Screening of antimicrobial and antioxidant activities of some selected herbs used as folk medicines in Darjeeling hills

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Certified that the work presented in the thesis entitled, *'Screening of antimicrobial and antioxidant activities of some selected herbs used as folk medicines in Darjeeling hills'* has been carried out by Ms Jayati Saha, MSc under my supervision at Microbiology Laboratory of the Department of Botany in the University of North Bengal. The results incorporated in the thesis have not been submitted for any other degree elsewhere.

Further certified that Ms Saha has followed the rules and regulations laid down by the University of North Bengal in carrying out this work.

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1 Introduction

1.1. Overview of herbal drugs

Amongst the estimated 250,000-500,000 of plant species on Earth (Borris, 1996), only a small percentage (1-10%) is used as foods by both humans and animals, but even more are used for medicinal purposes (Moerman, 1996). Hippocrates mentioned about 300-400 medicinal plants in the late fifth century B.C. (Schultes, 1978). In the first century A.D., Dioscorides wrote *De Materia Medica*, a medicinal plant catalogue which became the prototype for modern pharmacopoeias. The Bible offers descriptions of approximately 30 healing plants. Indeed, frankincense and myrrh were reported to have antiseptic properties, and even employed as mouthwashes. In 1887, alternative practitioners compiled their own catalogues, notably *The Homeopathic Pharmacopoeia of the United States*. While the Asian cultures compiled their own pharmacopoeia, in the West the Renaissance years saw a revival of ancient medicine, which was built largely on plant products.

Due to varied chemistry of these natural chemicals they are widely valued all over the world. About 25% of the drugs prescribed worldwide originate from plants, and 121 active compounds are currently in use. In 1997, it was estimated that the world market over-the-counter phytomedicinal products was 10 billion US$, with an annual growth rate of 6.5%. The World Health Organization (WHO) considers phytotherapy in its health programmes and suggests basic procedures for the validation of drugs from

plant origin in developing countries (Vulto and Smet, 1988). Of the 252 drugs considered as basic and essential by the WHO, 11% are exclusively of plant origin and a significant number of synthetic drugs are obtained from natural precursors. Examples of important drugs obtained from plants are digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* sp., vincristrine and vinblastine from *Catharanthus roseus*, atropine from *Atropa belladonna*, and morphine and codeine from *Papaver somniferum*. It is estimated that 60% of anti-tumour and anti-infectious drugs already in the market or under clinical trial are of natural origin. The vast majority of these cannot yet be synthesized economically and are still obtained from wild or cultivated plants. Natural compounds can be used as lead components, allowing design and rational planning of new semi-synthetic drugs.

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Countries, such as China and India, have a well-established herbal medicine industry, while Latin American countries have been investing in research programmes in medicinal plants and standardization and regulation of phytomedicinal products, following the example of European countries, such as France and Germany. Ayurveda, the traditional Indian medicinal system relies on using plant extracts, single and mixed preparations. The formulations often have lyrical names, such as ashwagandha (*Withania somnifera*) root, livo-vet etc. which are used to treat animals as well as humans (Dhuley, 1998).

Plants have a limitless ability to synthesize a wide range of secondary metabolites, such as terpenoids, steroids, alkaloids and predominantly phenols or their oxygen-substituted derivatives (Geissman, 1963). These compounds are widely spread in different parts of a plant, *viz.* fruits, seeds, leaves, flowers and barks. Plant phenolics mainly include derivatives and isomers of flavones, isoflavones, flavonoids, catechins, phenolic acids, quinines etc. However, the huge number of phenolics (~12,000), isolated from plants represents even less than 10% of their total natural abundance (Schultes, 1978). Besides phenolics, a diverse array of compounds, such as terpenoids, steroids and alkaloids also abounds in the plant kingdom. Some, such as terpenoids, give plants their odour; others (quinones and tannins) are responsible for the colour. Many compounds are responsible for plant flavour (*e.g.*, the terpenoid capsaicin from chili peppers), and several of the same herbs and spices used by humans to season food yield useful medicinal compounds (Table 1).

Amongst the diverse array of activities of the plant phenolics, major thrust is given to their antioxidant, antimicrobial and anticancer activities. The isoquinoline alkaloid, emetine, obtained from the underground part of *Cephaelis ipecacuanha* and related species, has been used for many years as amoebicidal drug as well as for the treatment of abscesses due to the spread of *Entamoeba histolytica* infections. Another important drug of plant origin with a long history of use is quinine. This alkaloid occurs naturally in

Table 1. Different classes of organic compounds and their medicinal properties

Class	Subclass	Example	Mechanism of action	Reference
phenolics	simple phenols	catechol, epicatechin	substrate deprivation and membrane disruption	Peres *et al.* (1997)
	phenolic acids, quinones	cinnamic acid, hypericin	binds to adhesion proteins and cell wall; inactivates enzymes	Duke (1985); King and Tempesta (1994)
	flavones, flavonoids	chrysin, abyssinone	binds to adhesion proteins and cell wall; inactivates enzymes; inhibits HIV reverse transcriptase	Brinkworth *et al.* (1992); Ono *et al.* (1989); Taniguchi and Kubo (1993)
	tannins	ellagitannis	binds to adhesion proteins and cell wall; inactivates enzymes; substrate deprivation; membrane disruption; metal ion chelation	Brownlee *et al.* (1990); Butler (1988); Haslam (1996); Scalbert (1991); Schultz (1988)
	coumarins	warfarin	interacts with eukaryotic DNA (antiviral activity)	Bose (1958); Hoult and Paya (1996); Keating and O'Kennedy (1997)
terpenoids, essential oil		capsaicin	disrupts cell membrane	Cichewicz and Thorpe (1996)
alkaloids		berberine, piperine	intercalates into cell wall and DNA	Burdick (1971); Houghton *et al.* (1994)
lectins, peptides		mannose-specific agglutinin, fabatin	blocks viral fusion or adsorption forming disulphide bridges	Meyer *et al.* (1997); Zhang and Lewis (1997)

the bark of *Cinchona* tree. Apart from its continued usefulness in the treatment of malaria, it can also be used to relieve nocturnal leg cramps. Currently, the widely prescribed drugs are analogues of quinine, such as chloroquine. Some strains of malarial parasites have become resistant to the quinines; therefore, antimalarial drugs with novel mode of action are required.

Similarly, higher plants have made important contributions in the areas beyond anti-infectives, such as cancer therapies. Early examples include the antileukaemic alkaloids, vinblastine and vincristine, which were both obtained from the Madagascan periwinkle (*Catharanthus roseus*) (Nelson, 1982). Other cancer therapeutic agents include taxol, homoharringtonine and several derivatives of camptothein. For example, a well-known benzylisoquinoline alkaloid, papaverine, has been shown to have a potent inhibitory effect on the replication of several viruses including cytomegalovirus, measles virus and HIV (Turano *et al.*, 1989). Three new atropisomeric naphthylisoquinoline alkaloid dimers, michellamines A, B and C were isolated from a newly described tropical liana species, *Ancistrocladus korupensis* from the rainforest of Cameroon. These compounds showed potential anti-HIV activity, where michellamine B being the most potent and abundant member of the series. These compounds were capable of complete inhibition of the cytopathic effects of HIV-1 and HIV-2 on human lymphoblastoid target cell *in vitro* (Boyd *et al.*, 1994).

The first generation-plant drugs were usually simple botanicals employed in more or less their crude form. Several effective medicines used in their natural state such as cinchona, opium, belladonna and aloe were selected as therapeutic agents based on empirical evidence of their clinical application by traditional societies from different parts of the world. Following the industrial revolution, a second generation of plant-based drugs emerged based on scientific processing of the plant extracts to isolate their active constituents. To isolate the active compounds, plant extracts are first qualitatively analysed by thin layer chromatography and screened for the biological activity. For purification and isolation, the active plant extracts are sequentially fractionated to yield pure compound(s). In general, a plant extract contains low

concentrations of active compounds, whose structures are determined by spectroscopic methods (NMR, IR, mass and UV). The second generation-phytopharmaceutical agents were pure molecules and some of the compounds were even more pharmacologically active than their synthetic counterparts. Notable examples were quinine from *Cinchona*, reserpine from *Rauvolfia*, and more recently taxol from *Taxus* spp. These compounds differed from the synthetic therapeutic agents only in their origin. They followed the same method of development and evaluation as other pharmaceutical agents.

In the development of third generation-phytotherapeutic agents, a top-bottom approach is usually adopted. This consists of first conducting a clinical evaluation of the treatment modalities and therapy as administered by traditional doctors or as used by the community as folk medicine. This evaluation is then followed by acute and chronic toxicity studies in animals. Studies should, when applicable, include cytotoxicity. It is only if the substance has an acceptable safety index; then it would be necessary to conduct detailed pharmacological/biochemical studies. Formulation and trial production of the dosage forms are structured to mimic the traditional use of the herbs. The stability of the finished product is given careful attention during the formulation of the final dosage form. This is a unique blend of the empiricism of the earlier first generation-botanicals with the experimental research used to prove the efficacy and safety of second generation-isolated pure compounds. Several pharmaceutical companies are engaged in the development of natural product drugs through the isolation of the so-called active molecules from plant extracts.

1.2. Aims of present investigations

Favourable climatic factors including the positive variations in humidity, temperature and altitude, and anthropogenic factors make the vegetation of Darjeeling Himalaya diverse and capable of sustaining a rich flora which serves as a storehouse of medicinal plants. The natives of this region are primarily surviving on folklore and cultural heritage. But the process of civilization is gradually eroding the knowledge of plants and their traditional usage in medicine. Many of the important information might have been lost or discarded *en route* to this age. The knowledge of folklore science, which remains limited within families of aborigines of such area, dies out with them. Recording of such knowledge of ethnobotany through surveys, recordings, specimen collection and examination along with interviews with the local tribal people and preservation of the acquired knowledge is the only means to restore the valuable ethnic information (Bhujel, 1996).

The health benefits of many of the plants/herbs may be related, at least in parts, to their antimicrobial and antioxidant properties. Also, these properties are often valued to increase the shelf-life of raw as well as semi-cooked food products. The plants used as ethnomedicines in Darjeeling Himalayan region are immense with great diversity. Thus, ascertaining the most suitable candidates with potential application as medicine and food preservative warrants extensive and proper screening. Hence, the objectives of the present investigation were to (1) survey various herbs used as ethnomedicines in Darjeeling hills, (2) evaluate their antioxidant activities using a series of *in vitro* assay and (3) screen the antimicrobial activities of the chosen herb extracts against a wide range of microorganisms (bacteria, yeasts and moulds).

The protocols adopted to fulfill these objectives were as follows:

(a) Surveying the three hilly subdivisions of Darjeeling district, documentation of the usage, local name etc. of the herbs and identification of their taxonomic status;

(b) Evaluation of antioxidant activity of a number of important herbs of this area using several *in vitro* assays;

(c) Screening of the above herbs for their inhibitory activity against a number of microbial strains; and

(d) Isolation of active fractions/compounds of some of the herbal extracts using various spectroscopic methods, and revalidation of their potency for the biological activity, mentioned above.

2

Review of literature

Based on the objectives mentioned in the previous chapter, herein a brief account of the (i) heritage medicine of the Darjeeling hills, (ii) implication of oxidative stress and its prevention/treatment, and (iii) utility of herbal antimicrobial agents is provided.

2.1. Overview of Darjeeling Himalaya

2.1.1. Geographic position

Darjeeling hills represent the eastern part of the Himalayan range and are situated in Darjeeling, the northernmost district of West Bengal, India. The district is surrounded by Bhutan in the east, Nepal in the west and Sikkim in the north and covers a total area of 3754.7 sq. km., *i.e.* about 3.68% of the total area of the state. It lies between 27°13'05" and 26°17'10" north latitude and between 88°53'00" and 89°59'30" east longitude. The hilly region occupies about 2320 sq. km. within the district, while the remaining 934.7 sq. km. in the Siliguri subdivision falls under terai and plain region. There are four subdivisions in the district *viz.* Darjeeling (935.5 sq. km), Kalimpong (1056.5 sq. km.), Kurseong (425.3 sq. km) and Siliguri (837.4 sq. km). The altitude variation in the district ranges from 150 m in Siliguri to 3636 m at Sandakphu with sharp physiographic contrast between the plain and the mountain regions (Rai and Bhujel, 2002).

2.1.2. Population and demography

The total population of this region is about 800,000, of which 72.77% lives in the rural areas; the male:female ratio is lopsided, with 982 females for every 1000 males (District Statistical Handbook, 2002). Due to similar environmental conditions and culture, the major inhabitants of Darjeeling hills and its surrounding areas are bonded together by the language Nepali, the medium of communication among the different ethnic groups *viz.* Lepchas, Bhutia, Rai, Sherpa, Tamang, Mangar, Gurung and Kagatay communities (Rai and Bhujel, 1999). Traditionally, the chief occupation of the people had been agriculture, agro-forestry, horticulture and animal husbandry. Presently tourism has also become a big industry. However, the tribal groups are still living in far-flung remote villages where the advantages of modern civilization are yet to arrive. With only 8 hospitals and 24 Public Health Centers, modern medical facilities are lacking in the Darjeeling hills. The doctor to people ratio is 1:4892 (Chhetri *et al.*, 2005). Hence, people treat their ailments using their own medicinal knowledge, mostly prepared from locally available herbs, animal parts, salts and other materials, locally termed as "jaributi" or "pahare dawai". The ethnic group of inhabitants has gathered this knowledge through trial and error, during their survival in such inhospitable environments for hundreds and thousands of years. Unfortunately, this traditional knowledge is ignored due to the advent of modern allopathic medicines. Recently the importance of traditional, especially the plant-based medicines have become popular worldwide, due to their perceived nontoxicity, efficacy and affordability. Surveying, recording and evaluating such herbal knowledge have assumed importance in various parts of world (Bhujel, 1996).

2.1.3. Vegetation

The most remarkable feature of natural vegetation of Darjeeling Himalaya is the presence of different types of forests within a small area. The forest can be classified into: (A) Sub-alpine or alpine: The region extends above 3500 m. The lower reaches show a dominance of shrubs and stunted dwarf trees, intermixed with grassy alpine meadows. Trees, mainly of the genus *Abies, Larix, Rhododendron* etc. are abundant, which gradually grades into the region of perpetual snow. (B) Temperate: This region extends from about 1800 to 3500 m, and can further be subdivided into lower and upper temperate regions. The lower temperate region consists of species of *Quercus, Cedrela* etc., while the upper temperate, cooler zone shows a dominance of conifers, *viz.*, *Juniper, Pinus, Abies* etc. (C) Tropical: It extends from the plains to about 1800 m altitude. The lower area comprises of rich forest of *Shorea robusta* along with *Terminalia, Albizia, Michelia, Bischofia, Toona, Dillenia* etc. Riverine succession, comprising an association of seasum and kair, is also common (Negi, 1993).

2.1.4. Knowledge of local medicinal plants

Scattered and sporadic work on the ethnobotanical studies and surveys in this area has been conducted during 1984 to 2003 (Das and Mandal, 2003; Nandi, 1997; Rai and Bhujel, 1999; Rai *et al.*, 1998; Yonzone *et al.*, 1984), but the knowledge gathered cannot be regarded as complete. Hence, an extensive and illustrative report on these aspects that will also ensure better documentation of Nepali "jaributi" system practiced by Baidya (herbal practitioner) and the locals is essential. The effort in this direction has been inadequate, and only haphazard information is available (Chhetri *et al.*, 2005), although the Tibetan herbal practice (Tsarong, 1986) has a deep impact on the tribals of the Darjeeling hills.

2.2. Overview of antioxidant biology and chemistry

2.2.1. Oxygen metabolism in aerobes

Oxygen, the source of aerobic respiration, is the main energy source of living organisms. Nature has chosen fuel substrates (carbohydrates and lipids) to undergo aerobic oxidation once the earth's atmosphere became rich in oxygen. Under aerobic conditions, substrates go through initial oxidation pathways in which the reducing equivalents (electrons) are harnessed by oxidation. Partially oxidized substrates from various pathways converge into Krebs cycle in mitochondria where complete oxidation takes place. The reducing equivalents, thus gathered, enter the electron transport chain (ETC) located in the inner mitochondrial membrane and yield adenosine triphosphate (ATP), the energy currency of the cell by the process called oxidative phosphorylation. Molecular and biochemical mechanisms ensure that molecular oxygen in the ETC always receives four electrons to form water, a harmless end product of catabolism. Thus, oxygen plays a vital role in oxidation of substrates and maintaining the flow of substrates through catabolic pathways, which otherwise would stop due to feedback inhibitory mechanisms.

2.2.2. Flip side of oxygen metabolism

However, aerobic/oxygen metabolism has its own disadvantages driven by the very chemical nature of oxygen. Molecular oxygen or dioxygen can also undergo reduction in sequential steps. Dioxygen contains two unpaired electrons with parallel spins in two degenerate orbitals and is a paramagnetic biradical molecule ($^{\bullet}O\text{-}O^{\bullet}$) with a triplet ground state. Hence, the sequence of one electron reductions of dioxygen yields (i) superoxide anion radical ($O_2^{\bullet-}$), a base with its conjugate acid hydroperoxyl radical, (ii) the peroxide ion, a very strong base, and (iii) a hypothetical (O_2^{3-}), precursor of oxide and oxene. Oxide is a conjugate base of water, while oxene is a conjugate base of the highly reactive hydroxyl radical ($^{\bullet}OH$). In addition, in the presence of various photo-sensitizers, dioxygen can undergo easy excitation to the singlet state to generate the singlet oxygen ($^1O_2$). The one electron reduction products of dioxygen, and the excited singlet forms of it are all predicted to be good oxidants with respect to water. Presence of catalytic metal ions recycles some of the oxidants generated from dioxygen and hastens their reactivity. These oxidants play very important roles in biology ranging from simple repairable damage to carcinogenesis/lethality in a multicellular organism. Interestingly, instances have been observed in aerobic organisms where oxidants are generated intentionally as well as accidentally. Typically around 2% of dioxygen in ETC undergo partial reduction to generate $O_2^{\bullet-}$, which subsequently gives rise to other oxygen-derived reactants, collectively called reactive oxygen species (ROS) (Chance *et al.*, 1979).

2.2.3. ROS

ROS can be defined as entities containing one or more oxygen atoms that meet the defining criteria for being chemically reactive. ROS consist of radical (an atom or molecule that contains one or more unpaired electrons) and nonradical species. A free radical is a radical that has moved out of the immediate molecular environment of its generation. Conversely, radicals that are retained within their sites of generation have been called "caged radicals" (Pryor, 1966). In biological systems most of the free radicals are derived from oxygen. Another group of reactive species contain both oxygen and nitrogen and include physiologically important nitric oxide and toxic peroxynitrite. These species are referred as reactive nitrogen species (RNS). Not all of these reactive species are radicals but in many cases the reactive nonradical species will end up as radicals, damaging biomolecules by one-electron oxidation. The danger of this type of reaction is that the oxidation products formed are radicals themselves, which are in many cases able to propagate the reaction, leading to extensive damages. The biologically important ROS and RNS, and their salient properties are listed in Table 2.

Table 2. Characteristics of different reactive oxygen species (ROS) and reactive nitrogen species (RNS)

Name of the reactive species	Symbol	Half life at 37°C	Remark
superoxide	$O_2^{\bullet}$	10^{-6} s	not very reactive
hydrogen peroxide	H_2O_2	min	not very reactive but yields potent species
hydroxyl	$^{\bullet}OH$	10^{-9} s	highly reactive
alkoxyl	$RO^{\bullet}$	10^{-6} s	reactive
peroxyl	$ROO^{\bullet}$	s	reactive
organic hydroperoxide	ROOH	stable	reacts with transient metal ions to yield reactive species
singlet oxygen	$^1O_2$	10^{-6} s	highly reactive
ozone	O_3	s	can react with biological molecules yielding $^1o_2$
nitric oxide	$NO^{\bullet}$	s	neurotransmitter and blood pressure regulator
peroxynitrite	$ONOO^{\bullet}$	10^{-3} s	highly reactive

2.2.3.1. Generation of ROS in living systems

2.2.3.1.1. Endogenous generation

A number of intracellular sources of ROS have been identified (Table 2). The importance of each source in any specific order is unknown, and the relative role each plays in tissue injury seems certain to vary with the specific experimental conditions employed.

(A) Phagocytes: Perhaps the best recognized biological sources of free radicals are phagocytic cells, *e.g.*, neutrophils and monocytes. When activated to begin phagocytosis, these cells exhibit a marked increase in oxygen consumption leading to "oxidative burst", which was shown to involve the rapid reduction of oxygen to $O_2^{\bullet-}$ (Babior *et al.*, 1973). Subsequent work demonstrated that this reaction is catalyzed by a plasma membrane-bound NADPH oxidase, with extracellular production of large amounts of ROS.

(B) Mitochondrial electron transport system: The mitochondrial ETC is a very efficient system ensuring complete oxidation of fuel molecules, but the very nature of the alternating one-electron oxidation-reduction reactions predisposes each electron carrier to side reactions with molecular oxygen (Chance *et al.*, 1979). There exists a tendency for an electron to pass directly to oxygen (generating $O_2^{\bullet-}$) instead to the next electron carrier in the chain (Cadenas *et al.*, 1992). About 1-2% of the total daily oxygen consumption goes to mitochondrial superoxide generation. The mitochondrial outer membrane enzyme, monoamine oxidase, generates a large source of hydrogen peroxide during oxidation of various xenobiotics, increasing the concentrations of the ROS in mitochondria and cytosol (Jackson *et al.*, 2002).

(C) Soluble oxidase enzymes: Xanthine oxidase, dopamine-β-hydroxylase, D-amino acid oxidase, urate oxidase, glucose oxidase, lipoxygenases, cyclooxygenases and fatty acyl CoA oxidase are some of the enzymes that can oxidize endogenous and exogenous substrates, and generate ROS.

(D) Transition metals: Endogenous redox active metal ions that form an integral part of normal function of life, notably iron and copper, can facilitate transfer of electrons to macromolecules like lipids, proteins and DNA. Metal ions also catalyze decomposition of existing organic hydroperoxides to generate reactive species.

2.2.3.1.2. Exogenous generation

Exogenous factors contributing to the generation of ROS are ionizing radiations, pollutants like industrial and cigarette smoke, certain drugs (doxorubicin, cyclophosphamide, 5-fluorouracil, methotrexate, and vincristine) and exposure to metal ions.

2.2.4. Oxidative stress

From the foregoing, the continuous generation of toxic ROS in cells is apparent. However, the excess ROS, that is harmful to cells, is kept under check by the cellular defense mechanisms, involving intracellular antioxidants such as vitamin C, vitamin E and selenium as well as antioxidant enzymes, such as catalase, superoxide dismutase and different glutathione-processing enzymes (glutathione-S-transferase, glutathione peroxidase and glutathione reductase), all of which neutralize the excess ROS (Fig. 1).

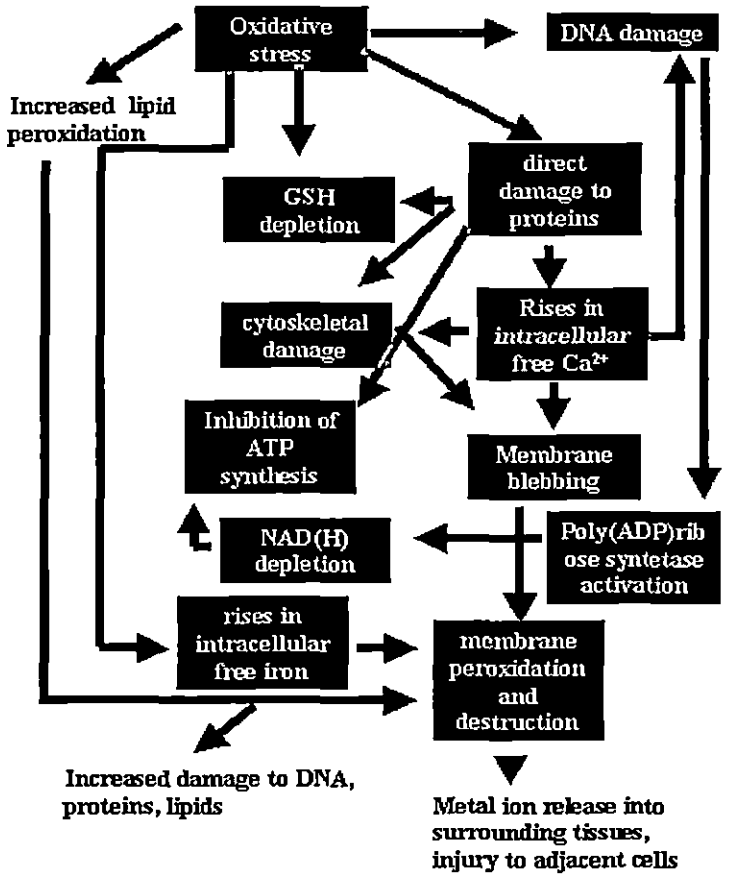


Fig. 1. Cellular events occurring under oxidative stress (Subramanian, 2005)

Under conditions of inflammation or disease, the precious balance is upset leading to overwhelming production of ROS or failure of endogenous antioxidant mechanisms - a condition, termed as 'oxidative stress' (Sies, 1991). It denotes an imbalance between the production of oxidants and the respective defense systems of an organism (Halliwell and Gutteridge, 1999), and can lead to the damage of important biomolecules (Fig. 2) and contribute to the origin and pathogenesis of several diseases.

2.2.5. ROS-mediated damages to biomolecules

2.2.5.1. DNA damage

ROS-mediated reactions can cause structural alterations in DNA (nicking, base-pair mutations, rearrangements, deletions, insertions and sequence amplification). The endogenous reactions that are likely to contribute to ongoing DNA damage are oxidation, methylation, depurination and deamination (Ames, 1995). Methylation of cytosines in DNA is important for the regulation of gene expression, and

normal methylation patterns can be altered during carcinogenesis (Weitzman *et al.*, 1994). Conversion of guanine to 8-hydroxyguanine, a frequent result of ROS attack (Box *et al.*, 1995; Dizdaroglu, 1993; Halliwell and Aruoma, 1993) has been found to alter the enzyme-catalyzed methylation of adjacent cytosines (Weitzman *et al.*, 1994). The chemistry of DNA damage by several ROS has been well-characterized *in vitro* (Box *et al.*, 1995; Dizdaroglu, 1993), although specific information about the changes produced by peroxyl, alkoxyl, ozone and several of the RNS is lacking. Different ROS affect DNA in different ways; for example, H_2O_2 does not react with DNA bases at all (Dizdaroglu, 1993), whereas •OH generates a multiplicity of products from all four DNA bases, and this pattern seems to be a diagnostic "fingerprint" of hydroxyl attack (Halliwell and Aruoma, 1993). By contrast, singlet oxygen selectively attacks guanine (Van den Akker *et al.*, 1994). The most commonly produced base lesion, and the one most often measured as an index of oxidative DNA damage, is 8-hydroxyguanine.

Damage to DNA by ROS/RNS seems to occur naturally, and low steady-state levels of base damage products have been detected in nuclear DNA from human cells and tissues (Musarrat and Wani, 1994; Richter, 1992). ROS/RNS can also damage mitochondrial DNA, which has been suggested to contribute to several human diseases, and aging (Harman, 1992; Shigenaga *et al.*, 1994). ROS, generated by the mitochondrial ETC, is perhaps the damaging agent (Ambrosio *et al.*, 1993). Although various repair enzymes can control DNA damage (Demple and Harrison, 1994.), they do not achieve complete removal of modified bases (Jaruga *et al.*, 1994)

2.2.5.2. Lipid peroxidation

Polyunsaturated fatty acids (PUFA) are prone to oxidation, resulting in the formation of alkanes, aldehydes, alcohols and hydroperoxides, among other products. The bis-allylic methylene hydrogens in PUFA are susceptible to abstraction by ROS to produce the lipid radicals (L•). These radicals can subsequently isomerize to produce conjugated radicals, which can furnish conjugated dienes (by loss of a H atom) or react with molecular oxygen forming the lipid peroxyl radicals (LOO•) or lipid peroxides (LOOH) (Wilcox and Marnett, 1993) in a set of chain reactions. The LOO• can also get degraded (by ransition metal ions) to produce alkoxy radicals (LO•) (Girotti, 1985) as well as various small organic molecules (primarily aldehydes, such as malondialdehyde, acrolein, 4-hydroxynonenal etc.) (Porter, 1984). The lipid peroxidation (LPO) leads to disruption of membrane architecture affecting cellular homeostasis and deactivation of membrane-bound key enzymes. Also, the LPO byproducts are strong hydrophobic electrophiles, and can cause further damages to DNA and other biomolecules, far away from the site of their generation. The reactions involved in LPO are shown in Fig. 2.

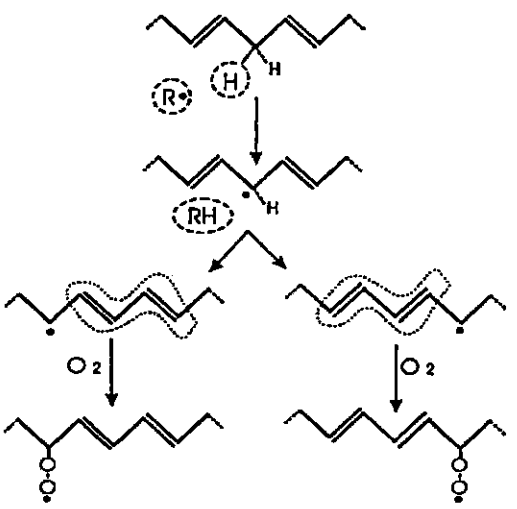


Fig. 2. Reactions involved in LPO (Hogg and Kalyanaraman, 1999)

In the absence of any additional reactions, the LPO chain reaction will terminate when two lipid radicals react to form nonradical products. Otherwise, compounds capable of donating a hydrogen atom to the peroxyl radical can also break or at least divert the chain reactions, provided a relatively inert radical is produced during the process. The phenolic compounds, such as the natural tocopherols are the most well-studied chain-breaking antioxidants (Liebler, 1993).

2.2.5.3. Protein oxidation

Intracellular proteins are also prone to oxidative modifications, which have been suggested to play a key role in the origin of senescence-associated losses in physiological functions, because oxidized proteins often lose catalytic function and undergo selective degradation (Giasson *et al.*, 2000; Pansarasa *et al.*, 1999). Oxidative damage to a specific protein, especially at the active site, can induce a progressive loss of a particular biochemical function. Several types of ROS-induced protein modifications, including the loss of sulfhyryl (SH) groups, formation of carbonyls, disulphide crosslinks, methionine sulfoxide, dityrosine cross-links, nitrotyrosine, glyoxidation and lipid peroxidation adducts etc. have been demonstrated (Giasson *et al.*, 2000). There is a large body of evidence implicating oxidative damage to proteins in the pathogenesis of both normal aging and neurodegenerative illnesses.

