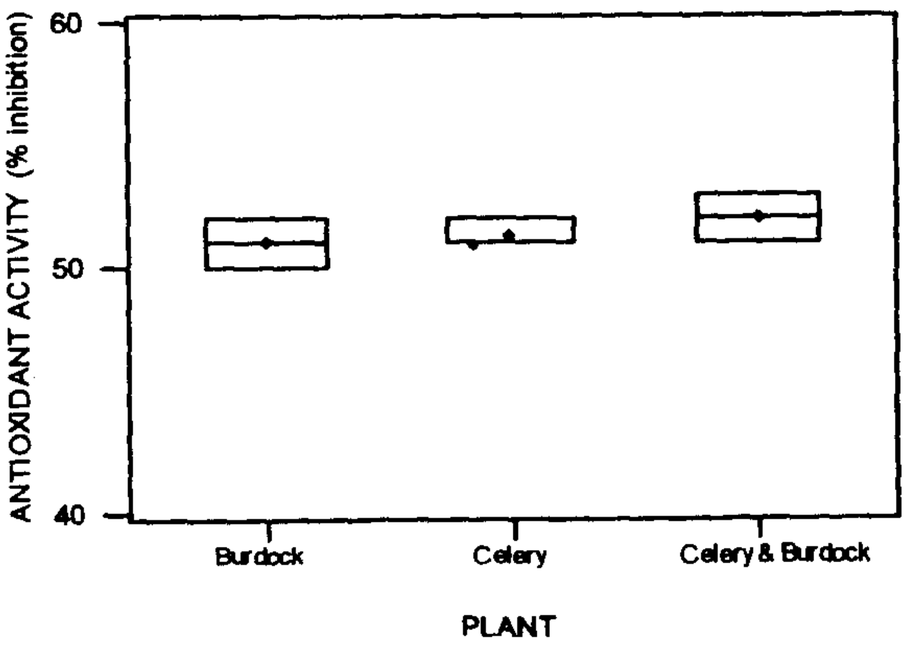


E. *Apium graveolens* (Celery) and *Crataegus laevigata* fruc. (Hawthorn berry)

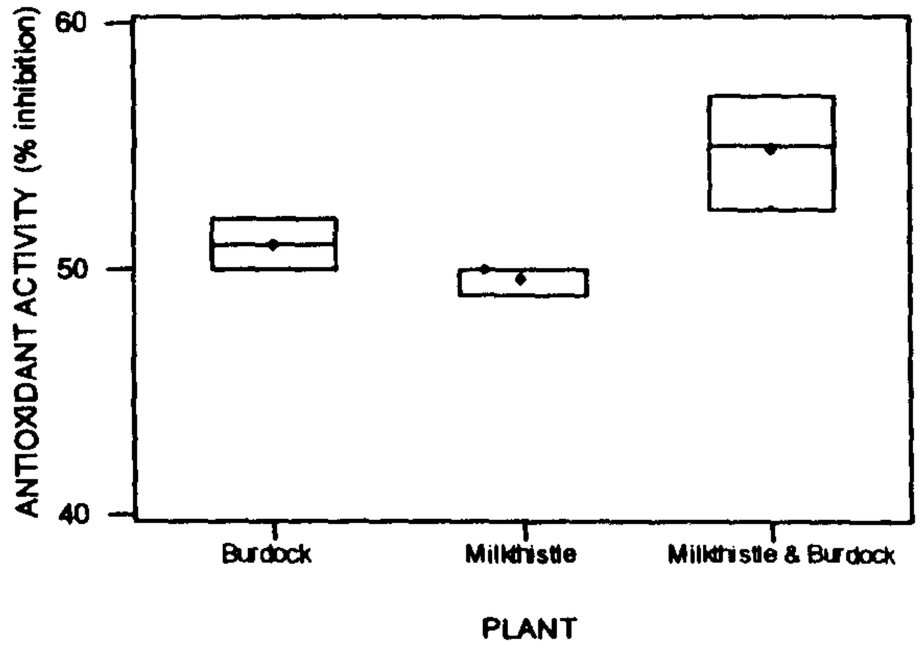


F. *Arctium lappa* (Burdock) and *Crataegus laevigata* (Hawthorn fol.)

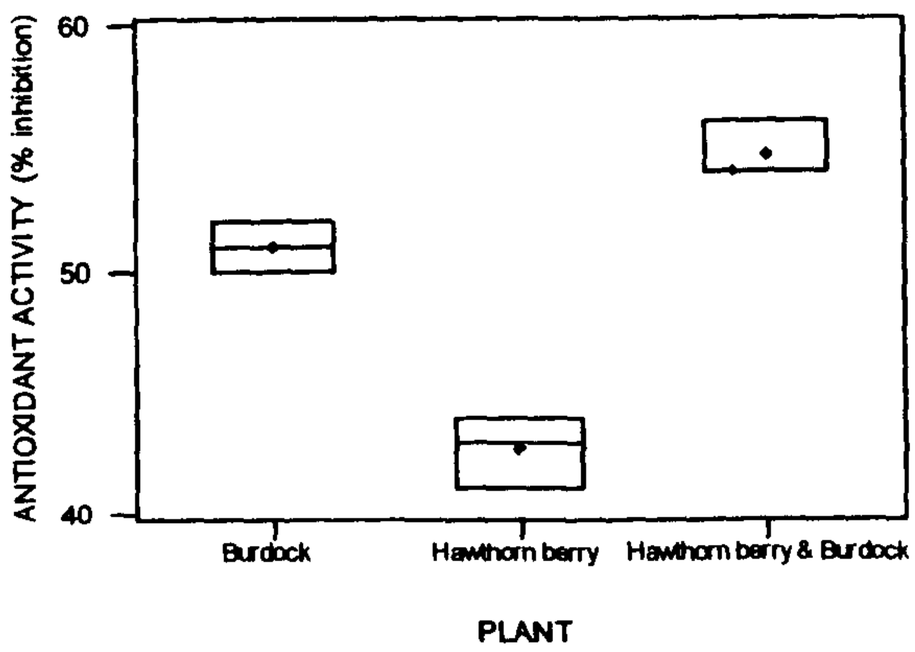
Figure 4.20(d) Boxplot graphs illustrating the % inhibition of linoleic peroxidation by crude plant extracts (final concentration 0.01mg/ml) of single herbs versus pairs of herbs. The median is shown by a line drawn across the box except where data is skewed and the median is either equal to, or too close to the upper/lower quartile to be visible on the graph in which case it is marked by a red dot. A black dot denotes the mean. Graph (C) increased activity (synergy, MW p = 0.1).



