Discussion

5. DISCUSSION

Reactive oxygen species (ROS) are constantly generated in living organisms as byproducts of cellular metabolism, but can also be produced as a consequence of ionizing radiation, chemotherapeutic drugs and environmental exposure to transition metals and chemical oxidants. ROS can randomly react with lipids, proteins and nucleic acids causing oxidative stress and damage in these macromolecules, leading to pathogenesis of age-related and chronic diseases, including cancer (Maynard *et al.*, 2009).

The term antioxidant typically refers to a large and heterogeneous group of compounds that function by preventing the formation, detoxifying or scavenging of oxidant species. Antioxidants can be grouped under different criteria, such as vitamins (e.g., α-tocopherol, ascorbic acid and β-carotene), synthetic (e.g., butylated hydroxytoluene), natural (e.g., plant-derived polyphenols) and inorganic (e.g., selenium). Some antioxidants act as chain-breaking molecules because they prevent the propagation of or stop radical chain reactions (i.e., α-tocopherol). By contrast, other antioxidants such as glutathione peroxidase and catalase detoxify hydrogen peroxide. This chemical reaction is crucial in cell biology because H_2O_2, in the presence of transition metals, such as Fe^{2+}, generates hydroxyl radical ($^{\bullet}OH$) for which there is no known detoxifying system (Pratico, 2008).

Living systems have developed mechanisms with which to control the harmful effects of ROS/RNS. These systems are mainly based on: (i) the presence of antioxidants (enzymatic and non-enzymatic); (ii) the repair of injured molecules; and (iii) the removal of damaged molecules. The wide

variety of antioxidant defence systems helps to prevent and repair ROS and RNS-induced damage (Mangialasche *et al.*, 2009).

The measure of the antioxidant properties of the herbal products and their active components is important. Different biochemical as well as chemical methods can be employed for these studies. *In vitro* animal model systems such as rat brain and liver mitochondria and various cell culture systems are some of the subjects where these studies are carried out. Several standard biochemical and molecular biological techniques are used to assess the damage caused by free radicals and its repair by the antioxidants. All these studies provide gross pictures about the events that take place involving free radicals and antioxidants in *in vitro* systems (Adhikari *et al.*, 2007).

The world is rich with natural and unique medicinal plants. Medicinal plants are now getting more attention than ever because they have the potential of myriad benefits to society, or indeed to mankind, especially in the line of medicine and pharmacology. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological actions on the human body (Akinmoladun *et al.*, 2007a).

Now-a-days, food industries need new food ingredients obtained from natural sources, to develop novel functional foods or neutraceuticals. Medicinal plants are an important alternative to extract natural antioxidant compounds to delay or prevent the oxidative damage caused by ROS (Athukorala *et al.*, 2006).

One such plant is *Artemisia vulgaris*, commonly called Mugwort, and Masipatchilai in Tamil. There are reports about the antidiabetic, antihelminthic, antiseptic, antispasmodic, antimalarial and antibacterial properties of the plant, but not much data regarding the antioxidant and apoptosis-modulating effects of

the leaves of *Artemisia vulgaris*. Hence, the present study was undertaken to conduct an elaborate and systematic study on the antioxidant and apoptosis-modulating effects of the leaves of *Artemisia vulgaris*.

The study was conducted in four phases. In the first phase, the fresh leaves were analysed for their antioxidant (both enzymic and non-enzymic) content and the different leaf extracts were tested for their antiradical effects. In phase II, the three selected extracts of the leaves (aqueous, methanol and chloroform) were tested for their biomolecular protective effect and its antioxidant effect in an *in vivo*-simulated *in vitro* system subjected to oxidative stress. In the third phase, the effect of the leaf extracts on apoptosis was analysed *in vitro* using yeast, primary chick embryo fibroblasts and cancer cell line. In the final phase, phytochemical analyses of the leaves were done to identify the active principle rendering the antioxidant and anticancer effects.

PHASE I

In this phase, the leaves of *Artemisia vulgaris* were analysed fresh for their antioxidant contents and the different solvent extracts of the leaves were tested for their radical quenching abilities. The results showed that the leaves of *Artemisia vulgaris* were rich sources of enzymic antioxidants, namely superoxide dismutase, catalase, peroxidase, glutathione reductase, glutathione S-transferase and polyphenol oxidase. The leaves were also found to contain considerable levels of non-enzymic antioxidants such as ascorbic acid, tocopherol, reduced glutathione, total phenols, flavonoids, total carotenoids, lycopene and chlorophyll.

These results revealed that *Artemisia vulgaris* leaves have a potential candidature as a source of antioxidants. The literature is rich with reports that correlate the antioxidant content of plants with their medicinal properties.

Metal treated *Bacopa monnieri* L. leaves exhibited increased superoxide dismutase and peroxidase activity (Sinha and Saxena, 2006). The leaf extract of *Plumbago zeylanica* L. was reported to have good catalase activity (Rout *et al.*, 2010).

Triadimefon treated rosea and alba varieties of *Catheranthus roseus* (L.) G. Don. expressed slight variation in superoxide dismutase, ascorbate peroxidase and catalase activities compared to untreated control plant (Jaleel *et al.*, 2006). The decreased catalase activity in the roots of NaCl-treated *Catherantus roseus* (L.) G. Don. recovered with paclobutrazol treatment (Jaleel *et al.*, 2007).

Boiling resulted in a drastic loss of catalase and peroxidase activities in green leafy vegetables (*Sauropus indicum*, *Moringa oleifera* and *Solanum nigrum*) (Padma and Anitha, 2005). The leaves of three under-exploited medicinal plants were found to exhibit moderate catechol oxidase and laccase activities (Nirmaladevi and Padma, 2008).

The total antioxidant, free radical scavenging and reducing powers of *Amaranthus* species, *Centella asiatica*, *Murraya koenigii* and *Trigonella foenum graecum* were attributed to the presence of ascorbic acid, total and β-carotene and total polyphenol (Gupta and Prakash, 2009). Both the fresh and dried form of *Pleurotus florida* and *Calocybe indica* possessed non-enzymic antioxidants namely vitamins C, E, A and GSH (Selvi *et al.*, 2007).

Ünver *et al.* (2009) reported that the plant species like *Mentha piperita* L., *Rhus coriaria* L., *Thymbra spicata*, *Salvia officinalis*, *Rosmarinus officinalis* L., *Capparis ovata* L., *Origanum vulgare* L., *Laurus nobilis* L., and *Capsicum annum* L. were good sources of phenolics. Compelling evidence from epidemiological studies suggest beneficial roles of dietary phytochemicals in protecting against chronic disorders such as cancer, inflammatory and cardiovascular diseases (Son *et al.*, 2008). The study of Ebrahimzadeh *et al.* (2008) have shown that the plant extracts with high phytochemicals and chealating activity can be observed as a good source of new agent for thalassemic patients.

The study carried out by Adedapo *et al.* (2009) revealed that the proanthocyanidins, total phenols, flavonoids and flavonols were higher in the leaves of *Celtis africana* than its stem. The chromatographic analyses of *Spirulina maxima* organic extracts showed that α-tocopherol, carotenoids and chlorophyll-derived phenol compounds were present as the major constituents, which correlated with its antioxidant and antimicrobial potentials (El-Baky *et al.*, 2008).

Khanavi *et al.* (2009) reported that the plants having polyphenols expressed higher antioxidant activity. Perumalsamy and Gopalakrishnakone (2008) reported that alkaloids, flavones (flavonoids, flavonols and quinines), essential oils, lectins, polypeptides, phenolics, polyphenols, tannins and terpenoids derived from medicinal plants tend to possess antimicrobial activities. The methanolic extract of the leaves and stems of *Calpurnia aurea* (Adedapo *et al.*, 2008a) and the methanolic extract of the stems of *Acokanthera oppositifolia* and *Adenia gummifera* (Adedapo *et al.*, 2008b) displayed considerable levels of non-enzymic antioxidants namely phenols, flavonoids,

flavonols and proanthocyanidins. These secondary metabolites are suggested to be responsible for the antioxidant and antimicrobial potentials.

Immature cashew nut-shell liquid was shown to be a unique natural source of unsaturated long-chain phenols which protected *Saccharomyces cerevisiae* cells against H_2O_2-induced oxidative damage, showing a clear and excellent antioxidant action (De Lima *et al.*, 2008). The methanolic extract of *Inula aucherana, Fumaria officinalis, Crocus sativus, Vicum album, Tribulus terestris, Polygonatum multiflorum, Alkanna tinctoria* and *Taraxacum officinale* showed differences in phenolic content (Sengul *et al.*, 2009).

In the present study, *Artemisia vulgaris* leaves proved to be a very good source of well-known antioxidants. Following this, the different solvent extracts of the leaves were tested for their ability to scavenge radicals.

In the present study, *Artemisia vulgaris* leaves were serially extracted into solvents of increasing polarity using Soxhlet apparatus and were tested for their ability to scavenge free radicals. The solvents used were petroleum ether, benzene, chloroform, ethyl acetate and methanol. A fresh crude aqueous extract of the leaves was also used.

DPPH RADICAL SCAVENGING EFFECTS

The different solvent extracts and the fresh aqueous extract of *Artemisia vulgaris* leaves were tested for their ability to scavenge DPPH radicals. The results of both the dot blot screening and spectrophotometric assays revealed that the methanolic extract, followed closely by the aqueous extract, exhibited the maximum scavenging activity.

DPPH is a molecule containing a stable free radical. In the presence of an antioxidant which can donate an electron to DPPH, the purple colour which is typical for free DPPH radical decays and the change in absorbance at 518nm is followed spectrophotometrically. This test could provide information on the ability of a compound to donate a hydrogen atom, on the number of electrons a given molecule can donate, and on the mechanism of antioxidant action (Mothana *et al.*, 2008). Because of the simplicity and reliability of this assay, it has become a routine analysis parameter in all studies of antioxidant potency.