2.2.6. Herbal antioxidants

It is by and large now clear that oxidative stress occurs due to inadvertent ROS generation and depletion of cellular antioxidant levels. This leads to various pathogenic conditions that can be reversed or prevented by external supplementation of antioxidants. A broader definition of an antioxidant is any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Halliwell, 1995). There have been several classifications of antioxidant molecules based on solubility (water/lipid soluble), chemical nature (vitamins, trace elements, proteins, polyphenols and polysaccharide), source (endogenous and exogenous/dietary), mechanism/mode of action (enzymatic and nonenzymatic; direct and indirect; sacrificial and interceptive; metal ion chelator and ROS scavenger) and molecular weight (low and high molecular weight). Hence, a comprehensive classification of antioxidants is difficult. A general view of antioxidants present in a cell is provided in Fig. 3. Although

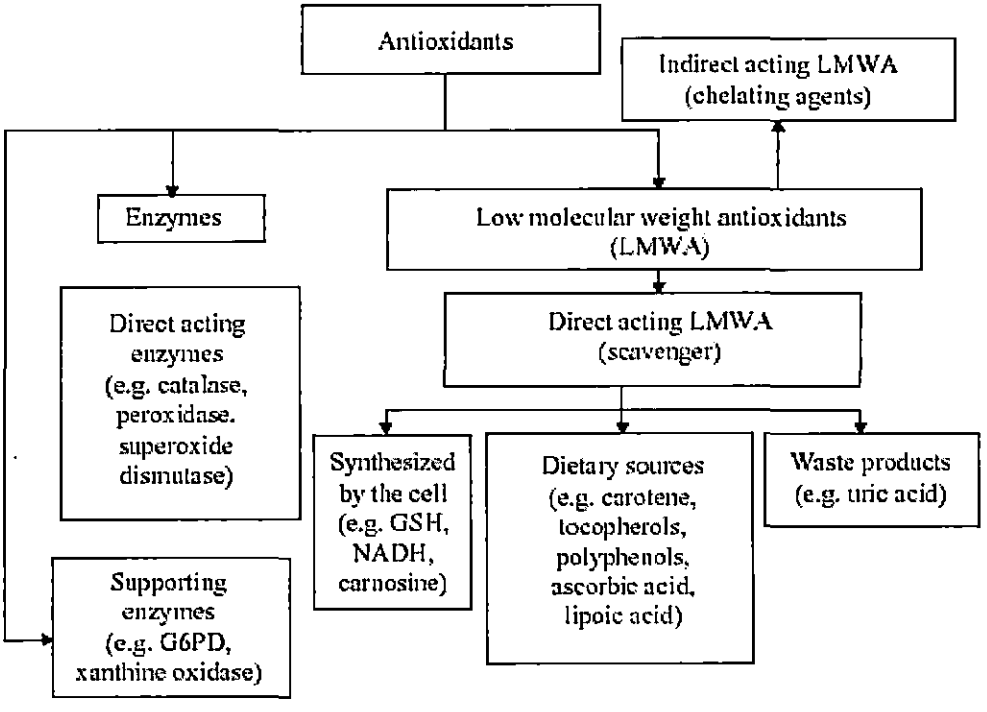


Fig. 3. Overview of cellular antioxidant defense system (Subramanian, 2005)

reported from several sources, plants remain the single major contributors of antioxidants. Functionally, antioxidants could act by one or more of the following mechanisms: (a) prevention of generation of ROS;

(b) scavenging of ROS, thereby averting oxidation of biological targets; (c) chemical repair of oxidized targets; and (d) up-regulation of endogenous defense and repair mechanisms.

2.2.6.1. Antioxidants as radioprotectors

As radiation-induced cellular damage is attributed primarily to the harmful effects of free radicals, molecules with direct free radical-scavenging properties are particularly promising as radioprotectors. The best-known radioprotectors are the sulfhydryl compounds, such as cysteine (Patt *et al.*, 1949) and cysteamine (Bacq *et al.*, 1953). However, these compounds produce serious side effects and are toxic at the doses required for radioprotection. Over the years, a number of compounds have been tested for their radioprotective efficacy with generally limited successes (Venkatachalam and Chattopadhyay, 2003). Thus, there is still an urgent need to identify novel, nontoxic, effective and convenient compounds to protect humans from the damaging effects of ionizing radiations (IR). Some antioxidant nutrients and phytochemicals have the advantage of low toxicity, although they are generally protective when administered at pharmacological doses (Weiss and Landauer, 2003). Many naturally occurring antioxidants exhibit a long window of protection, including post-irradiation protection against lethality and mutagenesis. A number of phytochemicals have multiple physiological effects as well as antioxidant activity, which result in radioprotection *in vivo*. The potential application of many of these antioxidants shows promise either prophylactically for anticipated exposures in emergency situations or therapeutically after radiation accidents/incidents. Many antioxidant nutrients and phytochemicals have antimutagenic properties, and their modulation of long-term radiation effects, such as cancer, needs further examination.

2.2.6.2. Nutraceutical antioxidants and health

Recent studies have shown that diets consisting of high content of phytochemicals can provide protection against various diseases. Approximately 90% of all cancer cases correlate with environmental factors, including one's dietary habit, and one-third of all cancer deaths are avoidable by changing dietary habits only (Milner, 1994; Willett, 1995). These discoveries have rapidly amplified the consumer awareness of the potential benefits of naturally occurring compounds from plants in health promotion and maintenance, and researches in nutraceuticals and functional foods as well as natural health products have become the hot topics in recent years. The term "nutraceutical" was coined from "nutrition" and "pharmaceutical" in 1989 by Stephen DeFelice. According to him, nutraceutical can be defined as, "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease" (Brower, 1998). The protective effects of fruits, vegetables and spices and herbs were found not only for cancer, but also other chronic diseases. Antioxidants such as vitamins C and E are essential for the protection against ROS. However, the majority of the antioxidant activity of botanical sources may be from compounds such as phenolic acids and flavonoids, rather than from vitamin C, E or β-carotene (Hanasaki *et al.*, 1994; Wang *et al.*, 1996). Intake of controlled diets rich in fruits and vegetables increases the antioxidant capacity of plasma significantly.

Antioxidant phytochemicals are, therefore, the focus of many recent studies. Besides radical scavenging, antioxidant phytochemicals also inhibit oxidation through a variety of mechanisms (Cao *et al.*, 1998; Kähkönen *et al.*, 1999; Steinmetz and Potter, 1991; Stich and Rosin, 1984). Polyphenolics, the major class of antioxidants is a highly inclusive term that covers many different subgroups of phenolic acids and flavonoids. More than 5000 polyphenolics, including over 2000 flavonoids have been identified, and the number is still growing (Harborne, 1993).

The polyphenolics vary in chemical structures: hydroxybenzoic acids and hydroxycinnamic acids have a single-ring structure, while flavonoids that can be further classified into anthocyanins, flavan-3-ols,

flavones, flavanones and flavonols, contain two rings fused together. Some of the flavonoids such as flavan-3-ols can even be found as dimers, trimers and even polymers. Many of the phenolics are often associated with sugar moieties that further complicate the phenolic profiles of plants (Merken and Beecher, 2000). Polyphenols are antioxidants, because of their high redox potentials, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kahkonen *et al.*, 1999). In addition, many of them show metal-chelating potential (Rice-Evans *et al.*, 1995). Their significance arises as the "life saving" gas, oxygen, has an adverse effect on metabolism and long-term storage of foods by forming several reactive oxygen radical species and other harmful chemicals. A brief overview of antioxidant polyphenols *vis-à-vis* their chemical classifications is provided below.

(A) Monophenols and phenolics acids: These compounds participate mainly via hydrogen donation and radical-scavenging to exert their antioxidant action. To serve as an active antioxidant, the *ortho-* or *para*-position of the phenol moiety must be substituted with electron donating groups to increase the electron density at the hydroxyl group and lower the oxygen-hydrogen bond energy. This increases its reactivity towards free radicals. The antioxidant activity of phenolics acids is mainly due to phenolics hydrogens. Some examples in this class include: (a) tocopherol and tocotrienol, (b) benzoic acid derivatives, *viz.* vanillic acid and gallic acid, and (c) cinnamic acid derivatives, like ferulic acid and caffeic acid.

(B) Flavonoids: These are a group of compounds characterized by a C6-C3-C6 unit, and several mechanisms such as hydrogen donation, radical-scavenging and metal chelation, individually or in combination, account for their superior antioxidant activity. Some of the well-known and widely occurring candidates in this class are: (a) flavones: apigenin, chrysin and luteolin; (b) flavonols: kaempferol, myricetin and rutin; (c) flavanones: taxifolin, naringin and naringenin, and (d) flavans: catechins.

(C) Isoflavones: These are structurally similar to flavonoids, and are mostly found in the members of Fabaceae family. Within this group, genistein and its 7-β-glucoside were found to have the highest antioxidant activity (Foti *et al.*, 2005).

(D) Anthocyanins and anthocyanidins: These are metabolic products of flavanones and are often placed in the flavanoid group. They lack the C4-carbonyl group, and show metal-chelating and radical-scavenging activities. While anthocyanins are cyanidin-3-glucoside and malvidin-3-glucoside, anthocyanidines are cyanidine and malvidine.

(E) Carotenoids: These are characterized by the presence of a conjugated polyene system which quenches singlet oxygen. They are generally coloured pigments widespread in plants and human foodstuff.

2.2.7. Antioxidant screening methods

The available assays to judge the antioxidant (AO) potential of test samples can be broadly divided under the following categories, *viz.* scavenging ROS and RNS, measuring oxygen uptake, measuring the inhibition of induced lipid auto-oxidation, hydrogen atom transfer (HAT) and electron transfer (ET) assays using molecular probes.

2.2.7.1. ET assays using molecular probe

The assays in this category include total phenol content (TPC), Trolox equivalent antioxidant capacity (TEAC) and ferric ion-reducing antioxidant power (FRAP). These are based on the following reaction and electron transfer:

$$\text{probe (oxidant)} + e^- \text{(from AO)} \longrightarrow \text{reduced probe} + \text{oxidized AO}$$

The oxidant probe extracts an electron from the AO, causing the probe to change colour, which is proportional to the AO capacity of the test sample. The slope of the linear plot of the change in absorbance *versus* AO concentration reflects the reducing capacity (expressed as Trolox equivalent (TE) or gallic acid equivalent (GAE)). As there is no oxygen radical in this equation, relating the parameters to the AO capacity is questionable in peroxidating food or under *in vivo* situations (Becker *et al.*, 2004). However, these offer very quick results.

2.2.7.1.1. TPC assay

This assay, originally intended to analyse protein, taking advantage of the phenol group in tyrosine has been extended to measure TPC in wine (Singleton *et al.*, 1999). The Folin–Ciocalteu reagent (FCR)-based assay actually measures the reducing capacity (ET) of the sample. The phenolic compounds can only react with FCR under basic conditions, where dissociation of the phenolic proton leads to a phenolate anion that can reduce FCR. This involved a complex redox reaction of the phenols with phosphotungstic and phosphomolybdic acids present in FCR. It is simple, reproducible, convenient and widely used when studying phenolic antioxidants. However, the lack of specificity of FCR to the phenolic compounds can lead to erroneous results (Escarpa and González, 2001). In addition, depending on the number of phenolic groups they have, phenolic compounds respond differently to the FCR. It is usually more correct to use HPLC analysis to determine phenolic concentrations, however, and this limits the quantification to one class of phenolic compounds (Becker *et al.*, 2004).

2.2.7.1.2. FRAP assay

The FRAP assay is also based on an ET reaction (Benzie and Strain, 1996) in which a ferric salt is used as an oxidant. The redox potential of the Fe(III) salt (~0.7 V) is similar to $ABTS^{\bullet+}$, and therefore the FRAP and TEAC assays differ in the operating pH values, *viz.* neutral pH (TEAC) and acidic pH, ~3.6 (FRAP). Although originally designed for the plasma samples, it is now extensively been for tea, wine and other plant extracts (Pulido *et al.*, 2000). The assay is not amenable to antioxidants, such as carotenoids that operate by HAT. Also, use of aqueous and alcoholic Fe(II) solutions leads to different intercepts and the slopes on the regression curves, even for the same analytes, like polyphenols. Therefore, care should be taken when non-aqueous samples are analysed, and comparison to the corresponding Fe(II) solutions should be made. Potential problems occur as the mixture contains other Fe(III) species, which can bind to chelators in the food extract, and these complexes are capable of reacting with the antioxidants. Results show that, similar to TEAC, there is no relation between the FRAP value and the number of electrons that an antioxidant can donate (Becker *et al.*, 2004).

2.2.7.1.3. 2,2-Diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$)-scavenging assay

It is one of the few stable and commercially available organic nitrogen radical assays, and is considered as an easy and accurate method for use with fruit and vegetable juice extracts (Sanchez-Moreno, 2002). Besides electron spin resonance monitoring, the $DPPH^{\bullet}$-scavenging is more conveniently quantified from the decrease in its absorbance at 515–528 nm in the presence of the antioxidants in ethanol/methanol. This assay is not suitable for measuring the antioxidant capacity of plasma, as proteins are precipitated in the presence of the alcoholic solvents. Also, antioxidants that may react quickly to the *in vivo* peroxyl radicals may react slowly with $DPPH^{\bullet}$. The reaction kinetics between $DPPH^{\bullet}$ and antioxidants is not linear to $DPPH^{\bullet}$ concentration; therefore, measurement of the EC_{50} is problematic. Although originally believed to be a HAT reaction, more recently, ET has been suggested as the operating mechanism (Foti *et al.*, 2004).

2.2.7.2. HAT assay using molecular probes

In general, these assays provide a steady flux of peroxyl radicals (from a radical initiator – usually 2,2-azobis(2-amidinopropane)hydrochloride (AAPH)) in air saturated solutions in the presence of a molecular probe that fluoresces when oxidized. The added antioxidants compete with the probes for oxidation. The difference between the methods in this category lies mostly in the approach to quantification of antioxidant capacity. The following methods are based on the antioxidant scavenging principle of hydrogen atom transfer.

2.2.7.2.1. Oxygen radical absorbance capacity (ORAC)

The ORAC assay has been used widely in measuring the net resultant antioxidant capacity (or peroxyl radical absorbance capacity) of botanical and other biological samples. Initially, β-phycoerythrin (β-PE) was used as the probe in this method, and the results were expressed with reference to a known amount of an antioxidant, Trolox (a water-soluble analogue of vitamin E) (Cao *et al.*, 1993). β-PE was chosen because of its excitation and emission wavelengths, high fluorescent yield, sensitivity to ROS, and water solubility, but this requires a FARA COBAS II analyser, which is not widely available. Also, the nonspecific protein binding of β-PE leads to its bleaching, even in the absence of any antioxidant. Subsequent replacement of β-PE with fluorescein (FL) led to overcome these problems, and the system was tested for precision and accuracy, ruggedness, specificity to antioxidant action and linearity of the relationship between the net area under curve (AUC) and antioxidant concentration (Meyer *et al.*, 1997). However, FL is pH sensitive, and this must be carefully monitored. Using AUC to measure the antioxidant capacity is advantageous, as it applies both to an antioxidant that has a lag phase and one that does not. This allows the unification of the lag time method and the initiator method of analysis. This is particularly useful in foods, as there is often a mix of activities that can be accounted for by using this method. The FL probe is inexpensive and the method can be speed up using a microplate reader.

2.2.7.3. Methods measuring inhibition of induced LPO

These methods induce auto-oxidation of linoleic acid or low-density lipoproteins (LDL) by Cu(II) or an azo initiator, and measures the UV absorbance at 234 nm (the λ_{max} of the conjugated diene peroxides from linoleic acid oxidation) (Pryor *et al.*, 1993). Problems arise because (1) it is difficult to measure the small lag times that occur and (2) many substances in foods also absorb at 234 nm. The reaction can occur in micelles or in organic solvents, but measuring the absorbance in micelles is not straightforward. Additionally, linoleic acid will form micelles in the presence of water. This is a critical issue, as the way an antioxidant performs between the two phases (aqueous and lipid) is important to its *in vivo* behaviour. The problem of micelle formation by linoleic acid can be overcome by using methyl esters. The percent inhibition of hexanal production was used as a measure of antioxidant capacity, since it is the major secondary byproduct of the peroxidation of n-6 fatty acids. However, it is only one of the secondary peroxidation products, and therefore, may not be a good marker of oxidation. Also, it has a relatively high boiling point and thus much of the product will be in the liquid phase and, therefore, not measured using this methodology.

2.2.7.4. Methods that measure uptake of oxygen

These methods require the measurement of the rate of O_2 consumption or the rate of conjugated diene peroxide formation in order to interprete the antioxidant's rate constant for inhibition, using a pressure transducer system under one atmospheric pressure of oxygen (Burton and Ingold, 1981). This method is not very popular as (1) it requires data collection under very high O_2 pressure, (2) it is difficult to accurately

measure the O_2 uptake, especially during the inhibition phase when the uptake is very low, (3) the antioxidant concentrations in foods are usually lower leading to insufficient sensitivity, and (4) the transition between inhibition and uninhibited O_2 uptake may not be distinct.

2.2.7.5. ROS and RNS scavenging

These methods rely on the estimation of physiological and food-related ROS and RNS using different techniques. Some of these are discussed below.

2.2.7.5.1. Measuring $O_2^{\bullet-}$-scavenging

The $O_2^{\bullet-}$ is formed by radiolysis of water in the presence of oxygen and formate, which allows accurate reaction rate constants to be measured (Halliwell, 1997). However, this reaction has slow rate constants, and requires appropriate equipments. In normal tissue, xanthine oxidase (XO), a dehydrogenase enzyme, transfers electrons to nicotinamide adenine dinucleotide (NAD), but during stress this enzyme is converted to an oxidase which produces $O_2^{\bullet-}$ and H_2O_2. Thus, XO plus hypoxanthine (or xanthine) at pH 7.4 can be used to generate the $O_2^{\bullet-}$, and quantified using its ability to reduce nitro blue tetrazolium (NBT) (Bull *et al.*, 1983) to formazan, which can be spectrophotometrically measured at 560 nm (Sanchez-Mareno, 2002). Use of microplate format (at 550 nm) and cytochrome c, in place of NBT (Quick *et al.*, 2000) has to improve the throughput and ease of this assay. An appropriate ratio of substrate (hypoxanthine) to enzyme is essential to ensure the production of optimum amounts of $O_2^{\bullet-}$. Too much of the substrate causes the formation of hydroperoxide resulting in undesirable side reactions that could potentially cloud the results. Selection of proper control reactions is essential for substances that may directly interfere with the action of the enzyme, or if the antioxidant itself directly reduces cytochrome c or NBT. Therefore, this method is not suitable for nonenzymatic antioxidants.

2.2.7.5.2. Measuring $^{\bullet}OH$-scavenging

Pulse radiolysis (Bielski, 1985) is one of the often-used techniques for measuring the reaction of the $^{\bullet}OH$ and antioxidants (Halliwell, 1990), but requires the specialized equipment and is a costly assay. The use of ESR spectrometry is also used for this purpose. Here, the decrease of the DMPO–$^{\bullet}OH$ ESR signal by the $^{\bullet}OH$ scavenger is used to measure the rate constant from a competition plot (Finkelstein *et al.*, 1980). The most popular assay, however, is based on oxidative degradation of 2-deoxyribose by the $^{\bullet}OH$, and spectrophotometric assay of the decomposed product by forming a chromogen with 2-thiobarbituric acid (TBA) (Halliwell *et al.*, 1987).

Electron transfer assays measure the reducing ability of the substrate (antioxidant); hydrogen transfer assays measure the hydrogen-donating ability of the substrate. It is clear that hydrogen atom donation is essential in the radical chain reaction stage of lipid peroxidation. Therefore, hydrogen transfer assays are relevant to the measurement of chain-breaking antioxidant capacity. Thus, in many cases the antioxidant capacity or the ability to trap radicals of a compound is related to the ease of hydrogen atom donation and not necessarily the redox potential of the compound. Therefore, electron transfer or reducing power assays are not relevant to antioxidant capacity *in vivo* and are more difficult to justify when looking for the antioxidant potential of new or novel compounds found in foods. Of course, in the case of some oxidants such as peroxynitrite and hypochlorite, reduction can render these compounds harmless and therefore reducing power has relevance in a few isolated cases. In general, it can be concluded that the assays that measure hydrogen atom transfer would be preferable to assays that measure electron transfer reactions.

2.3. Antimicrobials in plants

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immuno-compromised patients in developing countries (Al-Bari *et al.*, 2006). Worldwide, infectious disease is the number one cause of death accounting for approximately one-half of all the deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries, such as the United States. Death from infectious disease, ranked 5th in 1981, has become the 3rd leading cause of death in 1992, an increase of 58%. It is estimated that infectious disease is the underlying cause of death in 8% of the deaths occurring in the US. This is alarming given that it was once believed that infectious disease would be eliminated by the end of the millennium. The increases are attributed to increases in respiratory tract infections and AIDS. Furthermore, the most dramatic increases are occurring in the 25–44 years-old age group. Other contributing factors are an increase in antibiotic resistance in nosocomial and community-acquired infections. In third world countries, like Bangladesh, Nepal and Nigeria, irrational use of antimicrobial agents is a major cause of such resistance. Also, the public is becoming increasingly aware of problems with the overprescription and misuse of traditional antibiotics. Many people are interested in having more autonomy over their medical care.

These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. Proposed solutions are outlined by the Centers for Disease Control and Prevention (CDC) in the US as a multi-pronged approach that includes prevention (such as vaccination), improved monitoring and the development of new treatments. It is this last solution that would encompass the development of new antimicrobials.

The scientific discipline known as ethnobotany (or ethnopharmacology) utilizes the impressive array of knowledge assembled by indigenous people about the plant and animal products that have been used to maintain and promote health (Rojas *et al.*, 1992; Silva *et al.*, 1996). Exploration of indigenous plants is currently being considered essential in developing a safer antimicrobial principle against infectious diseases (Borris, 1996; Rahman *et al.*, 2001). Traditional healers have long used plants to prevent or cure infectious conditions; Western medicine is trying to duplicate their successes. A multitude of plant compounds (often of unreliable purity) is readily available over-the-counter from herbal suppliers and natural-food stores, and self-medication with these substances is commonplace. The use of plant extracts as well as other alternative forms of medical treatments is enjoying great popularity in the late 1990s.

Several plant constituents, such as tannins, terpenoids, alkaloids and flavonoids have been found *in vitro* to have antimicrobial properties. Historically, plants have provided a good source of anti-infective agents; emetine, quinine and berberine remain highly effective instruments in the fight against microbial infections. Phytomedicines derived from plants have shown a great promise in the treatment of intractable infectious diseases, including opportunistic AIDS infections. Plants containing protoberberines and related alkaloids, picralima-type indole alkaloids and garcinia biflavonones used in traditional African system of medicine, have been found to be active against a wide variety of microorganisms. The profile of known drugs, like *Hydrastis canadensis* (goldenseal), *Garcinia kola* (bitter kola), *Polygonum* sp. and *Aframomum melegueta* (grains of paradise) will be used to illustrate the enormous potential of anti-infective agents from higher plants. Newer drugs such as *Xylopia aethiopica*, *Araliopsis tabouensis*, *Cryptolepis sanguinolenta*, *Chasmanthera dependens* and *Nauclea* species will be reviewed.

2.3.1. Herbal antimicrobials

There are numerous illustrations of plant-derived antiinfective drugs. In many cases, these substances serve as plants' defense mechanism against predation by microorganisms, insects and herbivores. The

probable mechanism of phenolic toxicity towards microorganisms include enzyme inhibition by the oxidized compounds (possibly through reaction with SH groups) or through more nonspecific interactions with the proteins (Mason and Wasserman, 1987). Phenolic compounds possessing a C3 side chain at a lower level of oxidation containing no oxygen are classified as essential oils and are often cited as antimicrobial agents. Some selected examples, including those classified as, are presented below.

Garcinia kola Heckel (Clusiaceae): It is found in moist forest and grows as a medium-sized (up to 12 m high) tree, and is cultivated and distributed throughout west and central Africa. Medicinal uses include purgative, antiparasitic and antimicrobial. The seeds are used in the treatment of bronchitis and throat infections. They are also used to prevent and relieve colic, cure head or chest colds and relieve cough. Also the plant is used for the treatment of liver disorders and as a chewing stick. The constituents include biflavonoids, xanthones and benzophenones. The antimicrobial properties of this plant are attributed to the benzophenone and flavanones. This plant has shown anti-inflammatory, antimicrobial and antiviral properties. In addition, the plant possesses antidiabetic and antihepatotoxic activities (Iwu, 1993).

Aframomum melegueta K.Schum. (Zingiberaceae): This is a spicy edible fruit that is cultivated and occurs throughout the tropics. Its medicinal uses include aphrodisiac, measles, and leprosy, taken for excessive lactation and post partem hemorrhage, purgative, galactogogue and anthelmintic, and hemostatic agent (Iwu, 1993). The constituents are essential oils — such as gingerol, shagaol, paradol. Studies show antimicrobial and antifungal activity and effective against schistosomes.

Xylopia aethiopica A.Rich. (Annonaceae): An evergreen, aromatic tree growing up to 20 m high with peppery fruit is native to the lowland rainforest and moist fringe forest in the savanna zones of in Africa. The plant is used as a carminative, cough remedy, and a post partum tonic and lactation aid. Other uses are stomachache, bronchitis, biliousness and dysentery. It is also used externally as a poultice for headache and neuralgia. It is used with lemon grass for female hygiene. It is high in copper, manganese, and zinc. In studies, the fruit as an extracts has been shown to be active as an antimicrobial against gram positive and negative bacteria, except *E. coli* (Iwu, 1993). One of its constituents, xylopic acid shows activity against *Candida albicans* (Boakye-Yiadom and Fiagbe, 1977).

Cryptolepis sanguinolenta (Lindl.) Schltr. (Asclepiadaceae): A shrub that grows in the rainforest and the deciduous belt forest, found in the west coast of Africa. Its main medicinal use is for the treatment of fevers. It is used for urinary tract infections, especially *Candida.* Other uses are inflammatory conditions, malaria, hypertension, microbial infections and stomach colic (Iwu, 1993). Active principles identified are indoquinoline alkaloids that inhibit gram negative bacteria and yeast (Silva *et al.*, 1996). Additionally studies have shown its bactericidal activity. Clinical studies have shown extracts of the plant were effective in parasitemia. A study shows its potency against bacteria specifically, enteric pathogens, most notably *E. coli* (but also *Staphylococcus sp., E. coli, C. jejuni, Pseudomonous sp., Salmonella sp., Shigella sp., Streptococcus sp.* and *Vibrio* sp.) and some activity against *Candida* (Sawer *et al.*, 1995).

Chasmanthera dependens Hochst (Menispermaceae): A woody climber that grows wild in forest margins and savanna. The cultivated plant is used medicinally for venereal disease, topically on sprained joints and bruises and as a general tonic for physical and nervous debilities. The constituents include berberine type alkaloids, palmatine, colombamine, and jateorhizine. Studies show that the berberine sulfate in the plant inhibits lieshmania.

Nauclea latifolia Smith (Rubiaceae): It is a shrub or small spreading tree that is a widely distributed savanna plant. Its medicinal uses are as a tonic and fever medicine, chewing stick, toothaches, dental caries, septic mouth and malaria, diarrhea and dysentery (Lamidi *et al.*, 1995). The key constituents are

indole-quinolizidine alkaloids and glycoalkaloids and sapponins. The root has antibacterial activity against gram positive and negative bacteria, and antifungal activity (Iwu, 1993). It is most effective against *Corynebacterium diphtheriae*, *Streptobacillis* sp., *Streptococcus* sp., *Neisseria* sp., *Pseudomonas aeruginosa*, *Salmonella* sp. (Deeni and Hussain, 1991).

Araliopsis tabouensis Aubrév. & Pelleqr. (Rutaceae): It is a large evergreen tree found throughout west tropical Africa and used for the treatment of sexually transmitted diseases. The bark infusion is drunk for gonorrhea in the Ivory Coast (Irvine, 1961). Seven alkaloids have been isolated from the root and stem bark (Fish *et al.*, 1976).

Many of the plants with pronounced antidiarrhoeal (Manonmani *et al.*, 1991), immunomodulatory (Dhuley, 1998; Manonmani *et al.*, 1995), anticancer (Dwivedi and Abu-Ghazaleh, 1997), and psychotropic (Shah *et al.*, 1997) properties, are also well-known for antimicrobial activities. Two microorganisms against which Ayurvedic preparations have activity are *Aspergillus* spp. (Dhuley, 1998) and *Propionibacterium acnes* (Paranjpe and Kulkarni, 1995).

Indian plants: A large number of studies have been carried out using the great Indian biodiversity, the discussion of which is beyond the scope of this dissertation. Some representative examples are presented here. In an extensive screening, the antimicrobial activity of 105 Indian plant species was tested. Among them, 30 showed antibacterial activity; 20 of these exhibited antifungal action as well. Seeds of *Carum copticum*, stem of *Pinus longifolia*, roots of *Plumbago zeylanica* and *Saussurea lappa*, and rhizome of *Alpinia officinarum* showed considerable antifungal activity, especially against pathogenic fungi. Antibiotic activity against a wide variety of microorganisms - pathogenic and nonpathogenic Gram-positive and Gram-negative bacteria, yeasts and moulds - was also noted with leaves of *Lawsonia inermis* and fruits of *Tamarindus indica*, *Terminalia belerica* and *Emblica officinalis* (Ray and Majumdar, 1976).

In one of the recent studies with 20 plant extracts and 7 Gram-positive and 5 Gram-negative bacteria, maximum antibacterial activity was shown by the aqueous extracts of *Parthenium hysterophorus*. The aqueous extract of *P. hysterophorus* inhibited the growth of seven among the 12 bacterial strains studied. It showed maximum activity against *Pseudomonas aerugionsa.* The aqueous extracts of *Ficus benghalensis* and *Anethum graveolens* could not inhibit any of the bacterial strains, while the aqueous extracts *Boerhavia diffusa, Asparagus racemosus, Ficus religiosa, Commelina benghalensis, Ocimum sanctum* and *Zizyphus nummularia* showed minimum antibacterial activity. *Hibiscus sabdariffa* showed maximum activity against *Streptococcus fecalis*. But none of the plant extracts could inhibit *S. agalactiae, E. coli, C. freundii,* and *P. vulgaris* (Nair and Chanda, 2006).

In a related study, the ethanolic extracts of *Hemidesmus indicus* (roots), *Coscinium fenestratum* (stems), *Tephrosia purpurea* (roots), *Euphorbia hirta* (roots), *Symplocos racemosa, Curcubito pepo* and *Eclipta alba* showed strong inhibitory effects against *Propionibacterium acnes.* Amongst these, *C. fenestratum* was effective against both *P. acnes* and *Staphylococcus epidermidis* at low concentrations. Its constituent alkaloids have been suggested to be responsible for activity (Kumar *et al.*, 2007).