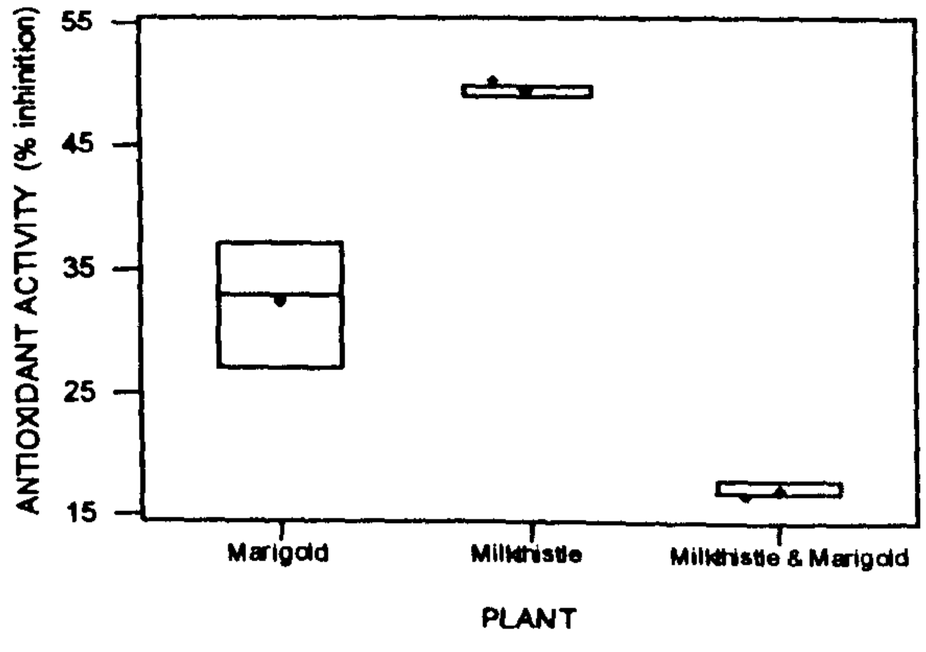
A. *Arctium lappa* (Burdock) and *Apium graveolens* (Celery)



B. *Arctium lappa* (Burdock) and *Silybum marianum* (Milkthistle) [Synergy]



C. *Arctium lappa* (Burdock) and *Crataegus laevigata* fruc. (Hawthorn berry) [Synergy]



D. Calendula officinalis (Marigold) and *Silybum marianum* (Milkthistle) [Inhibition]

Figure 4.20(e) Boxplot graphs illustrating the % inhibition of linoleic peroxidation by crude plant extracts (final concentration 0.01mg/ml) of single herbs versus pairs of herbs. The median is shown by a line drawn across the box except where data is skewed and the median is either equal to, or too close to the upper/lower quartile to be visible on the graph in which case it is marked by a red dot. A black dot denotes the mean.
Graph (B) increased activity (synergy, MW p = 0.03); Graph (C) increased activity (synergy, MW p = 0.03); Graph (D) decreased activity (inhibition, MW p = 0.03). A different scale is used for Graph D.

4.3.3 Discussion

The combination of crude plant extracts in pairs resulted in a demonstration of synergy against linoleic acid oxidation from five of the pairs and antagonism from three, as outlined in section 4.3.2, giving a total of eight interactions, approximately one third of the possible 28 two-herb combinations tested. Although in terms of magnitude the reactions displayed were fairly minor, it was observed by Berenbaum, (1987) in antibacterial studies, that minor interactions *in vitro* may result in significant synergism *in vivo* suggesting that the presence of relatively small degrees of synergy and indeed antagonism may well be of clinical significance.

No obvious trends emerged from the results by which any reasonable conclusions could be based. The following observations however may be relevant to further antioxidant research. All five two-herb combinations that demonstrated synergy against linoleic acid oxidation were combinations of 1) flavonoid-containing plants, i.e. flavonoids as shown by phytochemical analysis were the principal constituents, and 2) plants that primarily contained polyphenolic acids. *Arctium lappa* for example, shown by phytochemical analysis primarily to contain a range of caffeic acid derivatives was synergistic with the plants *Silybum marianum, Crataegus lavigata* (berry) and *Calendula officinalis,* whose primary constituents were confirmed as flavonoids, i.e. a range of flavanolignans; proanthocyanidins, flavonols and flavones and flavonols respectively. Significantly, *Arctium lappa* is a primary constituent of the Essiac formula (section 1.1.6.3) the potency of which is based anecdotally on the synergistic interaction of its four herbal constituents. There is no data on the possible synergistic effect of these individual herbs (Tamayo et al, 2000) however, since *Arctium lappa* has indicated a degree of synergy with three of the herbs tested, it possibly has an affinity for being synergistic with other herbs. *Calendula officinalis,* which showed the greatest propensity to pro-oxidant activity at low concentrations against lipid peroxidation (Fig. 4.8 section 4.2.1.1), was synergistic with *Arctium lappa,* but inhibitory with *Taraxacum officinale,* which was also shown by phytochemical analysis only to contain a range of caffeic acid derivatives. Both these herbs, as well as polyphenols, also contain a range of other chemical constituents (sections 2.3.1/2.3.3) not investigated in this study, that overall give each plant their own unique phytochemical profile, and may affect their interaction with other plant extracts quite differently.

Silybum marianum, whose principal constituents are flavanolignans, was synergistic with *Arctium lappa* but antagonistic with *Calendula officinalis* and *Crataegus laevigata* (leaves and flowers). Both plants were shown to contain a high proportion of flavonoids, especially flavonols and flavones. *Crataegus laevigata* (leaves and flowers) however, which also contained proanthocyanin flavonoids, was synergistic with *Salix alba* (bark), which, in addition to its range of salicylic acid derivatives, also contains proanthocyanins and some flavanones, perhaps suggesting that the combination of certain flavonoid types in some plants may affect their compatibility. *Apium graveolens* (seed), a flavonoid-containing plant consisting mainly of flavones, did not interact with any of the other seven plant parts tested.

Interactions in a combination may vary depending on the concentration and ratios between the constituents. Synergy apparent at one concentration may, at another, express a zero interactive response (Savelev et al., 2003). To substantiate the results already achieved therefore at a final concentration of 0.01mg/ml, dose related synergy should also be tested. Results for the antioxidant activity (expressed as percent inhibition of linoleic acid peroxidation) of the individual extracts showed no linear relation to dose and several anomalous results were observed. It is possible therefore that each pair of extracts may interact differently depending on the concentration. Since some of the pairs gave different results for both assays, i.e. the two assays used to test the antioxidant potential of our individual crude plant extracts, they cannot be extrapolated to other antioxidant systems. Pairs of extracts tested on the basis of IC_{50} values in the DPPH assay produced only additive effects, even in those combinations that had demonstrated either synergy or antagonism by the more complex process of inhibiting linoleic acid peroxidation. Although the concentrations used to test the crude plant extracts in both assays were different, this is unlikely to have affected the outcome for DPPH scavenging, which is quite simply based on the ability of a mixture to scavenge this radical via step-by-step hydrogen donation thus producing a purely additive effect. Consequentially, these results suggest that in the lipid peroxide assay, mechanisms other than hydrogen donation may be contributing to the antioxidant activity of some pairs of extracts.

Most studies on plant synergy have compared the activity of the natural plant extract with the activity of individual components combined in their naturally-occurring ratios (Savelev et al., 2003; Wagner and Steinke, 2004). Although several *in vivo* studies

using combinations of herbs have been carried out (Williamson, 2001), there are few *in vitro* studies on the interactivity of natural plant extracts in combination. Since statistical analysis is based on probability, more research is required to explore the interactions observed in this study. The method of Berenbaum, (1978; 1985) based on an assumption of zero activity of agents in a combination is the one most commonly used at present. Although this approach facilitates analysis of a combination of agents with different types of dose-response relation and permits combinations of any number of agents, according to Wagner (2004), this cannot plausibly be applied to herbal extract mixtures because all these agents must first be defined and isolated. Savalev et al (2003), commenting on methodological issues in relation to their investigations into the synergistic and antagonistic interactions of the anticholinesterase terpenoids of *Salvia lavandulaefolia* essential oil, demonstrate how two methods may be used to explore the interactions between compounds and how this may give different results when applied to the same set of data, so that a mixture may appear zero interactive according to one and synergistic to another. Thus, a suitable method for assessing combinations of herbal mixtures still appears to be elusive, especially since an inappropriate approach could lead to misleading information which could influence future research.