In the TLC-based qualitative antioxidant assay using DPPH spray, the methylene chloride fraction of *Allanblackia monticola* showed the presence of a yellow spot on a purple background on the TLC plates, indicating the free radical scavenging properties (Nguemfo *et al.*, 2009). *Filipendula ulmaria* (L.) *Maxim.*, *Mentha spicata* L., *Tanacetum parthenium* L., *Tanacetum balsamita* L., *Satureja pilosa Velen*, *Sideritis scardica* L., and *Chrysanthemum indicum* L. (yellow and rose type) exhibited strong DPPH scavenging activity (Nikolova and Dzhurmanski, 2009). The tannins extracted from leaves, twigs and stem bark of *Canarium album* showed strong DPPH scavenging activity (Zhang and Lin, 2008).

Green coffee conserves of *Coffea arabica* and *Coffea robusta* (Naidu *et al.*, 2008) and aqueous tea extracts of ten different herbal teas (Büyükbalci and Nehir, 2008) have been reported to have a noticeable effect on scavenging DPPH free radical. The DPPH radical scavenging ability was found to be more in fresh juices of orange and grape fruit than their concentrated form (Belaya *et al.*, 2009).

An ethanolic extract of *Morchella conica* Pers exhibited DPPH scavenging ability, which was fairly significant than the synthetic antioxidants BHA and α-tocopherol (Turkoglu *et al.*, 2006). Akinmoladun *et al.* (2007b) reported that the methanolic extract of *Chromolaena odorata* leaves have less DPPH scavenging effect and relatively low values of antioxidant indices.

Methanolic extract of *Lippia graveolens* (Martinez-Rocha *et al.*, 2008), *Phoenix theophrasti* (Liolios *et al.*, 2009) and semipolar extracts of the root bark of *Lannea velutina* A. (Maiga *et al.*, 2007) exhibited strong DPPH scavenging activities. The methanol extract of the leaves of *Mimusops elengi* Linn. exhibited a dose-dependent scavenging of DPPH radical (Saha *et al.*, 2008).

A methanol extract of *Conyza canadensis* exhibited good DPPH radical scavenging effect than its petroleum ether and ethyl acetate extracts (Hayet *et al.*, 2009). Our results also showed that the methanolic extract of *Artemisia vulgaris* leaves exhibited better DPPH scavenging activity than the other extracts tested.

Several studies provide evidence to the fact that DPPH-scavenging ability is a reliable index of antioxidant potential. Thus, our results showed that the *Artemisia vulgaris* leaves possess good antioxidant activity, which gets maximally extracted into methanol.

ABTS RADICAL SCAVENGING EFFECTS

The six different extracts of *Artemisia vulgaris* leaves were tested for their ability to scavenge the ABTS radical cation. Among all the extracts tested,

the methanolic extract showed higher ABTS scavenging effect than all the other extracts.

Radical scavengers can be evaluated by their reactivity toward the stable free radical ABTS. The DPPH and ABTS systems have both been commonly used to measure the total antioxidant status of various biological specimens because of their good reproducibility and easy quality control (Jang *et al.*, 2008). The ABTS radical scavenging test is widely used to determine the antioxidant activity of both hydrophilic and lipophilic compounds (Gao *et al.*, 2007).

The ABTS assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the stable ABTS radical. In the presence of antioxidants, the absorbance of this radical cation is quenched to an extent and on a time scale, dependent on the antioxidant capacity of the material under investigation (Drobiova *et al.*, 2009).

There are many studies reporting the ABTS inhibiting action of purified compounds/plant extracts. Matanjun *et al.* (2008) used ABTS assay as one of the methods to study the antioxidant effect of red, green and brown seaweeds. Polymeric proanthocyanidine fraction from *Pinus radiate* bark expressed a dose-dependent ABTS radical scavenging activity (Ku and Mun, 2008). Florek *et al.* (2009) used ABTS radical scavenging assay to assess the plasma antioxidant capacity in rats exposed to cigarette smoke.

The methanolic extract of *Caesalpinia digyna* root exhibited strong ABTS scavenging effect (Srinivasan *et al.*, 2007). The four different solvent extracts, namely chloroform, ethyl acetate, methanol and ethyl acetate / methanol extracts, and natural substances from *Rhus tripartitum* showed

differential ABTS radical scavenging effect (Mahjoub *et al.*, 2009). The ethyl acetate and methanol fractions of *Evax pygmaea* showed significant ABTS scavenging effects (Boussaada *et al.*, 2008).

The methanol/water and methanol/chloroform extracts of cereals and pseudocereals showed a high percentage of ABTS inhibition, which was close to BHA (Gorinstein *et al.*, 2007). The ABTS scavenging activity of 95% ethanolic extract of *Agrimonia pilosa* was superior to butylated hydroxytoluene, which was suggested to be due to its high phenol and flavonoid contents (He *et al.*, 2009).

Methanolic extracts of *Allium sativum* L., *Allium cepa* L., *Capsicum annum* L. and *Brassica oleraceavar* (Gorinstein *et al.*, 2009) and pressurized low polarity water extracts of cow cockle seed (Güçlü-Üstündağ and Mazza, 2009) showed antioxidant property by inhibiting $ABTS^{\bullet+}$. These reports are in agreement with our results, wherein the methanolic extract, followed by the aqueous extract, showed strong ABTS scavenging effects.

SUPEROXIDE RADICAL SCAVENGING EFFECTS

Superoxide radical is a physiological radical (Tuba and Gulcin, 2008). The leaf extracts of *Artemisia vulgaris* were tested for their scavenging effect on the *in vitro* generation of $SO^{\bullet}$. The results of our study indicated that the methanolic extract exhibited highest $SO^{\bullet}$ scavenging activity than the other five extracts tested.

Superoxide anion is an oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by non-enzymatic electron transfers that univalently reduce

molecular oxygen. It can also reduce certain iron complexes such as cytochrome (Mao *et al.*, 2006). Many reports in the literature associate the $SO^{\bullet}$ scavenging activity of plants and their components with strong antioxidant activity.

Sreelatha and Padma (2009) demonstrated that the extracts of both mature and tender leaves of *Moringa oliefera* have potent superoxide radical scavenging activity. Essential oil from *Mentha longifolia* and *Mentha pulegium* (Hajlaoui *et al.*, 2009) and *Ocimum minimum* (Telci *et al.*, 2009) expressed antioxidant activity by inhibiting $SO^{\bullet}$ production. The polysaccharide fraction from selenium enriched *Ganoderma lucidum* has also been reported to inhibit superoxide radical generation (Zhao *et al.*, 2008).

In another study, compounds from *Euphorbia heterophylla* exhibited good activity in inhibiting $SO^{\bullet}$ generation in a xanthine-xanthine oxidase system (Falodun *et al.*, 2008). Lee *et al.* (2007c) reported that esculin and esculetin from *Fraxinus chinensis* showed superoxide scavenging effect.

The hexane, chloroform, ethyl acetate, methanol and water extracts of bark and leaves of *Cassia siamea* and *Cassia javanica* (Kaur and Arora, 2009) and the methanolic extract of whole plant of *Bergia suffruticosa* (Anandjiwala *et al.*, 2007) showed significant inhibition of $SO^{\bullet}$ in a dose-dependant manner. The aqueous extract from the leaves of *Bauhinia forficata* and *Cissus sicyoides* showed their antioxidant effect on the extent of inhibition of superoxide radical generation (Khalil *et al.*, 2008).

An aqueous methanolic extract of *Bridelia molis* exhibited strong superoxide anion scavenging effect (Ndhlala *et al.*, 2006). Lopez *et al.* (2009) have demonstrated that the aqueous and methanol extracts from *Melissa*

officinalis were active in inhibiting SO$^{\bullet}$ production in a xanthine-xanthine oxidase system. With these available research findings, the ability of the *Artemisia vulgaris* leaf extracts to effectively scavenge superoxide radicals reveals the strong radical scavenging potential of the leaves.

NITRIC OXIDE SCAVENGING EFFECTS

Another radical which was studied in the present investigation was nitric oxide (NO). The maximum inhibition of NO generation was found in the methanolic extract of *Artemisia vulgaris*. The other extracts, except petroleum ether and benzene extracts, showed moderate activity against NO generation.

Nitric oxide is an inorganic reactive nitrogen species synthesized in organisms by different NO synthase isoforms. NO is considered as a fundamental intercellular and intracellular signaling molecule that is essential for the maintenance of homeostasis, acting either as a cytoprotective mediator or as an inducer of apoptosis (Zhao *et al*., 2009).

Moldzio *et al*. (2010) showed that green tea polyphenol epigallocatechin-3-gallate protected striatal slices by counteracting NO. Nitric oxide scavenging assay was adopted by Deepa *et al*. (2009) to compare the antioxidant effect of the ethanolic extracts of the leaves of *Commiphora caudata* and *Commiphora varpubescens*.

The aqueous extract of *Wasabia japonica* expressed their antioxidant property by strong scavenging activity towards NO in a cell free system (Lee *et al*., 2008). Pomegranate fruit extract was tested for its NO scavenging potential *in vivo* (Shukla *et al*., 2008). In another study conducted on the methanolic extracts of four different varieties of rice bran, namely Vasumathi,

Yamini, Jyothi and Njavara, the highest nitric oxide scavenging activity was observed in Njavara (Rao *et al.*, 2010).

The aqueous extract of *Helichrysum pedunculatum* showed appreciable NO scavenging activity, and this activity compared favourably with the standard butylated hydroxytoluene (Aiyegoro and Okoh, 2009). The ethyl acetate extract of *Phyllanthus emblica* possessed potent radical scavenging activity on NO generation (Kumaran and Karunakaran, 2006).

The ethyl acetate extract of *Desmodium gangeticum* moderately inhibited NO in a dose-dependent manner (Kurian *et al.*, 2010). In the present study, the leaves of *Artemisia vulgaris* showed moderate effect on NO generation, which reiterates their antioxidant activity.