The essential oils from 15 medicinal plants were also screened for their activity against *A. fumigatus* and *A. niger.* Maximum antimycotic activity was revealed by the oils of *Cymbopogon martini, Eucalyptus globulus* and *Cinnamomum zylenicum* which showed activity similar to miconazole nitrate. The oils of *Mentha spicata, Azadirachta indica, Eugenia caryophyllata, Withania somnifera* and *Zingiber officinale* exhibited moderate activity, while the activities of the oils of *Cuminum cyminum, Allium sativum, Ocimum sanctum, Trachyspermum copticum, Foeniculum vulgare* and *Elettaria cardamomum* were comparatively low than the control. Mixed oils showed the best maximum activity, supporting that the plant oils can be used as pharmaceutical and preservatives to cure mycotic infections (Bansod and Rai, 2008).

2.3.2. Benefits of herbal antimicrobials

The primary benefits of using plant-derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment.

2.3.2.1. Therapeutic benefits

Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin. Though most of the clinically used antimicrobials are produced by soil microorganisms, higher plants have also been as a source (Trease and Evans, 1972). Examples of these are the bacteriostatic and antifugicidal properties of lichens, the antibiotic action of allicin from *Allium sativum* (garlic), or the antimicrobial action berberines in goldenseal (*Hydrastis canadensis*). Plant-based antimicrobials have enormous therapeutic potential, since they are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. Many plants have tropisms to specific organs or systems in the body. Phytomedicines usually have multiple effects on the body. Their actions often act beyond the symptomatic treatment of disease. An example of this is *Hydrastis canadensis* which not only has antimicrobial activity, but also increases blood supply to the spleen promoting its optimal activity to release mediating compounds (Murray *et al.*, 1995).

2.3.2.2. Economic benefits

The renewed worldwide interest in natural products is a result of factors, such as consumer's belief that natural products are superior, consumer's dissatisfaction with conventional medicines, changes in laws allowing structure-function claims which result in more liberal advertising, aging baby boomers, and national concerns for health care cost. Sales of products in this market have increased dramatically in the last decade. Sales of botanical products in the US have reached $3.1 billion of the $10.4 billion dollar dietary supplement industry 1996, with the anticipated growth on the order of 15–20% into the new millennium. This growth rate will be maintained in an industry that is still considered to be in its infancy. Many plants that were previously wild crafted will need to be grown domestically to meet the demands of the consumer. This represents many opportunities for the cultivation of crops for this industry. A market-based illustration of the need for plant-based antimicrobials is demonstrated by the dissection of the herbal products market. In reviewing the top botanicals used as anti-infectives, the primary botanical used as an antimicrobial is *Hydrastis* with sales of 4.7% in 1995. A similar analysis of *Hypericum* (St John's wort) demonstrates the value of such an evaluation. Though *Hypericum* is primarily used as an antidepressant, *Hypericum* (St John's wort) is also an antiviral, ranking among the top-selling herbs. If the market dissection for anti-infectives is viewed in the same light as the *Hypericum* analogy, then perhaps this market is prime for receiving new plant-based antimicrobials.

The potential for developing antimicrobials into medicines appears rewarding, from both the perspective of drug development and the perspective of phytomedicines. The immediate source of financial benefit from plant-based antimicrobials is from the herbal products market. This market offers many opportunities for those cultivating new crops, as many of the plants that are wild-crafted today must be cultivated to match the demands of this market. Again *Hydrastis,* one of the top selling antimicrobials in the US herbal market, represents an example of a herb that has undergone domestication. Originally this plant, native to eastern North America, was wild-crafted. *Hydrastis,* has been used by Native Americans for many conditions, including as an antimicrobial for infections. Efforts to cultivate this plant were undertaken in order to supply the demands of the herbal products market and to battle its threatened extinction.

3
Materials and Methods

3.1. Materials

3.1.1. Culture media

Dulbecco's modified Eagle's medium (D5648) was procured from Sigma-Aldrich, Inc., St Louis, MO, USA), and malt extract (RM004), Müller-Hinton agar (M173), oat meal agar (M397), potato dextrose agar (M096), tryptone soya broth (M011) and yeast malt agar (M424) were obtained from HiMedia Laboratories Pvt Limited, Mumbai, India.

All media mentioned above were sterilized by autoclaving for 15 min, excepting Dulbecco's modified Eagle's medium which was filter-sterilized.

3.1.2. Reagents

Acetate buffer

Glacial acetic acid (20%; SRL 0129168)
NaOH (SRL 1949181) to pH 3.5

Dragendorff reagent

(Fluka 44578)

Fenton reagent (Tian and Hua, 2005)

H_2O_2	30 mM
Ascorbic acid	50 µM
$FeCl_3$	80 µM

Folin-Ciocalteau reagent

(SRL 62015)

Hepes buffer, pH 7.4

(Sigma 49897)

Krebs buffer (Lucas and Szweda, 1998)

NaCl	120 mM
KCl	4.8 mM
$CaCl_2$	2.0 mM
$MgCl_2$	1.25 mM
KH_2PO_4	1.25 mM
$NaHCO_3$	22 mM
Glucose	10 mM

Phosphate buffer (pH 6.6)

1 M K_2HPO_4 (SRL 1648212)	38.1 ml
1 M KH_2PO_4 (SRL1649201)	61.9 ml

Phosphate buffer (pH 7.4)

1 M K_2HPO_4 (SRL 1648212)	80.2 ml
1 M KH_2PO_4 (SRL1649201)	19.8 ml

3.1.3. Test microorganisms

Bacillus subtilis DK-W1, *Escherichia coli* MTCC119, *Klebsiella peumoniae* subsp. *ozaenae* MTCC2653, *Staphylococcus aureus* MTCC1430, *Listeria monocytogenes* MTCC839, *Salmonella typhi* MTCC733, *Bacillus pumilus* HWC86, *Bacillus licheniformis* HWC84, *Bacillus cereus* HWC88, *Candida albicans* MTCC183, *Saccharomyces cerevisiae* MTCC173, *Aspergillus niger* MTCC281 and *Alternaria alternata* MTCC1779, used in this study, were obtained from the Microbial Culture Collection of the Department of Botany, University of North Bengal.

3.2. Experimental

3.2.1. Survey

A moderate survey was conducted since April 2005 till December 2007 in villages of the three hilly subdivisions of the District of Darjeeling, taking the help of 70 villagers (Table 3).

Table 3. Areas under survey

Sub-division	Village
Kurseong	Singell tea estate, Ambotia tea estate, Chimney, Pankhabari, Gayabari, Sukna
Darjeeling	Aloobari, Botay basty, Happy Valley tea estate, Sukia-pokhari, Jorpokhari, Gorabari, Labda, Mungpur, Simlay
Kalimpong	Algarah, Charimaile, Lava, Rishyap, Kalimpong, Lolegaon

A semi-structured questionnaire (Table 4) was used to extract information on the medicinal plants or parts thereof and the types of ailments against which these were used.

Table 4. Questionnaire used for survey

Name:					Age:		Sex:
Village (sub-division):					Occupation:		
Date of collection	Scientific name	Local name	Family	Habit	Part used	Disease treated	Mode of administration

3.2.2. Sampling

Plant specimens were collected in air-tight polyethylene sampling bags and brought to the laboratory as soon as possible. They were cleaned, pressed and dried using blotting paper, followed by treatment with 8 g $HgCl_2$ l^{-1} ethanol, dried and mounted onto herbarium sheets. The herbs were identified, taking the help from the Plant Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal, and deposited at the National Gene Bank for Medicinal and Aromatic Plants (NGBMAP), Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India.

3.2.3. Preparation of methanolic extracts of samples

Samples of collected plant parts were dried in a hot air oven at 60°C for 24-48 h and pulverized using a waring blender. A 10-g powder was soaked in 10 vol. of methanol (Merck (India) 60600925001730) for 24 h with intermittent shaking, and the supernatant decanted. The extraction process was repeated thrice, using fresh solvent. The individual extracts were combined and filtered through a Whatman No. 1 paper, evaporated *in vacuo*, and lyophilized (Eyela FDU-506 freeze dryer). The lyophilized extracts were stored in a vacuum desiccator at 4°C. Prior to use, the lyophilized extracts were dissolved in methanol (1 mg ml^{-1} for assay of antioxidant activities, unless mentioned otherwise).

3.2.4. Assay of antioxidant activities of crude methanolic extracts

3.2.4.1. Total phenolics

Total soluble phenolics in the extracts were assessed using the method described by Singleton and Rossi (1965). A 100 µl-aliquot of lyophilized extract solution was added to 500 µl of 1:10 Folin-Ciocalteau's reagent and 400 µl of 75 g Na_2CO_3 (HiMedia RM861) l^{-1} aqueous solution. After incubating the reaction mixture at 24°C for 2 h, the absorbance was read at 765 nm (Jasco V-550 UV/VIS-spectrophotometer). The concentration of total phenolics was expressed as mg gallic acid equivalents (GAE) g^{-1} lyophilized extract, using the standard curve of gallic acid (HiMedia RM233).

3.2.4.2. Total flavonoids

Total soluble flavonoids in the extracts were quantified using the method described by Jia *et al.*, (1999). A 400 µl-aliquot of lyophilized extract solution was added with 30 µl of 50 g $NaNO_2$ l^{-1} aqueous solution. After incubation for 5 min at 25°C, 30 µl of 100 g $AlCl_3.6H_2O$ l^{-1} aqueous solution was added, followed by addition of 200 µl of 1 N NaOH after 6 min. The mixture was diluted with water to 1 ml and the absorbance was read at 510 nm. The total flavonoid content of the test samples was expressed as mg epicatechin equivalents (ECE) g^{-1} lyophilized extract, using the standard curve of epicatechin (Sigma E1753).

3.2.4.3. DPPH$^{\bullet}$-scavenging

The antioxidant activity of the extracts was measured in terms of hydrogen-donating or radical-scavenging

ability using the stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH•) method (Sanchez-Moreno *et al.*, 1998). A 0.1 ml methanolic solution of the lyophilized extract was added to 2.9 ml of 60 mM methanolic solution of DPPH• (HiMedia RM2798). The mixture was shaken immediately and allowed to stand at room temperature for 30 min in dark. The decrease in absorbance was measured at 517 nm using a spectrophotometer.

$$\%\text{scavenging} = \frac{A_0 - (A - A_b)}{A_0} \times 100$$

The inhibitory percentage of DPPH•, as calculated according to Shyu and Hwang (2002), is as follows:

3.2.4.4. $ABTS^{\bullet+}$-scavenging

This was carried out as described by Re *et al.* (1999). An aqueous stock solution (7 mM) of 2,2'-azinobis(3-ethylbenzothiazolline-6-sulfonic acid) (ABTS; Sigma A1888) was treated with potassium persulfate (final concentration, 2.45 mM; Aldrich 379824), and the mixture was allowed to stand in dark at room temperature for 15 h to produce $ABTS^{\bullet+}$. The $ABTS^{\bullet+}$ solution was diluted with ethanol (Merck (Germany) 1.00983.0511) to an absorbance of 0.70±0.02 at 734 nm and equilibrated at 30°C. After addition of 2 ml of the diluted $ABTS^{\bullet+}$ solution to 20 µl of the extracts, the absorbance at 734 nm was read 6 min after the initial mixing. The percentage scavenging was calculated as in 3.2.4.3.

3.2.4.5. •OH-scavenging

The method described by Halliwell and Gutteridge (1981) was followed. The reaction mixture in de-aerated water (1 ml) contained 2.8 mM 2-deoxyribose (HiMedia RM452), 20 µM $FcCl_3$ (SRL 64765), 100 µM EDTA (SRL 54448) (EDTA and $FeCl_3$ solutions were mixed prior to the addition of 2-deoxyribose), 200 µM H_2O_2 (Merck (India) 61868505001730), and methanolic extract solution in a 10 mM potassium phosphate buffer (pH 7.4). The reaction was triggered by adding ascorbic acid (300 µM; SRL 0149100) and subsequent incubation of the mixture for 1 h at 37°C. To this mixture, 1 ml of 10 g 2-thiobarbituric acid (TBA; HiMedia RM1594) l^{-1}, 50 mM NaOH and 1 ml of 28 g trichloroacetic acid (TCA; SRL 204842) l^{-1} aqueous solution were added. The mixture was heated in a boiling water bath for 15 min, and the amount of chromogen produced was spectrophotometrically measured at 532 nm.

3.2.4.6. Oxygen radical absorbance capacity (ORAC)

The assay was based on the method described by Aaby *et al.* (2004). The reaction mixture containing the extract (final concentration, 10 µg ml^{-1}) in 75 mM phosphate buffer (pH 7.4) and β-phycoerythrin (final concentration in the same buffer, 16.7 nM; Fluka 07367) was incubated at 37°C for 15 min. This was followed by the addition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH; final concentration, 4 mM; Aldrich 440914), and the fluorescence of the mixture was recorded (excitation, 540 nm; emission, 565 nm) up to 15 min. The extent of scavenging by the samples was calculated from the area under β-phycoerythrin decay curves, using gallic acid as the standard and expressed as µM GAE g^{-1} lyophilized extract.

3.2.4.7. Reducing power (RP)

The ability of the extracts to reduce Fe(III) was assessed according to the method of Oyaizu (1986). A 1.0-ml aliquot of lyophilized extract solution was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and

2.5 ml aqueous solution of 10 g potassium ferricyanide (HiMedia RM1034) l^{-1}. The mixture was incubated at 50°C for 20 min, added with 2.5 ml aqueous solution of 100 g TCA l^{-1} and centrifuged at 1200 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 1.0 g $FeCl_3$ l^{-1}, and the absorbance was measured at 700 nm. The reducing power was expressed as mg ascorbic acid equivalents (ASE) g^{-1} lyophilized extract, using the standard curve of ascorbic acid (SRL 149100).

3.2.4.8. Metal-chelating (MC) power

The Fe(II)-chealting ability by the extracts was carried out according to Carter (1971). The ability was monitored by measuring the formation of Fe(II)-ferrozine complex. A 200-µl aliquot of a methanolic solution of the lyophilized extract was added to 100 µl of 2.0 mM aqueous $FeCl_2$ (Merck 1.03861.0250) and 900 µl methanol. After incubation for 5 min, the reaction was initiated by adding 400 µl of 5.0 mM ferrozine (SRL 64956). The mixture was shaken and left at room temperature for 10 min to equilibrate. The absorbance of the resulting solution was recorded at 562 nm, using EDTA as the control. A lower absorbance indicates a stronger Fe(II)-chelating ability which was calculated as follows:

$$\text{Chelating effect (\%)} = [(1-A_s)/A_c] \times 100$$

where A_s was absorbance of the sample and A_c was that of the control.

3.2.5. Evaluation of protection to biological targets/whole cells

3.2.5.1. Preparation of mice liver homogenate

Samples of liver, removed from the freshly sacrificed mice, were immediately washed three times with cold Krebs buffer (pH 7.4), and homogenized using a ceramic glass homogenizer in 0.25 M sucrose solution and 1 mM EDTA. The mixture was centrifuged at 3000 g for 10 min and the sediment was washed three times with 0.05 M potassium phosphate buffer (pH 7.4). The pellet obtained was resuspended in the same buffer to a final concentration of 20 mg ml^{-1}, and the protein content was estimated by Lowry's method (Lowry *et al.*, 1951).

3.2.5.2. Anti-LPO assay

Lipid peroxidation (LPO) was measured by the TBA assay (Okhawa *et al.*, 1979). Briefly, to the reaction mixture containing mitochondrial fraction (4 mg protein ml^{-1}) and the test extracts in a phosphate buffer (100 mM; pH 7.4) was added to 100 µM ferrous ammonium sulphate (Sigma F1543) and 2 mM ascorbic acid to initiate LPO. After incubating the mixture for 2 h, 200 µl of the reaction mixture was added with 200 µl of 80 g sodium dodecyl sulphate (Sigma 436143) l^{-1}, 1.5 ml of 200 g acetate buffer l^{-1}, 1.5 ml of 8 g TBA l^{-1} and 400 µl distilled water. After heating at 100°C for 15 min, the tubes were immediately immersed in ice, added with 5 ml butanol-pyridine (15:1, v v^{-1}) and vortexed vigorously for 1 min, followed by centrifugation at 300 g for 10 min. The organic fraction in the supernatant was carefully collected, and its absorbance at 532 nm was read. Using the same protocol, a concentration dependent anti-LPO study was also carried out with the extract of 5-25 µg *Fragaria nubicola* root (FNR) ml^{-1}, and the IC_{50} value was determined.

Using 50 mM AAPH as the peroxidation initiator, the anti-LPO activity of FNR was also assessed and the IC_{50} value determined as above.

3.2.5.3. DNA protection assay

A 20.5 µl-reaction mixture, containing 0.25 µg pBR322 DNA (Genei 105848), FNR or furanocoumarins, Fenton reagent and 10 mM Hepes buffer (pH 7.4), was prepared in an Eppendorf tube. After incubating

the mixture at 37°C for 30 min, the resulting supercoiled, open-circular and linear forms of the plasmid DNA were separated by gel electrophoresis on 10 g agarose l^{-1} gel (Sigma A9539), stained with 0.5 μg ethidium bromide (Aldrich 160539) ml^{-1} for 30 min at 72 V. The gels were documented using a Kodak Gel Logic 200 Imaging System, and the intensity of the bands was quantified using a Kodak MI software.

3.2.5.4. Protein oxidation assay

Following the procedure of Tirosh *et al.* (1996), the inhibitory activity of FNR against γ-radiation-induced protein oxidation was measured from the loss of fluorescence in bovine serum albumin (BSA; Sigma A2153). The loss of fluorescence intensity was due to tryptophan and tyrosine oxidation. A 0.5 ml-reaction mixture, containing 0.12 mg BSA ml^{-1} 50 mM potassium phosphate buffer (pH 7.4) and with or without FNR was taken in tubes and irradiated at 25°C with γ-rays using a Co^{60} source up to a dose of 300 Gy (dose rate, 16 Gy min^{-1}). After irradiation, the content of each tube was diluted to 2 ml with the above buffer, and the fluorescence of each sample was estimated at λ_{ex} of 280 nm and λ_{em} of 345 nm, using a Jasco FP-6500 spectrofluorimeter.

3.2.6. Antimicrobial activity assay

3.2.6.1. Disc diffusion protocol

The assay was carried out with microbial cultures (bacteria, 24 h-old, yeasts, 2 days-old; moulds, 5 days-old). A loopful of each culture was inoculated into tryptone soya broth for bacteria and 25 g malt extract l^{-1} for yeasts. After 6-8 h-growth on a rotary shaker (200 rpm), the cell concentration was adjusted to 10^8 ml^{-1}, and used for surface spreading using a sterile swab on Müller-Hinton agar plates for bacteria and yeast malt agar for yeasts. In case of moulds, the spore concentration was adjusted to 10^5 ml^{-1} distilled water and plated onto oat meal agar and potato dextrose agar. After 15 min of drying, the plates were impregnated with sterile Whatman No. 1 filter paper discs (5.5 mm) containing desired concentrations of the herbal extracts (methanol was completely evaporated in the laminar airflow, and the discs were asceptically placed on the agar surfaces). The plates were then incubated at 37°C for 18-24 h for bacteria, at 30°C for 48 h for yeasts, and 25°C for 5 days for moulds. All the assays were carried out in duplicate. A clear zone of inhibition surrounding the discs with a diameter greater than 5.5 mm was considered to be positive (Bauer *et al.*, 1966; Murray *et al.*, 1995).

3.2.6.2. Broth dilution protocol

Bacterial cultures (24 h-old) were inoculated into tryptone soya broth and incubated at 37°C on a rotary shaker (100 rpm). After 18 h-incubation, the bacterial suspension was centrifuged at 12,000 g for 15 min. The pellet was resuspended in sterile distilled water, and the cell concentration was adjusted to 10^8 ml^{-1}. The antimicrobial assay was performed in a 96-well, sterile, flat bottom microtiter plate (Ali and Reddy, 2000; Suffredini *et al.*, 2004). Each well was filled with 200 μl of tryptone soya broth, 1 μl of test organism and 15 μl of different concentrations of furanocoumarins in dimethylsulfoxide (DMSO; Sigma D8418). After incubating at 37°C for 24 h, the plates were read at 550 nm using an ELISA microplate reader (Biotek Instruments ELX 800 MS).

3.2.7. HPLC analysis of *Fragaria nubicola* root (FNR) extract

The pulverized dried roots (100 mg) were hydrolyzed with 2 ml of 2 N HCl (Merck (India) 61752690251730) on a boiling water bath for 1 h. After cooling, 2 ml of 2 N NaOH and 6 ml methanol were added to the vial. The slurry was sonicated for 20 min, with occasional shaking, centrifuged at 12,000 g, and the supernatant was filtered through a 0.2 μm-membrane filter.

The supernatant (20 µl) was analyzed by HPLC (Jasco HPLC chromatogram with PU-2080 plus pump and UV-2075 plus detector) using an HiQ Sil C18 W column (4.6 mm x 250 mm, 5 mm) under isocratic elution with methanol:water:acetic acid (300:700:1) at a flow rate 1 ml min^{-1}. The compounds were detected at 280 nm and characterized from the chromatograms of the standards (gallic acid (Sigma G7384), *p*-coumaric acid (Fluka 28200), *o*-coumaric acid (Fluka 28170), ellagic acid (Sigma E2250), rosamarinic acid (Fluka 44699), caffeic acid (Fluka 60018), *p*-hydroxy-benzoic acid (Fluka 06940), dihydrocaffeic acid (Fluka 54130), ferulic acid (Fluka 46278), vanillic acid (Fluka 94770) and syringic acid (Fluka 86230)), under an identical condition. Each analysis was repeated three times.

3.2.8. Antiproliferative potential of the extracts

3.2.8.1. Cell Culture

The A-549 (human lung carcinoma), MCF-7 (human breast cancer) and INT-407 (human Caucasian intestine embryonic) cell lines were obtained from the National Centre for Cell Science (NCCS), Pune (India). The cells were routinely seeded at a density of 0.1-3 x 10^6 ml^{-1} and grown in DMEM medium supplemented with 10% heat-inactivated foetal calf serum (FCS; Sigma F7524), 2 mM glutamine (Sigma G7513), 100 µU penicillin (Sigma P3032) ml^{-1} and 100 mg streptomycin (Sigma S9173) ml^{-1} in a humidified 5% CO_2 atmosphere at 37°C. Cells were passaged every 3-4 days to keep the cell density below 0.4 ´ 10^6 ml^{-1}. The cell density and viability were determined by the trypan blue dye exclusion assay (Elia *et al.*, 1993). Subcultures were obtained by trypsinization (2.5 g trypsin (Sigma T4549) l^{-1}) in phosphate buffer saline (PBS; Sigma P5493).

3.2.8.2. Cytotoxic activity of FNR extract

The toxicity of various concentrations of FNR against the A-549, MCF-7 and INT-407 cell lines was evaluated by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma M5655) reduction assay (Mosmann, 1983). Briefly, cells (10^6) were seeded into 96 wells following trypsinization, incubated overnight with 10% FCS at 37°C in an atmosphere of 5% CO_2 and a relative humidity of 95%. After washing with PBS, different concentrations of FNR were added into each well, and incubated for 48 or 72 h (for INT-407 cells). After removing the medium, 100 µl of 0.5 mg MTT ml^{-1} solution was added to each well and kept at 37°C for 6 h. The formazan crystals in the viable cells were solubilized with 100 µl 0f 0.01 N HCl containing 100 g SDS l^{-1}, and the absorbance at 550 nm was read using an ELISA microplate reader.

3.2.9. Isolation of furanocoumarins from *Heracleum nepalense* fruits

Shade-dried and powdered fruits (14 g) of *H. nepalense* were refluxed for 4 h with 200 ml of *n*-hexane (Merck (India) 61783090251730). The hexane extract was filtered and concentrated *in vacuo* to yield a pale yellow residue, which was column chromatographed (silica gel (mesh size, 200), 0-30% (v v^{-1}) ethyl acetate (Merck (India) 60962325001730)/*n*-hexane) to yield several fractions. The fractions showing similar thin layer chromatogram (TLC) were pooled together. The three fractions, F1-F3, eluting with 15-25% (v v^{-1}) ethyl acetate/*n*-hexane was subjected to preparative TLC (PTLC) to obtain furopinnarin, sphondin and byakangelicol. The ^1H NMR spectra were recorded in d_4-MeOH with a Bruker AC-200 (200 MHz) spectrometer, and the data were provided in a δ-scale (ppm). The coupling constant (*J*) values are expressed in Hz.

3.2.10. Isolation of alkaloids from *Stephania hernandifolia* roots

The methanolic extract (0.05 g) of *S. hernandifolia* root was defatted by refluxing with hexane (2 x 10 ml) for 2 h each time, followed by extraction with diethyl ether (3 x 10 ml; Merck (India) 61764605001730).

The insoluble portion was triturated with 5 ml of 6 N HCl, the acid-soluble portions separated and subsequently basified with aqueous ammonia (Merck (India) 61779590251730) till pH 9.0. The aqueous layer was extracted with chloroform (3 x 5 ml; Merck (India) 60244590251730) to yield alkaloid-rich fraction (0.022 g) and identified by Dragendorff reagent. Reeated PTLC with 5% (v v^{-1}) ethyl acetate/*n*-hexane as the solvent system yielded a partially pure alkaloid (0.012 g).

3.2.11. Statistical analysis

The experimental results are expressed as mean ± SEM of three parallel determinations. Analysis of variance and significant differences among means were tested by one-way ANOVA. Principal component analysis was done using Minitab 15 software (Minitab Inc. USA).

4
Results

4.1. Survey of ethnomedicinal herbs of Darjeeling hills

The survey revealed that all the 106 herbs collected are commonly used by the local people for treating various ailments. Amongst the various parts of the plants, aerial ones (70%) are more extensively used than the underground ones (30%), for treating about 40 different types of ailments (Table 5), *viz.* cut/ wound, pneumonia, fever, diarrhoea, dysentery, food poisoning, fracture, tuberculosis etc. It was also evident that among the tribes of Darjeeling hills, herbs (65%) are preferred medicinal source than the shrubs (18%), and climbers (17%).

4.2. Screening of plants

Of 123 plants collected, 38 herbs/herbal parts were selected for the study of antioxidant and antimicrobial activities on the basis of their anticipated nontoxicity, since majority of the plant parts are consumed by the local people.

4.3. *In vitro* antioxidant activity screening

For preliminary screening, the DPPH$^{\bullet}$ assay for free radical-scavenging, reducing power and iron (II) chelating potential of the test samples were carried out to judge their relative antioxidant activity.