The scientific evaluation of herbal extracts is still very much in its infancy and there is much further knowledge to be gained. Investigations now show that most natural plant extracts exert multivalent effects and this multivalence of pharmacological actions can generate additive or over-additive potentiated synergistic effects (Wagner, 2004). Since this multivalent activity forms the main basis of prescribing in Western Herbal Medicine, the polyvalent or additive effects of the multiple active constituents of the two-herb combinations that did not show any obvious interaction cannot be undervalued. These experiments can only be viewed as a starting point.

CHAPTER 5: PRODUCT INVESTIGATION AND ANALYSIS

5.1. Introduction

In Chapter 4.2 plant extracts were investigated for their *in vitro* antioxidant activity against linoleic acid oxidation. During the course of these investigations some anomalous results were observed. Assays using certain batches of linoleic acid were found not to form hydroperoxides, indicated by a lack of colour change when the incubated substrate was measured by the ferrous thiocyanate method (section 3.2.2.1). This prompted further investigation to confirm the correct composition of linoleic acid and to show the presence of any contaminants that could retard oxidation.

Several lines of enquiry were used including the addition of a probable initiator, benzoyl peroxide, to the assay substrate to promote the production of hydroperoxides, discussions with the suppliers of the product and analysis of the different batches of linoleic acid, i.e. 'active' and 'inactive', by Gas Chromatography, Nuclear Magnetic Resonance Spectroscopy (NMR) and Infrared Spectral analysis.

5.2 Results and Discussion

5.2.1 Experimentation with benzoyl peroxide

Linoleic acid oxidation occurs as outlined in section 3.2.1.3 by autoxidation, i.e. oxidation by exposure to atmospheric oxygen. An important feature of this reaction is that it is autocatalytic. Unlike this method, some of the other assays used to test lipid antioxidant activity that use substances such as tissue homogenates, liposomes and lipoproteins, add an initiator to the substrate to start lipid peroxidation (Halliwell, 1990). Using the previously 'inactive' batch of linoleic acid, an initiator benzoyl peroxide (Revitt, 2004) was added to the assay substrate (section 3.2.2.6.1) to start lipid peroxidation. The use of this initiator failed to start the desired reaction, i.e. the formation of hydroperoxides in the assay substrate, measured as previously outlined by

the ferrous thiocyanate method (section 3.2.2.1). A slight colour change was detected, indicating the presence of a very low level of hydroperoxides. The generation of hydroperoxides at this level would have been insufficient to test the ability of an antioxidant to inhibit linoleic acid oxidation (see section 3.2.2.6).

5.2.2 Product Suppliers - investigations and discussions

The product suppliers, i.e. Sigma-Aldrich and Fisher Chemical Company, after their own investigations, confirmed that in regard to the linoleic acid product we were using there had been no changes to manufacturing procedures. The starting material, Safflower oil, remained the same and no chemical (e.g. preservatives) that could have inhibited oxidation of the linoleic acid, i.e. one that acted in the same way as an antioxidant, had been added. The linoleic acid was extracted from the base material by preparative High Performance Liquid Chromatography (HPLC). The presence of any oleic acid should not affect the oxidation potential of the linoleic acid under the selected assay conditions. Although the batch numbers were different the product itself, in the opinion of both suppliers, should be exactly the same. Linoleic acid subsequently supplied by Lancaster Synthetics Ltd., which was the product mainly used in this study was consistently reactive.

5.2.3 Instrumental analysis

5.2.3.1 Gas Chromatography

Gas chromatography was carried out on different batches of linoleic acid, i.e. 'active' and 'inactive' both at Middlesex University and at the Jodrell laboratory, Kew. Chromatograms of underivatised samples carried out at Middlesex University showed that the principal component of all the samples was linoleic acid with minor quantities of various low oxidation potential compounds present as identified by the chemical database. Gas chromatography carried out by the Jodrell laboratory, Kew on a selection of samples underivatised in hexane and by borontrifluoride-methanol derivatisation, gave the same results as above.

An observation by Kew staff that the two types of sample when mixed with solvent appeared to have slightly different shades of colour prompted the hypothesis that the difference in activity between the two samples could be related to *cis-trans* isomerism. Naturally occurring linoleic acid consists of an 18-carbon chain and 2 double bonds (C_{18} H_{32} $O_{2)}$ and is usually found in a *cis* configuration, i.e. *cis cis*. There are however, 4 isomers possible:- *cis cis; cis trans; trans cis and trans trans.* According to Sigma-Aldrich, the naturally occurring product, although primarily of a *cis cis* configuration, can, by a reversible reaction, go from *cis* to *trans* or *trans trans* (personal communication, technical dept.), and the product they supply may also contain these configurations. This is relatively unlikely however in a product manufactured by preparative HPLC. A consensus of speculative opinion indicated that the significance of isomerism in this instance was that the shape of the molecule could affect oxidation (Sigma-Aldrich, technical dept; Jodrell laboratory, Kew, personal communications). A *cis* configuration has a "V" shape and a *trans* configuration looks more like a straight line. The more open and straight the molecule the easier it is to oxidise because the double bonds are more physically accessible. To test this hypothesis NMR analysis was carried out on the 'active' and 'inactive' linoleic acid samples at the Jodrell Laboratory, Kew.

5.2.3.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

The NMR spectra of the two samples were found to be similar, making it difficult to identify any structural differences, Figs. 5.1 and 5.2.

5.2.3.3 Infrared Spectra

Infrared Spectral analysis, that could identify both the organic and inorganic components of our samples, was carried out to detect the possible presence of a contaminant that could retard oxidation if present in the assay substrate. The analysis was carried out on two samples of linoleic acid, i.e. 'active' and 'inactive' by Peter Whitton at Aston University. Infrared Spectra of the two samples are shown in Figs. 5.3 and 5.4. Although no obvious difference was observed in the previous analysis, i.e. Gas Chromatography and N.M.R., the infrared spectra of the two samples indicated a difference between the two samples. A large double peak on the infrared spectrum between band 650-700cm^{-1} in Fig. 5.3 is absent from the infrared spectrum in Fig. 5.4.

A review of possible compounds that absorb at a wavelength of 650-700 cm^{-1} showed sulphite as the most likely compound (Weast, 1980, page F-263; Williams & Fleming, 1989). This corroborates the opinion of the NMR staff at the Jodrell laboratory, Kew that an inorganic contaminant like sulphite might be the causative factor.

The compound interpretation shown with each IR spectrum relates to the best match the software found in the database, albeit the algorithm is far from foolproof. In Fig. 5.3, the compound suggested by the computer's chemical database i.e. phenylhexanoic acid, was rejected because a benzene ring usually appears in infrared spectra between bands 1500-1600 cm^{-1} (Williams & Fleming, 1989).), so clearly does not relate to the peak between bands 500 – 750 cm^{-1}. This would also be at variance with the N.M.R. spectrum and the retention time and spectra from Gas Chromatography.

Sulphite is a considerably more powerful reducing agent than linoleic acid and would preferentially scavenge any oxidants present, thus inhibiting linoleic acid peroxidation via autoxidation (Weast, 1980, page D-146). Since sulphite absorbs at a frequency band of 650-700 cm^{-1}, it is the author's contention that in view of the limited evidence available, it is possible that the bulk containers in which the linoleic acid is stored by the suppliers may have been cleaned with a sulphite solution as an antibacterial precaution. This is not uncommon industrial practice. This idea was compounded by the discovery that linoleic acid from Lancaster Synthetics Ltd. with the same batch number but supplied from different bulk containers, was both effective and ineffective depending on which container it was supplied from.