HYDROXYL RADICAL SCAVENGING EFFECTS

Oxygen-derived free radicals are thought to be involved in several diseases, such as cancer, cardiovascular diseases and diabetes. Among the various oxygen-derived free radicals, the hydroxyl radical is one of the most highly reactive and harmful oxygen-derived free radicals in a living organism. When $^{\bullet}OH$ is generated in excess and the cellular antioxidant defense is deficient, some free radical chain reactions can attack lipids, nucleic acids and proteins, leading to cellular damage (Ogasawara *et al.*, 2007). The presence of antioxidants can block the initiation of $^{\bullet}OH$ formation and break free radical chain reactions (Nobushi and Uchikura, 2010).

In the present study, the effect of *Artemisia vulgaris* leaf extracts was tested for $^{\bullet}OH$ scavenging activity. The results imply that all the three extracts of *Artemisia vulgaris* leaves (aqueous, methanolic and chloroform) exhibited

strong scavenging effect against $^{\bullet}OH$. The methanolic extract was found to be better than the other two extracts.

There are many literature reports to support this observation. Prakash *et al.* (2007) tested around 25 medicinal plants for their antioxidant and radical scavenging abilities and the tested plants showed better inhibition of hydroxyl radical-induced deoxyribose degradation. Jimenez *et al.* (2008) demonstrated that the raw and processed apricots (*Prunus armeniaca v. bulida*) samples showed very good scavenging effect against $^{\bullet}OH$, which was better than the standard antioxidants BHA and BHT.

The β-carbolline alkaloids showed protective effect against H_2O_2 and paraquat-induced oxidative stress in yeast cells, where their antimutagenic and antioxidant effects were suggested to be due to their ability to scavenge hydroxyl radicals (Moura *et al.*, 2007). The tincture prepared from *Pedilantus tithymaloides* was found to be a good scavenger of hydroxyl radicals (Abreu *et al.*, 2006).

The methanolic extract of *Cassia grandis* showed better hydroxyl radical scavenging effect but no profound effect by chloroform and petroleum ether extract (Meena *et al.*, 2009). The ethyl acetate, ethanol, methanol and water extracts of the leaves of *Lagerstroemia speciosa* L. were found to possess high hydroxyl radical scavenging effect (Priya *et al.*, 2008).

The aqueous extract of *Teucrium polium* L. (Ljubuncic *et al.*, 2006), 70% methanolic extract of *Caesalpinia crista* leaves (Mandal *et al.*, 2009) and the 70% hydroalcoholic extract of *Parkinsonia aculeate* (Mruthunjaya and Hukkeri, 2008) have been reported to inhibit hydroxyl radical mediated deoxyribose degradation. DNA damage by hydroxyl radicals could also be effectively

prevented in the presence of ethyl acetate and butanol fractions of *Ballota nigra* (Matkowski *et al.*, 2008). The ESR spectroscopy method revealed a decreased hydroxyl radical signal by the treatment of KIOM-4 (mixture of extracts from *Magnolia officinalis, Pueraria lobata, Glycyrrhiza uralensis* and *Euphorbia pekinensis*) in a cell-free system (Kang *et al.*, 2010).

It is evident from our results that the extracts of *Artemisia vulgaris* leaves possess very good hydroxyl radical scavenging activity. In the light of the available literature, these findings gain significance in establishing the antioxidant potential of the leaves.

HYDROGEN PEROXIDE SCAVENGING EFFECTS

Hydrogen peroxide is a relatively unstable metabolic product responsible for the generation of hydroxyl radicals and singlet oxygen from Fenton reaction (Queval *et al.*, 2008). Together with the other reactive oxygen species, hydrogen peroxide can damage several cellular components. Therefore, it is crucial for cells to remove hydrogen peroxide as an antioxidant defense (Wang *et al.*, 2007).

The ability of *Artemisia vulgaris* leaf extracts to scavenge hydrogen peroxide in an *in vitro* system was assessed in the present study. The results revealed that, out of the three extracts used, the methanolic extract exhibited a stronger scavenging effect against hydrogen peroxide than the other two extracts.

The activity of curcumin to scavenge hydrogen peroxide was found to be higher than that of the most commonly used antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, α-tocopherol and trolox (Gülcin,

2008). Gülcin *et al.* (2010) reported that tannic acid exhibited strong H_2O_2 scavenging activity.

The active constituents from various berry types showed radical scavenging ability including H_2O_2 (Stoner *et al.*, 2008). The alcalase hydrolysate of *Arca subcrenata* Lischke showed strong antioxidant activity by inhibiting H_2O_2, than the neutrase hydrolysate and papain hydrolysate (Song *et al.*, 2008).

The fraction extracted with methanol/water (50:50) from *Pedilanthus tithymaloides* presented a high H_2O_2 scavenging activity (Abreu *et al.*, 2008). The 70% methanol extract of *Spondias pinnata* exhibited poor H_2O_2 scavenging effect (Hazra *et al.*, 2008).

B-76 genotype of *Vaccinium stamineum* L. (deerberry) was found to exhibit potent H_2O_2 scavenging ability (Wang and Ballington, 2007). Gomez *et al.* (2007) studied the hydrogen peroxide generation in rat liver mitochondria.

An aqueous extract of *Melothria maderaspatana* was capable of scavenging H_2O_2 in a dose-dependent manner and the scavenging activity was better than the standard antioxidant α-tocopherol (Raja and Pugalendi, 2009). H_2O_2 was also scavenged in a dose-dependent manner by the aqueous extract of *Terminalia chebula* Retz. (Mahesh *et al.*, 2007a).

In the context of these literature reports, the observation made in the present study of *Artemisia vulgaris* leaf extracts exhibiting strong H_2O_2-scavenging activity gains significance in strengthening the antioxidant potential of the leaves.

PHASE II

The first phase of the study revealed that the leaf extracts of *Artemisia vulgaris* possess potent antioxidant and antiradical effects. In this phase, the selected leaf extracts (aqueous, methanol and chloroform) were tested for their ability to protect biomolecules (lipids and DNA) from oxidative damage and to analyse their influence on the antioxidant status in H_2O_2-treated liver slices.

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE OXIDATIVE DAMAGE TO LIPIDS

Oxidative stress induced by reactive oxygen species is implicated in the pathogenesis of a variety of diseases. Lipid peroxides are derived from the oxidation of polyunsaturated fatty acids of membranes and are capable of further lipid peroxidation by a free radical chain reaction. Malondialdehyde is a breakdown product of peroxidation of long chain fatty acids that accumulates when lipid peroxidation increases (Kaur *et al.*, 2008).

In the present study, the lipid peroxidation was estimated by quantifying malondialdehyde. Three different membrane preparations were exposed to oxidant and the effect of the leaf extracts to inhibit LPO was analysed. The membrane lipid preparations used were goat RBC ghosts (plasma membrane devoid of intracellular membranes), goat liver homogenate (mixture of plasma membrane and internal membranes) and goat liver slices (intact cells). All the three extracts of *Artemisia vulgaris* caused a significant decrease in the extent of LPO in all the three membrane preparations.

The extent of protection was much better in the liver homogenate than in the other two systems. These results suggest interesting insights into the nature

of the active components involved in the protection of lipids. The fact that higher protection is rendered to the system where the internal membranes are directly exposed to the antioxidants, suggests that the nature of the lipids influences the extent of protection. Additionally, the observation also suggests that some component(s) in the leaf extracts may not be readily membrane permeable, as the extent of LPO in the liver slice (intact cells) was lower. A similar finding was reported by Sumathi and Padma (2009), who postulated the involvement of an endogenous factor that acts in conjugation with the components of *Withania somnifera* to render a better protection to the intracellular membrane lipids.

The ability of herbal components to inhibit LPO, as a reflection of their protective antioxidant capacity, has been a routine investigation in determining the antioxidant effect. Amifostine, triphosphate free oxygen scavenger showed significant inhibition of LPO in spinal cord homogenate (Chronidou *et al.*, 2009). In another study, DOPET, a natural olive oil antioxidant was able to completely reverse the cyclosporine-A induced effect on lipid peroxidation in rat kidney (Capasso *et al.*, 2008). Silibinin from *Silibum marianum* showed decreased nonalcoholic steatohepatitis-induced lipid peroxidation in rat (Haddad *et al.*, 2009).

Ascorbic acid showed a dose-dependent decrease in H_2O_2-induced LPO, which was assayed by measuring the TBARS in cultured peripheral human blood lymphocytes (Siddique *et al.*, 2009). Polyphenols present in *Cinnamomum zeylanicum* and *Acacia catechu* were suggested to be predominantly responsible for the LPO inhibitory action and the antioxidant activity of the plants (Yadav and Bhatnagar, 2009).

A decoction from the flowers of *Echium amoenum* showed potent inhibition of plasma lipid peroxidation, which property was attributed to the probable presence of rosmarinic acid and flavonoids (Ranjbar *et al.*, 2006). Both *Spinacia oleraceae* L. and *Perilla frutescens* L. tended to decrease LPO significantly in plasma (Schirrmacher *et al.*, 2009).

An essential oil of *Canarium schweinfurthii* displayed antioxidant activity *in vitro*, reflected by its capacity to reduce lipid peroxidation (Obame *et al.*, 2007). Lavitschka *et al.* (2007) showed that the alcoholic fraction obtained from *Agaricus subrufescens* gave a dose-dependent protection against lipid peroxidation in rat mitochondrial preparation.

Abu-Rizq *et al.* (2008) reported that the turmeric crude extract from *Curcuma longa*, both at low and high doses, were found to be more efficient against lipid peroxidation in CCl_4-induced rat liver tissues as compared to purified curcumin. An aqueous extract of *Mentha piperita* leaves showed inhibition of LPO in mice liver and lung tissues (Samarth *et al.*, 2006).

The methanol and chloroform extracts of *Lysichiton camtschatcense* (L.) were potent inhibitors of LPO induced by Fe^{2+} and ascorbate in rat kidney and brain homogenate (Takatsu *et al.*, 2009). The methanol extract of *Peltigera rufescens* significantly lowered the LPO induced by carrageenan and cotton pellet granule *in vivo* (Tanas *et al.*, 2010).

Our results are in accordance with these reports. The methanolic extract of *Artemisia vulgaris* leaves protected all the three membranes from lipid damage more effectively than the aqueous and chloroform extracts.