Table 5. Collection of medicinally important plants/ plant parts from Darjeeling hills

Date of collection	Scientific name	Local name	Family	Habit[a]	Part used	Disease treated	Mode of administration	CIMAP[b] Accn No.
11.05.05	*Adhatoda vasica* Nees in Wall.	basak	Acanthaceae	s	leaf	cough, cold	chewed in empty stomach	5985
11.05.05	*Costus speciosus* (Koen.) J.E.Smith.	betlouri	Costaceae	s	root	jaundice	fresh roots are chewed for 10 days	5983
11.05.05	*Drymaria cordata* (L.) Willd.	abijalo	Caryophyllaceae	h	leaf	sinusitis, pnuemonia	juice is consumed twice	5991
11.05.05	*Elsholtzia blanda* (Benth.)	mrigay-jhar/ mirey-jhar	Lamiaceae	h	leaf	diarrhoea	filtered juice is consumed twice for 2 days	5999
11.05.05	*Eupatorium glandulosum* Kunth.	banmara	Asteraceae	s	leaf	cut, wound	paste is applied	5987
11.05.05	*Houttuynia cordata* Thunburgh.	gandey-jhar	Saururaceae	h	leaf	tuberculosis	decoction (2 tsp) taken for 15 days	5984
11.05.05	*Hydrocotyle himalaica* P.K. Mukherjee	botuke-jhar	Apiaceae	h	leaf	tonsillitis	decoction is used for gargle	5990
11.05.05	*Ocimum sanctum* L.	babariphool	Lamiaceae	h	leaf	fever and cold	juice is mixed with ginger and consumed at bed time	5986
11.05.05	*Pouzolzia hirta* (Blume) Hassk.	chipley	Urticaceae	h	root	bone fracture	paste is bandaged around the affected area	6026
11.05.05	*Solanum nigrum* L.	khursane-jhar	Solanaceae	h	aerial part	sedative	juice is consumed	5993
11.05.05	*Stephania rotunda* sensu Hook.f. et Thoms.	seto-tamarke	Menispermaceae	c	root	diabetes	decoction is consumed in empty stomach	6001
18.05.05	*Asparagus racemosus* Willd.	satamuli	Asparagaceae	h	root	diabetes	powder is consumed	6004
18.05.05	*Astilbe rivularis* D. Don.	bansupari	Saxifragaceae	h	root	bleeding after childbirth	dried and chewed	6015

Date of collection	Scientific name	Local name	Family	Habit[a]	Part used	Disease treated	Mode of administration	CIMAP[b] Accn No.
18.05.05	*Cardamine hirsuta* L.	simrayo	Brassicaceae	h	aerial part	low pressure, in cardiac problems	cooked and consumed	6082
18.05.05	*Clematis buchananiana* Wall.	pinase lahara	Ranunculaceae	c	leaf, root	sinusitis	burnt and its smoke inhaled	6005
18.05.05	*Duchesnea indica* (Andrews) Focke.	thare oonew	Rosaceae	h	leaf	cut, wound	paste is applied externally	5999
18.05.05	*Fragaria daltoniana* J.Gay	bhui-ka-phal	Rosaceae	h	root	toothache	smoked as cigar	
18.05.05	*Heracleum nepalense* D. Don	chimping	Apiaceae	h	seed	fever, influenza	dried seeds are chewed	6014
18.05.05	*Lycopodium* sp.	nagbeli	Lycopodiaceae	h	spore	bleeding after childbirth	powdered spores are consumed	6008
18.05.05	*Potentilla kleiniana* Wright & Arn.	kauwa ka phal	Rosaceae	h	root	burn	root pasted with coconut oil is applied externally	6006
18.05.05	*Rubia manjith* Roxb. Ex Fleming.	magito	Rubiaceae	c	root	skin disease	paste applied externally	6010
18.05.05	*Rubus ellipticus* Smith.	ainselu	Rosaceae	s	fruit	gastritis	rind is consumed	6003
18.05.05	*Solanum khasianum* Clarke.	beek ara	Solanaceae	h	thorn	toothache	powder is put in the affected tooth	6002
18.05.05	*Zanthoxylum acanthopodium* DC.	boke timboor	Rutaceae	s	fruit	indigestion	eaten	6007
19.06.05	*Bergenia ciliata* (Hwarth) Sternberg.	pakhanbet	Acanthaceae	h	leaf	dysentery	paste is consumed	6029
19.06.05	*Diplazium esculentum* (Retz.) SW.ex Schrad	ningro	Woodsiaceae	h	frond	constipation, maintains good stomach condition	eaten as curry	

Date of collection	Scientific name	Local name	Family	Habit[a]	Part used	Disease treated	Mode of administration	CIMAP[b] Accn No.
19.06.05	*Equisetum debile* Roxb. Ex Vaucher.	kurkure jhar	Equisetaceae	h	aerial part	mouth sore	paste applied externally	6020
19.06.05	*Hypericum patulum* Thunb.	urilo	Clusiaceae	s	bark	cut, wound	paste applied externally	6022
19.06.05	*Lygodium flexnosum* (L.) Sw.	bahun lahara	Schizaeaceae	h	young frond	rheumatism, sprain	Fronds soaked in mustard oil and externally applied on affected areas	6035
19.06.05	*Stephania elegans* Hook. f. & Thomson.	tamarke	Menispermaceae	c	root	diabetes	soaked overnight in water and the water is drunk	
19.06.05	*Tectaria coadunata* (J. Sm.) C.Chr.	kali oonew	Aspidaceae	h	whole plant	dysentery	boiled and taken as soup	6091
19.06.05	*Tinospora cordifolia* (Willd.) Miers.	gurjo-lahara	Memispermaceae	c	fruit	tuberculosis	boiled in milk and drunk for 10-15 days	6064
02.10.05	*Abrus pulchellus* L.	lalgeri	Fabaceae	c	fresh root	hill dysentery	filtered juice is consumed	6058
02.10.05	*Acorus calamus* L.	bojho	Acoraceae	h	rhizome	osteoarthritis	crushed, boiled with salt and the decoction is massaged	6051
02.10.05	*Desmodium triflorum* (L.) DC.	sano-charmeli	Fabaceae	h	leaf	diarrhoea, dysentery	filtered juice is consumed	6096
02.10.05	*Geranium sp.*	ragatgeri	Geraniaceae	h	whole plant	renal disease, dysentery	filtered juice is consumed	5997
02.10.05	*Girardinia diversifolia* (Link).	bhangre-sisnu	Urticaceae	s	root, inflorescence	bone fracture, high pressure, diabetes	paste eaten	6090
02.10.05	*Gloriosa superba* L.	bikh-phool	Colchicaceae	h	leaf	rheumatism, skin disease, leprosy, piles	paste is used for external application	

Date of collection	Scientific name	Local name	Family	Habit[a]	Part used	Disease treated	Mode of administration	CIMAP[b] Accn No.
02.10.05	*Hyptis suaveolens* L.Poit	gange-jhar	Lamiaceae	h	leaf	lice, parasitic infestations	juice is warmed and applied	6070
02.10.05	*Mimosa pudica* L.	buari-jhar	Mimosaceae	s	root	toothache	powder is used for tooth cleaning	6057
02.10.05	*Osbeckia nepalensis* Hooker.	angeri	Melastomataceae	s	young leaf or tender shoot	pneumonia, fever, common cold	extract is applied on forehead and chest	6053
02.10.05	*Sida acuta* Burm. f.	khareto	Malvaceae	s	stem	bone fracture	paste is applied externally	6042
22.07.06	*Achyranthes bidentata* Blume.	ankhlay-jhar	Amaranthaceae	h	root	rheumatism, gout	juice with salt massaged	6040
22.07.06	*Acmella calva* (DC.) Jansen.	kalijhar	Asteraceae	h	flower, inflorescence	toothache, decay, mouth sore	chewed to relieve pain	6066
22.07.06	*Ageratum conyzoides* L.	jiamae-jhar	Asteraceae	h	fresh leaf or young shoot	cut, injuries for blood clotting, sores	paste applied externally	6038
22.07.06	*Canna indica* L.	phool tarul	Cannaceae	h	root	gonorrhoea	paste applied on the genitals	6068
22.07.06	*Cannabis sativa* L.	ghanja / gagina	Cannabaceae	h	root	indigestion, acidity	chewed	6032
22.07.06	*Cynodon dactylon* (L.) Pers.	dubo	Poaceae	h	aerial part	nose bleeding, cut, indigestion, body swelling	juice is used	
22.07.06	*Dioscorea tuberosa* Vell.	gittha	Dioscoreaceae	c	tuber	gastritis	filtered juice is consumed	6047
22.07.06	*Eupatorium odoratum* L.	banmara	Asteraceae	s	leaf	cut, wound	paste applied externally	6086

Date of collection	Scientific name	Local name	Family	Habit[a]	Part used	Disease treated	Mode of administration	CIMAP[b] Accn No.
22.07.06	*Kaempferia rotunda* L.	bhuichampa	Zingiberaceae	h	root	bone fracture, joint dislocation, sprain, gout, rheumatism	paste is bandaged in soft cloth	
22.07.06	*Mussaenda roxburghii* Hook.f.	dhobinikath	Rubiaceae	s	root	jaundice	filtered juice is consumed orally	6049
22.07.06	*Pouzolzia indica* (L.) Wight.	chipley	Urticaceae	h	root	bone fracture	plastered with *P. hirta* and *Curcuma longa*	6095
22.07.06	*Scoparia dulcis* L.	chinijhar	Scrophulariaceaa	h	leaf	diabetes	eaten	
02.09.06	*Abroma augustum* (L.) L.f.	shringraj	Sterculiaceae	s	young shoot	discharges in females	decoction is consumed, in which shoots are soaked overnight	6037
02.09.06	*Flemingia strobilifera* Roxb.	barkaulijhar	Leguminosae	s	root	indigestion, insomnia, epilepsy	juice 2 teaspoon twice	6080
02.09.06	*Oxalis corniculata* L.	chariamilo	Oxalidaceae	h	leaf	eye infection	filtered juice is applied	
02.09.06	*Paederia foetida* L.	biri-lahara	Rubiaceae	c	leaf	diarrhoea, dysentery	cooked and eaten	6062
02.09.06	*Persicaria capitata* (D. Don) h. Gross.	ratnowlo	Polygonaceae	h	leaf	insect sting	paste applied externally	6019
02.09.06	*Physalis minima* L.	phak-phakay	Solanaceae	h	leaf	earache, boils, diuretic	juice is applied externally	
02.09.06	*Plantago erosa* Wall. In Roxb.	jibre-jhar	Plantaginaceae	h	seed	dysentery	juice is consumed	
02.09.06	*Pupalia atropurpurea* Moq. In DC.	ulta-kuro	Amaranthaceae	h	leaf	dysentery tonsillitis	decoction is consumed gargled	6033

Date of collection	Scientific name	Local name	Family	Habit[a]	Part used	Disease treated	Mode of administration	CIMAP[b] Accn No.
02.09.06	*Solanum torvum* Swartz.	ban-bihi	Solanaceae	s	leaf	toothaches	rolled into cigars and smocked	6036
02.09.06	*Sonchus arvensis* auct.non.L.	ban-rayo	Asteraceae	h	root	toothache	paste is applied on affected tooth	6023
02.09.06	*Trichosanthes lepiniana* Naudin.	indraynee	Cucurbitaceae	c	mature fruit	diabetes	dry powder is consumed after lunch	6039
02.09.06	*Tupistra nutans* Wall. ex Lindl.	nakima	Convallariaceae	h	inflorescence	food-poisoning	boiled, cooked and consumed	6089
27.01.07	*Leucas indica* (L.) Sm.	dulphe jhar	Lamiaceae	h	leaf , flower	rheumatism, headache, common cold, sores	burnt and the ash is smelled	
02.09.06	*Urtica ardens* Blume.	sisnu	Urticaceae	h	root	kidney stones	juice is consumed	6018
06.05.07	*Amaranthus spinosus* L.	lonre	Amaranthaceae	h	leaf	burns, boils, as laxative	eaten as curry	6094
06.05.07	*Amaranthus viridis* L.	lude	Amaranthaceae	h	leaf	stomach colic, as laxative	filtered juice is consumed with sugar	6000
06.05.07	*Andrographis paniculata* (Burm.f.) Wallich.	kalmegh	Acanthaceae	h	leaf	constipation, for blood purification	made pills and consumed in empty stomach in morning	6073
06.05.07	*Bauhinia vahlii* wight and Arn.	malu	Leguminosae	c	bark	dysentery	filtered juice is consumed	6040
06.05.07	*Blumea hieracifolia* (Don) D.G.	sahasrabooti	Asteraceae	h	leaf	asthma	dried and smelled	6075
06.05.07	*Cissus quadrangularis* L.	harhjorha	Vitaceae	c	whole plant	bone fracture	paste applied externally	6083
06.05.07	*Cyperus rotundus* L.	mothe	Cyperaceae	h	root	analgesic, sedative	decoction is consumed	6055

Date of collection	Scientific name	Local name	Family	Habit[a]	Part used	Disease treated	Mode of administration	CIMAP[b] Accn No.
06.05.07	*Euphorbia adenophorum* Sprengel	kalo banmara	Asteraceae	h	leaf	external injuries, cut	paste applied externally	6084
06.05.07	*Euphorbia sikkimensis* Boiss.	dudhe	Euphorbiaceae	h	root	boils	applied externally	5989
06.05.07	*Mentha arvensis* L.	padina	Lamiaceae	h	leaf	digestion	chewed	6030
06.05.07	*Piper longum* L.	pipla	Piperaceae	c	fruit	cold, cough	fried and eaten	
06.05.07	*Piper nigrum* L.	pipla	Piperaceae	c	fruit	prolonged cough	milled with ginger and honey, and consumed	6078
17.06.07	*Calamintha umbrosa* (M.Bieb.) Fisch & Mey. Ind.Sem. Hort.Petrop.	bilajor	Lamiaceae	h	whole plant	good health	eaten as vegetable to ensure good health	6016
17.06.07	*Centella asiatica* (L.) Urban.	golpatta	Apiaceae	h	leaf	cough	washed and juice is consumed	6012
17.06.07	*Chenopodium album* L.	bethe	Chenopodiaceae	h	leaf	gastritis, body pain	consumed as soup	6100
17.06.07	*Clerodendrum viscosum* Vantenat.	bhat	Lamiaceae	s	leaf	leucoderma, hydrophobia	juice is consumed	6034
17.06.07	*Coccinia* sp.	kundri	Cucurbitaceae	c	fruit	diabetes	eaten raw	6087
17.06.07	*Curcuma longa* L.	hardi	Zingiberaceae	h	root	cough, cold, as an antiseptic in sores and wounds	decoction is consumed	6081
17.06.07	*Cymbopogon pendulus* (Nees ex Steud.) Will. Watson	kagati ghas	Poaceae	h	whole plant	fever	filtered juice is consumed	6092
17.06.07	*Drymaria diandra* (Blume)	pothe	Caryophyllaceae	h	leaf	headache, throat pain,	juice is consumed	6067

Date of collection	Scientific name	Local name	Family	Habit[a]	Part used	Disease treated	Mode of administration	CIMAP[b] Accn No.
17.06.07	*Enhydra fluctuens* Loureiro.	hincha	Asteraceae	h	leaf	skin disease, liver problem, diabetes, bronchitis	aqueous extract is used	
17.06.07	*Euphorbia hirta* L.	ratulo	Euphorbiaceae	h	latex	warts, cut	applied externally	6046
17.06.07	*Hedyotis corymbosa* (L.) Lam.	bakhri lahara	Rubiaceae	s	root	stomach colic, gastritis, food poisoning	juice is drunk thrice a day till recovery	6063
17.06.07	*Luffa aegyptiaca* Miller	ghyura	Cucurbitaceae	c	fruit	stomach disorder	pulp is consumed	6052
17.06.07	*Melastoma malabathricum* L.	chulasi	Melastomataceae	s	stem bark, root	wounds, skin diseases	paste applied externally	6065
17.06.07	*Physalis peruviana* L.	jangli mewa	Solanaceae	s	leaf	fever, pneumonia, cold	paste is used on neck, forehead	
17.06.07	*Phytolacca acinosa* Roxburgh.	jaringo	Phytolaccaceae	h	leaf	high blood pressure	juice is consumed	6025
17.06.07	*Pratia nummularia* Benth. ex Kurz.	lanka-sanay	Campanulaceae	h	leaf and	dysentery, tonsillitis	juice is used	6098
					root	snakebite	paste	
01.07.07	*Fragaria nubicola* Lindl.	bhui-ainselu	Rosaceae	h	root	cough, cold, toothache, anticonvulsive, high altitude sickness	juice 4 teaspoon twice a day	6103
					fruit	digestive and laxative	ripe fruit is chewed	

Date of collection	Scientific name	Local name	Family	Habit[a]	Part used	Disease treated	Mode of administration	CIMAP[b] Accn No.
01.07.07	*Panax pseudo-ginseng* N. Wallich.	panch-patey	Araliaceae	h	root	liver disorder, stomach colic, antipyretic, menstrual disorder	juice is consumed (2 tsp thrice a day for 7 days)	6102
25.08.07	*Vitex negundo* L.	simali	Lamiaceae	c	stem	body- swelling, common cold and influenza and bone fracture	steam bath paste is bandaged	6045
25.08.07	*Passiflora foetida* L.	Sano jhar	Passifloraceae	c	leaf	insomia, hysteria and epilepsy and as painkiller	infusion of leaves are consumed	6079
25.08.07	*Commelina benghalensis* L.	kane jhar	Commelinaceae	h	leaf	conjunctivitis	applied 2 drops for 4 days on eyes	6041
25.08.07	*Nephrolepis cordifolia* (L.)Presl.	pani amala	Davalliaceae	h	tuber	burning urination, diabetes, high blood pressure	extract is consumed	
25.08.07	*Ocimum tenuiflorum* L.	babari-phool	Lamiaceae	h	inflorescence	influenza, cold, asthma, bronchitis	chewed in empty stomach	
25.08.07	*Plantago major* L.	chamche-jhar	Plantaginaceae	h	leaf, flower and fruit	throat pain, cut, wounds	juice in consumed in case of throat pain and flower and fruit juice are applied externally	6099
25.08.07	*Pteris* sp.	oonew	Pteridaceae	h	frond	dysentery	consumed	6088
25.08.07	*Stephania hernandiifolia* Walp.	panhelo tamarke	Menispermaceae	c	tuber	diabetes	chewed	6093

[a]s, shrub; h, herb; c, climber
[b]CIMAP, Central Institute of Medicinal and Aromatic Plants, Lucknow, India

4.3.1. DPPH•-scavenging

The assay was carried out with a fixed concentration of all the extracts (1 mg ml^{-1}). *F. nubicola* root (FNR), *P. hirta, E. fluctuens, O. tenuiflorum, Pteris* sp., *C. buchananiana, P. major, C. album, D. indica* and *G. superba* showed excellent DPPH•-scavenging ability (>80%), while the activities of *S. hernandifolia, L. indica, A. rivularis* and *C. rotundus* were good (60-76%) (Table 6). Amongst these, the extracts of FNR, *P. hirta* and *E. fluctuens* showed comparable activity as that (96%) of 1 mg butylated hydroxyanisole (BHA) ml^{-1}. Interestingly, *F. nubicola* fruit extract showed only moderate (43%) DPPH•-scavenging.

Table 6. Preliminary antioxidant parameters of the plant extracts[a]

Plant	Antioxidant parameter			SA[e]
	DPPH[b]	RP[c]	MCA[d]	
Acmella calva	46.3 ± 2.32[1]	553.7 ± 24.81[38]	10.0 ± 0.41[10,13]	m
Amaranthus spinosus	24.2 ± 2.14[2]	164.3 ± 12.42[37]	51.0 ± 0.61[12]	p
Amaranthus viridis	5.0 ± 0.34[3]	121.3 ± 9.27[36]	48.7 ± 0.52[12]	p
Astilbe rivularis	66.8 ± 3.48[4]	762.3 ± 9.77[35]	2.0 ± 0.11[3,5]	g
Cardamine hirsuta	12.8 ± 1.21[5,8]	118.0 ± 8.24[34,36]	29.3 ± 0.44[7,11]	p
Chenopodium album	86.6 ± 1.44[6]	418.1 ± 4.80[33]	94.8 ± 7.50[6]	e
Clematis buchananiana leaf	87.9 ± 6.22[6,7]	202.7 ± 5.92[32]	10.6 ± 0.62[10]	e
Clematis buchananiana roots	11.3 ± 0.80[5]	39.7 ± 3.52[31]	17.1 ± 0.91[1]	p
Cyandon dactylon	16.6 ± 1.17[8]	22.7 ± 1.88[30,31]	7.2 ± 0.24[4,5,13]	p
Cyperus rotundus	60.7 ± 2.24[9]	394.1 ± 4.51[29]	4.3 ± 1.42[3,4,5]	g
Desmodium triflorum	23.6 ± 1.77[2]	16.9 ± 0.92[28,30]	8.5 ± 0.38[4,10]	p
Diplazium esculentum	25.4 ± 2.11[2]	112.0 ± 7.40[34,36]	58.8 ± 3.08[9]	p
Duchesnea indica	84.8 ± 0.86[6,10]	251.1 ± 11.55[26]	43.1 ± 3.12[8]	e
Enhydra fluctuens	91.5 ± 0.51[7,11]	822.5 ± 5.27[25]	72.4 ± 4.66[6]	e
Equisetum debile	15.0 ± 0.92[8]	60.7 ± 4.80[24]	40.1 ± 2.50[8]	p
Fragaria nubicola fruit	43.0 ± 3.79[1,12]	477.0 ± 22.56[23]	7.5 ± 0.92[4,5,10]	m
Fragaria nubicola root	92.3 ± 5.32[11]	927.0 ± 11.04[22]	25.0 ± 1.18[7]	e
Gloriosa superba	81.4 ± 2.25[10,13]	388.1 ± 3.08[21]	2.5 ± 0.11[3,5]	e
Heracleum nepalense	34.0 ± 2.48[14]	502.3 ± 34.11[20]	14.1 ± 0.25[1]	m
Hottuynia cordata	45.3 ± 3.74[1]	719.0 ± 32.87[19]	10.6 ± 0.64[4,10]	m
Leucas indica	77.2 ± 2.46[3,13]	327.2 ± 2.28[18]	9.8 ± 0.56[4,10]	g
Nephrolepis cordifolia	2.88 ± 0.09[15]	9.6 ± 0.85[17,28,30]	1.6 ± 0.08[3,5]	p
Ocimum tenuiflorum	90.8 ± 0.74[6,11]	868.1 ± 7.40[16]	73.0 ± 5.21[6]	e
Paederia foetida	46.6 ± 5.28[1]	489.6 ± 22.50[20,23]	0.6 ± 0.01[3,5]	m
Panax pseudoginseng	5.8 ± 0.31[3,15,16]	10.9 ± 0.32[14,17,28,30]	32.3 ± 2.45[2,11]	p
Perilla fructescence	21.3 ± 1.68[2]	288.7 ± 15.55[13]	7.9 ± 0.92[4,5,10]	p
Physalis minima	29.9 ± 1.52[14]	22.8 ± 1.28[12,14,17,28,30,31]	92.8 ± 2.11[6]	p
Physalis peruviana	39.7 ± 2.71[12]	27.0 ± 2.80[12,14,17,28,30,31]	2.9 ± 0.28[3,5]	m
Plantago major	87.1 ± 1.68[6]	297.0 ± 5.66[10,13]	6.2 ± 0.35[4]	e
Pouzolzia hirta	92.0 ± 2.11[7,11]	409.0 ± 2.73[29,33]	4.9 ± 0.32[3,4,5]	e
Pouzolzia indica	26.8 ±1.50[2,14,17]	80.7 ± 5.23[8]	4.9 ± 0.45[3,4,5]	p
Pratia numularia	9.9 ± 0.62[5,16]	19.0 ± 0.60[7,11,12,14,17,28,30]	27.5 ± 1.12[7]	p
Pteris sp.	89.1 ± 4.50[6,11]	300.3 ± 17.36[10,13]	6.6 ± 0.38[4]	e
Rubia manjith	37.2 ± 2.42[12,14]	82.3 ± 4.55[8]	6.7 ± 0.32[4,13]	m
Sonchus arvensis	24.6 ± 1.18[2,17]	26.9 ± 2.61[4,7,11,12,14,17,28,30,31]	2.8 ± 0.32[3]	p
Stephania hernandifolia	77.4 ± 6.10[13]	165.2 ± 11.41[37]	32.8 ± 2.23[2]	g
Tectaria coadunata	9.9 ± 0.28[5,16]	45.0 ± 2.21[4,11,24,31]	31.4 ± 2.36[2,11]	p
Tupistra nutans	11.8 ± 0.76[5]	141.0 ± 7.82[1]	16.8 ± 0.58[1]	p

[a]Values are mean with standard error of measurements (n = 3).
[b]% scavenging of DPPH• by the test extracts (1 mg ml^{-1}) after 30 min incubation.
[c]mg ascorbic acid equivalent (AAE) g^{-1} dry weight of the extract.
[d]% inhibition of Fe (II)-ferrozine complex formation by the test extracts (1 mg ml^{-1}).
[e]SA, scavenging activity; e, excellent (≥ 80%); g, good (60-<80%); m, moderate (30-<60%); p, poor (<30%).
Values of means with same superscript in any given column are same at $P<0.05$.

Based on DPPH•-scavenging results, the test extracts were categorized into four broad classes, *viz.* excellent, good, moderate and poor radical-scavengers (Table 6). It is well-known that DPPH• is a *N*-centred stable free radical, resembling the biologically relevant lipid peroxyl radical. DPPH•-scavenging activity

often correlates with the ability of the test sample with its anti-lipid peroxidation (LPO) activity. Hence, for the categorization of the antioxidant property of the 38 extracts, emphasis was given on their DPPH•-scavenging ability.

4.3.2. Reducing power (RP)

The respective RP values (Table 6) of the test samples revealed that FNR, *O. tenuiflorum*, and *E. fluctuens* were the most potent followed by *A. rivularis* that also showed good DPPH•-scavenging activity. The correlation between reducing power and DPPH•-scavenging activity was found to be quite high, in most of the categories.

4.3.3. MC power

Besides oxygen metabolism, various reactive oxygen species (ROS) can be formed in cells by the transition metals, especially Fe(II)-mediated reactions and radiation exposure leading to deleterious effects on membrane lipids and DNA. Endogenously available metal ions are known to initiate and propagate LPO chain process via decomposition of the initially formed lipid hydroperoxides (Braughler *et al.*, 1987). Therefore, Fe(II)-chelating ability is an added advantage for antioxidants. Amongst the plants in the excellent category, significant MC was found with *C. album* > *O. tenuiflorum* ~ *E. fluctuens* > *D. indica* > FNR. In the good category, *S. hernandifolia* showed better MC property, while *P. foetida* (moderate class) was almost inactive (Table 6). Surprisingly, some of the plants with poor DPPH•-scavenging ability showed a significant MC property. Amongst the test samples, *C. album* showing moderate DPPH•-scavenging was the best Fe(II)-chelator (95%), better than EDTA (1 mg ml^{-1}) that showed 70% chelation under identical conditions.

4.4. Phenolic and flavonoid contents

The plant phenolics act as defense against various stresses and often account for the antioxidant and antimicrobial activities of the plants. In addition, the class of phenolics, known as flavonoids, is also credited with antioxidant activity and various other health benefits. Hence, the total phenolic and flavonoid contents (designated as TPC and TFC, respectively) of the extracts were also determined and the results are presented in Table 7. The relative order of the plants with high TPC was *H. cordata* >> *E. debile* > *Pteris* sp. > *C. buchananiana* leaf. Likewise, the plants, *Pteris* sp., FNR, *P. peruviana*, *S. hernandifolia* and *P. foetida* showed impressive TFC values. Notably, *Pteris* sp. scored high in both the assays, while FNR was enriched with flavonoids that might account for its excellent DPPH•-scavenging property. Grossly, the pteridophytes were found to contain high phenolics. Although *C. album* and *G. superba* contained a very low amount of phenol and flavonoids, those exhibited a very good antioxidant activity.

4.5. Principal Component Analysis (PCA) of antioxidant parameters

To understand the interrelationships among the four measured antioxidant parameters *viz.* DPPH•-scavenging, iron chelation, and RP with TPC plus TFC of the plant extracts, PCA was performed. The loadings of variables in the analyses are summarized in Table 8, while Fig. 4 depicting the PCA plot provides a clear picture of the correlation of the PC with the original variables and between antioxidant activity parameters.

A factor rotation using the Varimax method was performed and three factor loadings were obtained that accounted for the 81% of the total variance of the plant extracts, chosen on the basis of their eigen values (>1). The loading in the PCA plot expresses the correlation of the PC with the original variables and between antioxidant parameters with TPC and TFC. TFC, DPPH•-scavenging ability and RP were shown to be highly loaded on factor 1 (PC1) with loadings 0.555, 0.929 and 0.795, respectively. DPPH•-scavenging

Table 7. Total phenolic and flavonoid contents of the lyophilized extracts[a]

Plant	TPC[b]	TFC[c]
Acmella calva	137.5 ± 5.4[24]	21.6 ± 0.7[33]
Amaranthus spinosus	103.5 ± 8.1[23]	7.3 ± 0.2[32]
Amaranthus viridis	82.6 ± 1.3[22]	6.8 ± 0.06[31,32]
Astilbe rivularis	26.6 ± 2.2[21]	16.2 ± 1.4[30]
Cardamine hirsuta	78.8 ± 0.6[22]	10.0 ± 0.06[29,31,32]
Chenopodium album	8.6 ± 0.6[20]	7.5 ± 0.5[28,29,31,32]
Clematis buchananiana leaf	472.4 ± 11.7[19]	51.7 ± 0.8[13]
Clematis buchananiana roots	108.6 ± 8.7[18,23,24]	7.3 ± 0.03[28,29,31,32]
Cyandon dactylon	44.1 ± 3.4[17]	4.2 ± 0.3[27,28,31,32]
Cyperus rotundus	107.4 ± 3.1[16,23]	14.1 ± 2.9[26,29,30]
Desmodium triflorum	21.1 ± 1.7[15,21]	10.9 ± 0.9[25,25,28,29,31,32]
Diplazium esculentum	82.5 ± 4.9[14]	19.0 ± 1.6[24,30,33]
Duchesnea indica	31.5 ± 1.9[13,21]	28.3 ± 1.6[23]
Enhydra fluctuens	16.2 ± 0.3[11,15,19]	14.1 ± 0.4[22,25,26,29,30]
Equisetum debile	532.5 ± 3.9[12]	33.9 ± 0.9[21]
Fragaria nubicola fruit	38.1 ± 2.1[1,13,17]	9.3 ± 0.5[20,25,28,29,31,32]
Fragaria nubicola root	130.1 ± 9.4[5]	106.7 ± 8.8[19]
Gloriosa superba	13.0 ± 0.8[11,19]	10.2 ± 0.7[18,22,25,26,28,29,31,32]
Heracleum nepalense	38.7 ± 0.1[1,17]	24.8 ± 0.1[17,23,33]
Hottuynia cordata	2275.5 ±1.6[10]	28.1 ± 0.9[16,17,23]
Leucas indica	26.9 ± 2.1[9,13,15,21]	25.4 ± 1.5[16,17,23,33]
Nephrolepis cordifolia	32.5 ± 0.08[1,8,9,13,21]	0.8 ± 0.02[15,27]
Ocimum tenuiflorum	26.4 ± 2.4[4,8,9,13,15,21]	25.0 ± 1.8[14,16,17,23,33]
Paederia foetida	125.9 ± 10.2[5]	49.9 ± 3.6[13]
Panax pseudoginseng	16.6 ± 1.1[4,7,11,15]	2.9 ± 0.1[12,15,27,31]
Perilla fructescence	18.9 ± 0.3[4,11,15,21]	0.1 ± 0.01[11,12,15,27]
Physalis minima	16.6 ± 1.8[4,7,11,15]	8.4 ± 0.4[10,18,20,25,27,28,29,31,32]
Physalis peruviana	164.1 ± 5.8[8]	96.0 ± 1.2[9]
Plantago major	19.9 ± 2.2[4,7,9,11,15,21]	14.4 ± 1.1[8,18,22,25,26]
Pouzolzia hirta	35.0 ± 2.6[1,13]	21.0 ± 1.6[7,14,17,24,33]
Pouzolzia indica	14.4 ± 0.6[7,11,15,19]	10.9 ± 1.4[2,8,10,18,20,22,25,26,28,29,31]
Pratia numularia	13.4 ± 1.5[7,11,15,19]	13.1 ± 1.6[2,8,18,20,22,25,26,29,30]
Pteris sp.	502.2 ± 4.8[6]	178.3 ± 9.7[6]
Rubia manjith	107.5 ± 9.5[5,16]	18.2 ± 1.2[5,7,8,22,24,26,30,33]
Sonchus arvensis	24.2 ± 1.4[4,9,13,15,21]	23.9 ± 2.1[4,7,14,16,17,33]
Stephania hernandifolia	175.6 ± 8.5[3]	86.1 ± 5.5[3]
Tectaria coadunata	258.8 ± 9.1[2]	11.0 ± 0.8[2,8,10,18,20,22,25,26,28,29,31,32]
Tupistra nutans	34.3 ± 1.0[1,9,13,21]	6.5 ± 0.1[1,10,12,18,20,27,28,29,31,32]

[a]Values are mean with standard error of measurements (n = 3).
[b]mg GAE g^{-1} dry weight of the extract.
[c]mg ECE g^{-1} dry weight of the extract.
Values of means with same superscript in any given column are same at $P<0.05$.

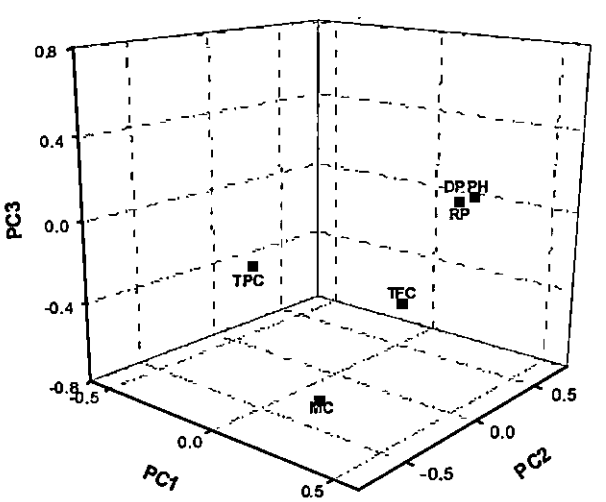


Fig. 4. Loading plot of the different antioxidant parameters and phenol and flavonoid contents of the 38 herbs, analysed using principal component analysis.

ability and RP were closely loaded, which indicated the two properties are intimately related to the antioxidant activity. However, the high loading (-0.978) of TPC on factor 3 (PC3) where other parameters have low loading indicated its independence from the other variables. On the other hand, the Fe(II)-chelating ability of the plant extracts shows a loading of -0.853, while TFC has a loading of 0.622 on Factor 2 (PC2), which showed low loading of the other parameters.