5.3 Conclusions

The linoleic acid assay provides a relatively quick and effective method of measuring the antioxidant activity of a plant extract. The exposure of the linoleic acid-alcohol-water substrate to oxygen during incubation however, is crucial to successful lipid peroxidation. Thus, due to the sensitivity of this assay, a contaminant like sulphite, even at a very low level that could interfere with this oxidative process will retard linoleic acid oxidation with a subsequent lack of measurable peroxides. The next step would be to test this hypothesis by carrying out further analysis for the presence of sulphite by using ion chromatography or other suitable method.

KEW783 SAMPLE A, 1D 1H SPECTRUM ACQUIRED ON AVANCE400

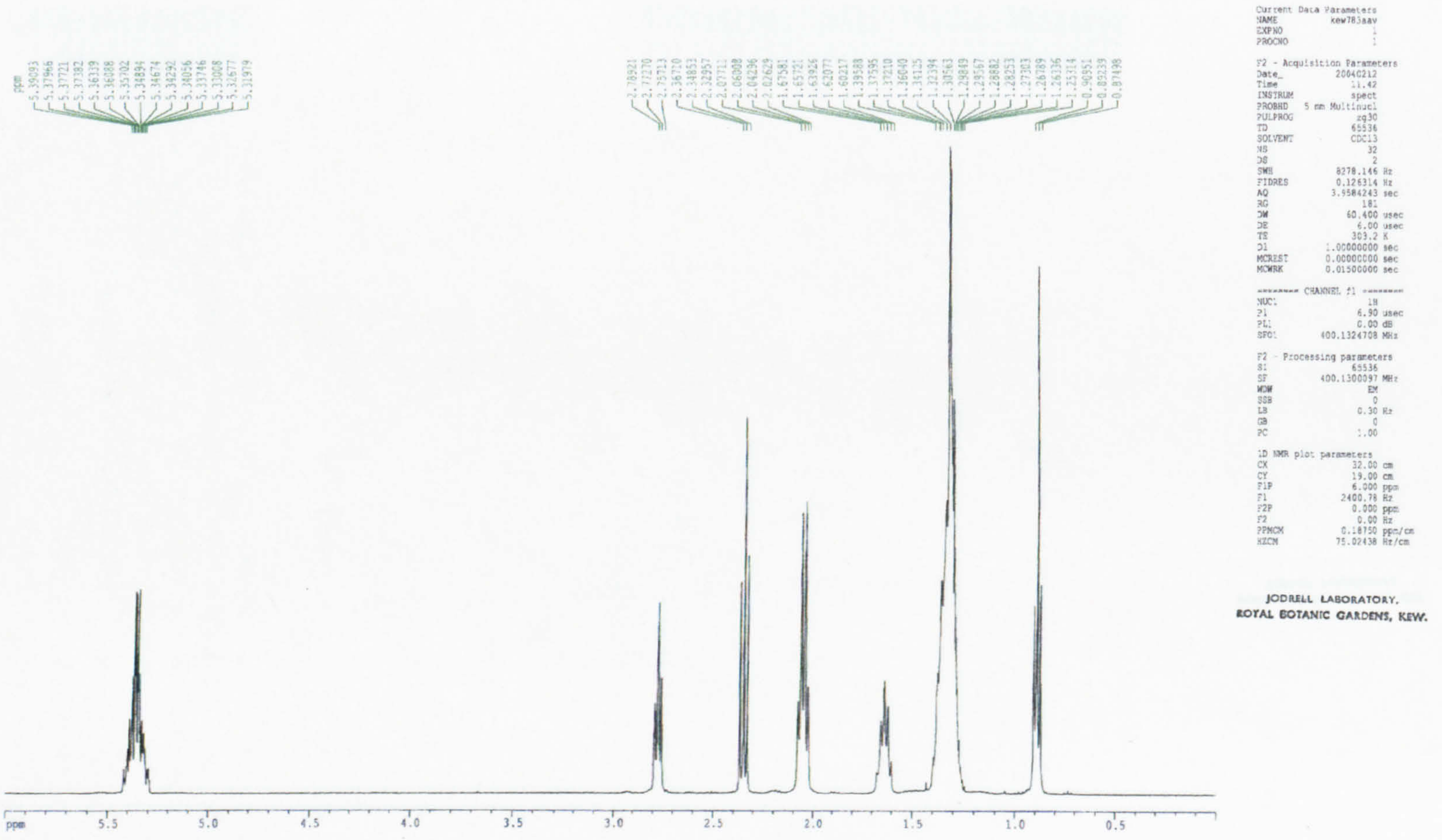
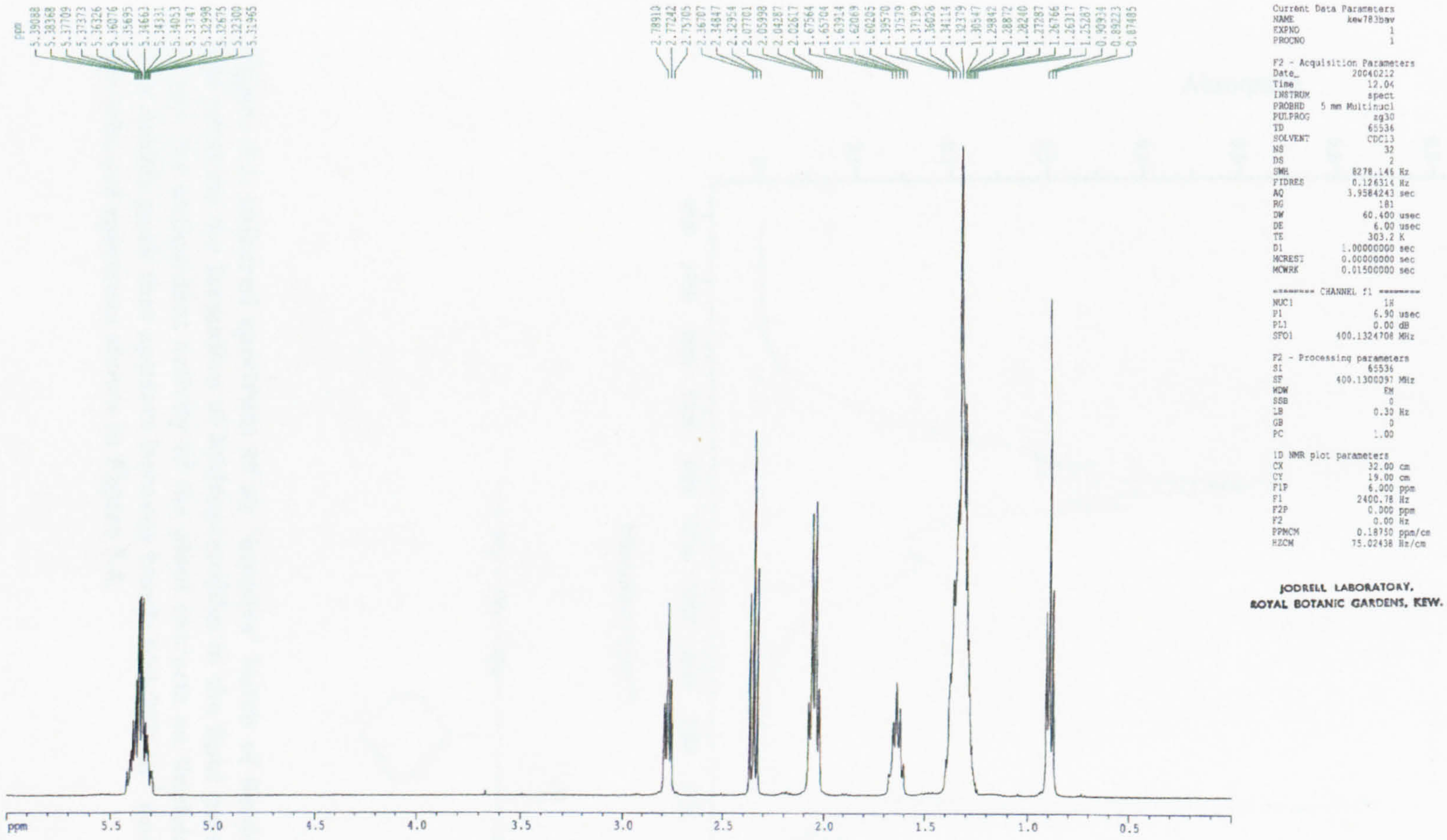