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE OXIDATIVE DAMAGE TO DNA

Oxidative stress is considered as an imbalance between the production of oxidizing molecules and the presence of cellular antioxidants. Free radicals are highly reactive oxidant molecules that cause damage to cells by inducing DNA strand breaks, purine oxidation, protein-DNA cross linking and cell membrane damage. Accumulation of such damage may cause cell death (Gharib, 2009). With this backdrop, in the present study, the DNA-protective effects of *Artemisia vulgaris* leaf extracts (aqueous, methanol and chloroform) were studied.

Three different hierarchical sources of DNA (pUC18, λ DNA and herring sperm DNA) were tested. The results showed that all the three leaf extracts of *Artemisia vulgaris* exhibited protection against hydrogen peroxide-induced damage, of which the methanol extract rendered the maximum protection, in all the DNA samples used.

The literature is rich with reports of the DNA-protective effects of plant extracts and their components. The protective effect of *Azadirachta indica* A. against H_2O_2-induced DNA damage in pBR322 was attributed to the presence of more polar compounds such as nimbolide and quercetin, which also exhibited radical scavenging effects (Manikandan *et al.*, 2009). Estrogenic compounds (17b-estradiol methoxychlor)-induced DNA damage in ovarian surface epithelium was prevented by the antioxidant vitamin E (Symonds *et al.*, 2008).

Alpha lipoic acid showed protective effect against Cr^{6+}-induced DNA damage in both mouse peripheral blood mononuclear and bone marrow cells,

which was dose-dependent (Kumar *et al.*, 2009). Studies have revealed that some amino acids are able to protect DNA damage caused by ROS formation under conditions of moderate oxidative stress (Shtarkman *et al.*, 2008).

The kojizyme enzyme extract from *Ishige okamurae* exerted protection to DNA damage induced by H_2O_2 (Heo and Jeon, 2008). Karawita *et al.* (2007) showed that the enzymic extract of the microalgae *Pediastrum duplex* (also other species) was associated with a reduction in H_2O_2-induced oxidative damage.

Chaudhary *et al.* (2009) reported that various polyphenols and flavonoids present in the extract of *Podophyllum hexandrum* might contribute to the scavenging of radiation-induced free radicals, thereby preventing DNA damage and stimulating its repair. The phenolic fraction from *Zingiber officinale* caused 90% recovery from DNA damage in H_2O_2-exposed calf thymus DNA (Nanjundaiah *et al.*, 2009).

Miranda *et al.* (2008) described that an aqueous extract of *Ilex paraguariensis* could protect H_2O_2-induced DNA damage in liver, kidney and bladder cells and also enhance the DNA repair activity, which may be afforded by the antioxidant activity of its bioactive compounds. An aqueous extract of *Cyanthillium cinereum* inhibited H_2O_2-induced damage to pBR322 DNA (Guha *et al.*, 2009). The water extract of *Allium sativum* L. prevented H_2O_2-induced DNA damage in normal human leukocytes, which was attributed to the presence of phenolic constituents (Park *et al.*, 2009).

The water extract of *Acanthopanax senticosus* protected the DNA damage induced by H_2O_2 in human leucocytes (Park *et al.*, 2006). The results of the present study showed that the leaves of *Artemisia vulgaris* were able to

protect DNA against oxidative damage in pUC18 DNA, λ DNA and herring sperm DNA, proving that the leaves contain components that can protect DNA against oxidant-induced mutagenic lesions.

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE ANTIOXIDANT STATUS OF GOAT LIVER SLICES SUBJECTED TO OXIDATIVE STRESS

The results obtained thus far demonstrated that the leaf extracts of *Artemisia vulgaris* were efficient in protecting biomolecules from the damage caused by oxidants. Subsequently, the influence of leaf extracts were tested on the antioxidant status of cells maintained in their tissue architecture. For this, thin slices of liver were exposed to oxidant and co-treated with plant extracts.

Precision-cut liver slices are one of the most powerful tools used in metabolic studies, as they have a complex structure as well as are rapid and simple in preparation. Thus, this *in vitro* tool functions in a relatively similar way to the situation *in vivo* concerning complex metabolic processes (Glöckner *et al.*, 2008).

In the present study, the precision-cut liver slices were employed as an *in vitro* tool and the antioxidant effect of the leaf extracts was studied in hydrogen peroxide-induced oxidative stress *in vitro*. Enzymic and non-enzymic antioxidants were analysed in the liver slices subjected to oxidative stress in the presence and the absence of leaf extracts.

ENZYMIC ANTIOXIDANTS

The leaf extracts of *Artemisia vulgaris* were tested for their antioxidant effects in the liver slices. The enzymic antioxidants analysed were superoxide

dismutase, catalase, peroxidase, glutathione reductase and glutathione S-transferase.

SUPEROXIDE DISMUTASE

The H_2O_2-exposure caused a significant decrease in SOD activity, whereas, the leaf extracts of *Artemisia vulgaris* significantly elevated the enzyme activity. Among the three extracts used, the methanolic extract exhibited the maximum increase in SOD activity.

Superoxide dismutase is considered as the first line of defense against the deleterious effects of oxy radicals in the cell by catalyzing the dismutation of superoxide radicals to hydrogen peroxide. Molecular oxygen and catalase have been shown to be responsible for the detoxification of H_2O_2 (Chandra *et al.*, 2007).

Antioxidant enzymes, especially superoxide dismutase, have been demonstrated to reduce tumorigenesis both *in vivo* and *in vitro*. Liu *et al.* (2009a) reported that a diet enriched with protandium, a combination of five phytochemicals from medicinal plants, improved SOD activities and suppressed tumor promoter-induced oxidative stress. The trans-ferulic acid and gamma-oryzanol-treated mice recovered from an ethanol-induced decrease in hepatic glutathione level by enhancing SOD activity (Chotimarkorn and Ushio, 2008).

A study conducted by Ghosh *et al.* (2006) reported that the 43kD protein fraction from the leaves of *Cajanus indicus* significantly improved SOD, catalase and glutathione S-transferase activities in chloroform administrated mice liver *in vivo*. A water-soluble polysaccharide MP-I isolated from *Mytilus coruscus* increased the hepatic total superoxide dismutase activity, and

improved hepatic damage in the CCl_4-induced liver injury in mice in a dose-dependent manner (Xu *et al.*, 2008).

Yang *et al.* (2007) reported that the pretreatment with three *Angelica sinensis* polysaccharide fractions improved the activity of SOD in murine peritoneal macrophage intoxicated with tert-butylhydroperoxide. The ethanolic extract of cinnamon enhances enzymic antioxidants namely SOD and catalase in CCl_4-intoxicated rats (Moselhy and Ali, 2009).

Wang *et al.* (2010c) demonstrated that the pretreatment with methanol extract of *Gentiana manshurica* Kitagawa significantly alleviated acetaminophen-induced oxidative stress by increasing GSH content, and retaining the activity of SOD in the liver. Our results indicate that the leaf extracts of *Artemisia vulgaris* improve the SOD activity in the liver slices exposed to oxidant, indicative of their antioxidant potential.

CATALASE

In the present study, the catalase activity was found to be decreased when exposed to H_2O_2. This was effectively reverted by the administration of leaf extracts. The methanolic extract exhibited the maximum activity compared to the aqueous and chloroform extracts.

The effective detoxification mechanism of an organism comprises SOD and catalase, which work in a sequential manner in the disposal of superoxide radical and the conversion of hydrogen peroxide to water (Avti *et al.*, 2006). Co-administration of a protein from *Cajanus indicus* improved the activity of catalase in $HgCl_2$-treated mice (Ghosh and Sil, 2008). The tocotrienol-rich fraction, a main active ingredient of palm oil, showed significantly higher

catalase activity in exercise-induced oxidative stress in forced swimming rats (Lee *et al.*, 2009a).

Saravanan and Pugalendi (2006) reported that ursolic acid improved the activity of catalase in ethanol-treated rat heart. Gupta *et al.* (2007) have reported that the methanol extract of *Oldenlandia umbellate* exerts a protective effect on hepatic injury by CCl_4 by increasing the activity of catalase. Our results showed that the decreased activity of catalase was effectively reverted by the administration of methanolic extracts of *Artemisia vulgaris* leaves.

The chloroform and ethanol extract of *Ichnocarpus frutescens* produced significant hepatoprotection by increasing catalase activity and decreasing the activity of serum enzymes in a dose-dependent manner in paracetamol-administered rats (Dash *et al.*, 2007). The activity of catalase decreased by streptozotocin was effectively increased by the aqueous extract of *Annona muricata* leaves (Adewole and Caxton-Martins, 2006). The ethanol:water (7:3) extract of *Coriandrum sativum* significantly increased SOD, CAT and GPx activities in CCl_4-treated rat liver (Sreelatha *et al.*, 2009).

The ethanol extract of *Aquilegia vulgaris* showed hepatoprotective effect on CCl_4-induced male Wistar rats by increasing antioxidant enzymes (Jodynis-Liebert *et al.*, 2009). The increased activity of catalase by the *Artemisia vulgaris* leaf extracts in H_2O_2-exposed liver slices in the present study also indicates the antioxidant potential of *Artemisia vulgaris* leaves.

PEROXIDASE

The activity of peroxidase decreased by H_2O_2 was effectively reverted by the administration of *Artemisia vulgaris* leaf extracts. The maximum activity

was found in the methanolic extract treated group.

Jayanthi *et al.* (2009) reported that melatonin improved glutathione peroxidase and catalase levels in the brain, heart and blood of oxidatively challenged rats. Lee *et al.* (2009b) reported that the administration of garlic improved the activity of peroxidase in diabetic mice.

The administration of Triphala significantly improved enzymic antioxidants namely SOD, CAT and GPx against cold stress-induced oxidative damage (Dhanalakshmi *et al.*, 2006). Lee *et al.* (2009c) suggested that the supplementation of 8% red beet leaf (*Beta vulgaris* L.) in high fat, high cholesterol diet could prevent lipid peroxidation and improve antioxidant defense system in the plasma and tissue of C57BL/6J mice.