Table 8. Loadings of variables (antioxidative parameters and phenol and flavonoid contents) by individual principal components (PCs)

Variable	Loading			Communality
	PC1	PC2	PC3	
TPC	0.058	0.115	-0.978	0.973
TFC	0.555	0.622	-0.081	0.701
DPPH	0.929	0.128	0.119	0.893
RP	0.795	-0.212	-0.250	0.740
MCA	0.135	-0.853	0.084	0.753

4.6. Further antioxidant screening

The extracts, showing good to excellent DPPH•-scavenging ability (Table 6) were chosen for further evaluation of their antioxidant activity. To this end, their ABTS•+ and hydroxyl radical (•OH)-scavenging activity as well as oxygen radical absorption capacity (ORAC) were assessed. The collective results are shown in Table 9.

Table 9. Antioxidant activities of lyophilized extracts of some selected plants[a]

Plant	ABTS•+ scavenging (%)	ORAC (mg GAE g^{-1} extract)	•OH scavenging (%)
Astilbe rivularis	42.6 ± 4.3**	509.8 ± 6.14	33.9 ± 3.1$
Chenopodium album	60.4 ± 3.1**	ND	67.5 ± 1.4$
Clematis buchananiana leaf	59.8 ± 5.6**	919.4 ± 8.52#	0
Cyperus rotundus	59.5 ± 3.5**	515.1 ± 5.53	72.7 ±2.5$
Duchesnea indica	52.4 ± 2.5**	506.7 ± 6.89	54.8 ± 2.7$
Enhydra fluctuens	63.2 ± 2.7*	263.1 ± 8.28#	32.1 ± 2.1$
Fragaria nubicola root	66.43 ± 6.1	1387.6 ± 11.1#	85.4 ± 2.4$
Gloriosa superba	55.3 ± 2.9**	409.9 ± 4.68#	49.1 ± 4.5$
Leucas indica	51.4 ± 3.1**	ND	59.1 ± 3.6$
Ocimum tenuiflorum	61.7 ± 2.9*	352.3 ± 5.7#	11.5 ± 0.8$
Plantago major	57.7 ± 3.4**	ND	60.9 ± 3.5$
Pouzolzia hirta	64.8 ± 4.5	ND	29.1 ± 1.9$
Pteris sp.	60.5 ± 6.6**	606.9 ± 9.57#	46.5 ± 3.3$
Stephania hernandifolia	60.8 ± 6.2**	547.6 ± 4.56#	52.6 ± 2.4$

[a]All the assays were carried out using 1 mg ml^{-1} of test samples. Values are mean with standard error of measurements (n = 3).
*$P < 0.05$, compared to BHA.
**$P < 0.01$, compared to BHA.
$$P<0.01$, compared to mannitol.
#Values are different with each other at $P<0.01$.

4.6.1. ABTS•+-scavenging ability

Except for *A. rivularis* extract that showed 43% scavenging, other test samples showed similar and good activity 60-67% in this assay (Table 9).

4.6.2. •OH-scavenging activity

The extracts of FNR and *C. rotundus* showed excellent •OH-scavenging (85% and 73%, respectively), while *P. major*, *C. album*, *D. indica*, *S. hernandifolia* and *L. indica* showed 55-60% activity. *C. buchananiana* leaf extract was inactive and *O. tenuiflorum* showed marginal activity (Table 9). Although *P. hirta*, *E. fluctuens* and *A. rivularis* showed moderate activity, the phenol content and preliminary antioxidant screening were found to be powerful.

4.6.3. ORAC

The ORAC assay gives a measure of the capacity of a test sample to scavenge peroxyl radicals, generated by spontaneous decomposition of AAPH. This assay has been successfully used to assess the antioxidant property of a wide variety of edible plant extracts containing a diverse array of phytochemicals such as

alkaloids, coumarins, flavonoids, phenylpropanoids, terpenoids and phenolics acids (Aruoma, 2003; Domínguez *et al.*, 2005). Hence, ORAC values of the plant extracts were estimated in terms of gallic acid equivalents. In this assay, FNR extract showed a superior capacity, and the extracts of *C. buchananiana* leaf, *Pteris* sp., *D. indica*, *S. hernandifolia*, *A. rivularis*, and *C. rotundus* were found potent (Table 9).

4.6.4. Protection against LPO

LPO, which often results by the Fenton-mediated process, is one of the most damaging events in free radicals-mediated cellular injury. Hence, the protective activity of a fixed concentration (1 mg ml^{-1}) of the test samples against iron-mediated LPO was examined, and the results are summarized in Table 10. Amongst the test samples, *P. major* showed insignificant protection against LPO, while *E. fluctuens* showed moderate protection (34%). The activities of the other extracts were impressive. FNR, *G. superba* and *S. hernandifolia* showed equivalent potency as that of BHA. Although the results with FNR and *G. superba* correlated well with their DPPH•-scavenging activities, the poor protection offered by *E. fluctuens*, an excellent DPPH• scavenger, was unexpected.

Table 10. Protective activity of selected plant extracts against lipid peroxidation[a]

Treatment	Malonaldialdehyde[b]	% protection[c]
experimental control	49.9 ± 5.3	0
Astilbe rivularis	14.1 ± 1.1	71.74***
Chenopodium album	13.5 ± 0.9	72.95***
Clematis buchananiana leaf	14.4 ± 1.7	71.14***
Cyperus rotundus	12.3 ± 0.8	75.35***
Duchesnea indica	9.6 ± 1.5	80.76***
Enhydra fluctuens	32.9 ± 3.5	34.07*
Fragaria nubicola root	5.2 ± 0.6	89.66***
Gloriosa superba	5.1 ± 0.3	89.78***
Leucas indica	13.6 ± 1.2	72.75***
Ocimum tenuiflorum	9.7 ± 0.7	80.56***
Plantago major	46.5 ± 3.8	6.81
Pouzolzia hirta	17.2 ± 2.1	65.53**
Pteris sp.	21.2 ± 2.5	57.52**
Stephania hernandifolia	7.2 ± 0.9	85.57***
BHA	4.5 ± 0.6	90.98***

[a]All the assays were carried out using 1 mg ml^{-1} of test samples. Values are mean with standard error of measurements (n = 3).
[b]nmol MDA mg^{-1} protein.
[c]% reduction of MDA formation with respect to the experimental control.
*$P<0.05$, compared to BHA.
**$P<0.01$, compared to BHA.
***$P<0.001$, compared to BHA.

4.7. Comprehensive analyses of the antioxidant capacity of FNR

The above results clearly revealed that the extract of FNR possessed the best antioxidant activity. Hence, a comprehensive analysis of its antioxidant property was carried out using several *in vitro* methods. In addition, its antiproliferative activity was also investigated.

4.7.1. $ABTS^{•+}$-scavenging

FNR and BHA (positive control) showed a dose-dependent scavenging of the $ABTS^{•+}$ with the IC_{50} values of 276 µg ml^{-1} and 187 µg ml^{-1}, respectively (Fig. 5). The percentage of scavenging increased sharply up to 250 µg ml^{-1} of both the samples, beyond which the increase was gradual. At the lower concentration range (up to 250 µg ml^{-1}), this corresponds exactly ($P < 0.01$) with the activity of BHA.

4.7.2. DPPH•-scavenging

FNR and BHA also showed a concentration-dependent DPPH•-scavenging activity throughout the entire range of test concentrations (up to 100 µg ml^{-1}). From this, the respective IC_{50} values of FNR and BHA for DPPH•-scavenging were found as 49.5 and 45.7 µg ml^{-1}, respectively (Fig. 6). In separate experiments, It was also found that the reactions with $ABTS^{•+}$ and DPPH• were quite fast and almost in all cases complete in 1-3 min and ~15 min, respectively (data not shown).

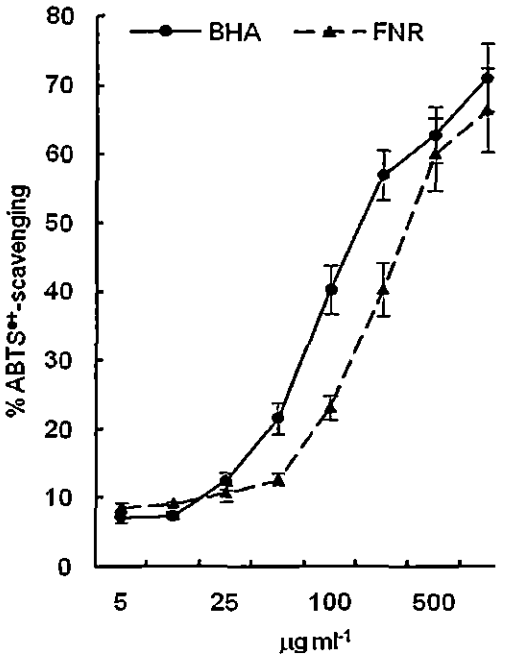


Fig. 5. $ABTS^{\bullet+}$-scavenging potential of *Fragaria nubicola* root extract (FNR) and butylated hydroxyanisole (BHA). The values are mean ± SEM (n = 3).

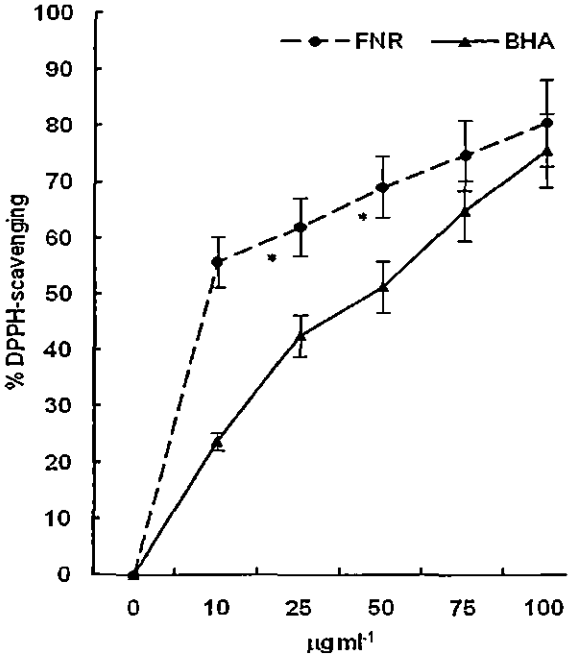


Fig. 6. $DPPH^{\bullet}$-scavenging potential of *Fragaria nubicola* root extract (FNR) and butylated hydroxyanisole (BHA). The values are mean ± SEM (n = 3). $^{*}P<0.01$, compared to BHA.

4.7.3. RP

The reducing power of FNR (final concentration, 10-100 μg ml^{-1}), was measured in terms of mg AAE g^{-1} lyophilized extract, determined from its iron(III)-reducing potential which also showed a concentration-dependent increase (Fig. 7).

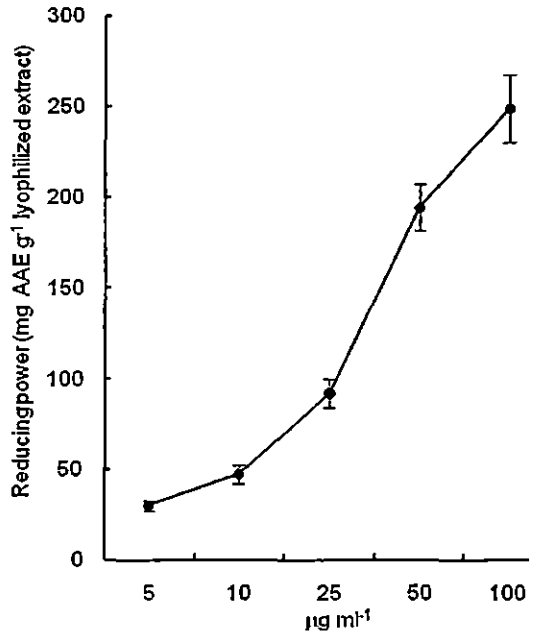


Fig. 7. Reducing power of *Fragaria nubicola* root extract. The values are mean ± SEM (n = 3). The values at 10-100 μg ml^{-1} were significantly different ($P<0.01$).

4.7.4. Anti-LPO

The protective capacity of FNR against LPO was assessed using mice liver mitochondria as a convenient lipid source, and the Fenton reagent (Fe(II)/ascorbic acid) or AAPH as the radical initiator. The end product of LPO was measured in terms of malondialdehyde (MDA).

In unstimulated experiments, the amount of MDA in mice liver mitochondria was marginal, which increased to 22.7 nmol mg^{-1} protein on stimulation with Fe(II)/ascorbic acid. FNR inhibited LPO in a concentration-dependent manner (Fig. 8), with an IC_{50} value of 10.1 μg ml^{-1}. Under similar conditions, 1 μM BHA (positive control) provided 50.2% protection against LPO (not shown in the graph). In the AAPH-induced LPO, the protective activity of FNR was significantly less ($P < 0.05$) with an IC_{50} value of 13.8 μg ml^{-1}.

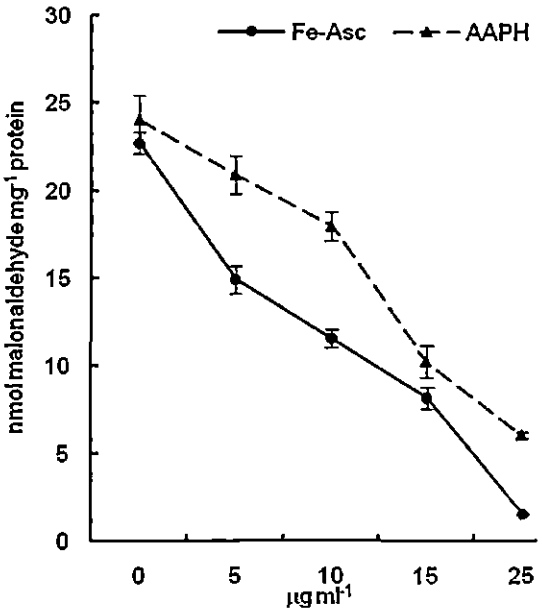


Fig. 8. Protective activity of *Fragaria nubicola* root extract (FNR) against Fe(II)-ascorbic acid (Fe-Asc) and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced lipid peroxidation. The values are mean ± SEM (n = 3). The concentrations of FNR, reducing the malonyldehyde concentration by 50% (IC_{50}) compared to the experimental control were calculated from the graph. The IC_{50} values of FNR in the AAPH or Fe-Asc-induced lipid peroxidation were significantly different ($P<0.05$).

4.7.5. DNA protection

The protective effect of FNR against Fenton-induced damage to pBR322 DNA was studied by separating the different DNA bands by electrophoresis. A representative gel electrophoresis photograph showing the protective activity of FNR is presented in Fig. 9. The quantitative protection offered by FNR revealed its remarkable protecting ability even at very low concentrations (Table 11). For example, 25 µg FNR ml^{-1} extract showed 94.9% protection against the Fenton-induced DNA nicking.

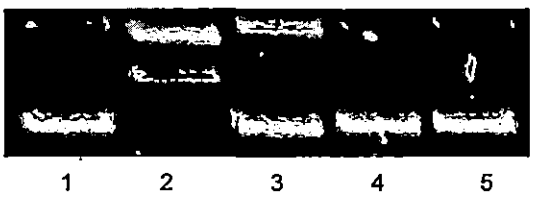


Fig. 9. Concentration dependent protective activity of *Fragaria nubicola* root extract (FNR) against Fenton-mediated DNA nicking as revealed by DNA gel electrophoresis. Lane 1, native DNA; Lane 2, DNA + Fenton reagent; Lanes 3-5, DNA + Fenton reagent + FNR (0.5, 2.5 and 5 µg ml^{-1}, respectively).

Table 11. Concentration-dependent protective activity of *Fragaria nubicola* root (FNR) against Fenton-mediated damage to plasmid DNA[a]

Sample	% of supercoiled DNA	% of open-circular DNA	% of linear DNA	% protection
Native DNA	94.93	5.07	0	100
DNA + Fenton reagent (A)	0	14.4	85.6	0
A + FNR (0.5 µg ml^{-1})	40.5	59.5	0	62.7
A + FNR (2.5 µg ml^{-1})	91.2	8.8	0	96.1
A + FNR (5 µg ml^{-1})	92.91	7.09	0	97.9

[a]Values are mean of duplicate sets.

4.7.6. Radioprotection of proteins

For this assay, γ-rays were used for the oxidation of bovine serum albumin (BSA) as a model protein. The protective activity of FNR was examined by comparing the fluorescence of unirradiated protein samples *vis-à-vis* that of those, irradiated in the absence and presence of FNR (2.5-10 µg ml^{-1}) and the positive control, Trolox (2.5 µM).

Exposure of BSA to γ-rays at a dose of 300 Gy reduced the protein fluorescence by 76%. Addition of FNR to BSA, prior to γ-irradiation, increased the protection dose-dependently, although at 2.5 µg ml^{-1}, it was inactive (Table 12). Under similar conditions, Trolox, a water-soluble analogue of vitamin E, did not show any protective effect.

4.8. Toxicity of FNR

Toxicity of various concentrations of FNR to the normal cells was assessed by the methyl tetrazolium salt (MTT) using INT-407 as the model system. As revealed by the MTT results (Fig. 10), FNR was nontoxic to

Table 12. Concentration-dependent protective activity of *Fragaria nubicola* root (FNR) against γ-ray-mediated oxidation of bovine serum albumin (BSA)[a]

Treatment	fluorescent unit	% protection
BSA (unirradiated)	2.16 ± 0.16	100
γ-irradiated BSA (A)	0.52 ± 0.07*	0
A + FNR (2.5 μg ml^{-1})	0.54 ± 0.05	1.2
A + FNR (5 μg ml^{-1})	1.22 ± 0.09**	42.5
A + FNR (10 μg ml^{-1})	1.54 ± 0.14**	62.6
A + Trolox (2.5 μM)	0.52 ± 0.06	0

[a]BSA was γ-irradiated at 25°C at a dose of 300 Gy (dose rate, 16 Gy min^{-1}) and the fluorescence of the samples was estimated at λ_{ex} = 280 nm and λ_{em} = 345 nm. Values are presented as mean with standard error of measurements (n = 3). The % protection was calculated from the reduction of fluorescence intensity with respect to irradiated control.
*P<0.001, compared to unirradiated sample.
**P<0.001, compared to irradiated sample.

antiproliferative capacity towards both the cell lines. Although the effect was dose-dependent, the sensitivities towards the cell lines were different. Thus while its toxicity towards the A-549 cells increased monotonously, its efficacy against the MCF-7 cells was comparatively less at the higher concentration range. Under identical conditions, the IC_{50}-values of the positive control, curcumin, against A-549 was 26.7 μM (data not shown). The nontoxicity of the vehicle (0.1% DMSO) against the cell lines was also confirmed.

4.10. HPLC of FNR

HPLC analysis of acid hydrolysate of FNR showed the presence of 11 compounds. The peaks were detected at the λ_{max} (280 nm) of FNR (Fig. 11). Based on comparison of the HPLC profile with those of standards, seven of the constituents were identified as ascorbic acid and six hydrolyzable tannins as shown in Table 13. FNR was found to be rich in ascorbic acid, gallic acid, and dihydrocaffeic acid, besides containing small amounts of ellagic acid, genistic acid, caffeic acid and *o*-coumaric acid.

the INT-407 cells up to 200 μg ml^{-1} after incubation for 72 h. Hence, it promises to be a good antioxidant for further investigation in cellular systems.

4.9. Antiproliferative activity of FNR

Cytotoxic effect of FNR at various concentrations on two different human cancer cell lines *viz.* lung (A-549) and breast (MCF-7) cells was also examined. The MTT assay results at 48 h (Fig. 10) established its pronounced toxicity against both the cell lines. Even at low concentrations (upto 25 μg ml^{-1}), FNR showed good

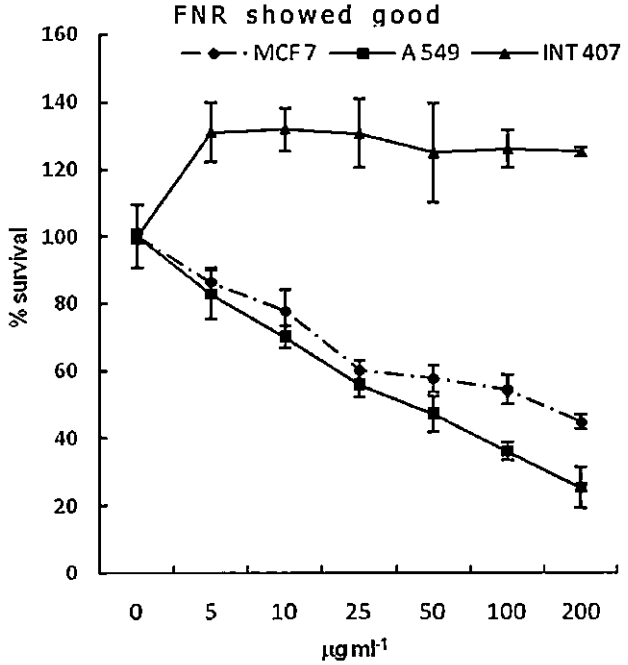


Fig. 10. Influence of *Fragaria nubicola* root extract against normal (INT-407) and lung (A549) and breast (MCF-7) cancer cell lines. Values (mean ± SEM; n = 18) are expressed in percentage considering that of the untreated control cells as 100. In normal cell line values were not significantly different, compared to control cells. In cancerous cell lines, the IC_{50} values were significantly different (P<0.01).

4.11. Antimicrobial assay of plant extracts

Disc diffusion method is extensively used to assay the antimicrobial activity of natural substances and plant extracts. The assay is based on the use of discs as reservoirs containing solutions of substances to be examined (Gülcin *et al.*, 2003). Using the assay, the antimicrobial activity of 38 extracts of 36 different herbs was examined. For this, three Gram-negative bacteria (*E. coli*, *K. pnuemoniae* and *S. typhi*), and six

Table 13. Chemical composition[a] of *Fragaria nubicola* root extract, as revealed in HPLC analysis (vide Fig. 11)

Peak no.	Compound	Retention time (min)	Quantity (μg mg^{-1} extract)
1	unidentified	2.88	nd[b]
2	ascorbic acid	2.99	97.9 ± 3.45
3	unidentified	3.18	nd
4	unidentified	3.52	nd
5	gallic acid	4.36	51.4 ± 2.61
6	unidentified	5.03	nd
7	genistic acid	5.48	1.9 ± 0.16
8	dihydrocaffeic acid	6.44	11.3 ± 1.42
9	ellagic acid	8.36	5.3 ± 0.67
10	caffeic acid	9.05	4.4 ± 0.32
11	*o*-coumaric acid	12.30	1.1 ± 0.15

[a]Values are mean with standard error of measurements
[b]nd, not determined.

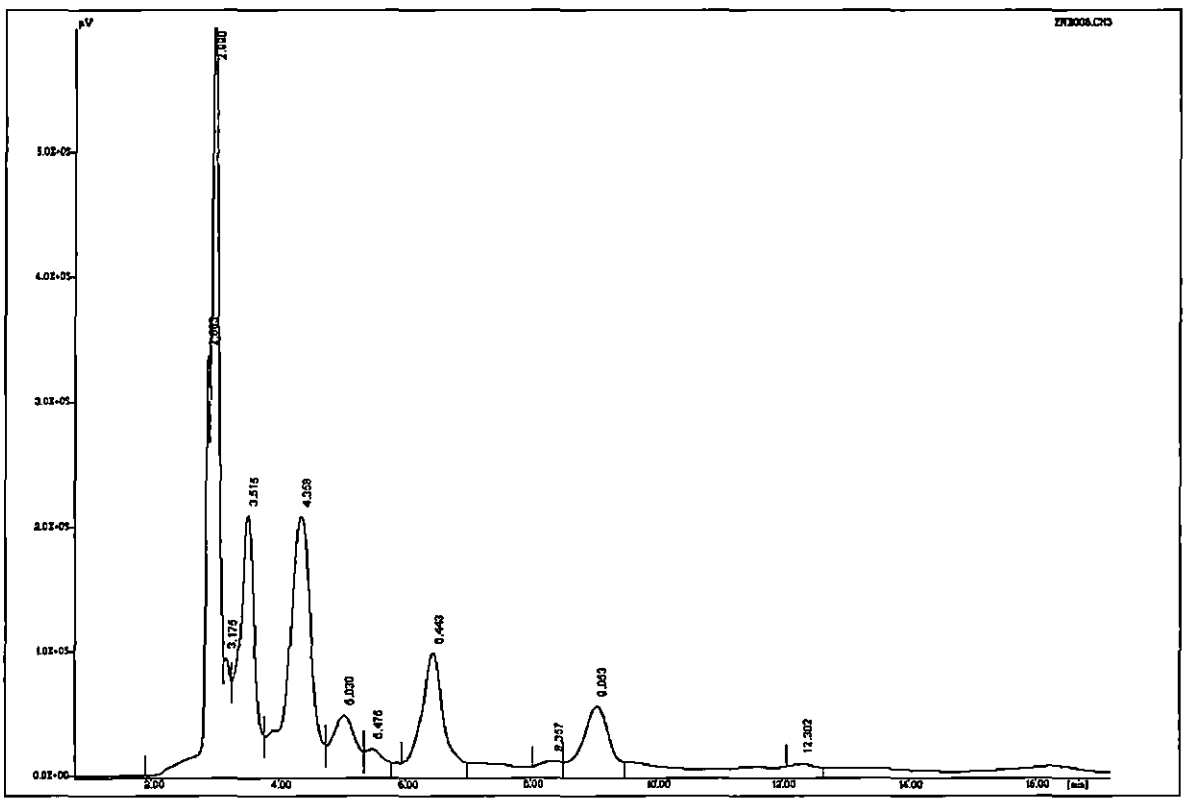


Fig. 11. HPLC profile of *Fragaria nubicola* root extract. Numerical values represent the peak numbers, mentioned in Table 13.

Gram-positive bacteria (*B. subtilis*, *B. cereus*, *B. pumilus*, *B. licheniformis*, *L. monocytogenes* and *S. aureus*), two yeasts (*C. albicans* and *S. cerevisiae*) and two moulds (*A. niger* and *A. alternata*) were chosen as the target organisms. The results of the studies are shown in Tables 14-16.

Thirty of the tested extracts were active against most of the Gram-positive bacteria. Especially, the extracts of *D. indica*, FNR, *L. indica*, *P. minima*, *P. peruviana*, *Pteris* sp. and *S. hernandifolia* inhibited all the test bacteria in this category, and many of these showed very low MIC values (<1 mg ml^{-1}). The extracts were not very active against the Gram-negative bacteria tested; the most potent ones are *R. manjith* which showed inhibitory activity against *K. pnuemoniae*, *A. rivularis* and *D. indica* which showed inhibitory activity against *S. typhi*, and *A. viridis* which showed inhibitory activity against *E. coli* (Table 14). On the other hand, *Physalis* extracts were active against Gram-positive bacteria (Table 15), but did not

Table 14. Antimicrobial activities of the herbs against Gram-negative bacteri

Plant	MIC (mg disc^{-1})		
	Escherichia coli	*Klebsiella pnuemoniae*	*Salmonella typhi*
Acmella calva	>8.0	>8.0	4.0-5.0
Amaranthus spinosus	7.0-8.0	>8.0	>8.0
Amaranthus viridis	2.0-3.0	>8.0	4.0-5.0
Astilbe rivularis	>8.0	>8.0	1.0-2.0
Cardamine hirsuta	>8.0	>8.0	>8.0
Chenopodium album	>8.0	>8.0	>8.0
Clematis buchananiana leaf	2.0-3.0	>8.0	7.0-8.0
Clematis buchananiana root	>8.0	>8.0	7.0-8.0
Cyandon dactylon	>8.0	>8.0	>8.0
Cyperus rotundus	>8.0	>8.0	7.0-8.0
Desmodium triflorum	>8.0	6.0-7.0	>8.0
Diplazium esculentum	>8.0	>8.0	>8.0
Duchesnea indica	5.0-6.0	6.0-7.0	1.0-2.0
Enhydra fluctuens	>8.0	>8.0	>8.0
Equisetum debile	>8.0	7.0-8.0	>8.0
Fragaria nubicola fruit	6.0-7.0	1.0-2.0	6.0-7.0
Fragaria nubicola root	>8.0	6.0-7.0	4.0-5.0
Gloriosa superba	3.0-4.0	>8.0	>8.0
Heracleum nepalense	>8.0	>8.0	>8.0
Hottuynia cordata	6.0-7.0	>8.0	>8.0
Leucas indica	>8.0	>8.0	>8.0
Nephrolepis cordifolia	>8.0	>8.0	>8.0
Ocimum tenuiflorum	7.0-8.0	>8.0	>8.0
Paederia foetida	>8.0	>8.0	6.0-7.0
Panax pseudoginseng	6.0-7.0	>8.0	>8.0
Perilla fructescence	>8.0	>8.0	>8.0
Physalis minima	>8.0	>8.0	6.0-7.0
Physalis peruviana	7.0-8.0	>8.0	>8.0
Plantago major	>8.0	>8.0	>8.0
Pouzolzia hirta	>8.0	>8.0	>8.0
Pouzolzia indica	>8.0	>8.0	>8.0
Pratia numularia	>8.0	>8.0	>8.0
Pteris sp.	>8.0	>8.0	6.0-7.0
Rubia manjith	>2.0	1.0-2.0	>2.0
Sonchus arvensis	>8.0	>8.0	>8.0
Stephania hernandifolia	>8.0	7.0-8.0	5.0-6.0
Tectaria coadunata	>8.0	>8.0	5.0-6.0
Tupistra nutans	>8.0	7.0-8.0	>8.0

show appreciable activity against the Gram-negative ones. *Listeria monocytogenes*, an important foodborne pathogen, was strongly inhibited by *S. hernandifolia*, *H. nepalense*, *E. fluctuens* and *R. manjith*. The results revealed that the MIC values of the plant extracts were much lower for the Gram-positive bacteria, compared to the Gram-negative ones.

The yeasts, *C. albicans* and *S. cerevisiae*, were inhibited by eight extracts; *Candida albicans*, a potential human pathogen, was best inhibited by *H. nepalense* and *T. nutans* (Table 16). The moulds were quite resistant to the extract. *A. alternata* was inhibited by 45% of the extracts, while *A. niger* was susceptible to 24% only of the extracts used. *H. nepalense* and *T. nutans* could inhibit the growth of all the tested organisms at 2-5 mg ml^{-1} and 0.5-4 mg ml^{-1}, respectively.

4.12. Isolation of active principles from extracts

4.12.1. Furanocoumarins from *H. nepalense* fruits

Besides showing moderate antioxidant activity, the methanol extract of *H. nepalense* fruit possessed significant activity against various bacteria, yeasts and moulds. Hence, the phytochemical investigation was pursued to isolate and identify some of its major components.