Figure 5.1. Proton NMR spectrum of an 'inactive' sample (A) of linoleic acid. Comparison with a standard sample of 99% linoleic acid from Aldrich confirmed the identity of this sample. There were no obvious structural differences between sample (A) above and sample (B) shown in Figure 5.2 .

KEW783 SAMPLE B, 1D 1H SPECTRUM ACQUIRED ON AVANCE400

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PROCNO                  1

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PULPROG             zg30
TD                 65536
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NS                     32
DS                      2
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FIDRES             0.126314 Hz
AQ              3.9584243 sec
RG                    181
DW                 60.400 usec
DE                  6.00 usec
TE                303.2 K
D1             1.00000000 sec
MCREST         0.00000000 sec
MCWRK          0.01500000 sec

======== CHANNEL f1 ========
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P1                  6.90 usec
PL1                 0.00 dB
SFO1         400.1324708 MHz

F2 - Processing parameters
SI                 65536
SF          400.1300097 MHz
WDW                   EM
SSB                    0
LB                  0.30 Hz
GB                     0
PC                  1.00

1D NMR plot parameters
CX                 32.00 cm
CY                 19.00 cm
F1P                6.000 ppm
F1                2400.78 Hz
F2P                0.000 ppm
F2                   0.00 Hz
PPMCM            0.18750 ppm/cm
HZCM            75.02438 Hz/cm
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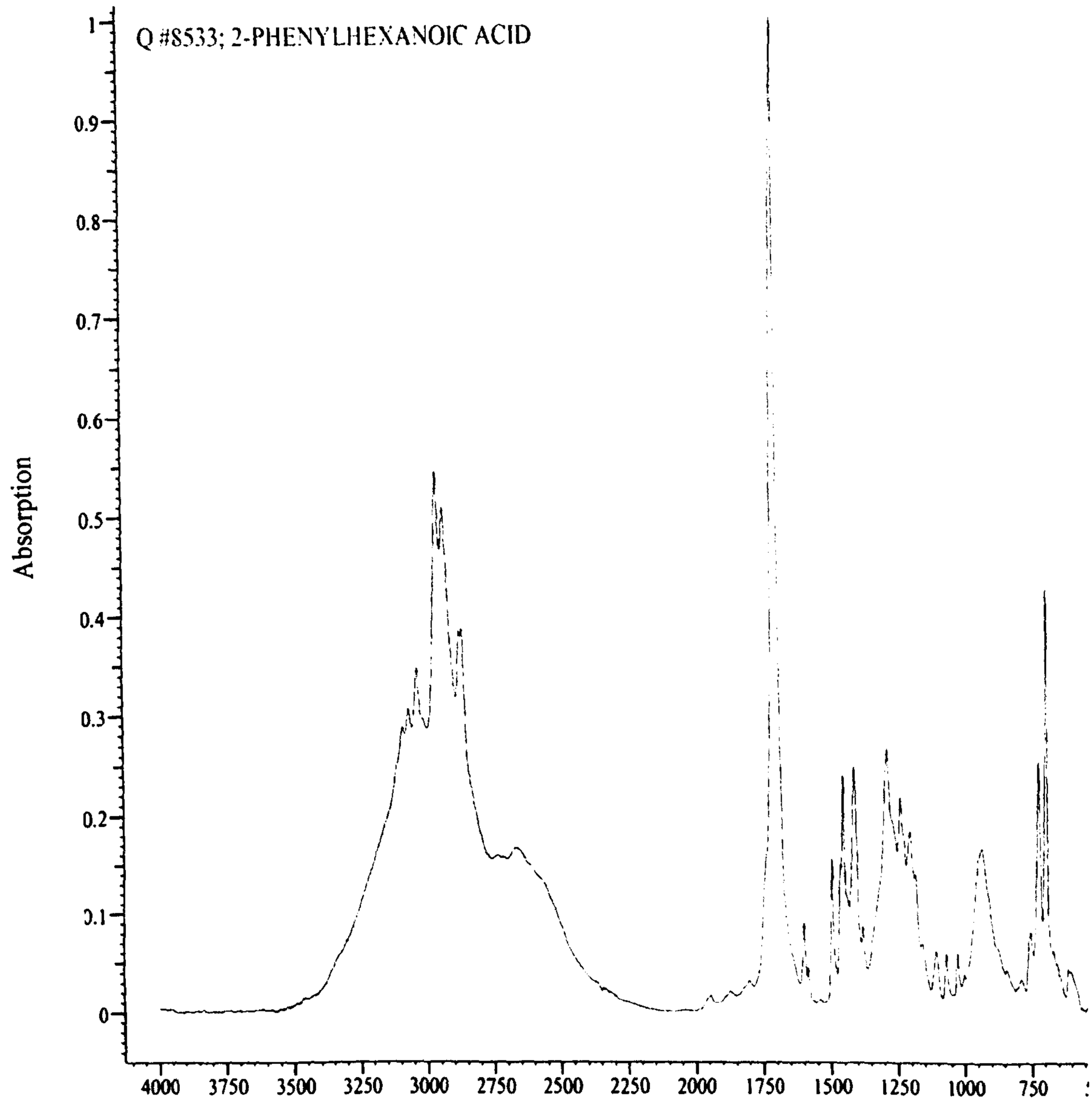
JODRELL LABORATORY,
ROYAL BOTANIC GARDENS, KEW.

Figure 5.2. Proton NMR spectrum of an 'active' sample (B) of linoleic acid. Comparison with a standard sample of 99% linoleic acid from Aldrich confirmed the identity of this sample. There were no obvious structural differences between sample (B) above and sample (A) shown in Figure 5.1.