Mahesh *et al.* (2007b) demonstrated that the administration of an aqueous extract of *Terminalia chebula* showed marked increase in GPx activity in aged rat brain, which was suggested to be due to the protection of sulphydryl groups in glutathione from oxidative damage. Treatment with the alcoholic extract of *Momordica charantia* increased the peroxidase activity in ammonium chloride treated rats (Thenmozhi and Subramanian, 2010).

Dehydrocavidine, a main active ingredient of *Corydalis saxicola* Bunting (Yanhuanglian) significantly prevented the depletion of glutathione peroxidase in the liver of CCl_4-intoxicated male Sprague-Dawley rats (Wang *et al.*, 2008a). The oral administration of an aqueous extract of *Annona squamosa* leaf combated the streptozotocin-induced oxidative stress by increasing enzymic antioxidants like SOD, CAT and GPx (Kaleem *et al.*, 2006). Our results are corroborative with these reports, emphasizing the antioxidant response evoked by *Artemisia vulgaris* leaves.

GLUTATHIONE REDUCTASE

The standard oxidant H_2O_2 caused a reduction in GR activity in the liver slices, which was effectively counteracted by the leaf extracts of *Artemisia vulgaris*. The effect of the methanolic extract was found to be maximal in this regard.

The flavoenzyme glutathione reductase is a dimeric disulfide oxido-reductase that converts oxidized glutathione (GSSG) to two molecules of reduced glutathione (GSH) using NADPH as cofactor and an FAD prosthetic group. GR plays a critical role in maintaining the cell's reducing environment and battling oxidative stress (Berkholz *et al.*, 2008).

The activities of glutathione reductase and glutathione S-transferase were decreased in CCl_4 and N-nitrosodiethylamine injured rat liver, which were significantly preserved by the synergistic effect of silymarin and garlic (Shaaraway *et al.*, 2009). The intraperitoneal administration of 43 kD protein from *Cajanus indicus* L. significantly elevated the activity of glutathione reductase during sodium fluoride stress in mice (Manna *et al.*, 2007).

In the present study, the antioxidant potential of *Artemisia vulgaris* leaves was evident by the increased glutathione reductase activity in oxidant-induced goat liver slices.

GLUTATHIONE S-TRANSFERASE

In the present study, during H_2O_2 intoxication, the GST activities were found to be significantly reduced. Treatment with the leaf extracts, however, enhanced the status. The effect was more pronounced in the groups treated with the methanolic extract of *Artemisia vulgaris*.

Oral pretreatment of betaine significantly prevented isoprenaline-induced alterations in the activity of superoxide dismutase, catalase, glutathione peroxidase and glutathione S-transferase in the plasma of experimental rats (Ganesan *et al.*, 2010). Acetaminophen reduced the levels of antioxidant enzymes including GST in the liver and kidney of Swiss albino male mice. Treatment with the 43 kD protein from *Cajanus indicus* significantly reversed these changes (Ghosh and Sil, 2007).

An aqueous extract of *Ocimum sanctum* leaves with vitamin E resulted in a significant increase in GST activity in the tissue of streptozotocin-induced diabetic rats (Eshrat and Mukhopadhyay, 2006). An aqueous extract of *Terminalia arjuna* increased the activities of SOD, CAT and GST in the liver and kidney tissue homogenates of CCl_4-treated mice (Manna *et al.*, 2006). Our results also showed that in addition to methanolic extract, the aqueous extracts of *Artemisia vulgaris* improved GST activity in H_2O_2-exposed goat liver slices.

Prasad *et al.* (2008) reported that lupeol/crude extract of mango pulp treatment resulted in a decrease in ROS levels with restoration in the levels of lipid peroxidation and antioxidant enzymes namely CAT, SOD, GR and GST. Our results indicate that the *Artemisia vulgaris* leaf extracts are able to protect the precision-cut liver slices against oxidant-induced stress by elevating the antioxidant enzyme activities.

NON-ENZYMIC ANTIOXIDANTS

Oxidative stress induced by reactive oxygen species are effectively counteracted by the presence of enzymic and non-enzymic antioxidants in the body. They act as a defense to protect the body from the pathogenic effects of

radicals. The non-enzymic antioxidants analysed in precision-cut liver slices were vitamin C, vitamin E, vitamin A and reduced glutathione.

VITAMIN C

Exposure of liver slices to H_2O_2 caused a significant decrease in the ascorbic acid level, which was improved by treating the tissues with the leaf extracts of *Artemisia vulgaris*. The methanolic extract offered the maximum increase.

Vitamin C is a water-soluble antioxidant occurring in the organism as an ascorbic anion. It also acts as a scavenger of free radials and plays an important role in the regeneration of α-tocopherol (Flora *et al*., 2008).

Visavadiya and Narasimhacharya (2009) reported that *Asparagus racemosus* root powder improved the status of antioxidants namely ascorbic acid, SOD and CAT in hypercholesterolemic rats. Concentrations of vitamin C in plasma were not affected by the mineral wool exposure (Staruchova *et al*., 2008).

An ethanolic extract of the leaves of *Rhododendron arboretum* prevented a decrease in the excretion of ascorbic acid in CCl_4-induced hepatotoxicity in rats. Their antioxidant and hepatoprotective activity was attributed to the presence of quercetin related flavonoids, saponins and phenolic compounds (Prakash *et al*., 2008).

Jia *et al*. (2009b) reported that the polysaccharides of *Ganoderma lucidum* significantly increased enzymic and non-enzymic antioxidants in STZ-induced diabetic rats in a dose-dependent manner. Thus, it is perceivable that

the increase in vitamin C levels by the leaf extracts of *Artemisia vulgaris* proves their ability to improve the antioxidant status in the tissues.

VITAMIN E

Hydrogen peroxide exposure decreased the levels of tocopherol in the goat liver slices. This reduction was effectively reversed by the treatment of *Artemisia vulgaris* leaf extracts. Among the three extracts tested, the methanolic extract caused the maximum increase in the levels of vitamin E.

Vitamin E is an excellent lipid soluble, chain breaking antioxidant in the presence of the co-operative antioxidants like vitamin C and carotenoids. Vitamin E prevents lipid peroxidation and this prevents membrane damage and modification of low-density lipoproteins (Ramprasath *et al.*, 2006).

Morin, a kind of flavonoid, maintained the level of ascorbic acid and vitamin E in 1,2-dimethylhydrazine-induced colon carcinogenesis in a rat model (Sreedharan *et al.*, 2009). According to the work of Kamalakkannan and Prince (2006), oral administration of rutin improved the vitamin E level in streptozotocin-induced diabetic rat tissues, which was attributed to their antioxidant effects.

Excessive TBARS formation and decreased antioxidant status are the effects observed in alcohol-exposed rats. *Trigonella foenum graecum* seed polyphenol extract successfully prevented the damage caused by alcohol, and enhanced the vitamin E level in alcohol-exposed rat (Kaviarasan *et al.*, 2008).

The ethanol extract of *Terminalia arjuna* bark significantly elevated the vitamin E level in hepatocellular carcinoma in rats (Sivalokanathan *et al.*, 2006). Co-administration of vitamin E with the extracts of *Withania somnifera*,

Ocimum sanctum and *Zingiber officinalis* enhanced the protective effects against the oxidative stress imposed in rats (Misra *et al.*, 2005). The *Artemisia vulgaris* leaves can render protection to the membranes by increasing the levels of vitamin E and improving the levels of vitamin C.

VITAMIN A

In the preset study, liver slices treated with H_2O_2 showed decreased vitamin A level, which was reverted by the presence of *Artemisia vulgaris* leaf extracts. Among the three different extracts used, the methanolic extract exhibited the maximum antioxidant activity.

About 600 different carotenoids are presently known, many of which perform a number of vital functions in a living organism, among which the main task is the conversion of carotenoid to vitamin A (Antipov *et al.*, 2007). The chemopreventive potential of vitamin A and β-carotene during early hepatocarcinogenesis induced by 5-azacytidine in Wistar rats has been demonstrated (Sampaio *et al.*, 2007).

Carotenoids have the capacity to act against reactive oxygen species, protecting the organism against oxidative stress and, consequently, preventing damages and tissue lesions related to several chronic diseases (Chaves *et al.*, 2008). Gezginci-Oktayoglu *et al.* (2009) demonstrated that the combined treatment with vitamin C, vitamin E, and selenium improved the levels of reduced glutathione and inhibited LPO against the hepatotoxicity produced by STZ-induced diabetes.

Crocetin carotenoids increased GR and GSH levels in benzo(a)pyrene induced lung cancer-bearing mice (Magesh *et al.*, 2006). Soussi *et al.* (2006)

demonstrated that the pretreatment with green tea (*Camellia sinensis*) significantly improved the levels of vitamins E and A in the liver and kidney of ammonium metavanadate-induced toxicity in rats. Murthy *et al.* (2005) reported that the carotenoids obtained from *Spirulina platensis* exerted higher antihepatotoxic effect.

Vitamin A administration has been reported to prevent the hepatic injury caused by CCl_4 treatment (Noyan *et al.*, 2006). Thus, it is perceivable that the increase in vitamin A levels by the leaf extracts of *Artemisia vulgaris* proves their ability to improve the antioxidant status in the tissues.

REDUCED GLUTATHIONE

In the present study, the liver slices treated with the standard oxidant showed depleted levels of GSH. The liver slices treated with the leaf extracts of *Artemisia vulgaris* along with the oxidant improved the GSH levels, indicating their antioxidant activity in oxidant exposed liver slices.

One of the most important intracellular antioxidant systems is the glutathione redox cycle. Glutathione is one of the essential compounds for maintaining cell integrity because of its reducing properties and participation in the cell metabolism (Yanardag and Tunali, 2006).

Daidzein isoflavonoids improved the GSH content during mammary carcinogenesis in prepubertal animals (Mishra *et al.*, 2009). A 30kD glycoprotein isolated from the stem bark of *Acanthopanax senticosus* increased the level of GSH in alcohol-induced hepatotoxicity (Choi *et al.*, 2006).