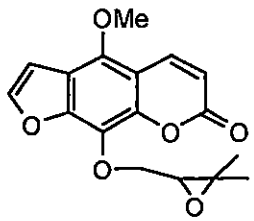
Earlier, several furanocoumarins have been isolated from *Heracleum* spp. (Banerjee *et al.*, 1980; Kavli *et al.*, 1983; Sajjadi and Noroozi, 2007; Tosun *et al.*, 2008). Due to the wide occurrence of coumarins, including furanocoumarins in plants, and their diverse biological activities (Borges *et al.*, 2005; Fylaktakidou *et al.*, 2004; Kulkarni *et al.*, 2006), they are valued in medicinal chemistry. Considering their high solubility in nonpolar organic solvents, the 1.4 g-hexane extract (10% w w^{-1}) of *H. nepalense* fruits was subjected to column chromatography over silica gel, and the three fractions eluting with 15-25% ethyl acetate/ hexane furnished three furanocoumarins after PTLC.

These were identified as byakangelicol (BA), sphondin (SD) (Tosun *et al.*, 2008)) and furopinnarin (FP) (Banerjee *et al.*, 1980) from their respective ^{1}H NMR spectrum that matched with reported values.

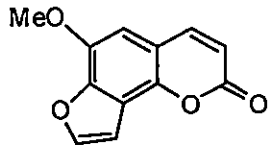
Table 15. Antimicrobial activities of the herbs against Gram-positive bacteria

Plant	MIC (mg disc^{-1})					
	Bacillus subtilis	*Bacillus cereus*	*Bacillus pumilus*	*Bacillus licheniformis*	*Listeria monocytogenes*	*Staphylococcus aureus*
Acmella calva	7.0-8.0	5.0-6.0	>8.0	4.0-5.0	3.0-4.0	>8.0
Amaranthus spinosus	>8.0	6.0-7.0	>8.0	>8.0	>8.0	7.0-8.0
Amaranthus viridis	>8.0	5.0-6.0	>8.0	4.0-5.0	4.0-5.0	6.0-7.0
Astilbe rivularis	0.5-1.0	1.0-2.0	7.0-8.0	7.0-8.0	2.0-3.0	1.0-2.0
Cardamine hirsuta	>8.0	>8.0	>8.0	>8.0	>8.0	>8.0
Chenopodium album	7.0-8.0	3.0-4.0	>8.0	>8.0	>8.0	>8.0
Clematis buchananiana leaf	1.0-2.0	>8.0	>8.0	2.0-3.0	5.0-6.0	0.5-1.0
Clematis buchananiana root	0.5-1.0	7.0-8.0	>8.0	4.0-5.0	3.0-4.0	1.0-2.0
Cyandon dactylon	>8.0	7.0-8.0	>8.0	>8.0	>8.0	>8.0
Cyperus rotundus	6.0-7.0	3.0-4.0	>8.0	7.0-8.0	>8.0	2.0-3.0
Desmodium triflorum	4.0-5.0	3.0-4.0	0.25-0.5	>8.0	>8.0	0.5-1.0
Diplazium esculentum	6.0-7.0	>8.0	>8.0	>8.0	>8.0	5.0-6.0
Duchesnea indica	3.0-4.0	1.0-2.0	0.125-0.25	0.5-1.0	>8.0	0.125-0.25
Enhydra fluctuens	1.0-2.0	>8.0	0.5-1.0	0.25-0.5	1.0-2.0	0.25-0.5
Equisetum debile	7.0-8.0	7.0-8.0	>8.0	>8.0	>8.0	>8.0
Fragaria nubicola fruit	7.0-8.0	6.0-7.0	5.0-6.0	>8.0	>8.0	1.0-2.0
Fragaria nubicola root	0.5-1.0	0.25-0.5	0.125-0.25	0.5 -1.0	5.0-6.0	<0.125
Gloriosa superba	6.0-7.0	7.0-8.0	6.0-7.0	2.0-3.0	>8.0	1.0-2.0
Heracleum nepalense	1.0-2.0	3.0-4.0	2.0-3.0	6.0-7.0	2.0-3.0	2.0-3.0
Hottuynia cordata	6.0-7.0	>8.0	4.0-5.0	>8.0	4.0-5.0	>8.0
Leucas indica	0.125-0.25	0.5-1.0	0.25-0.5	0.25-0.5	7.0-8.0	0.25-0.5
Nephrolepis cordifolia	>8.0	>8.0	>8.0	>8.0	>8.0	>8.0
Ocimum tenuiflorum	6.0-7.0	2.0-3.0	1.0-2.0	1.0-2.0	5.0-6.0	0.5-1.0
Paederia foetida	>8.0	>8.0	>8.0	>8.0	7.0-8.0	>8.0
Panax pseudoginseng	7.0-8.0	7.0-8.0	2.0-3.0	7.0-8.0	>8.0	0.125-0.25
Perilla fructescence	>8.0	>8.0	>8.0	>8.0	>8.0	7.0-8.0
Physalis minima	0.5-1.0	>8.0	0.25-0.5	0.125-0.25	4.0-5.0	0.125-0.25
Physalis peruviana	0.125-0.25	0.5-1.0	0.5-1.0	0.25-0.5	>8.0	2.0-3.0
Plantago major	7.0-8.0	1.0-2.0	1.0-2.0	5.0-6.0	>8.0	6.0-7.0
Pouzolzia hirta	5.0-6.0	3.0-4.0	>8.0	>8.0	>8.0	0.5-1.0
Pouzolzia indica	>8.0	6.0-7.0	7.0-8.0	>8.0	>8.0	6.0-7.0
Pratia numularia	7.0-8.0	>8.0	>8.0	>8.0	2.0-3.0	0.125-0.25
Pteris sp.	0.25-0.5	0.5-1.0	1.0-2.0	0.5-1.0	>8.0	0.25-0.5
Rubia manjith	1.0-2.0	>2.0	>2.0	<0.125	0.125-0.5	0.125-0.25
Sonchus arvensis	>8.0	>8.0	>8.0	7.0-8.0	>8.0	3.0-4.0
Stephania hernandifolia	0.125-0.25	0.125.25	0.5-1.0	0.25-0.5	0.5-1.0	0.5-1.0
Tectaria coadunata	0.5-1.0	5.0-6.0	6.0-7.0	>8.0	>8.0	>8.0
Tupistra nutans	>8.0	>8.0	2.0-3.0	>8.0	>8.0	6.0-7.0

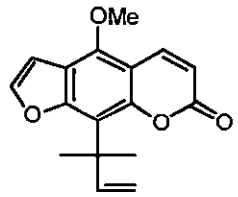
Amongst these, BA and SD were earlier isolated from *Heracleum crenatifolium* fruits (Tosun *et al.*, 2008), while FP was obtained from *Heracleum thomsoni* roots (Banerjee *et al.*, 1980). The ^{1}H-NMR spectral data of the compounds are presented below, while their chemical structures are shown in Fig. 12.



Byak-angelicol



Sphondin



Furopinnarin

Fig. 12. Chemical structures of the furanocoumarins, isolated from *Heracleum nepalense*

The ^{1}H NMR spectrum (Fig. 13) of BA showed three 3-protons singlets at δ 1.21, 1.29 and 4.15 for the *gem*-dimethyl and methoxy groups, along with a 1H-triplet at δ 3.26 (J = 5.6 Hz, epoxy proton) and a 2H-doublet at δ 4.39 (J = 5.6 Hz, -CH_2O). The pair of 1H-doublets at δ 6.23 and δ 8.08, each with

Table 16. Antimicrobial activities of the herbs against yeasts and moulds

Plant	MIC (mg disc^{-1})			
	Candida albicans	*Saccharomyces cerevisiae*	*Aspergillus niger*	*Alternaria alternata*
Acmella calva	7.0-8.0	>8.0	>8.0	3.0-4.0
Amaranthus spinosus	>8.0	>8.0	>8.0	4.0-5.0
Amaranthus viridis	>8.0	>8.0	>8.0	>8.0
Astilbe rivularis	>8.0	>8.0	>8.0	1.0-2.0
Cardamine hirsuta	>8.0	>8.0	1.0-2.0	7.0-8.0
Chenopodium album	7.0-8.0	5.0-6.0	>8.0	>8.0
Clematis buchananiana leaf	>8.0	>8.0	>8.0	1.0-2.0
Clematis buchananiana root	>8.0	>8.0	4.0-5.0	7.0-8.0
Cyandon dactylon	>8.0	>8.0	>8.0	>8.0
Cyperus rotundus	>8.0	>8.0	>8.0	5.0-6.0
Desmodium triflorum	>8.0	>8.0	>8.0	>8.0
Diplazium esculentum	>8.0	>8.0	2.0-3.0	4.0-5.0
Duchesnea indica	>8.0	>8.0	>8.0	>8.0
Enhydra fluctuens	>8.0	>8.0	>8.0	>8.0
Equisetum debile	>8.0	>8.0	2.0-3.0	6.0-7.0
Fragaria nubicola fruit	>8.0	>8.0	0.5-1.0	0.25-0.5
Fragaria nubicola root	>8.0	>8.0	3.0-4.0	2.0-3.0
Gloriosa superba	>8.0	>8.0	>8.0	>8.0
Heracleum nepalense	4.0-5.0	4.0-5.0	2.0-3.0	4.0-5.0
Hottuynia cordata	5.0-6.0	7.0-8.0	>8.0	3.0-4.0
Leucas indica	6.0-7.0	7.0-8.0	>8.0	>8.0
Nephrolepis cordifolia	>8.0	>8.0	>8.0	>8.0
Ocimum tenuiflorum	5.0-6.0	5.0-6.0	>8.0	>8.0
Paederia foetida	>8.0	>8.0	>8.0	>8.0
Panax pseudoginseng	>8.0	>8.0	>8.0	>8.0
Perilla fructescence	>8.0	>8.0	1.0-2.0	>8.0
Physalis minima	>8.0	>8.0	>8.0	>8.0
Physalis peruviana	7.0-8.0	>8.0	>8.0	>8.0
Plantago major	>8.0	5.0-6.0	>8.0	>8.0
Pouzolzia hirta	>8.0	>8.0	>8.0	0.5-1.0
Pouzolzia indica	>8.0	>8.0	>8.0	1.0-2.0
Pratia numularia	>8.0	>8.0	>8.0	>8.0
Pteris sp.	>8.0	>8.0	>8.0	>8.0
Rubia manjith	>2.0	>2.0	>2.0	>2.0
Sonchus arvensis	>8.0	>8.0	>8.0	>8.0
Stephania hernandifolia	>8.0	6.0-7.0	>8.0	>8.0
Tectaria coadunata	>8.0	>8.0	>8.0	4.0-5.0
Tupistra nutans	0.5-1.0	1.0-2.0	0.5-1.0	3.0-4.0

coupling constant 9.8 Hz accounted for the coumarin double bond, while another pair of 1H-doublets at δ 6.98 and δ 7.59 with a low *J*-value (2.4 Hz) establised its disubstituted furan moiety.

For SD, similar ^{1}H NMR resonances for the furanocoumarin moiety were present. The additional 1-proton singlet at the aromatic region (δ 6.77) and the absence of the more up field signals established that one of carbons of the coumarin phenyl group was unsubstituted by the epoxyhydroxymethyl group as in BA.

On the other hand, the ^{1}H NMR spectral features of furocoumarin moiety in furopinnarin (FP) were similar to that of BA. However, the pair of ^{1}H NMR resonances at δ 5.58 (t, 1H) and δ 4.82 (d, 2H) established the presence of a terminal olefin group, while that of a *gem*-dimethyl group was inferred from the two 3-protons singlets at δ 1.68 and 1.71.

9-((3,3-Dimethyloxiran-2-yl)methoxy)-4-methoxy-7*H*-furo[3,2-g]chromen-7-one [(±)-byakangelicol]: Yield: 29.5 mg (0.21%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.08 (d, *J* = 9.8 Hz, 1H), 7.59 (d, *J* = 2.4 Hz, 1H), 6.98 (d, *J* = 2.4 Hz, 1H), 6.23 (d, *J* = 9.8 Hz, 1H), 4.39 (d, *J* = 5.6 Hz, 2H), 3.26 (t, *J* = 5.6 Hz, 1H), 4.15 (s, 3H), 1.29 (s, 3H), 1.21 (s, 3H).

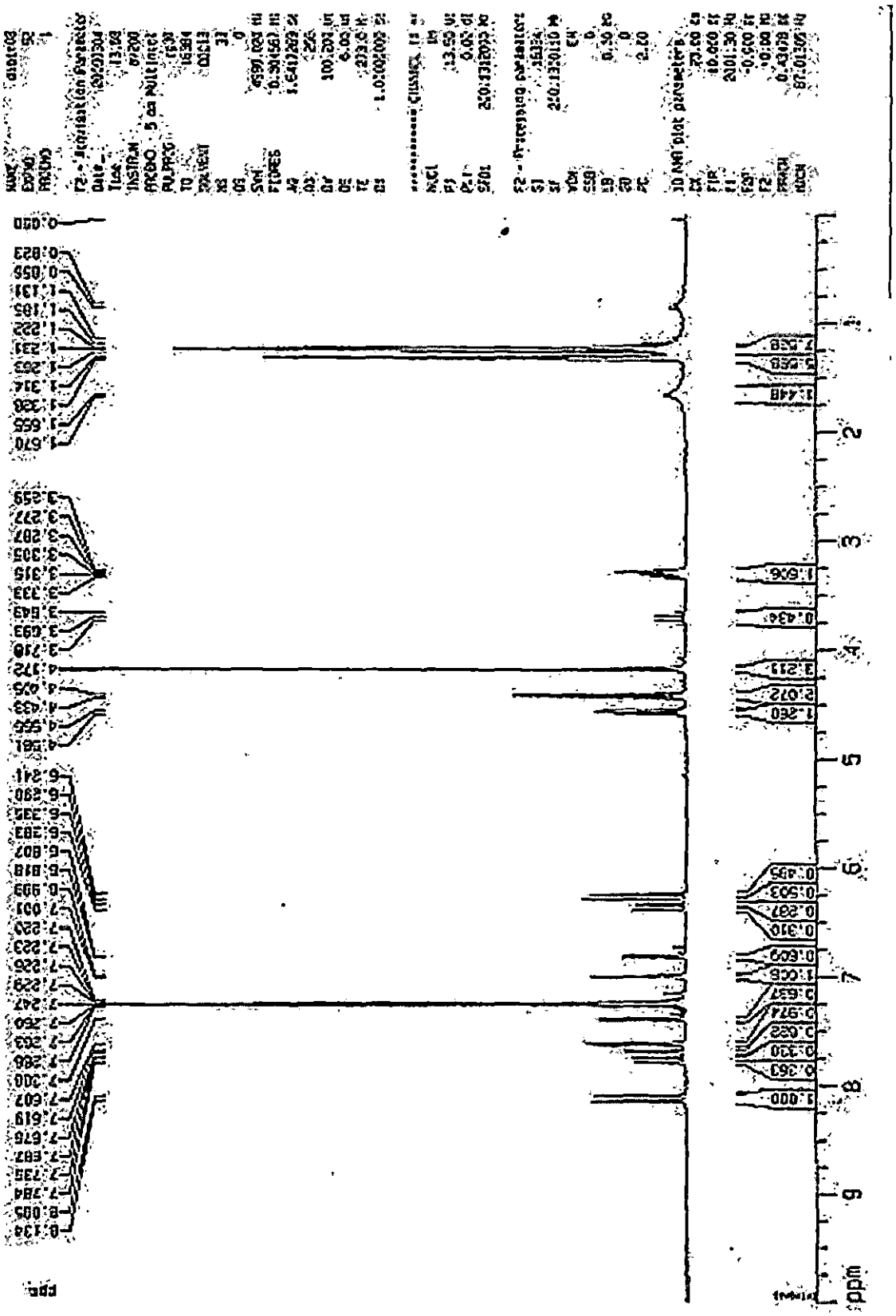


Fig. 13. ^{1}H NMR spectrum of byak-angelicol

6-Methoxy-2*H*-furo[2,3-*h*]chromen-2-one (sphondin): Yield: 10.5 mg (0.075%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.75 (d, *J* = 9.6 Hz, 1H), 7.69 (d, *J* = 2.2 Hz, 1H), 6.99 (d, *J* = 2.2 Hz, 1H), 6.77 (s, 1H), 6.39 (d, *J* = 9.6 Hz, 1H), 3.93 (s, 3H).

4-Methoxy 9-(2-methylbut-3-en-2-yl)-7*H*-furo[3,2-g]chromen-7-one (furopinnarin): Yield: 15.6 mg (0.11%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.09 (d, *J* = 9.8 Hz, 1H), 7.60 (d, *J* = 2.3 Hz, 1H), 6.97 (d, *J* = 2.4 Hz, 1H), 6.24 (d, *J* = 9.8 Hz, 1H), 5.58 (t, *J* = 7.2 Hz, 1H), 4.82 (d, *J* = 7.2 Hz, 2H), 4.15 (s, 3H), 1.71 (s, 3H), 1.68 (s, 3H).

4.12.2. Alkaloids from *S. hernandifolia* roots

The root extract of *S. hernandifolia* also showed a good antioxidant activity, as well as an excellent antibacterial activity against all Gram-positive and Gram-negative (except *E. coli*) bacteria. Especially, its activity against the Gram-positive bacterium *L. monocytogenes* was exceptionally good. Hence, a preliminary phytochemical investigation of the extract was carried out. The extract was found to be enriched with several alkaloids as revealed by the Dragendorff test. From the acid soluble portion of the extract, a mixture of alkaloids was isolated. However, due to the close R_f of the constituents, the compounds could not be purified.

4.12.3. Antioxidant and antimicrobial activities of *H. nepalense* furanocoumarins

4.12.3.1. DPPH•-scavenging

None of the furanocoumarins showed significant DPPH•-scavenging activity. Amongst these, BA was marginally (15%) active at 0.2 mM, which did not improve at the higher concentrations. SD and FP, on the other hand, showed 21% scavenging at 1 mM.

4.12.3.2. Anti-LPO

All the compounds showed a dose-dependent protection against Fenton-mediated LPO. BA and SP showed a better protection, while the anti-LPO of FP was manifested at the higher concentrations.

4.12.3.3. •OH-scavenging

Amongst the furanocoumarins, FP showed some (25%) •OH-scavenging activity only at 0.8 mM. The other two compounds scavenged the radical dose-dependently. BA was the most active amongst the tested furanocoumarins. The entire antioxidant data are summarized in Table 17.

4.12.3.4. DNA protection

Exposure of pBR322 plasmid DNA to Fenton reagent led to the formation of open circular and linear forms. BA restored the supercoiled (SC) form up to 5 μg ml^{-1}, but led to drastic DNA damage beyond 5 μg ml^{-1}, as revealed from the reduction of the SC form (data not shown).

On the other hand, FP restored the SC form dose-dependently, as revealed from the increase in the SC form that was reduced (80.6%) drastically on exposure to the Fenton reagent. The results are summarized in Fig. 14 and 15 and Tables 18 and 19.

4.12.4. Antimicrobial activity of isolated furanocoumarins

All the compounds showed moderate antibacterial activity against the Gram-positive and Gram-negative bacteria. BA showed better inhibition (100 μM) against all the tested bacteria. Interestingly, they were also

Table 17. Concentration-dependent DPPH·-scavenging, Fenton-mediated lipid peroxidation inhibitory and hydroxyl radical-scavenging activities of the isolated byak-angelicol (BA), sphondin (SD) and furopinnarin (FP)[a]

Furanocoumarin	%DPPH·-scavenging[a]			nmol malonaldialdehyde mg^{-1} protein[b]			%OH·-scavenging[c]		
(mM)	BA	SD	FP	BA	SD	FP	BA	SD	FP
0.2	14.6±0.94[1]	4.8±0.58[1]	2.3±0.50[1]	15.7±1.44[1]	16.4±2.20[1]	1.5±1.07[1]	28.7±1.78[1]	15.9±0.60[1]	2.5±1.40[1]
0.4	14.2±0.52[1]	6.6±0.22[1]	10.2±1.16[2]	21.3±1.78[2]	27.5±0.80[2]	7.4±0.62[2]	32.2±2.52[1,2]	18.5±0.68[2]	6.7±1.84[2]
0.6	14.1±1.08[1]	9.6±0.14[2]	11.4±1.62[2,3]	39.3±0.81[3]	38.3±2.45[3]	15.7±0.90[3]	34.9±2.11[2]	22.6±1.22[3]	10.4±1.31[3]
0.8	15.2±0.71[1]	12.8±1.48[3]	12.6±1.76[3]	41.4±1.62[3,4]	42.5±1.84[3]	26.2±1.88[4]	44.2±1.66[3]	34.6±1.54[4]	24.8±2.12[4]
1.0	15.4±1.40[1]	20.8±1.82[4]	18.8±0.91[4]	44.2±2.12[4]	52.2±3.54[4]	49.2±2.67[5]	0	0	0

[a] Values are mean with standard error of measurements (n = 3). Means with same superscript in a given column are the same at $P<0.05$

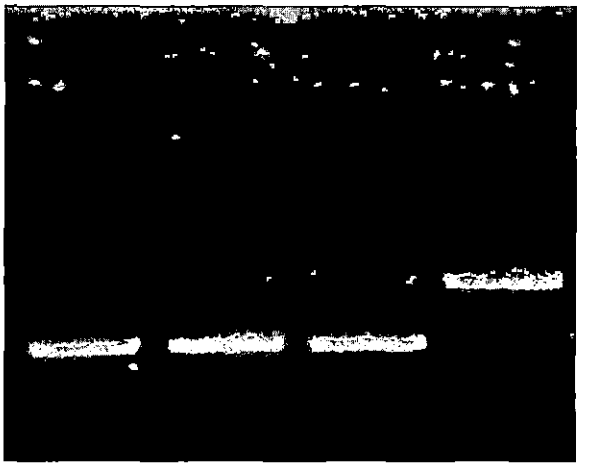


Fig.14. Gel electrophoresis pattern exhibiting the effect of byak-angelicol (BA) on Fenton-mediated plasmid DNA damage. Lane 1, native DNA; Lanes 2-3, native DNA + Fenton reagent + BA (2.5 and 5μg ml^{-1}, respectively); Lane 4, native DNA + Fenton reagent

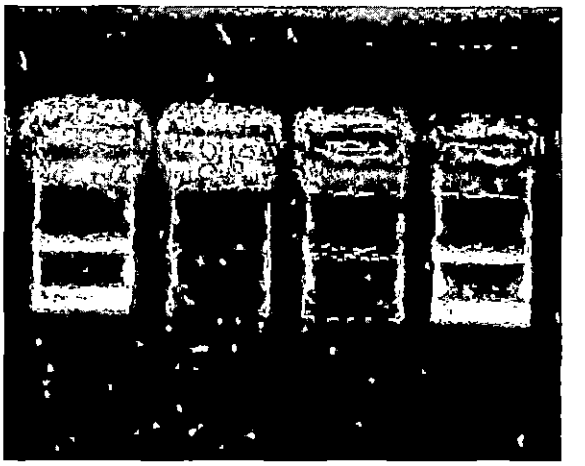


Fig. 15. Gel electrophoresis pattern of plasmid pBR322 exposed to Fenton reagent in the presence and absence of different concentrations of furopinnarin (FP). Lane 1, native DNA; Lane 2, native DNA + Fenton reagent; Lanes 3-5, native DNA + Fenton reagent + FP (2.5 and 5 μg ml^{-1}, respectively).

Table 18. Quantification of DNA bands in different lanes of Fig. 14

Sample	% supercoiled DNA	% protection
native DNA	95.26	100
DNA + Fenton reagent (A)	8.48	0
A + byak-angelicol (2.5 μg ml^{-1})	89.71	93.60
A + byak-angelicol (5 μg ml^{-1})	78.56	80.76

Table 19. Quantification of DNA bands in different lanes of Fig. 15

Sample	% supercoiled DNA	% protection
native DNA	88.73	100
DNA + Fenton reagent (A)	12.03	0
A + furopinnarin (2.5 μg ml^{-1})	42.91	40.20
A + furopinnarin (5 μg ml^{-1})	80.64	89.45

effective against two Gram-negative bacteria at the same concentration range; *E. coli* being more resistant to all of them. In general, BA and FP were more potent than SD (Table 20).

Table 20. Concentration-dependent antibacterial activities of the isolated byak-angelicol (BA), sphondin (SD) and furopinnarin (FP)[a]

Furano-coumarin (μM)	*Listeria monocytogenes*			*Escherichia coli*			*Salmonella sp.*			*Bacillus subtilis*		
	BA	SD	FP	BA	SD	FP	BA	SD	FP	BA	SD	FP
100	56.3±1.4	52±0.7	63.7±2.1	34.3±1.8	38.8±2.8	48.3±1.1	70.5±0.4	51.1±0.9	73.7±0.4	75.6±0.4	68.6±1.6	70.1±0.5
80	42.6±0.8	34±1.5	55.5±1.4	22.4±2.5	26.7±2.5	30.4±2.4	52.6±0.8	47.5±0.6	55.5±0.7	62.1±0.7	53.9±0.5	52.5±0.4
60	20.6±1.6	30±1.8	42.8±1.7	23.7±2.2	13.1±1.6	26.6±0.8	42.5±0.8	35.7±0.5	32.5±0.8	56.3±0.9	44.4±0.8	46.8±0.6
40	14.3±0.6	15±1.1	20.4±2.2	10.1±1.5	6.8±1.2	14.2±0.4	36.2±0.6	29.1±0.4	11.3±0.3	41.3±0.3	26.2±0.6	35.4±0.5
20	0	0	5.7±0.8	0	0	8.1±0.9	23.7±0.3	8.4±0.7	9.6±0.2	25.8±0.2	15.7±0.5	11.2±0.3

[a]Values (% inhibition) are mean with standard error of measurements (n = 3).

5
Discussion

5.1. Overview

Amongst the various deadly diseases of the 21st century, the most dangerous ones are caused either by microbial infections or by free radicals. With an increase in the drug resistance among microbes, our age-old "magic bullets" are failing sharply, and medical researchers all over the globe are struggling hard to develop multiple drug therapy or screening new sources for antimicrobial agents. On the other hand, oxidative stress, due to various factors including atmospheric pollutants, cigarette smoking, ultraviolet rays, radiation, toxic chemicals, over-nutrition and advanced glycation end products (AGEs) in diabetes, shifts the delicately maintained balance between generation of free radicals and antioxidant defense in favour of pro-oxidants. Oxidative stress has been implicated in the etiology of several (>100) human diseases and in the process of ageing (Halliwell, 1997; Harman, 1992; Shigenaga et al., 1994).

In the present effort to develop some inexpensive and nontoxic antioxidant and antimicrobial formulation, 38 parts from 36 indigenous herbs of Darjeeling Himalaya were chosen based through a survey, and their extracts/constituent chemicals were screened for their *in vitro* antioxidant and antimicrobial activities. Antioxidant activity of the most potent extracts was subsequently examined in further details, using some well-accepted, chemically well-defined and biologically relevant *in vitro* models. The chemical

compositions of the two most potent antioxidant and antimicrobial plants were also analyzed by chromatographic techniques, and a preliminary assessment of the anticancer activity of one of the extracts was made using two human cancer cell lines.

5.2. Survey

The main aim of the survey of the three hilly subdivisions of the District of Darjeeling in the State of West Bengal was to unfold the ethnic uses of the plants by the local tribals in curing various ailments. It was found that the traditional drugs are prepared as paste, decoction, water extract and pills/tablets using the plant parts, in combination with additives like animal portions, salt etc., and factors such as day or patient's posture during drug administration are believed to be crucial in their efficacy. However, no script of this system is available. Since the younger generations are reluctant to learn this practice, the knowledge usually perishes with the death of the practitioners. In this context, Baidya Chewang Pakhrin's name is worth-mentioning for his successful practice in herbal drugs. He has been engaged in treating bone fracture with herbal medicine of Darjeeling hills for over the last 50 years and claims to have inherited the knowledge from his forefathers (Bhujel, 1996).

The modern infrastructure developmental activities have led to uncontrolled grazing and uprooting of the plants by the locals or external agencies, posing a great threat to the flora of medicinal plants of Darjeeling area (Rai *et al.*, 1998). Plants like *Panax pseudoginseng, Gloriosa superba, Clematis buchchaniana, Zanthoxylum acanthopodium* etc. are nearing extinction. Mass awareness of the threat and replanting programmes by the forest department and government authorities appear to be the only solution for the problem. Further, authentication of the plants and validation of their medicinal properties based on accepted scientific methodologies is also essential to strengthen the 'evidence-based alternative medicine system'. The present survey, aimed at some of these aspects, revealed 106 medicinal plants (Table 5). From this, 36 herbs were selected for the bio-evaluation based on the three following rationale:

- Easy availability, mostly throughout the year.
- Extensive medicinal use among the villagers.
- Probable nontoxicity due to their wide consumption in different forms as foods.

5.3. Screening of antioxidant activity

A growing body of research is focused on nutritional and pharmaceutical prevention of the oxidative stress-related diseases, and testing for antioxidant protection has become a major focus in the dietary and natural product industries. Hence, the screening of antioxidant activity of the herbs was carried out. Initially five parameters, *viz.* DPPH•-scavenging, reducing power (RP) and metal-ion chelation (MC) capacities of the herbs as well as their total phenolic and flavonoid contents (TPC and TFC, respectively) were chosen for the screening. The choice of the parameters was based on the following considerations.

The DPPH• is considered biologically relevant due its comparable stability with that of lipid peroxyl radical that constitutes one of the major biological markers produced during oxidative stress. The DPPH•-scavenging assay is a simple and reliable protocol for measuring the antioxidant activity of fruits and vegetables, juices or extracts, and even pure compounds. This has been applied to assess the antioxidant activities of hydrolyzable tannins (Yoshida *et al.*, 1989), pure polyphenolic compounds (Brand-Williams *et al.*, 1995; Yokozawa *et al.*, 1998) in *Salvia officinalis* (Wang *et al.*, 1998), pure phenolic acids and derivatives (Silva *et al.*, 2000) and flavonoids (Burda and Oleszek, 2001; Lebeau *et al.*, 2000), and as constituents of fresh orange juices (Rapisarda *et al.*, 1999), nontannin phenolics including resveratrol (Tadolini *et al.*, 2000), curcumin (Noguchi *et al.*, 1994), gallic acid, catechin and stilbenes from *Polyginum multiflorum* extracts

(Chen *et al.*, 1999) and other plants (Amarowizc *et al.*, 2000). The antioxidant properties of processed foods, such as grains (Minamiyama *et al.*, 1994), jams from red raspberry (Zafrilla *et al.*, 2001), storage protein from *Dioscorea batata* (Hou *et al.*, 2001), pomegranate juice (Gil *et al.*, 2000) and aged red wines (Larrauri *et al.*, 1999) are also analysed by this method.

Apart from radical-scavenging, the ability of the extracts to reduce Fe(III) to Fe(II), known as reducing power (RP), correlates well with its capacity to reduce free radicals. It has been reported that RP is a significant reflection of the antioxidant activity of various extracts/compounds and with good correlation (Pin-Der-Duh, 1998; Tanaka *et al.*, 1988). Compounds with high RP would behave as strong electron donors and can reduce the oxidized intermediates of lipid peroxidation (LPO), so that they can act as primary and secondary antioxidants (Yen and Chen, 1995). The reducing properties are generally associated with the presence of reductones (Pin-Der-Duh, 1998), which exert antioxidant action by breaking the free radical chains, via hydrogen atom donation (Gordon, 1990). Reductones are also reported to prevent peroxide formation, by reacting with certain precursors of peroxide.