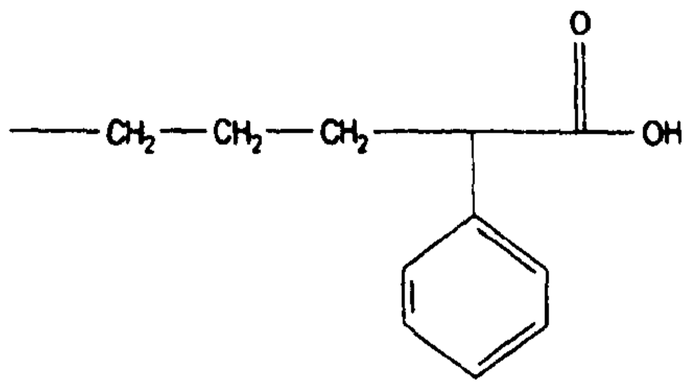
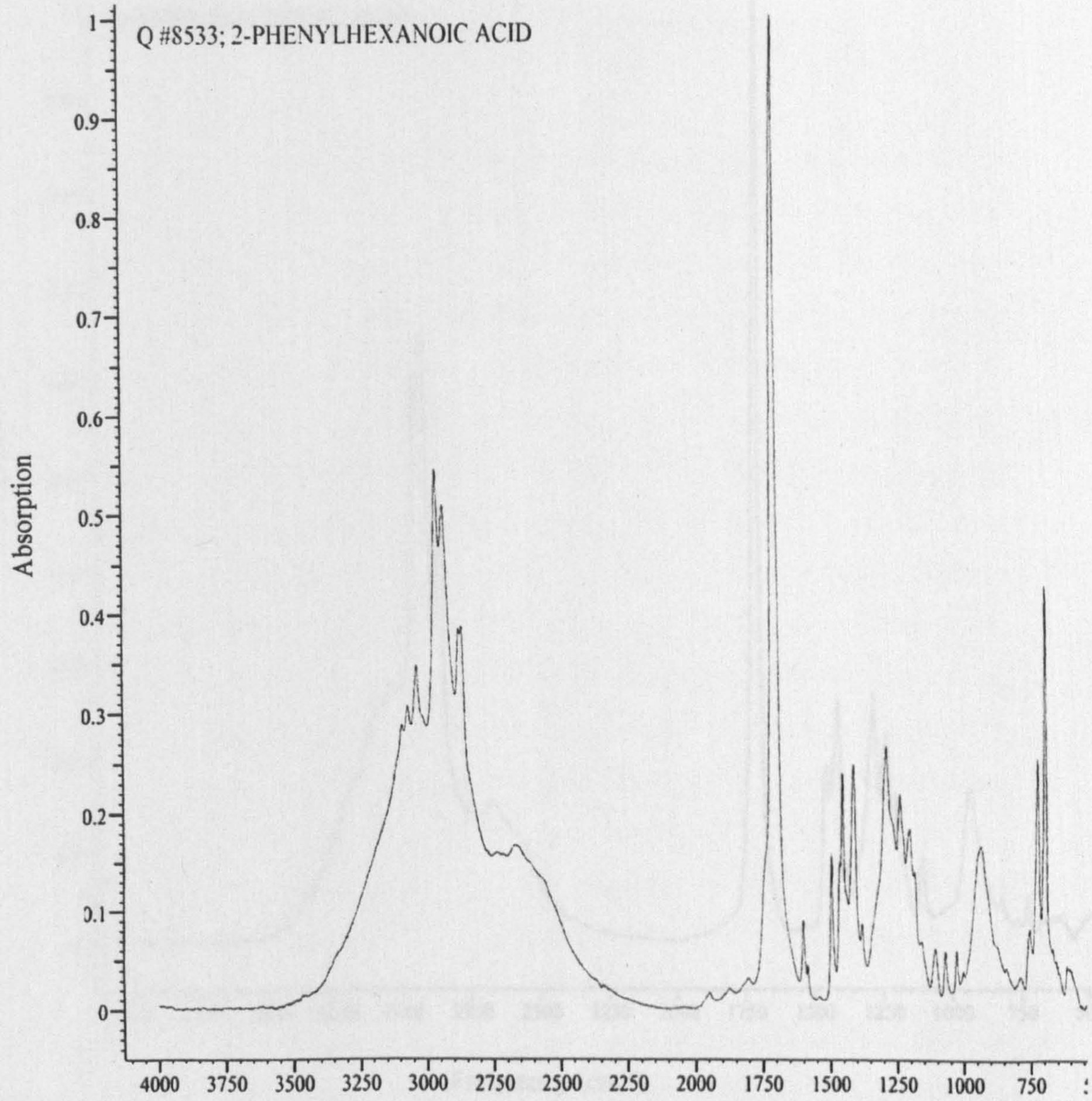


Figure 5.3. Infrared spectrum of an 'inactive' batch of linoleic acid that did not generate the formation of hydroperoxides in the lipid peroxide assay used to test the antioxidant activity of the plant extracts on linoleic acid oxidation. The double peak that appears between bands 650-750cm^{-1} does not appear on the infrared spectrum shown in Figure 5.4.



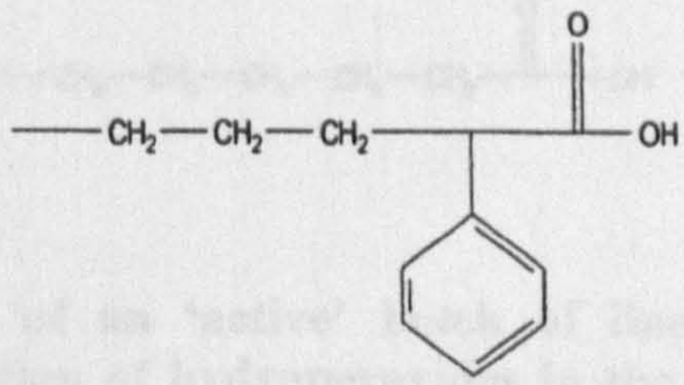
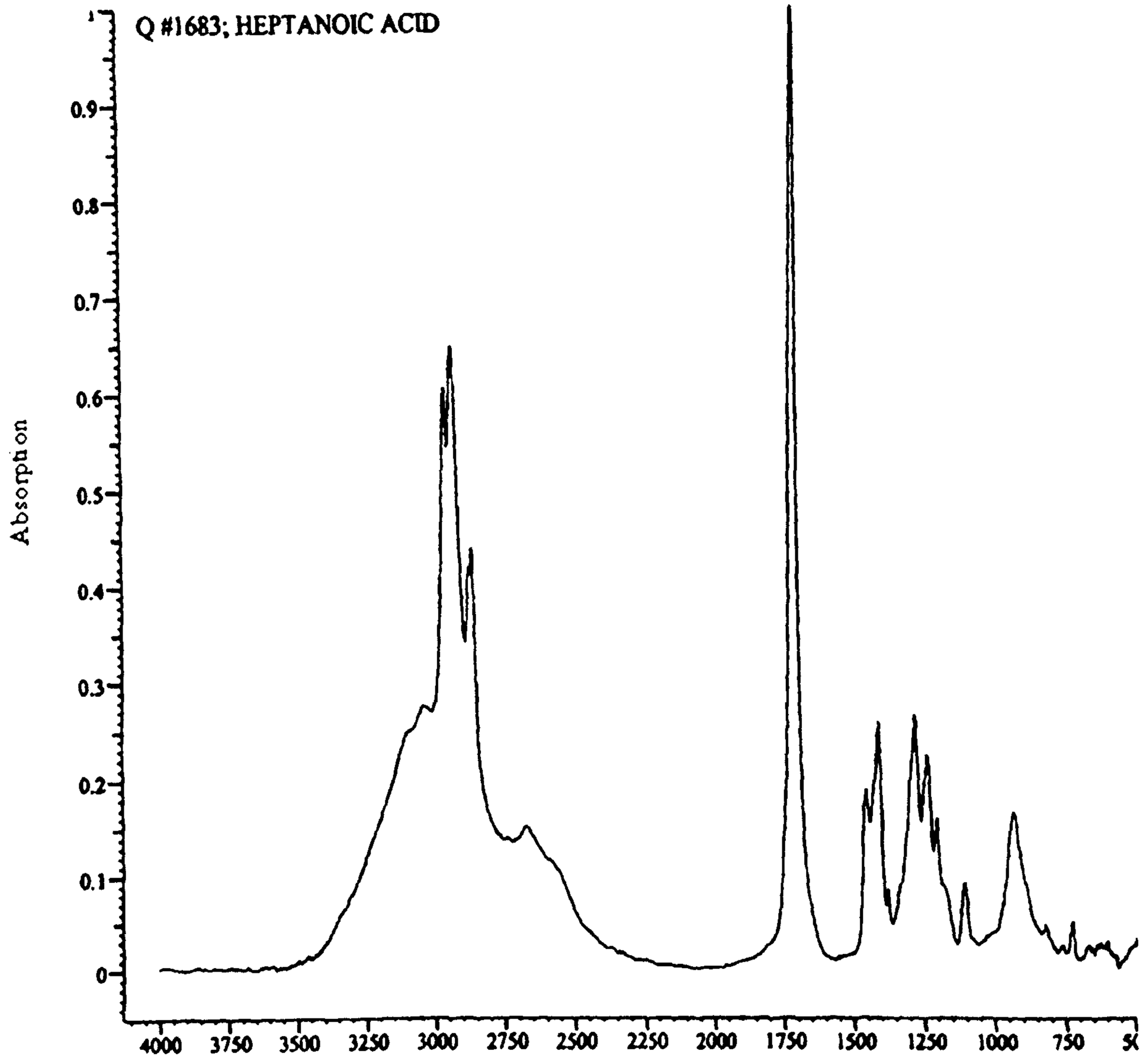