When human Chang liver cells were treated with ethanolic and polyphenolic extracts of *Trigonella foenum graecum* seed, the reduced

glutathione/oxidized glutathione ratio was maintained and this action was comparable to that of the standard antioxidant silymarin (Kaviarasan *et al.*, 2006). The results of the work conducted by Ratheesh *et al.* (2010) demonstrated that the alkaloid extract of *Ruta graveolens L.* increased the GSH level in carrageenan-induced acute inflammation and acted as potential antioxidants.

Treatment of polarized hepatic (WIF-B) cells with L-buthionine sulfoximine leads to depletion of GSH, abrogation of caspase-3 activation as well as reversal of ethanol-induced morphological changes (McVicker *et al.*, 2009). An aqueous extract of *Ocimum sanctum* increased the level of GSH in alcohol treated rats (Shetty *et al.*, 2008).

In view of these reports, an increase in reduced glutathione levels by the leaf extracts of *Artemisia vulgaris* in the present study presents very significant implications in the manifestation of the antioxidant defense by the leaves.

PHASE III

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON OXIDATIVE STRESS-INDUCED APOPTOTIC DEATH IN VARIOUS TYPES OF CELLS

ROS are well known to be cytotoxic and have been implicated in the etiology of a wide array of human diseases, including cancer (Waris and Ahsan, 2006). During critical illness, a number of cell types in different tissues undergo apoptosis. Excessive cell death, including apoptotic cell death, contributes to the pathogenesis of traumatic brain injury and organ failure. Paradoxically, insufficiency of cell death may also be associated with the pathogenesis of

critical illnesses (Bayir and Kagan, 2008). Antioxidants are scavenging free radicals and prevent the body from the pathogenic effects of ROS. Hence, understanding the role of antioxidant as key mediators in apoptosis modulation may provide various opportunities for pharmacological intervention.

With this backdrop, in phase III of the study, the effect of *Artemisia vulgaris* leaf extracts on oxidative stress-induced apoptosis was analysed in untransformed (*Saccharomyces cereviciae* and chick embryo fibroblasts) and transformed (Hep2 laryngeal carcinoma) cells. The two different oxidants used in this phase were H_2O_2 and etoposide.

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE H_2O_2-INDUCED DEATH IN UNTRANSFORMED CELLS

Results of the apoptotic studies revealed that hydrogen peroxide caused significantly increased apoptotic death in yeast cells and primary chick embryo fibroblasts. *Artemisia vulgaris* leaf extracts were not toxic to untransformed cells, by themselves. The stress induced by H_2O_2 was effectively reverted when the cells were co-administered with *Artemisia vulgaris* leaf extracts. These findings clearly demonstrated the apoptosis inhibiting effects of *Artemisia vulgaris* leaf extracts on untransformed cells.

EFFECT OF *Artemisia vulgaris* LEAF EXTRACTS ON THE ETOPOSIDE-INDUCED DEATH IN UNTRANSFORMED AND TRANSFORMED CELLS

The results demonstrated that etoposide induced a significant number of both transformed and untransformed cells (Hep2 and primary chick embryo fibroblasts respectively) to commit to apoptosis. The leaf extracts, by

themselves, did not cause apoptosis in normal cells, whereas they induced apoptosis in Hep2 cells.

In primary chick embryo fibroblasts, the apoptosis-inducing effect of etoposide was effectively counteracted by the administration of leaf extracts. But in the case of Hep2 cells, the apoptosis-inducing effect of etoposide was further increased by the presence of *Artemisia vulgaris* leaf extracts.

Thus, the results suggest that the *Artemisia vulgaris* leaves show differential response depending on the cell type. In normal cells (*Saccharomyces cerevisiae* and primary chick embryo fibroblasts), the leaf extracts did not induce apoptosis, whereas the number of apoptotic cells were found to be more when the Hep2 cells were treated with the plant extracts. The results also showed that the leaf extracts increased the apoptosis-inducing action of etoposide on Hep2 cells. These results show that the extracts enhanced the cytotoxicity of etoposide on cancer cells, while protecting the normal cells.

MTT VIABILITY ASSAY

The oxidants (H_2O_2/etoposide) used in the present study significantly decreased the viability of both untransformed and transformed cells. The oxidant-induced apoptotic death was significantly reverted by the *Artemisia vulgaris* leaf extracts in the untransformed cells, whereas the oxidant-induced apoptotic death was further increased by the leaf extracts in cancer cells.

MTT assay is being used as a powerful tool to study the cytotoxicity of the phytocompounds/extracts. The literature is rich with reports that correlate the cytotoxicity of the compounds.

Wali *et al.* (2009) analysed the cell viability in neoplastic mouse +SA mammary epithelial cells by the MTT assay, wherein the cell viability was significantly decreased by α-tocotrienol in a dose- and time-dependent manner. In another study (Ramanouskaya *et al.*, 2009), the same assay was used to analyse the cytotoxicity of 16 aglycon flavonoids on human K562 chronic myelogenous leukemia cells, out of which flavones and flavonols exhibited the strongest cytotoxicity.

MTT assay revealed that khaweol an cafestol improved the cell viability in a dose-dependent manner in H_2O_2-treated NIH3T3 cells (Lee and Jeong, 2007). Selenium inhibited the proliferation of melanoma cells in a dose-dependent manner as assessed by MTT assay (Song *et al.*, 2009). An *in vitro* antiproliferative activity of rosamine derivatives against a promyelocytic leukemia cell line (HL-60) was determined using the MTT assay (Lim *et al.*, 2009).

Chew *et al.* (2010) used the MTT assay to investigate the cytotoxic effects of cinnamaldehydes (trans-cinnamaldehyde and its analogs 2-hydroxycinnamaldehyde and 2-benzoyloxycinnamaldehyde) on HCT 116 colon carcinoma cells, wherein a dose-dependent cell killing effect was observed. MTT assay also showed the antiproliferative effects of curcumin and novel structural analogues in three independent human colorectal cancer cell lines (Cen *et al.*, 2009).

The MTT assay showed the cytotoxic effect of paeonol from *Pycnostelma paniculatum* K.S. and the root of *Paeonia suffruticosa* on the human hepatocellular carcinoma cell lines BEL-7404, SMMC-7721 and MHCC97-H *in vitro* in a dose-dependent manner (Chunhu *et al.*, 2008).

Terpenoids from the root bark of *Ailanthus excelsa* Roxb induced death in a variety of cancer cell lines of different origin such as B16F10, MDA-MB- 231, MCF-7 and PC3 cells, but showed minimum growth inhibition in normal HEK 293 cell, which were assessed by the MTT assay (Lavhale *et al.*, 2009).

Quercetin-3-*o*-glucoside from *Prangos ferulaceae* reduced the viability of McCoy cell line, as assessed by the MTT assay (Razavi *et al.*, 2009). A potential antimitotic agent, 1-(2,4-dimethoxyphenyl)-3-(1-methylindolyl) propenone, decreased the viability of bladder cancer cells as determined by the MTT assay (Martel-Frachet *et al.*, 2009).

Emblica officinalis extracts did not cause any cytotoxic effect on the total cell populations of human primary osteoclasts as assessed by MTT assay (Penolazzi *et al.*, 2008). A similar trend was found in our study also, where the *Artemisia vulgaris* leaf extracts were not toxic to yeast and primary chick embryo fibroblasts.

The methanolic extracts of *Hygrophila auriculata* and *Hibiscus tiliaceous* and the aqueous extract of *Limnophila indica* showed no toxicity against healthy mouse fibroblasts (NIH3T3). But they showed selective cytotoxicity against breast cancer cells (gastric: AGS; colon: HT-29; and breast: MDA-MB-435S) (Uddin *et al.*, 2009). This report correlates with our results. The leaf extracts of *Artemisia vulgaris* showed differential effect. The leaf extracts induced apoptosis in transformed cells but not in untransformed cells.

MTT assay results revealed that *Pogostemon cablin* prevents mitochondrial dysfunction induced by H_2O_2 in human neuroglioma cell line A172 (Kim *et al.*, 2008). Pterogynidine alkaloid from *Alchornea glandulosa* decreased the cell viability in human umbilical vein endothelial cells (HUVEC)

in a dose-dependent manner as evaluated by the MTT assay (Lopes *et al.*, 2009).

It can be inferred from our results of the MTT assay that the leaf extracts of *Artemisia vulgaris* induced more of the cancerous cells to commit to death in the presence of an anticancer agent. The same extracts protected the non-cancerous cells from its toxic effects.

SRB ASSAY

Several reports in the literature have validated the SRB assay as a relevant tool in quantifying the extent of cell survival. In the presence study, the decreased cell survival (of cancerous cells) and improvement in cell survival (of non-cancerous cells) were observed in the presence of the leaf extracts using SRB assay.

SRB assay was employed to understand the synergistic growth inhibitory effect of zoledronic acid-docetaxel on prostate cancer cell line (Fabbri *et al.*, 2008) and xanthorrhizol-curcumin towards human breast cancer cells (MDA-MB-231) (Cheah *et al.*, 2009). Hsiao *et al.* (2008) reported that Mana-Hox, a synthetic derivative of β-carboline, screened for cytotoxic activity against five human cancer cell lines using SRB assay, showed anticancer effect.

Marchal *et al.* (2007) measured the *in vitro* proliferation of human breast cancer cell line MCF-7 by SRB colorimetric assay and observed a dose-dependent action when treated with (RS)-1-(2,3-dihydro-5H-1,4-benzodioxepin-3-yl) uracil. The cell growth inhibitory effects of the drugs indomethacin and cyclooxygenase-2 were determined by SRB colorimetric assay on HT-29 colorectal cancer cell line (Réti *et al.*, 2009).

The cytotoxic effect of berberine was evaluated in K1735-M2 mouse melanoma cells and WM793 human vertical growth phase melanoma cells using the SRB assay (Serafim *et al.*, 2008). The cytotoxic effect of inflammatory cytokines and S-nitroso-glutathione was determined by SRB in human cholangiocarcinoma cell line and HeLa cells (Prawan *et al.*, 2009).