Besides oxygen metabolism, various reactive oxygen species (ROS) can be formed in cells by transition metal (especially Fe(II))-mediated reactions (Waling, 1975) leading to deleterious effects on membrane lipids and DNA. The transition metal ions, such as iron and copper ions, are omnipresent in cellular systems. Consequently, MC was chosen as an important parameter for the antioxidant screening. Various cellular reducing agents such as ascorbic acid, NADPH and NADH can easily convert them to the lower oxidation states (Fe(II) and Cu(I)) that can take part in the Fenton reaction to generate the most active ROS, hydroxyl radicals ($^{\bullet}OH$). This, in turn, initiates the radical chain reaction to cause LPO, one of the major factors in pathophysiology (Nawar, 1996). Agents that chelate transition metals may inhibit the Fenton-mediated radical generation and inhibit/reduce free radical damage. Some phenolic compounds exhibit antioxidant activity primarily through the chelation of metal ions. Essentially, chelation of the metals renders them less/nonreactive in the Fenton process, reducing the ROS formation, and thereby creating oxidative stress. For example, the iron chelator, 1,10-phenanthroline, is known to follow the latter mechanism in its antioxidant action (Mello-Filho and Meneghini, 1991). Iron chelators, such as desferoxamine, are often the only options against the iron-overload diseases.

Thus, overall, all the chosen assays provide basic measure of the primary and secondary antioxidant activities of the test samples, and have been used to ascertain antioxidative potential of the 38 herb extracts. Amongst the herbs, *F. nubicola* root (FNR), *P. hirta, E. fluctuens, O. tenuiflorum, Pteris* sp., *C. buchchaniana, P. major, C. album, D. indica* and *G. superba* were found to show >80% DPPH$^{\bullet}$-scavenging capacity, and hence was classified as "excellent" (Table 6). On the other hand, *S. hernandifolia, L. indica, A. rivularis* and *C. rotundus* with DPPH$^{\bullet}$-scavenging activity of 60-79% were categorized as "good". Most of the herbs falling under these categories also showed high RP, although some herbs in the "moderate" category also possessed a good RP. On the other hand, the iron chelation capacity of the herbs was scattered among the different categories. The highest chelation was shown by *C. album* (excellent scavenging) > *P. minima* (poor scavenging) > *O. tenuiflorum* (excellent scavenging) > *E. fluctuens* (excellent scavenging).

It is well-known that plant phenolics, in general, are highly effective free radical-scavengers and antioxidants. Consequently, the antioxidant activities of herbal extracts are often explained with their total phenolic contents (Skerget *et al.*, 2005). Amongst the phenolics, the flavonoids are possibly valued most because of their potential beneficial effects on human health. Most of our dietary sources such as fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks) are enriched with flavonoids and many of these are reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumour and antioxidant activities (Grotewold, 2006).

Hence, TPC and TFC of the extracts were also determined. The results revealed that, in general, the pteridophytes contained high phenolics (Table 7). Notably, *Pteris* sp. had excellent TPC and TFC, while the phenolics present in FNR were primarily flavonoids. Qualitatively, the excellent DPPH•-scavenging property of them might be accounted by the TPC and TFC parameters. However, similar correlation of the scavenging activity is hard to be established with plants such as *H. cordata*, *E. debile*, *C. buchchaniana* leaf (high TPC) as well as *P. peruviana*, *S. hernandifolia* and *P. foetida* (good TFC). Interestingly, *C. album* and *G. superba* exhibited a very good antioxidant activity, despite showing low TPC and TFC. This called for a better correlation amongst the chosen antioxidant parameters by a statistical analysis. Hence, the relationship between the three antioxidant parameters (DPPH•-scavenging, RP and MC) of the plant extracts with their TPC and TFC contents (Tables 6 and 7) was attempted using the principal component analysis (PCA) (Table 8). This was felt to establish the inter-relationship among these five parameters.

5.4. PCA

PCA of the five antioxidative parameters of 38 herb extracts resulted in three main principal components (PCs). For each PC a large loading of a specific variable indicates that it contributes strongly to the value of PC (Fig. 4). PC1 presented a large loading (>0.7) by DPPH•-scavenging and RP. MC has a large negative loading on PC2, while TFC is loaded on both PC1 and PC2. TPC, on the other hand, was the one contributing most to PC3, with >0.9 loading. It seems that TPC and TFC were not closely loaded on the same PC. This may be because the method employed for the estimation of TPC of the herbs using Folin Ciocalteau (FC) reagent lacks specificity. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids, present in the FC reagent, resulting in lack of specificity. Besides the polyphenols, the assay can also incorporate other substances that could be oxidized by the FC reagent. Various researchers have reported poor specificity of the assay (Escarpa and González, 2001; Singleton *et al.*, 1999). Also, depending on the number of phenolic groups present in them, compounds respond differently to the FC reagent (Singleton *et al.*, 1999). The opposite high loading of TFC and MC on PC2 clearly indicated that a high amount of phenolic contents does not necessary translates into a high Fe(II)-sequestering activity. This means that the secondary antioxidant properties are not directly related to the primary antioxidant property.

So, it is envisaged that compared to RP and MC, the DPPH•-scavenging assay results might be a better diagnostic tool of the antioxidant activity of the plant extracts. Based on the results of this assay, the 38 herbal extracts were classified in four categories (Table 6), and 14 herbs, belonging to the "excellent" category were chosen for more elaborate bio-evaluation.

5.5. *In vitro* antioxidant activity-based selection of herbs

Due to chemical diversity of the antioxidant compounds present in herbs, comprehensive databases on herb antioxidant content are not yet available. The levels of single antioxidants in herbs do not necessarily reflect their total antioxidant capacity (TAC) since this also depends on the synergic and redox interactions among the different molecules present in the plants. Several methods, differing in their chemistry (generation of different radicals and/or target molecules) and detection of end points, have been developed for measuring the TAC of plants. Of these, the prevailing methods such as oxygen radical absorption capacity (ORAC) (Cao *et al.*, 1993) and total radical trapping antioxidant parameter (TRAP) (Ghiselli *et al.*, 1995) apply various probes (fluorescein, $ABTS^{\bullet+}$, pyrogallol red, phycoerythrin, crocin and pyranine) as reference compounds, and the antioxidant capacity is assessed by the extent of consumption of the probe, measured by spectrophotometry or fluorescence. Lag phase and rate of probe decay as well as the area under the curve (AUC) of the probe-decay, compared to that of the blank are measured (Prior *et al.*, 2005). The use

of an azo initiator (AAPH) is most convenient to generate free radicals at a known, constant and controlled rate which enables quantitative assessment. Because different antioxidant compounds may act *in vivo* through different mechanisms, no single method can fully evaluate the TAC of plants.

Herbal antioxidants often broadly include radical chain breakers, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors. The antioxidant action of a test sample is mediated by hydrogen atom transfer (HAT) and single electron transfer (SET) reactions. Hence, both HAT-based (DPPH•-scavenging, ORAC and TRAP) and SET-based (RP and TPC) methods as well as iron chelation were selected in the present studies. The SET-based assays measure the reducing capacity, while the HAT-based assays quantify hydrogen atom donating capacity of the antioxidants. Thus, herbs belonging to the designated excellent and good categories were tested for their •OH and $ABTS^{\bullet+}$-scavenging, ORAC and anti-LPO properties.

The $ABTS^{\bullet+}$-scavenging is extremely useful for the estimation of the antioxidant activity of both lipophilic and water-soluble pure compounds as well as complex mixtures. An improved version of it was adopted in this study, where the $ABTS^{\bullet+}$ was generated by persulfate oxidation (Re *et al.*, 1999). Also with the same assay, relative antioxidant activities in the aqueous phase of plant-derived polyphenolic flavonoids and phenolic acids are measured (Rice-Evans *et al.*, 1995; Salah *et al.*, 1995). The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink were evaluated by the former assay (Miller and Rice-Evans, 1997). The result of $ABTS^{\bullet+}$-scavenging assay by the tested herbs were consistent with their DPPH•-scavenging capacity (Gil *et al.*, 2000).

For the ORAC assay, the AUC approach is equally suitable for antioxidants that exhibit distinct or no lag phases. This approach unifies the lag time and initial rate methods, and is particularly useful for herb/food samples, which often contain multiple ingredients and have complex reaction kinetics. The ORAC assay has provided substantial information regarding the antioxidant capacity of pure compounds such as flavonoids (Cao *et al.*, 1997) and caffein (Lee, 2000), food complex matrix, such as common vegetables (Caldwell, 2001; Cao *et al.*, 1996; Guo *et al.*, 1997; Kalt *et al.*, 1999), fruits (Wang *et al.*, 1996), berries (Wang and Lin, 2000), cocoa and chocolate (Adamson *et al.*, 1999), tea (Cao *et al.*, 1996; Prior and Cao, 1999), and oat (Handelman *et al.*, 1999). Also, ORAC assay is useful to evaluate the influence of cultivar and storage temperatures on the antioxidant activity of cranberries (Wang and Stretch, 2001) and in bioavailability human studies of vitamin C, carotenoids, anthocyanins and other phenolic compounds from berries, fruits, vegetables and wine (Cao and Prior, 1999; Cao *et al.*, 1998; Ehlenfeldt and Prior, 2001). In this study, extracts with top three ORAC values belonged to the "excellent" category. In addition, the herbs, *S. hernandifolia*, *A. rivularis*, and *C. rotundus* showed "good" results in both ORAC and DPPH• assays, while *D. indica* (excellent DPPH• scavenger) scored "good" ORAC value.

Due to their high reactivity, the •OH has a very short biological half-life and is the most damaging ROS (O'Neill and Fieden, 1993). The •OH-scavenging ability of a test sample can be conveniently evaluated by the Fenton-mediated 2-deoxyribose (2-DR) assay, and was used in the present work. Among the best four •OH scavengers, three herbs, *viz.* FNR, *C. album* and *P. major* belonged to the "excellent" category, while *D. indica*, *S. hernandifolia* and *L. indica* belonging to the "good" category were also impressive •OH scavengers (Table 9). The poor/non-activity *of C. buchchaniana* leaf and *O. tenuiflorum* indicated that these may be more effective in lipophilic radicals.

Owing to high levels of unsaturation and increased consumption of oxygen, mitochondrial lipids are susceptible to oxidative damage. Lipid peroxidation can inactivate cellular components and play a major role in oxidative stress in biological systems. Several toxic byproducts of the peroxidation can, in turn, damage other biomolecules, including DNA, far away from the site of their generation (Box and Maccubbin

1997; Esterbauer, 1996). Therefore, compounds possessing anti-LPO activity are extremely important for health benefit and food preservation. Hence, anti-LPO activities of the herbal extracts were studied using Fe(II)/ascorbic acid-induced peroxidation of mice liver homogenate as the model system. The anti-LPO potential was measured by the thiobarbituric acid reactive substances (TBARS) assay which revealed comparable protective activity of the extracts of FNR, *G. superba*, *S. hernandifolia*, *O. tenuiflorum* and *D. indica*, as that of BHA (Table 10).

These stepwise screening experiments finally confirmed the antioxidant activity of the designated samples and established FNR as the most potent extract.

5.6. Antioxidant activities of FNR

Till date there is no report on the antioxidant activity of *F. nubicola*, although the antioxidant, anti-allergic and antimicrobial activities of the fruits and leaves of its close relative, *F. ananassa* (Masahiro *et al.*, 2007; Wang and Lin, 2000), and the berry phenols (Heinonen, 2007) have been evaluated. Hence, a comprehensive analysis of the antioxidant property of FNR was taken up using several biological targets like lipids, proteins and DNA, as discussed below.

5.6.1. Free radical-scavenging

The activity was tested against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ at various concentrations (Fig. 5 and 6). Amongst these, bleaching of $DPPH^{\bullet}$ absorption by the test compound is representative of its capacity to scavenge free radicals which are generated independent of any enzymatic or transition metal-based systems. THe $DPPH^{\bullet}$ assay results revealed excellent scavenging activity of FNR compared to BHA, which was also confirmed by the $ABTS^{\bullet+}$ assay.

5.6.2. RP

The iron-reducing power was found to increase in a concentration-dependent manner (10-100 μg ml^{-1}). Therefore, the single electron transferring capacity of FNR was found impressive through the assay (Fig. 7).

5.6.3. Anti-LPO

Owing to high levels of unsaturation, lipids are susceptible to oxidative damage. LPO can inactivate cellular components and play a major role in oxidative stress in biological systems. Several toxic byproducts of the peroxidation can, in turn, damage other biomolecules including DNA, far away from the site of their generation. Therefore, compounds possessing anti-LPO activity are extremely important for health benefit and food preservation. It was revealed that consistent with their relative TAC, FNR was significantly active against Fenton-mediated LPO, showing almost equal potency as BHA (Fig. 8).

The excellent anti-LPO activity of FNR might be due to their better radical-scavenging and/or Fe(II)-chelating abilities. Consequently, for a better understanding of the operative mechanism, the anti-LPO activity of FNR against the AAPH-induced LPO was investigated. AAPH is an efficient free radical generator and is extensively used for inducing LPO (Visioli *et al.*, 2000). The alkyl peroxyl radicals produced from AAPH, which causes LPO, are very similar to radicals produced in biological systems. Thus, the preventive capacity of a test compound against the AAPH-induced LPO provides a good measure of its anti-LPO activity in an iron-independent system. Interestingly, in the AAPH-mediated assay, FNR showed less potency than that in the Fenton-mediated process (IC_{50}, 10 and 14 μg ml^{-1}, respectively). These results revealed that the anti-LPO activity of FNR may be due to both radical-scavenging and Fe(II)-chelation properties.

5.6.4. DNA protection

The ROS generated by the Fenton process can react with DNA due to the presence of various reactive sites (base and sugar) in them (Breen and Murphy, 1995). The pathophysiological implication of the ROS-mediated DNA damage is well-known. In view of this, the protective capacity of FNR against Fe(II)-induced DNA nicking was also assessed. Exposure of the supercoiled plasmid DNA to the Fenton reagents led to extensive DNA nicking, producing a significant amount of the linear form. The results revealed an extraordinary potency of FNR in protecting oxidative DNA damage (Fig. 9), since even at a very low concentration (0.5 $\mu g\ ml^{-1}$), it prevented the formation of the linear form and restored the supercoiled DNA form by 62.7% of the control value (Table 11).

5.6.5. Protein protection

Protein oxidation is inherent to aerobic life. Oxidation of membrane proteins by ROS, a process independent of LPO, is also a highly damaging event whose significance has been realized more recently (Dean *et al.*, 1997; Stadtman, 1992). Activated oxygen species and other free radicals, generated as byproducts of cellular metabolism or by photochemical reaction, modify amino acids of proteins. Subsequently, loss of protein structure and function can occur through denaturation, fragmentation and aggregation. It has been established that most amino acids are susceptible to oxidation by $^{\bullet}OH$ or $O_2^{\bullet-}$ (Davies, 1987). Once oxidized, proteins are degraded by the proteosome complex and by lysosomal hydrolases. Alternatively, they can be repaired by antioxidants.

In the present studies, the protective activity of FNR against γ-radiation-induced protein oxidation of BSA was investigated in terms of quenching of its fluorescence. Loss of tryptophan and tyrosine fluorescence is an early event of protein oxidation, and was used in the present studies (Rampon *et al.*, 2001). The reduction in the protein fluorescence at 345 nm revealed appreciable protein oxidation on exposure of BSA to γ-rays of 300 Gy (Table 12). Even at a low concentration (10 $\mu g\ ml^{-1}$), FNR could provide significant protection. Body exposure to ionizing radiation leads to radiolysis of cellular water, giving rise to the formation of various ROS ($^{\bullet}OH$, superoxide radical and H_2O_2). These, in turn, cause the damages in biological systems, while the contribution of direct radiation-induced damage is much less (von Sonntag, 1987). The excellent protective property of FNR against the γ-rays-induced protein oxidation confirmed FNR as a powerful $^{\bullet}OH$ scavenger.

5.6.6. Antiproliferative activity

Redox dysregulation originating from metabolic alterations and dependence on mitogenic and survival signaling through ROS represents a specific vulnerability of malignant cells that can be selectively targeted by redox chemotherapeutics. Differential redox set points in cancer versus nontransformed normal cells provide a therapeutic window of sufficient width permitting redox intervention that selectively targets cancer cells with constitutively upregulated levels of ROS. Also, simultaneous modulation of multiple redox sensitive targets by these agents can overcome drug resistance originating from redundancy of oncogenic signaling and fast mutation (Cabello *et al.*, 2007; Desagher and Martinou, 2000). Natural products, including plants, microorganisms and marine lives provide rich resources for anticancer drug discovery (Schwartsmann *et al.*, 2002). Higher plants have long been shown to be an excellent and reliable source of novel anticancer drugs. Earlier, strawberry and raspberry were shown to inhibit the growth of certain cancer cell lines *in vitro* (Liu *et al.*, 2002; Meyers *et al.*, 2003; Wedge *et al.*, 2001). Hence, the cytotoxicity of FNR against human breast and lung cancer cell lines was checked. Because different components in a herb may potentiate the power of the active constituent (synergism) or buffer its toxic effect, the crude FNR extract was used for the study.

FNR showed a good potency in inhibiting proliferation of both the cells (Fig. 10). Interestingly, it was more effective against the lung cancer (A-549) cells, showing similar potency as that of curcumin. A separate examination with the human intestinal (INT-407) cells established it to be nontoxic to normal cells. These results warrant further exploration of FNR as a chemopreventive agent.

5.7. HPLC profile of FNR

The chemical composition of herbs is known to vary depending on their origin, parts, stage of maturity, season of collection, agronomic conditions etc. Hence, it is mandatory to identify and quantify the major chemical constituents in any herbal preparation. Earlier, strawberry (*Fragaria ananassa*) fruit was found to be an excellent source of antioxidants, such as flavonols, anthocyanins, flavanols, hydroxycinnamic and benzoic acid derivatives, ellagic acid glycosides and ellagitannins (Aaby et al., 2005). Many of these phenolic compounds, present in the extracts of berries of the Rosaceae family, show strong antioxidative potential (Kähkönen *et al.*, 2001; Liu *et al.*, 2002; Wang and Lin, 2000; Wang and Zheng, 2001) and also inhibit the growth of pathogenic bacteria (Puupponen-Pimiä *et al.*, 2001; Rauha *et al.*, 2000).

To this end, the HPLC analysis of the acid hydrolyzed products of FNR was carried out. The results (Fig. 11 and Table 13) revealed FNR to be rich in ascorbic acid, gallic acid and dihydrocaffeic acid, besides containing small amounts of ellagic acid, genistic acid, caffeic acid and *o*-coumaric acid.

5.8. Antimicrobial activity of herbs

Plants are known to produce an enormous variety of small-molecule (MW, <500) antibiotics, generally classified as 'phytoalexins'. Their structural space is diverse having terpenoids, glycosteroids, flavonoids and polyphenols. Be that as it may, it is interesting to note that most of these small molecules have weak antibiotic activity – several orders of magnitudes less than that of common antibiotics, produced by bacteria and fungi. In spite of the fact that plant-derived antibacterials are less potent, plants fight infections successfully. Hence, it becomes apparent that plants adopt a different paradigm – "synergy" – to combat infections (Hemaiswarya, 2008).

The antimicrobial effect of the medicinal plants is well-documented (Valero and Salmeron, 2003). The antimicrobial activity of found in folk medicines (Ngwendson *et al.*, 2003), essential oils (Alma *et al.*, 2003) or isolated compounds such as alkaloids (Klausmeyer *et al.*, 2004), flavonoids (Sohn *et al.*, 2004), sesquiterpene lactones (Lin *et al.*, 2003), diterpenes (El-Seedi *et al.*, 2002), triterpenes (Katerere *et al.*, 2003) or naphthoquinones (Machado *et al.*, 2003) has been in major focus recently. Recio *et al.* (1989) compiled a list of 75 plant species in which the authors had established the activity of the extract along with both the spectrum and the principles responsible for this activity. The results of different studies provide evidence that some medicinal plants might indeed be potential sources of new antibacterial agents even against some antibiotic-resistant strains (Koné *et al.*, 2004).

Phenolics are the predominant active chemical in plants. Gram-positive bacteria are more sensitive than the Gram-negative ones, but the major problem is the lack of uniformity in the criteria selected to study the activity. Ríos *et al.* (1988) proposed the use of diffusion methods (polar compounds of small or medium molecular size) and solid dilution method (polar, nonpolar substances and all types of complex extracts) for testing the relative potency of extracts by facilitating the use of different strains against the extract on the same plate. However, liquid dilution method is the best way to establish the potency of a pure compound.

Surprisingly, despite its vast potential, the antimicrobial property of the natural flora of Darjeeling Himalaya has not been extensively screened yet, although specific region or country-wise studies are quite

popular. Examples include studies of medicinal plants from Brazil (Duarte *et al.*, 2005), Thailand (Wannissorn *et al.*, 2005), Turkey (Uzun *et al.*, 2004), Lebanon (Barbour *et al.*, 2004), Argentina (Salvat *et al.*, 2004), Colombia (López *et al.*, 2001) and India (Jeevan Ram *et al.*, 2004), to cite a few. In this study, using the disc diffusion method the antimicrobial activity of the 38 herbal extracts of Darjeeling Himalaya was checked against bacteria, yeasts and moulds (Tables 14-16).

Surveillance data from the International Antimicrobial Therapy Group (IATG) of the European Organisation for Research and Treatment of Cancer (EORTC) show a shift in the etiology of infection and in the patterns of resistance (Viscoli, 2002). Before the mid-1980s Gram-negative bacilli were the predominant pathogens associated with bacteraemia. In trials undertaken during the late 1980s there was a shift to Gram-positive cocci which became the dominant isolates until the turn of the century when the Gram-negative bacilli re-emerged, notably *Pseudomonas aeruginosa, Escherichia coli* and the other enteric Gram-negative bacilli. This shift may be partially explained by a decrease in the use of uoroquinolone as prophylaxis. In terms of Gram-negative bacilli, Surveillance and Control of Pathogens of Epidemiologic (SCOPE) data reports *E. coli, Klebsiella* spp. and *P. aeruginosa* as the most commonly isolated organisms.

The results of the present studies revealed that 30 of the 38 extracts were active against the Gram-positive bacteria (Table 15). Amongst these, *L. indica, S. hernandifolia, T. cicutaria, P. minima*, and *P. peruviana* showed excellent activity (very low MIC) against all the tested strains in this category. Some *Bacillus* spp., connected to food poisoning, have been shown to produce heat-stable toxins, (Mikkola *et al.*, 2000, 2004; Salkinoja-Salonen *et al.*, 1999; Suominen *et al.*, 2001). *Bacillus* strains producing heat-stable toxins introduce a potential safety risk to dairy products because both the endospores and the toxins can survive current dairy processes. So, strains of *B. cereus, B. subtilis, B. licheniformis* and *B. pumilus* were used for the screening. *B. subtilis* was less resistant than *B. cereus*, while majority of the extracts restricted *B. licheniformis* compared to *B. pumilus*. The other Gram-positive bacterium, *Staphylococcus aureus*, employed for the study, is a potential pathogen, since it causes skin and post-operative wound infections (Skinner and Keefer, 1941). Interestingly, many of the extracts were effective against *S. aureus*.

The extracts of *R. manjith, F. nubicola* fruit, *D. indica, A. rivularis* and *A. viridis* showed maximum potency, being effective against both Gram-negative and Gram-positive bacteria. However, most of the extracts are more effective on Gram-positive bacteria than their counterparts (Tables 14 and 15). The differences of susceptibility between these two sets of bacteria against the extracts are consistent with the literature reports (Ceylan and Fung, 2004; Lopez *et al.*, 2005; Zaika, 1988).

A possible explanation for the better efficacy of the extracts against the Gram-positive bacteria may lie in the significant differences in the outer layers of Gram-negative and positive bacteria. Between the two, only the Gram-negative bacteria possess an outer membrane and a unique periplasmic space (Duffy and Power, 2001; Nikaido, 1996). The resistance of Gram-negative bacteria towards antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules and is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside (Gao *et al.*, 1999; Nikaido, 1994; Russell, 1991). On the other hand, antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane of the Gram-positive bacteria and result in a leakage of the cytoplasm and its coagulation (Kalemba and Kunicka, 2003).

Amongst the yeasts and moulds, *Candida albicans* is the most frequently encountered among *Candida* spp. associated with man as a commensal, and acts as an opportunistic pathogen (Calderone, 2002). Although, the extracts, in general did not show much activity against the yeasts and moulds, the

extracts of *A. calva, C. album, H. cordata, H. nepalense, L. indica, O. tenuiflorum* and *T. nutans* could successfully control *C. albicans* (Table 16).

Fungi can contaminate foods anytime from cultivation to harvest, during transportation and storage, and in various production phases (Frisvad and Samson, 1991). Both the mould speciess used for screening of the herb extracts are potential producers of mycotoxins, which, depending on their concentrations in foods and feeds, may pose serious problems to human and animal health (Moss, 1998). In the present screening, the extracts showed insignificant antimould activity. *C. hirsuta, C. buchananiana* root, *D. esculentum, E. debile, F. nubicola* (root and fruit), *H. nepalense* and *T. nutans* could inhibit both the mould species, at the tested concentrations.

5.9. Isolation of phytochemicals from *H. nepalense* and *S. hernandiifolia*

S. hernandifolia is rich in alkaloids, and furanocoumarins are the principal constituents of *H. nepalense*. Hence, an effort was given to isolate them and study their activity.

The above results showed that besides showing moderate antioxidant activity, the methanol extract of *H. nepalense* fruit possessed significant activity against various bacteria, yeasts and moulds. Likewise, the *S. hernandiifolia* also inhibited the growth of Gram-positive and Gram-negative bacteria, while inhibition to the foodborne pathogens and food spoilage bacteria might be important in the safety and preservation of processed foods. Hence, the phytochemical investigation of these extracts was pursued to isolate and identify some of its major components.

5.9.1. Furanocoumarins and alkaloids from *H. nepalense* and *S. hernandiifolia*, respectively

Three furanocoumarins, *viz.*, byakangelicol (BA), sphondin (SD) (Tosun *et al.*, 2008)) and furopinnarin (FP) (Banerjee *et al.*, 1980) were isolated from the hexane extract of *H. nepalense* fruits by column chromatography, and identified using the ^{1}H NMR spectrum (Fig. 12 and 13). In a similar manner, Dragendorff test of the acid-soluble portion of the *S. hernandiifolia* extract revealed it to consist of a mixture of alkaloids only. However, the mixture was too intricate, and the individual compounds could not be purified.

5.9.2. Antioxidant and antimicrobial activities of isolated furanocoumarins

The results showed significant DPPH$^{\bullet}$-scavenging by the furanocoumarins, BA being the best scavenger (Table 17). However, all the compounds, especially BA and SP showed reasonably good protection against Fenton-mediated LPO. This may be due to the better affinity of furanocoumarins to lipid and/or lipid peroxyl radicals. Further, the LPO inhibition capacity is dictated not only by the free radical-scavenging capacity but also by the actions of antioxidant-derived radicals and interactions between antioxidants, which are not measured by a probe method. In addition, both BA and FP prevented Fenton-induced plasmid DNA damage up to 5 μg ml^{-1} (Tables 18 and 19; Fig. 14 and 15). It is well-known that the $^{\bullet}$OH is the most toxic amongst the ROS and is primarily responsible for the DNA damage, caused by the Fenton system. Hence, the $^{\bullet}$OH-scavenging potential of the compounds was also checked and a good correlation was obtained.

With regard to the antimicrobial activity, all the furanocoumarins were effective against the Gram-positive and Gram-negative bacteria, albeit less for the latter type. Interestingly, the relative order of their potency, *viz.* BA > FP > SD matched with the trend about their antioxidant property (Table 20).

6

Summary

Darjeeling Himalayan region possesses close similarities among multilingual and multiethnic populations from Nepal to Sikkim and Bhutan. Different ethnic communities, *viz.* Nepalese (Gorkha, in general), Bhutia, Lepcha, Limboo, Manger etc. form the main inhabitants of this belt. The knowledge of traditional medicinal plants, used by the natives is entirely surviving on folklores, and recording of such knowledge is the only means of restoration of this valuable ethnic information (Das and Mandal, 2003). This can be achieved by surveys and interviews with the local tribals along with specimen collection and their preservation followed by proper identification. The plants, used as ethnomedicine in this region, have a great potential, and can be exploited to scientific propagation for serving a greater spectrum of human lives. The medicinal plants, used for long by various tribal folks, are known to produce several important therapeutic compounds. Hence, the study was taken up with an attempt of a moderate survey of the plants of Darjeeling Himalayan region imparting a special focus on their folklore use against different diseases by the local people, belonging to the Nepalese community mainly. Another objective was to rationalize the medicinal attributes in terms of the antioxidant property of the plants, since antioxidants are considered beneficial in combating various reactive oxygen species (ROS), which induce oxidative damage to biomolecules that lead to several degenerative diseases, *viz.* artherosclerosis, heart diseases, cancer, malaria etc. In addition, the plants were also tested for their efficacy in preventing potential infection which is becoming an important cause

of health hazard in the developing countries. The antimicrobial activities of various plant extracts and essential oils have vast potential for raw and processed food preservation, as pharmaceuticals and as alternative medicines for natural therapies. These are regarded as GRAS (Generally Recognized As Safe) substances. For a comprehensive view of the outcome of the results are summarized below.

A total of 106 plants were surveyed to be commonly used by the local people inhabiting three hilly Subdivisions of the district of Darjeeling for treating different ailments. These were taxonomically identified and sample specimens were kept in a reference repository (National Gene Bank for Medicinal and Aromatic Plants (NGBMAP), Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India), and their traditional uses have been documented. This would be helpful in identifying the problems of erosion of the knowledge about the ethnic system of medicine and taking remedial measures.

Methanolic extracts of 38 different plant parts were screened for their *in vitro* antioxidant activity (DPPH$^{\bullet}$-scavenging, reducing power and metal-chelating power) which was correlated with their chemical composition (polyphenol and flavonoid contents) using principal component analysis. Flavonoid content, DPPH$^{\bullet}$-scavenging ability and reducing power were shown to be highly loaded on factor 1 (PC1) with loadings 0.555, 0.929 and 0.795, respectively. The statistical analysis of the results suggested the key components responsible for the antioxidant activity.

Using the most promising 14 plants, their antioxidant activity was analysed further using a wide range of well-accepted protocols (ABTS$^{\bullet+}$-scavenging, $^{\bullet}$OH-scavenging, anti-lipid peroxidation and oxygen radical absorbance capacity). *Fragaria nubicola* root (FNR) extract showed a superior capacity, in all the tests.