Figure 5.3. Infrared spectrum of an 'inactive' batch of linoleic acid that did not generate the formation of hydroperoxides in the lipid peroxide assay used to test the antioxidant activity of the plant extracts on linoleic acid oxidation. The double peak that appears between bands 650-750cm^{-1} does not appear on the infrared spectrum shown in Figure 5.4.



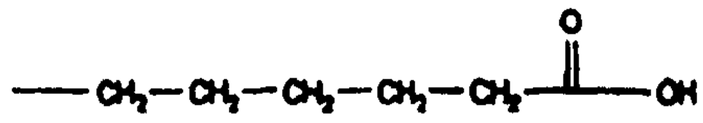


Figure 5.4. Infrared spectrum of an 'active' batch of linoleic acid that successfully generated the formation of hydroperoxides in the lipid peroxide assay used to test the antioxidant activity of the plant extracts on linoleic acid oxidation. The chemical formula shown relates to the best match the software found in the database, i.e. the match index was only 815.32 out of a possible 1000. At this level compound identification becomes speculative. The double peak between bands 650-750cm^{-1} in Figure 5.3 is absent from the above infrared spectrum.

CHAPTER 6: GENERAL DISCUSSION

Osteoarthritis is the most common form of joint disease strongly associated with ageing (Doherty, 2001). Cartilage degeneration is a hallmark of cartilage-ageing and osteoarthritis (Tiku et al, 2000). Degeneration of articular cartilage in osteoarthritis is accompanied by chronic pain and significant disability. Reactive oxygen species are implicated in both cartilage ageing and the pathogenesis of osteoarthritis (Tiku et al, 2000). Conclusions from a systematic review (Long et al, 2001) on herbal medicine used for the treatment of osteoarthritis and a pilot study (Bell et al, 1999) investigating herbal treatment outcomes present promising evidence for the effective use of some herbal preparations for the treatment of this condition. Since *in vitro* evidence linking chondrocyte lipid peroxidation to cartilage matrix protein (collagen) oxidation and degradation suggests that chain breaking antioxidants have a preventive role (Tiku et al, 2000), the effectiveness of herbal remedies traditionally used in its treatment may be linked to their antioxidant potential. Phytochemical analysis of the crude plant extracts of the selected plants all showed the presence of a number of phenolic compounds including flavonoids, identified firstly by TLC via comparison with authentic samples and then by HPLC. Preliminary results indicate that all the crude plant extracts have promising antioxidant activity on both the DPPH radical and linoleic acid oxidation, thus making them ideal defence agents against peroxyl radical-mediated pathological disorders including osteoarthritis, where lipid peroxidation-linked damage plays a causative role (Tiku et al, 2000).

The everyday practice of herbal medicine, as discussed in Chapter 2, involves a multi-faceted approach to treatment by means of a prescription containing herbs which have a broad range of appropriate actions. Although the plants selected for this study would be included in an herbal prescription for their range of different actions (section 2.2.2), their collective antioxidant properties may fulfil one of the main treatment objectives for osteoarthritis, the reduction of inflammation. The superoxide radical is overproduced in inflammation as a result of the bactericidal action of activated phagocytes (Javanovic et al, 1998). Although the healthy cell is generally capable of maintaining the balance of production and inactivation of the superoxide radical, when it is overproduced, cell damage can occur if the excess radicals are not inactivated. A study by Kucera (1998) investigating a link between inflammation and the inadequate breakdown of superoxide

revealed a severe defect in superoxide degeneration in the articular exudates of patients with active synovitis. One of the working hypotheses in the beneficial effects of herbal extracts in inflammation is that the excess superoxide is eliminated by the flavonoids (Jovanovic et al, 1998) and the inactivation of the superoxide radical by flavonoids has been thoroughly studied (Tsujimotov et al., 1993; Jovanovic et al, 1994). Flavonoids reduce the superoxide radical to produce hydrogen peroxide and the flavonoid radical (Jocanovic et al, 1994). The mechanism of these reactions appears to be electron transfer (Jocanovic et al, 1994) and this was the mechanism of action tested in this study by the DPPH assay. Since all the extracts of the selected plants tested were shown significantly to reduce the DPPH radical, they may also be effective in the reduction of inflammation associated with osteoarthritis, thus supporting their traditional use in the treatment of this condition. A number of methods are available for the measurement of superoxide radicals (Halliwell, 1990). Having established a possible link between the antioxidant and anti-inflammatory activity of the selected plant extracts, it is now important to carry out further work to evaluate the influence of these extracts on the generation of the superoxide radical in conjunction with their ability to scavenge hydrogen peroxide, one of its primary metabolites.

In spite of the widely acclaimed antioxidant effect of flavonoids, in Western Herbal Medicine the therapeutic potential of most flavonoid-containing plants is not attributed to the flavonoid fraction alone, but rather to the synergistic interaction of a complex mixture of chemically different compounds. As a result, although the crude plant extracts tested in this study have shown marked antioxidant activity and given that this is not the form in which herbs are generally administered as medicines, the antioxidant effects of these extracts were compared with tinctures commonly used by herbalists to investigate if they exhibit similar levels of antioxidant activity. The results suggest that the crude plant extracts in almost all cases appeared to be more active as antioxidants than the tinctures *in vitro*. Whether this is the case *in vivo* is a matter of conjecture. In recent years there has been intensive discussion regarding the comparability of herbal medicine products (Vierling et al, 2003). For the production of equally effective herbal medicines, it is recommended that not only the active substance, but also the following parameters of the finished product are equivalent: mass of native extract, auxiliary substances and administered form and daily dose (Vierling et al. 2003). At least this goes some way to producing comparable products, but no specific information appears to be available on the optimal alcohol-water mixtures required to facilitate the efficient