The results of SRB assay indicated that the nitrogen containing synthetic compounds namely FMA762 and FMA796 decreased tert-butylhydroperoxide-induced cell death of H9c2 cardiomyoblasts (Silva *et al.*, 2010). The cytotoxicity of a coumarin derivative (RKS262) was analysed by SRB assay in ovarian cancer cells (Singh *et al.*, 2009).

Batzellins from *Batzells* species showed increased cytotoxicity in four different pancreatic cell lines (Panc-1, AsPC-1, BxPC-3, MIA-PaCa2) but they exhibited much less cytotoxicity in the Vero cells (normal cells) suggesting a preferential cytotoxic ability towards tumor cell lines (Guzmán *et al.*, 2009). Our results are in agreement with this report.

Jada *et al.* (2008) reported that the andrographolides (SRJ09 and SRJ23) from *Andrographis paniculata* tested for cytotoxicity against two human cancer cell lines (MCF-7 and HCT-116) using SRB assay showed good cytotoxicity. The cytotoxic effects of the purified compounds from *Streptoverticillium luteoverticillatum* on cell growth and viability of K-562 human erythromyeloblastoid leukemia cell line were established by SRB assay (Dehai *et al.*, 2007).

The antiproliferative effects of compounds isolated from *Diospyros lotus* L. were analysed by SRB assay, where phenolics exhibited higher activity (Loizzo *et al.*, 2009). From the cell viability assays, it can be inferred that the

oxidants caused significant cell death in both untransformed and transformed cells. *Artemisia vulgaris* leaves showed their protective effect against oxidant in untransformed cells, while enhancing the cytotoxic effect of the anticancer drug in cancer cells.

MORPHOLOGICAL CHANGES ASSOCIATED WITH APOPTOSIS

Cell shrinkage, plasma membrane blebbing, rounding and poor adhesion of the cells are the morphological changes associated with apoptosis, which were studied by giemsa staining. The observation clearly indicated that the leaf extracts of *Artemisia vulgaris* exerted anticancer effect towards cancer cells and rendered protection towards non-cancerous cells.

LaBarbera *et al.* (2009) reported that the marine alkaloid naamidine A-treated A431 epidermoid carcinoma cells showed significant changes in cell morphology. Liu *et al.* (2009b) showed that the MCF-7 cells exposed to 3-bromopyruvic acid showed the typical morphological characteristics of apoptosis.

The cytotoxicity of F4 fraction of *Petiveria alliacea* was dose-dependent, inducing significant morphological changes, such as cell deformation and elongation in the tested tumor cell lines (A375-human melanoma cells, and K562 - a human erythroleukemia) (Urueña *et al.*, 2008). The typical apoptotic morphological changes were observed under microscope when mammalian tsFT210 cells were treated with isolated compounds from marine-derived *Streptomyces* (Ren *et al.*, 2006).

HT-29 human colon adenocarcinoma cells treated with panduratin A isolated from *Boesenbergia pandurata* showed features of apoptosis (Kirana

et al., 2007). Typical apoptotic morphologies could be observed in the mouse liver cancer cell line (H22) treated with *Scutellaria barbate* (Dai *et al.*, 2008).

Apoptotic cell death with respect to morphological changes was observed in WM793B (a melanoma cell line) by parthinolide from *Tanacetum parthenium* (Lesiak *et al.*, 2010). An ethanolic extract of *Piper sarmentosum* induced apoptosis-related morphological changes in human hepatoma cell line (HepG2) following May-Grunwald-Giemsa staining (Ariffin *et al.*, 2009).

An ethanolic extract of *Chrysanthemum indicum* induced apoptosis in human hepatocellular carcinoma MHCC97H cell line as shown by apoptotic morphological changes without cytotoxicity to normal cells, namely rat hepatocytes and human endothelial cells (Li *et al.*, 2009b). Our results show a similar effect for *Artemisia vulgaris* leaves.

It is evident from the present study that the leaf extracts of *Artemisia vulgaris* exerts a differential response against the oxidative damage-induced apoptosis in different types of cells. Among the three extracts used, the methanolic extract exhibited the maximum activity, followed by the aqueous and chloroform extracts.

NUCLEAR CHANGES ASSOCIATED WITH APOPTOSIS

Apoptosis is a form of programmed cell death and is an important natural process used by the human body to remove unwanted cells (Fesik, 2005). The characteristic nuclear changes of apoptosis such as chromatin condensation, nuclear fragmentation and chromosomal DNA fragmentation were studied by PI, EtBr and DAPI staining.

The results of the nuclear staining assays revealed that oxidative stress caused an increased extent of apoptosis in all cell types used. The leaf extracts of *Artemisia vulgaris* showed no cytotoxicity to untransformed cells but showed cytotoxicity towards cancer cells. The co-administration of leaf extracts rendered protection to the untransformed cells against etoposide-induced death, whereas it enhanced apoptosis in the cancer cells. Thus, the leaves protect normal cells and render the cancer cells more susceptible to chemotherapy.

PROPIDIUM IODIDE STAINING

Propidium iodide is a fluorochrome that intercalates into double-stranded nucleic acids and helps to determine the nuclear changes of apoptosis. There are many reports with propidium iodide staining.

PI staining was employed to test the synergistic anticancer effect of hydrazine analogue (SC144) with etoposide in two different cancer cell lines (Oshima *et al*., 2009). Annexin/PI staining of human melanoma cell line (A375) showed apoptosis brought about by oxymatrine, a natural quinolizidine alkaloid in a dose-dependent manner (Zhang *et al*., 2010a).

The extent of propidium iodidie staining along with other techniques has been used to study the effect of dihydroartemisinin and artemisinin dimers on proliferation and apoptosis in prostate cancer cells (Morrissey *et al*., 2010). PI staining was adopted by Kundu *et al*. (2009) to study the cadmium-induced toxicity in mice lung cells.

The effect of oral cancer overexpressed 1 (ORAOV1) siRNA on cell apoptosis was evaluated by flow cytometry using PI (Jiang *et al*., 2010). The diterpenes (foliol and linearol) exerted protection against the apoptosis triggered

by LPS/IFN-γ in mouse macrophage cell line, RAW 264.7, as determined by PI staining (de las Heras *et al.*, 2007).

Two cytolysins (toxin Bc2 and equinatoxin) enhanced the vincristine action (anticancer drug) in human glioblastoma cells as assessed by PI staining (Soletti *et al.*, 2008). In the present study, the methanolic extract of *Artemisia vulgaris* leaf extracts enhanced the activity of etoposide in laryngeal carcinoma cells. In another study (Zhu *et al.*, 2008), propidium iodide staining was used to measure vincristine-induced apoptosis in two different human melanoma cell lines (Mel-RM and IgR3).

The dose dependency of bisindolylmaleimide-IX on TNF-α-mediated apoptosis of human colon adenocarcinoma (COLO 205) cells was determined using Hoechst 33342/PI staining (Pajak *et al.*, 2008). Yedjou *et al.* (2009) have reported that the ascorbic acid co-treatment induces more apoptosis of HL-60 cells than did arsenic trioxide alone, as determined by flow cytometry assessment using Annexin V FITC/PI. PI and annexin V–fluorescein isothiocyanate staining of osteosarcoma cell line (MG-63 and U2O2) showed increased apoptosis brought about by genistein alone and with gemcitabine (Zhang *et al.*, 2010b).

Thymoquinine, a component of black seed essential oil, inhibited the proliferation of a panel of human colon cancer cells (Caco-2, HCT-116, LoVo, DLD-1 and HT-29), without exhibiting cytotoxicity to normal human intestinal (FHs74Int) cells, as assessed by PI staining (El-Najjar *et al.*, 2009). Similarly, plumbagin, isolated from the root of *Plumbago zeylanica* L, inhibited prostate cancer cell invasion and selectively induced apoptosis in prostate cancer cells but not in immortalized non-tumorigenic prostate epithelial RWPE-1 cells (Aziz

et al., 2008). These results are similar to our results, where the leaf extracts of *Artemisia vulgaris* caused apoptosis in Hep2 cells but not in normal cells.

Curcurbitacin E isolated from *Cucurbitaceae* and *Wilbrandia ebracteata* inhibited the growth of human leukemia HL-60 cells as investigated by propidium iodide staining (Li *et al.*, 2010). The results of PI stained MCF-7 cells revealed that, compared to the unsteamed extract of *Panax quinquefolius*, the 2-hour steamed extract significantly increased the antiproliferative activity and significantly reduced the number of viable cells (Wang *et al.*, 2008b).

Dihydroartemisinin from *Artemisia annua* induced apoptotic cell death in a dose- and time-dependent manner in human lung adenocarcinoma ASTC-a-1 cells, as assayed by PI (Lu *et al.*, 2009). Flow cytometric and annexin V/ propidium iodide immunofluorescence double-staining techniques was adopted by Yen *et al.* (2010) to prove the apoptosis-inducing potential of 4β-hydroxywithanolide E from *Physalis peruviana*. H_2O_2-induced damage in rabbit bone marrow stromal cells was augmented by the treatment with *Panax notoginseng* saponins (Qiang *et al.*, 2010).

In the light of these reports, the results of PI staining in the present study are highly validated.

ETHIDIUM BROMIDE STAINING

EtBr is an intercalating agent, which has been used as a staining agent to quantify the number of cells showing apoptotic changes in several studies. Arsenic-induced apoptosis in NB4 cells were studied by ethidium bromide staining, reported by Mozdarani and Asghari (2010).

Wei *et al.* (2008) demonstrated that the co-administration of all-trans retinoic acid with 5-fluorouracil and mitomycin C increased the apoptotic nuclei of LoVo cells as measured by acridine orange/ethidium bromide staining. A combined acridine orange/ethidium bromide staining was adopted by Kim *et al.* (2009) to study the etoposide-induced apoptosis in human leukemia U937 cells by heptelidic acid, a sesquiterpene lactone.