extraction of the active constituents from the individual medicinal plants (Bilia et al, 2001). This was clearly reflected in most of the results obtained from the phytochemical analysis of the commercial tincture extracts, which generally contained fewer detected compounds than their crude plant extract counterparts. Although this problem has been recognised, phytochemical research is still in its infancy and there is only a small amount of published literature addressing this issue (Bilia et al, 2001; Vierling et al, 2003). Although the crude plant extracts generally demonstrated higher levels of antioxidant activity than the commercial tinctures at identical doses *in vitro*, pharmacological effects *in vivo* may, or may not be comparable. A study by Vierling (2003), investigating the pharmaceutical and pharmacological equivalence of different Hawthorn extracts obtained by the same method but using different solvents, showed that the extract with a lower quantity of the active compounds measured had a comparatively lower potency but similar efficacy on cardiotonic activity in laboratory animals. Because efficacy cannot be scientifically proven by the parameters accepted by orthodox medicine, the relationship between efficacy and dosage is difficult to evaluate in herbal medicine. Providing herbal medicines are pharmaceutically comparable, the question of efficacy may be an issue of clinical observation via controlled clinical trials. The results of this study show that the solvent-water mixtures used as the extraction solvents affected the resulting spectra of constituents, particularly the commercial tincture extracts. There is no statutory method of tincture production in the U.K. however, so the results reported cannot be extrapolated to other products or manufacturers. As the use of complementary therapies, especially herbal remedies, increases in the U.K., there is a need to increase efforts to develop more standardisation of methods for the preparation of phytomedicines, particularly tinctures that are administered as the ingredients of many liquid formulations.

In traditional holistic healthcare, the use of herbal formulae has been the practice for hundreds of years. For example, in both traditional Chinese and Western herbal medicine single herbs are rarely prescribed but combinations are routinely used for the treatment of patients. The concept of combining herbs in this way is not only the polyvalent action of the multi-constituents but the assumed synergistic interaction of all the compounds. Until very recently, outside traditional medicine, the use of single-substance therapy was the accepted philosophy of classic medicine. During the last decade, this treatment approach has seen a shift of emphasis to the treatment of patients with drug combinations that have been shown to be therapeutically synergistic (Wagner,

2004), with the result that the concept of synergy, a vital feature of phytotherapy, is gradually being more widely accepted. The therapeutic superiority of many plant extracts over single isolated constituents is now being recognised along with the potency and efficacy of mixtures of herbs (Williamson, 2001; Wagner, 2004). Much of our knowledge of plant interactions is still theoretical rather than practical and the acceptance of the concept of plant synergy requires scientific evidence. Although the limitations of method are appreciated, the results of this study imply synergistic interactions may have occurred, both positively and negatively between some pairs of crude plant extracts that involved an increase or decrease in antioxidant activity against lipid peroxidation, demonstrated *in vitro* via linoleic acid oxidation. The combination of crude plant extracts containing certain flavonoid classes appeared to be less compatible than others. It cannot be predicted however, how these combinations would interact in mixtures greater than the two extracts tested, especially if the additional extracts also contained further types of flavonoid. To explore the interactions already demonstrated, the use of dose-response curves now used by many workers in the field may be the next appropriate step (Williamson, 2001; Savalev et al, 2003). Since it is not possible to translate *in vitro* observations into the reality of the human situation however (Rice-Evans, 2001), the best evidence for synergy is demonstrated by results obtained from clinical trials.

Thus, the proof of efficacy of the selected plants investigated in this study may now need to be substantiated by scientific evidence via controlled clinical trials that conform to the standards outlined by the House of Lords Select Committee Report on Complementary and Alternative Medicine (2000).

CONCLUSION

Knowledge in regard to the aetiology of disease has substantially increased over the last 50 years, particularly in identifying the role of reactive oxygen species, now thought to be critically involved in the causation and the progression of numerous diseases, and the role of antioxidants as a defence system against them. Due to a paucity of scientific research, Medical Herbalists still, to a large degree, rely on anecdotal evidence in their prescribing of plant remedies. Osteoarthritis is a common degenerative disease where free radical damage has now been partly implicated. This study chose to establish a link between the aetiology of osteoarthritis, and the therapeutic efficacy of a small selection of plants traditionally used by herbalists in its treatment. These results show that the extracts of all the plants tested contained a selection of phenolic compounds including flavonoids known to possess antioxidant activity and also demonstrated their antioxidant activity *in vitro*. Although it is not always feasible to extrapolate *in vitro* results to *in vivo*, it is possible that these medicinal plants, when administered as medicines, may be acting therapeutically as antioxidants by contributing to anti-inflammatory activity and by halting the destructive process of lipid peroxidation. Since the antioxidant assays used in this study represent only two antioxidant mechanisms, it may be important to understand how these plant extracts also perform in assays for superoxide scavenging, scavenging of hydrogen peroxide, hydroxyl radical scavenging activity and chain reaction termination of lipid peroxidation using another method such as the liposome model system.

Herbal remedies are most commonly administered by herbalists as tinctures. These results show that the selected plants tested as crude plant extracts were more effective as antioxidants *in vitro* than the tincture extracts. The method of extraction clearly affected the resultant constituent profile of the extract raising concerns not only of potency but also of efficacy in terms of the possible levels of dosages required to achieve a therapeutic effect. Additionally, in regard to quality, with currently no standardised methods of production or accepted measure of quality in place for the manufacture of tinctures for use by herbalists in their prescribing, there is no guarantee of either their potency or efficacy. There is an urgent need for work to be carried out in regard to the optimization of the alcohol ratio and extraction procedure of the herbs formulated as tinctures that are popularly used by Herbal Practitioners. Traditional methods of preparation should be explored as a possible baseline for production. Higher standards

of quality control are required which include some phytochemical analysis and exploration of the chemical stability of the finished product during storage, to ensure the production of an efficacious product. Augmentation of the recently introduced EU Directive on Traditional Herbal Medicine Products should improve manufacturing quality control generally. The author strongly recommends encouragement of Herbal Practitioners to the use of standardised extracts to ensure both consistency of quality and dosage and to make treatment outcomes easier to evaluate.

Our understanding of connections between the antioxidant compounds in herbs, their antioxidant capacity and their impact on the body's ability to resist oxidative damage is still poorly understood. Although the results of this *in vitro* study suggest that higher doses of the tincture formulations may be required than the crude plant extracts to obtain a therapeutic effect, it is not clear if reduced potency will necessarily affect their therapeutic efficacy *in vivo*. Further research is warranted into the efficacy of different types of herbal extracts and their pharmacological effects *in vivo* particularly in regard to their bioequivalence.

Phytochemical analysis showed that the plants in this study have the potential to provide humans with a broad range of antioxidant compounds and it is probable that, with such a mixture of antioxidants, they will enhance a diverse number of physiological activities. Administering the plants as whole plant extracts is likely to be associated with the best promotion of health, especially since research suggests that these compounds are likely to work synergistically. It is also probable that, due to the complex nature of a natural plant extract, some compounds may still be unidentified. Statistical analysis of the results of experimental work carried out to compare the antioxidant activity of single plant extracts against a combination of two extracts tested together indicated that interactions of both a synergistic and antagonistic nature may have occurred. For more conclusive results, all the two herb combinations should now be tested at a range of concentrations to confirm the initial observations. Interactions between key compounds isolated from the pairs of extracts exhibiting synergy or antagonism can then be explored.

Herbalists use combinations of medicinal plant extracts in the belief that synergy between the various components of the mixture occurs. Due to the variety of phytochemicals in each plant extract however, and the possibility that they may act

simultaneously, the interactive effects of these substances would appear to be complex and difficult to unravel. Given that the efficacy of some herbal mixtures may in part be attributed to synergy, the answer to why or how this occurs is likely to be the subject of research and conjecture for some time to come.

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