In the human breast cancer cell line MCF-7, diallyl disulfide, a component of garlic, significantly increased cell death and attenuated cell apoptosis as measured by acridine orange/ethidium bromide staining (Lei *et al.*, 2008). AO/EtBr staining showed apoptotic morphology when McCoy cells were treated with a dichloromethane extract of *Prangos uloptera* (Zahri *et al.*, 2009). Raspberry extract caused significant protective effects against DNA damage induced by hydrogen peroxide in HT29 colon cancer cells stained by ethidium bromide (Coates *et al.*, 2007).

In the present study, *Artemisia vulgaris* leaf extracts significantly protected the yeast cells and primary chick embryo fibroblasts from H_2O_2-induced damage. The methanolic extract exhibited better protection, followed by the aqueous and chloroform extracts. The same extracts not only caused increased death in cancer cells, but also made them more susceptible to etoposide action. The methanolic extract was the most effective in this respect also.

DAPI STAINING

DAPI staining has been adopted by many apoptotic studies to evaluate the nuclear changes of apoptosis. Dithiocarbamate and Cu treatment resulted in

nuclear changes consistent with apoptosis that was visualized using DAPI stain in M-14, WM-278 and WM-1552c cells (Morrison *et al.*, 2010).

Apogossypolon treated U937 cells (human leukemic monocyte lymphoma cell line) exhibited obvious apoptotic characteristics after staining with DAPI (Sun *et al.*, 2008). Hydrogen peroxide-induced apoptosis was studied in Human B (BJAB) and T (Jurkat) lymphoma cells, studied by DAPI staining (Son *et al.*, 2009).

Nagendraprabhu and Sudhandiran (2009) used DAPI, the nucleus specific counter stain, to detect nuclear changes in 1,2-dimethyl hydrazine-induced rat colon carcinogenesis treated with astaxanthin. DAPI staining was used to assess etoposide- and/or cordycepin-induced nuclear changes of apoptosis in HL-60 (human acute myeloid leukemia), U937 (human histiocytic lymphoma), K562 (human chronic myeloid leukemia in blast crisis), Molt-4 (human T-cell acute lymphoblastic leukemia) and Daudi (human Burkitt lymphoma) cell lines (Thomadaki *et al.*, 2008).

Co-treatment of A2780/DX3 cells with doxorubicin and nifedipine-like compounds 1 and 2 showed that the treatment caused a significant increase in the number of DAPI stained apoptotic nuclei (Viale *et al.*, 2009). Zhang *et al.* (2008) adopted DAPI staining to quantify the apoptosis in gastric cancer cell lines SGC-7901 and BGC-823 after treatment with cisplatin. Cisplatin induced relatively high levels of apoptosis in SGC-7901 cells; in contrast, BGC-823 cells seemed to be resistant to cisplatin-induced apoptosis.

DNA fragmentation, a hallmark of apoptosis was studied in HL 60 human leukemia cells by DAPI staining. Compound K isolated from *Panax*

ginseng showed DNA fragmentation in a time and dose-dependent manner (Cho *et al.*, 2009).

The normal human bone cells in primary culture were found to be less sensitive to the effects of taurolidine than human osteosarcoma cell line (Walters *et al.*, 2007). Our results are in agreement with this. In the present study, the Hep2 cells are more susceptible to the cytotoxic effect of *Artemisia vulgaris* leaf extracts than the primary chick embryo fibroblasts.

Thus, in the present study, all the parameters of cytotoxicity and apoptotic cell death showed that *Artemisia vulgaris* leaves can alleviate the oxidative stress imposed on normal (untransformed) cells, while causing an increased extent of death in the cancer (transformed) cells. This is a very significant observation, as it strengthens the candidature of *Artemisia vulgaris* leaves as a support to cancer chemotherapy, to reduce the toxic side effects of the chemotherapeutic agents on normal cells, and to target and focus the cytotoxicity specifically to cancer cells.

PHASE IV

Having found the strong, exciting protective effects of *Artemisia vulgaris* leaf extracts, an attempt was made in the last phase of the study to identify the nature of phytochemical components in the leaves. This was done in order to pave the way for the development of powerful pharmaceuticals.

In recent times, the field of modern medicine, which usually uses synthesized drugs, has increasingly focused interest in drugs of plant origin. Efforts have been made in many countries to identify plants having medicinal properties effective against various modern diseases (Govindaraj *et al.*, 2008).

Traditional medicinal plants have the ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. The useful major groups of phytochemicals can be divided into several categories that include alkaloids, flavones (flavonoids, flavonols, quinines), essential oils, lectins, polypeptides, phenolics, polyphenols, tannins and terpenoids (Perumalsamy and Gopalakrishnakone, 2008).

In recent years, there is a tremendous interest in the possible role of nutrition in the prevention of disease. In this context, antioxidants especially those derived from natural sources such as medicinal plants and herbal drugs derived from them, require special attention. Antioxidants neutralize the toxic and volatile free radicals. Antioxidants have many potential applications, especially in relation to human health, both in terms of prevention of disease and therapy (Vaidya and Devasagayam, 2007).

The preliminary phytochemical screening and HPTLC analysis revealed the presence of phenols, flavonoids and sesquiterpenoids in the leaves of *Artemisia vulgaris*. To get a clear picture about the chemical nature of the active components present in the leaves of *Artemisia vulgaris*, spectral analysis (HPLC, IR and GC-MS) were carried out, which confirmed the presence of phenols, flavonoids and terpenoids.

Several studies on the analysis of phytochemicals are portrayed in favour of our findings. The UV spectra of purified compounds from ethyl acetate fractions of *Tecoma radicans* F. revealed the presence of a major flavonoid and apigenin, which showed characteristic absorption maxima at 274nm and 296nm respectively (Hashem, 2007). The UV spectra of eluted compounds from the

methanolic extract of *Coleus* tissues revealed the presence of the most abundant phenolics namely hydroxylcinnamic acid derivatives (Rasineni *et al.*, 2008).

The HPTLC analysis of *Annona squamosa* L. revealed the presence of linalol, borneol, eugerol, fernsol and geraniol (Patel and Kumar, 2008). The HPTLC method was adopted by Kumar *et al.* (2007) to quantify teraxerol in *Clitoria ternatea* L. In the HPTLC screening of *Strychnos potatorum* L. the ether fraction showed seven peaks, the unsaponifiable fraction showed five peaks and the alkaloid fraction showed seven peaks (Priya and Venkataraman, 2010). Ferulic acid, a potent antioxidant present in *Lycopodium clavatum*, has been identified by HPTLC analysis (Srivastava *et al.*, 2007).

HPLC of the methanolic extracts of *Syzygium cummini* and *Terminalia bellerica* gave peaks at retention times 4.58 and 4.61 minutes respectively, which were similar to the retention time of gallic acid (4.6) (Sharma *et al.*, 2009). Quercetin, a flavonoid identified from *Citrullus colocynthus*, showed a characteristic peak at a retention time of 3.475 minutes (Meena and Patni, 2008).

The IR spectrum of subamolide D purified from *Cinnamomum subavenium* showed absorption bands for a hydroxyl group at 3450 cm^{-1} and α,β-unsaturated γ-lactone moiety at 1770 and 1680 cm^{-1} (Kuo *et al.*, 2008). The flavonoids, sophoraflavanone G and kurarinone, from *Sophora flavescens* were suggested to be responsible for their antioxidant and kidney protective effects (Piao *et al.*, 2006). The IR spectrum of compound 1 isolated from *Capparis himalayensis* displayed absorption bands for amide (3436 cm^{-1}), carbonyl (1715 cm^{-1}) and aromatic groups (1615 cm^{-1}, 1505 cm^{-1}, 1463 cm^{-1}) respectively (Li *et al.*, 2008).

The IR spectrum of chakasaponin VI from *Camellia sinensis* showed absorption bands at 3452, 1714, 1647, 1078 and 1048cm-1, ascribable to hydroxyl, carbonyl, α,β-unsaturated ester and ether functions. Chakasaponin I from the above mentioned plant showed absorption bands at 3414, 1694 and 1078 cm^{-1} due to hydroxyl, carbonyl and ether functions in the IR spectrum (Yoshika *et al.*, 2008).

The GC-MS analysis of *Gymnema sylvestre* R. Br. revealed the presence of terpenoids, glycosides, saturated and unsaturated fatty acids and alkaloids (Sathya *et al.*, 2010). GC-MS analysis of the ethanolic extract of *Aloe vera* showed 26 bioactive phytochemical compounds (Arunkumar and Muthuselvam, 2009). GC-MS results of the chloroform extract of *Andrographis paniculata* revealed phenols, aromatic carboxylic acids and esters, which are the molecules that are implied to be responsible for their antimicrobial activity (Roy *et al.*, 2010).

The chloroform extract of the roots of *Ferula ferulago* showed the presence of two monoterpene coumarin and three sesquiterpene lactones (El-Razek *et al.*, 2001). The coumarins have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antiviral and anticarcinogenic activities (Kostova, 2005).

HPLC analysis showed that quercetin 3,7,3'-trimethyl ether is the most abundant compound found in *Artemisia vulgaris* (Nikolova *et al.*, 2004). The GC-MS results of the fresh leaves of *Artemisia vulgaris* has revealed the presence of terpenes (Barney *et al.*, 2005). The GC-MS spectrum reported by another group (Tigno *et al.*, 2000) showed the leaves of *Artemisia vulgaris* to contain two sesquiterpene lactones and a novel aromatic compound. The

dichloromethane extract of the air-dried leaves of *Artemisia vulgaris* afforded a new sesquiterpene, caryophyllene oxide, phytyl fatty acid esters, squalene, stigmasterol and sitosterol (Ragasa *et al*., 2008). These results are in agreement with our results, wherein phenols, flavonoids and terpenoids were found to be the major components identified in the leaves of *Artemisia vulgaris*. In this study, eight sesquiterpenoid bands were identified by HPTLC.

The outcome of the present study, thus, highlights the protective effects rendered by the leaf extracts of *Artemisia vulgaris* upon oxidative stress. The phytochemical analysis of the methanolic extract revealed the presence of polyphenols, flavonoids and terpenoids as the major active components, which probably are the main players in the antioxidant effects evoked.

The findings of the present study are summarized and the conclusions that can be drawn from them are elaborated in the next chapter.