The best candidate, FNR was then assessed for its antioxidant properties (DPPH$^{\bullet}$-scavenging, ABTS$^{\bullet+}$-scavenging and reducing power) and for relevant biological models, *viz.* lipid, protein and DNA in a concentration dependent manner. Lipid peroxidation was induced by Fenton reagent as well as by free radical, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH); protein (bovine serum albumin) damage from radiation, while Fenton induced DNA damage was found to be successfully controlled by FNR.

It was also screened for its chemopreventive activity against two human cancerous cell lines (A-549 and MCF-7) and found promising. It prevented proliferation of both lung and breast carcinoma cells (being more toxic to lung cancer cell line), without being toxic to the normal cells (INT-407). The medicinal property of the extract may be due to its constituent hydroxyl acids (gallic acid, dihydrocaffeic acid, caffeic acid etc.), as revealed by the HPLC analysis of its acid-hydrolyzed product.

Most of the extracts of plants (*Leucas indica*, *Tectaria cicutaria*, *Physalis minima*, *Physalis peruviana* etc.) also showed "good" to "excellent" inhibitory activity against Gram-positive bacteria, while a few were also active against Gram-negative ones. The extracts of *Rubia manjith*, *Fragaria nubicola* fruit and root, *Duchesnea indica*, *Astilbe rivularis* and *Amaranthus viridis* showed effectiveness against both Gram-negative and Gram-positive bacteria. Besides FNR, the extracts of *Heracleum nepalense* seeds and root tubers of *Stephania hernandifolia* showed good antioxidant as well as antimicrobial properties. *H. nepalense* was found to contain several furanocoumarins of which three were isolated chromatographically and identified. The furanocoumarins were found to be byak-angelicol, sphondin and furropinnarin. The identification was achieved by comparing their 1H-NMR with the available literature. All these compounds showed a reasonably good antimicrobial activity. On the contrary, *S. hernandifolia* appeared to be a good source of alkaloids, although their complete identification could not be carried out.

The antimicrobial and antioxidant activities of the purified furanocoumarins were screened individually following chemical models (DPPH$^{\bullet}$-scavenging and $^{\bullet}$OH-scavenging) and using biologically relevant models (anti-lipid peroxidation and DNA protection), in a concentration dependent manner. Among the isolated

compounds, sphondin responded poorly in all the antioxidant tests in both the models as well as in the antimicrobial assays, while byak-angelicol was the best candidate. Furopinnarin, possessed the intermediate potential.

The study, thus, provided us with information about the common ethnomedicinal resource of Darjeeling Himalayan region. Among the 38 selected plants, three were analysed for their phenolic constituents and found prospective in alleviating human sufferings.

7

Bibliography

Aaby, K., Hvattum, E. and Skrede, G. (2004) Analysis of flavonoids and other phenolic compounds using high-performance liquid chromatography with coulometric array detection: relationship to antioxidant activity. *Journal of Agricultural and Food Chemistry* **52**, 4595-4603

Aaby, K., Skrede, G. and Wrolstad, R.E. (2005) Phenolic composition and antioxidant activities in flesh and achenes of strawberries (*Fragaria ananassa*). *Journal of Agricultural and Food Chemistry* **53**, 4032-4040

Adamson, G.E., Lazarus, S.A., Mitchell, A.E., Prior, R.L., Cao, G., Jacobs, P.H., Kremers, B.G., Hammerstone, J.F. Rucker, R.B., Ritter, K.A. and Schmitz, H.H. (1999) HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *Journal of Agricultural and Food Chemistry* **47**, 4184–4188

Al-Bari, M.A.A., Sayeed, M.A., Rahman, M.S. and Mossadik, M.A. (2006) Characterization and antimicrobial activities of a phenolic acid derivative produced by *Streptomyces bangladeshiensis* - a novel species collected in Bangladesh. *Research Journal of Medicine and Medical Sciences* **1**, 77-81

Ali, G.C. and Reddy, A.S.N. (2000) Inhibition of fungal and bacterial plant pathogens by synthetic peptides: In vitro growth inhibition, Interaction between peptides and inhibition of disease progression. *Molecular Plant-Microbe Interactions* **13**, 847-859

Alma, M.H., Mavi, A., Yildirim, A., Digrak, M. and Hirata, T. (2003) Screening chemical composition and in vitro antioxidant and antimicrobial activities of the essential oils from *Origanum syriacum* L. growing in Turkey. *Biological and Pharmaceutical Bulletin* **26**, 1725–1729

Amarowizc, R., Nacsk, M. and Shahidi, F. (2000) Antioxidant activity of various fractions of non-tannin phenolics of canola hulls. *Journal of Agricultural and Food Chemistry* **48**, 2755–2759

Ambrosio, G., Zweier, J.L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P.P., Tritto, I., Cirillo, P., Condorelli, M. and Chiariello, M. (1993) Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *Journal of Biological Chemistry* **268**, 18532-18541

Ames, B.N., Gold, L.S. and Willet, W.C. (1995) The causes and prevention of cancer. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 5258-5265

Aruoma, O.I. (2003) Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutation ResearchFundamental and Molecular Mechanisms of Mutagenesis* **523–524**, 9–20.

Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *The Journal of Clinical Investigation* **52**, 741-744

Bacq, Z.M., Dechampsm, G., Fischer, P., Herve, A., Lebihan, H., Lecomte, J., Pirotte, M. and Rayet, P. (1953) Protection against X-rays and therapy of radiation sickness with β–mercaptoethylamine. *Science* **117**, 633-636

Banerjee, S.K., Gupta, B.D. and Atal, C.K. (1980) Coumarins of *Heracleum thomsoni* and claisen rearrangement of lanatin. *Phytochemistry* **19**, 1256-1258

Bansod, S. and Rai, M. (2008) Antifungal activity of essential oils from Indian medicinal plants against human pathogenic *Aspergillus fumigatus* and *A. niger*. *World Journal of Medical Sciences* **3**, 81-88

Barbour, E.K., Al Sharif, M., Sagherian, V.K., Habre, A.N., Talhouk, R.S. and Talhouk, S.N. (2004) Screening of selected indigenous plants of Lebanon for antimicrobial activity. *Journal of Ethnopharmacology* **93**, 1–7

Bauer, A.W., Kirby, W.M.M., Scherris, J.C. and Turck, M. (1966) Antibiotic susceptibility testing by a standardized single disk method. *The American Journal of Clinical Pathology*, **45**, 493-496

Becker, E.M., Nissen, L.R, and Skibsted, L.H. (2004) Antioxidant evaluation protocols: Food quality or health effects. *European Food Research and Technology* **219**, 561–571

Benzie, I.F.F. and Strain, J.J. (1996) The ferric reducing ability of plasma as a measure of 'antioxidant power': the FRAP assay. *Analytical Biochemistry* **239**, 70–76

Bhujel, R.B. (1996) Studies on the dicotyledonous flora of Darjeeling district. Ph.D. Thesis, University of North Bengal, Siliguri, p. 966

Bielski, B.H.J. (1985) Reactivity of HO_2/O_2^- radicals in aqueous solution. *Journal of Physical and Chemical Reference Data* **14**, 1041–1100

Boakye-Yiadom, K.N. and Fiagbe, S.A. (1977) Antimicrobial properties of some West African medicinal plants IV. Antimicrobial activity of xylopic acid and other constituents of the fruits of *Xylopia aethiopica* (Annonaceae). *Lloydia* **40**, 543–545

Borges, F., Roleira, F., Milhazes, N., Santana, L. and Uriarte, E. (2005) Simple coumarins and analogues in medicinal chemistry: Occurrence, synthesis and biological activity. *Current Medicinal Chemistry* **12**, 887-916

Borris, R.P. (1996) Natural products research: Perspectives from a major pharmaceutical company. *Journal of Ethnopharmacology* **51**, 29–38

Bose, P.K. (1958) On some biochemical properties of natural coumarins. *Journal of the Indian Chemical Society* **58**, 367–375

Box, H.C. and Maccubbin, A.E. (1997) Lipid peroxidation and DNA damage. *Nutrition* **13**, 920-921

Box, H.C., Freund, H.G., Budzinski, E., Wallace, J.C. and Maccubbin, A.E. (1995) Free radical-induced double base lesions. *Radiation Research* **141**, 91-94

Boyd, M.R., Hallock, Y.F., Cardellina II, J.H., Manfredi, K.P., Blunt, J., McMahon, J.B., Buckheit Jt, R.W., Bringmann, G., Schaffer, M., Cragg, G.M., Thomas, D.W. and Jato, J.G. (1994) Anti-HIV michellamines from *Ancistrocladus korupensis*. *Journal of Medicinal Chemistry* **37**, 1740–1745

Brand-Williams, W., Cuvelier, M.E. and Berset, C. (1995) Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft und-Technologie* **28**, 25–30

Braughler, J.M., Chase, R.L. and Pregenzer, J.F. (1987) Oxidation of ferrous ion during peroxidation of various lipid substrates. *Biochimica et Biophysica Acta* **921**, 457-464

Breen, A.P. and Murphy, J.A. (1995) Reactions of oxyl radicals with DNA. *Free Radical Biology & Medicine* **18**, 1033-1077

Brinkworth, R.I., Stoermer, M.J. and Fairlie, D.P. (1992) Flavones are inhibitors of HIV-1 proteinase. *Biochemical and Biophysical Research Communications* **188**, 631– 637

Brower, V. (1998) Nutraceuticals: poised for a healthy slice of the healthcare market? *Nature Biotechnology* **16**, 728-731

Brownlee, H.E., McEuen, A.R., Hedger, J. and Scott, I.M. (1990) Antifungal effects of cocoa tannin on the witches' broom pathogen *Crinipellis perniciosa*. *Physiological and Molecular Plant Pathology* **36**, 39–48

Bull, C., McClune, G.J. and Free, J.A. (1983) The mechanism of Fe- EDTA catalyzed superoxide dismutation. *Journal of the American Chemical Society* **105**, 5290–5300

Burda, S. and Oleszek, W. (2001) Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry* **49**, 2774–2779

Burdick, E.M. (1971) Carpaine, an alkaloid of *Carica papaya*. Its chemistry and pharmacology. *Economic Botany* **25**, 363–365

Burton, G.W. and Ingold, K.U. (1981) Autoxidation of biological molecules. 1. The autoxidation of vitamin E and related chain breaking antioxidants *in vitro*. *Journal of the American Chemical Society* **103**, 6472–6477

Butler, L.G. (1988) Effects of condensed tannin on animal nutrition. p. 553. In: *Chemistry and Significance of Condensed Tannins*, R.W. Hemingway and J.J. Karchesy, eds. Plenum Press, New York, N.Y.

Cabello, C.M., Bair, W.B. and Wondrak, G.T. (2007) Experimental therapeutics: Targeting the redox Achilles heel of cancer. *Current Opinion in Investigational Drugs* **8**, 1022-1037

Cadenas, E., Hochstein, P. and Ernster, L. (1992) Pro- and antioxidant functions of quinones and quinone reductases in mammalian cells. *Advances in Enzymology and Related Areas of Molecular Biology* **65**, 97-146

Calderone, R.A., ed. (2002) *Candida and Candidiasis*. ASM Press, Washington, D.C., p. 67

Caldwell, C.R. (2001) Oxygen radical absorbance capacity of the phenolic compounds in plant extracts fractionated by high-performance liquid chromatography. *Analytical Biochemistry* **293**, 232–238

Cao, G.H., Alessio, H. and Cutler, R.G. (1993) Oxygen-radical absorbency capacity assay for antioxidants. *Free Radical Biology & Medicine* **14**, 303–311

Cao, G., Booth, S.L., Sadowski, J.A. and Prior, R.L. (1998) Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *American Journal of Clinical Nutrition* **68**, 1081-1087

Cao, G. and Prior, R.L. (1999) Anthocyanins are detected in human plasma after oral administration of an elderberry extract. *Clinical Chemistry* **45**, 574–576

Cao, G., Prior, R.L., Cutler, R.G. and Yu, B.P. (1997) Effect of dietary restriction on serum antioxidant capacity in rats. *Archives of Gerontology and Geriatrics* **25**, 245–253

Cao, G., Sofic, E. and Prior, R.L. (1996) Antioxidant capacity of tea and common vegetables. *Journal of Agricultural and Food Chemistry* **44**, 3426–3431

Carter, P. (1971) Spectrophotmetric determination of serum iron at the submicrogram level with a new reagent (ferrozine). *Analytical Biochemistry* **40**, 450-458

Ceylan, E. and Fung, D.Y.C. (2004) Antimicrobial activity of spices. *Journal of Rapid Methods and Automation in Microbiology* **12**, 1–55

Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs. *Physiological Reviews* **59**, 527-605

Chen, Y., Wang, M., Rosen, R.T. and Ho, C.T. (1999) 2,2-Diphenyl-1-picrylhydrazyl radical-scavenging active components from *Polygonum multiflorum* Thunb. *Journal of Agricultural and Food Chemistry* **47**, 2226–2228

Chhetri, D.R., Basnet, D., Chiu, P.F., Kalikotay, S., Chhetri, G. and Parajuli, S. (2005) Current status of ethnomedicinal plants in the Darjeeling Himalaya. *Current Science* **89**, 264-268

Cichewicz, R.H. and Thorpe, P.A. (1996) The antimicrobial properties of chile peppers (*Capsicum* species) and their uses in Mayan medicine. *Journal of Ethnopharmacology* **52**, 61–70

Das, A.P. and Mandal, S. (2003) Some *Medicinal Plants of Darjeeling Hills.* WWF-India, West Bengal State Office, Kolkata, p. 95

Davies, J.A. (1987) Protein damage and degradation by oxygen radicals. I. General aspects. *Journal of Biological Chemistry* **262**, 9895-9901

Dean, R.T., Fu, S., Stocker, R. and Davies, M.J. (1997) Biochemistry and pathology of radical mediated protein oxidation. *Biochemical Journal* **324**, 1-18

Deeni, Y. and Hussain, H. (1991) Screening for antimicrobial activity and for alkaloids of *Nauclea latifolia. Journal of Ethnopharmacology* **35**, 91–96

Demple, B. and Harrison, L. (1994) Repair of oxidative damage to DNA: Enzymology and biology. *Annual Review of Biochemistry* **63**, 915-948

Desagher, S. and Martinou, J.C. (2000) Mitochondria as the central control point of apoptosis. *Trends in Cell Biology* **10**, 369–377

Dhuley, J. (1998) Therapeutic efficacy of ashwagandha against experimental aspergillosis in mice. *Immunopharmacolgy and Immunotoxicology* **20**, 191–198

District Statistical Handbook, Darjeeling (2002) Bureau of Applied Economics and Statistics, Government of West Bengal, Kolkata

Dizdaroglu, M. (1993) Chemistry and free radical damage to DNA and nucleoproteins. In: *DNA and Free Radicals,* B. Halliwell and O.I. Aruoma, eds. Ellis Harwood, Chichester, UK, pp. 19-39

Dominguez, M., Nieto, A., Marin, J.C., Keck, A.-S., Jeffery, E. and Céspedes, C.L. (2005) Antioxidant activities of extracts from *Barkleyanthus salicifolius* (Asteraceae) and *Penstemon gentianoides* (Scrophulariaceae). *Journal of Agriculture and Food Chemistry* **53**, 5889-5895

Duarte, M.C., Figueira, G.M. Sartoratto, A., Rehder, V.L. and Delarmelina, C. (2005) Anti-*Candida* activity of Brazilian medicinal plants. *Journal of Ethnopharmacology* **97**, 305–311

Duffy, C.F. and Power, R.F. (2001) Antioxidant and antimicrobial properties of some Chinese plant extracts. *International Journal of Antimicrobial Agents* **17**, 527–529

Duke, J.A. (1985) *Handbook of Medicinal Herbs.* CRC Press, Inc., Boca Raton, FL, p. 677

Dwivedi, C. and Abu-Ghazaleh, A. (1997) Chemopreventive effects of sandalwood oil on skin papillomas in mice. *European Journal of Cancer Prevention* **6**, 399–401

Ehlenfeldt, M.K. and Prior, R.L. (2001). Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of high bush blueberry. *Journal of Agricultural and Food Chemistry* **49**, 2222–2227

Elia, M.C., Storer, R.D., Harmon, L.S., Kraynak, A.R., McKelvey, T.W., Hertzog, P.R., Keenan, K.P., DeLuca, J.G. and Nichols, W.W. (1993) Cytotoxicity as measured by trypan blue as a potentially confounding variable in the in vitro alkaline elution/rat hepatocyte assay. *Mutation Research* **291**, 193-205

El-Seedi, H.R., Sata, N., Torssell, K.B. and Nishiyama, S. (2002) New labdene diterpenes from *Eupatorium glutinosum*. *Journal of Natural Products* **65**, 728–729

Escarpa, A. and González, M.C. (2001) Approach to the contents of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. *Analytica Chimica Acta* **427**, 119-127

Esterbauer, H. (1996) Estimation of peroxidative damage: a critical review. *Pathologie. Biologie* **44**, 25-28

Finkelstein, E., Rosen, G.M. and Rauckman, E.J. (1980) Spin trapping: Kinetics of the reaction of superoxide and hydroxyl radicals with nitrones. *Journal of the American Chemical Society* **102**, 4994–4999

Fish, F., Meshal, I. and Waterman, P. (1976) Minor alkaloids of *Araliopsis tabouensis*. *Planta Medica* **29**, 310–317

Foti, M.C., Daquino, C. and Geraci, C. (2004) Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH radical in alcoholic solutions. *The Journal Organic Chemistry* **69**, 2309–2314

Foti, P., Erba, D., Riso, P., Spadafranca, A., Criscuoli, F. And Testolin G. (2005) Comparison between daidzein and genistein antioxidant activity in primary and cancer lymphocytes. *Archives of Biochemistry and Biophysics* **433**, 421-427

Frisvad, J.C. and Samson, R.A. (1991) Filamentous fungi in foods and feeds: Ecology, spoilage and mycotoxins production. In: *Handbook of Applied Mycology: Foods and Feeds, D.K.* Arora, K.G. Mukerji and E.H. Marth, eds, vol. 3. Marcel Dekker, New York, N.Y., pp. 31–68

Fylaktakidou, K.C., Hadjipavlou-Litina, D.J., Litinas, K.E. and Nicolaides, D.N. (2004) Natural and synthetic coumarin derivatives with anti-inflammatory/ antioxidant activities. *Current Pharmaceutical Design* **10**, 3813-3833

Gao, Y., van Belkum, M.J. and Stiles, M.E. (1999) The outer membrane of Gram negative bacteria inhibits antibacterial activity of brochocin-C. *Applied and Environmental Microbiology* **65**, 4329–4333

Geissman, T.A. (1963) Flavonoid compounds, tannins, lignins and related compounds, p. 265. In: Pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol. 9, M. Florkin and E.H. Stotz, eds. Elsevier, New York, N.Y.

Ghiselli, A., Serafini, M., Maiani, G., Azzini, E. and Ferroluzzi, A. (1995) A fluorescense-based method for measuring total plasma antioxidant capability. *Free Radical Biology & Medicine* **18**, 29-36

Giasson, B.I., Duda, J.E., Murray, IV, Chen, Q., Souza, J.M., Hurtig, H.I., Ischiropoulos, H., Trojanowski, J.Q. and Lee, V.M. (2000) Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* **290**, 985-989

Gil, M.I., Tomás-Barberán, F.A., Hess-Pierce, B., Holcroft, D.M. and Kader, A.A. (2000) Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry* **48**, 4581–4589

Girotti, A.W. (1985) Mechanism of lipid peroxidation. *Free Radical Biology & Medicine* **1**, 87-95

Gordon, M.H. (1990) The mechanism of antioxidant action in vitro, pp. 1–18 In: *Food Antioxidants,* B.J.F. Hudson, ed. Elsevier Applied Science, London

Grotewold, E. (2006) *The Science of Flavonoids*. Springer, New York, N.Y., p. 273

Gülçin, I., Oktay, M., Kireçci, E. and Küfreviolu, O. (2003) Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum* L.) seed extracts. *Food Chemistry* **83**, 371–382

Guo, C., Cao, G., Sofic, E. and Prior, R.L. (1997) High performance liquid chromatography coupled with coulometric array detection of electroactive components in fruits and vegetables: Relationship to oxygen radical absorbance. *Journal of Agricultural and Food Chemistry* **45**, 1787–1796

Halliwell, B. (1990) How to characterize a biological antioxidant. *Free Radical Research Communications* **9**, 1–32

Halliwell, B. (1995) Antioxidant characterization: Methodology and mechanism. *Biochemical Pharmacology* **49**, 1341-1348

Halliwell, B. (1997) Antioxidants and human diseases: A general introduction. *Nutrition Reviews* **55**, S44-S52

Halliwell, B. and Aruoma, O.I., eds. (1993) *DNA and Free Radicals.* Ellis Harwood, New York, N.Y., p. 332

Halliwell, B. and Gutteridge, J.M.C. (1981) Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: The role of superoxide and hydroxyl radicals. *FEBS Letters* **128**, 347-352

Halliwell, B. and Gutteridge, J.M.C. (1999) *Free Radicals in Biology and Medicine,* 3rd edn. Oxford University Press, Oxford, U.K.

Halliwell, B., Gutteridge, J.M. and Aruoma, O.I. (1987) The deoxyribose method: a simple 'test tube' assay for determination of rate constants for reactions of hydroxyl radicals. *Analytical Biochemistry* **165**, 215–219

Hanasaki, Y., Ogawa, S. and Fukui, S. (1994) The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radical Biology & Medicine* **16**, 845-850

Handelman, G.J., Cao, G., Walter, M.F., Nightingale, Z.D., Paul, G.L., Prior, R.L. and Blumberg, J.B. (1999) Antioxidant capacity of oat (*Avena sativa* L.) extracts. 1. Inhibition of low-density lipoprotein oxidation and oxygen radical absorbance capacity. *Journal of Agricultural and Food Chemistry* **47**, 4888–4893

Harborne, J.B., ed. (1993) *The Flavonoids: Advances in Research Since 1986.* Chapman and Hall, London

Harman, D. (1992) Free radical theory of aging. *Mutation Research* **275**, 257-266

Haslam, E. (1996) Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *Journal of Natural Products* **59**, 205–215

Heinonen, M. (2007) Antioxidant activity and antimicrobial effect of berry phenolics - a Finnish perspective. *Molecular Nutrition & Food Research* **51**, 684-691

Hemaiswarya, S., Kruthiventi, A.K. and Doble, M. (2008) Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine* **15**, 639-652

Hogg, N. and Kalyanaraman, B. (1999) Nitric oxide and lipid peroxidation. *Biochimica et Biophysica Acta (BBA) – Bioenergetics* **1411**, 378-384

Hou, W.C., Lee, M.H., Chen, H.J., Liang, W.L., Han, C.H., Liu, Y.W. and Lin, Y.H. (2001) Antioxidant activities of dioscorin, the storage protein of yam (*Dioscorea batatas* Decne). *Journal of Agricultural and Food Chemistry* **49**, 4956–4960

Houghton, P.J., Woldemariam, T.Z., Khan, A.I., Burke, A. and Mahmood, N. (1994) Antiviral activity of natural and semi-synthetic chromosome alkaloids. *Antiviral Research* **25**, 235–244

Hoult, J.R.S. and Paya, M. (1996) Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. *General Pharmacology* **27**, 713–722

Irvine, F.R. (1961) *The Woody Plants of Ghana.* Oxford Press, UK, p. 868

Iwu, M.M. (1993) *Handbook of African Medicinal Plants.* C.R.C. Press, Boca Raton, FL

Jackson, M.J., Papa, S., Bolanos, J., Bruckdorfer, R., Carlsen, H., Elliott, R.M., Flier, J., Griffiths, H.R., Heales, S., Holst, B., Lorusso, M., Lund, E., Moskaug, J. O., Moser, U., Di Paola, M., Polidori, M.C., Signorile, A., Stahl, W., Vina-Ribes, J. and Astley, S.B. (2002) Antioxidants, reactive oxygen and nitrogen species, gene induction and mitochondrial function. *Molecular Aspects of Medicine* **23**, 209-285

Jaruga, P., Zastawny, T.H., Skokowski, J., Dizdaroglu, M. and Olinski, R. (1994) Oxidative DNA base damage and antioxidant enzyme activities in human lung cancer. *FEBS Letters* **341**, 59-64

Jeevan Ram, A., Bhakshu, L.M. and Venkata Raju, R.R. (2004) In vitro antimicrobial activity of certain medicinal plants from Eastern ghats, India, used for skin diseases. *Journal of Ethnopharmacology* **90**, 353–357

Jia, Z., Tang, M. and Wu, J. (1999) The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry* **64**, 555-599

Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S. and Heinonen, M. (1999) Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food. Chemistry* **47**, 3954-3962

Kähkönen, M.P., Hopia, A.I. and Heinonen, M. (2001) Berry phenolics and their antioxidant activity. *Journal of Agricultural and Food Chemistry* **49**, 4076-4082

Kalemba, D. and Kunicka, A. (2003) Antibacterial and antifungal properties of essential oils. *Current Medicinal Chemistry* **10**, 813–829

Kalt, W., Forney, C.F., Martín, A. and Prior, R.L. (1999) Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *Journal of Agricultural and Food Chemistry* **47**, 4638–4644

Katerere, D.R., Gray, A.I., Nash, R.J. and Waigh, R.D. (2003) Antimicrobial activity of pentacyclic triterpenes isolated from African Combretaceae, *Phytochemistry* **63**, 81–88

Kavli, G., Raa, J., Johnson, B.E., Volden, G. and Haugsbo, S. (1983) Furocoumarins of *Heracleum laciniatum*: isolation, phototoxicity, absorption and action spectra studies. *Contact Dermatitis* **9**, 257–262

Keating, G.J. and O'Kennedy, R. (1997) The chemistry and occurrence of coumarins, p. 23-26. In: *Coumarins: Biology, Applications and Mode of Action*, R. O'Kennedy and R.D. Thornes, eds. John Wiley & Sons, Inc., New York, N.Y.

King, S.R. and Tempesta, M.S. (1994) From shaman to human clinical trials: the role of industry in ethnobotany, conservation and community reciprocity. *Ciba Foundation Symposium* **185**, 197–206

Klausmeyer, P., Chmurny, G.N., McCloud, T.G., Tucker, K.D. and Shoemaker, R.H. (2004) A novel antimicrobial indolizinium alkaloid from *Aniba panurensis*. *Journal of Natural Products* **67**, 1732–1735

Koné, W.M., Kamanzi Atindehou, K., Terreaux, C., Hostettmann, K, Traoré, D. and Dosso, M. (2004) Traditional medicine in North Côte-d'Ivoire: Screening of 50 medicinal plants for antibacterial activity. *Journal of Ethnopharmacology* **93**, 43–49

Kulkarni, M.V., Kulkarni, G.M., Lin, C.H. and Sun, C.M. (2006) Recent advances in coumarins and 1-azacoumarins as versatile biodynamic agents. *Current Medicinal Chemistry* **13**, 2795-2818

Kumar, G.S., Jayaveera, K.N., Kumar, C.K.A., Sanjay, U.P., Swamy, B.M.V. and Kumar, D.V.K. (2007) Antimicrobial effects of Indian medicinal plants against acne-inducing bacteria. *Tropical Journal of Pharmaceutical Research* **6**, 717-723

Lamidi, M., Ollivier, E., Faure, R., Debrauwer, L., Nze-Ekekang, L. and Balansard, G. (1995) Quinovic acid glycosides from *Nauclea diderichii*. *Planta Medica* **61**, 280–281

Larrauri, J.A., Sánchez-Moreno, C., Rupérez, P. and Saura-Calixto, F. (1999) Free radical scavenging capacity in the aging of selected red Spanish wines. *Journal of Agricultural and Food Chemistry* **47**, 1603–1606

Lebeau, J., Furman, C., Bernier, J.L., Duriez, P., Teisser, E. and Cotelle, N. (2000) Antioxidant properties of di-tertbutyl-hydroxylated flavonoids. *Free Radical Biology & Medicine* **29**, 900–912

Lee, C. (2000) Antioxidant ability of caffeine and its metabolites based on the study of oxygen-radical absorbing capacity and inhibition of LDL peroxidation. *Clinica Chimica Acta* **295**, 141–145

Liebler, D.C. (1993) The role of metabolism in the antioxidant function of vitamin E. *Critical Reviews in Toxicology* **23**, 147-169

Lin, F., Hasegawa, M. and Kodama, O. (2003) Purification and identification of antimicrobial sesquiterpene lactones from yacon (*Smallanthus sonchifolius*) leaves. *Bioscience Biotechnology, and Biochemistry* **67**, 2154–2159

Liu, M., Li, X.Q., Weber, C., Lee, C.Y., Brown, J. and Liu, R.H. (2002) Antioxidant and antiproliferative activities of raspberries. *Journal of Agricultural and Food Chemistry* **50**, 2926-2930

López, A., Hudson, J.B. and Towers, G.H. (2001) Antiviral and antimicrobial activities of Colombian medicinal plants. *Journal of Ethnopharmacology* **77**, 189–196

Lopez, P., Sanchez, C., Batlle, R. and Nerin, C. (2005) Solid- and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. *Journal of Agricultural and Food Chemistry* **53**, 6939–6946

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275

Lucas, D.T. and Szweda L.I. (1998) Cardiac reperfusion injury: Aging, lipid peroxidation, and mitochondrial dysfunction. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 510–514

Machado, T.B., Pinto, A.V., Pinto, M.C., Leal, I.C., Silva, M.G., Kuster, R.M.A. and Santos, K.R.N. (2003) In vitro activity of Brazilian medicinal plants, naturally occurring naphthoquinones and their analogues, against methicillin-resistant *Staphylococcus aureus. International Journal of Antimicrobial Agents* **21**, 279–284

Manonmani, S., William, S., Subramanian, S. and Govindasamy, S. (1991) Biochemical studies on the antidiarrhoeal effects of Cauvery-100, an ayurvedic formulation, in rats. *Biochemistry International* **24**, 701–708

Masahiro, T., Takeshi, M., Akie, T., Hisayuki, K., Ken'ya, S., Tomoko, F., Hiroyuki, K., Eiji, I., Sayaka, N. and Norio, M. (2007) Antioxidant activity and anti-allergic activity of *Fragaria ananassa* leaf. *Japanese. Journal of Pharmacognosy* **61**, 18-23

Mason, T.L. and Wasserman, B.P. (1987) Inactivation of red beet betaglucan synthase by native and oxidized phenolic compounds. *Phytochemistry* **26**, 2197–2202

Mello-Filho, A.C. and Meneghini, R. (1991) Iron is the intracellular metal involved in the production of DNA damage by oxygen radicals. *Mutation Research* **251**, 109-113

Merken, H.M. and Beecher, G.R. (2000) Measurement of food flavonoids by high-performance liquid chromatography: A review. *Journal of Agricultural and Food Chemistry* **48**, 577-599

Meyer, A.S., Yi, O.S., Pearson, D.S., Waterhouse, A.L. and Frankel, E.N. (1997) Inhibition of low density lipoprotein oxidation in relation to composition of phenolic antioxidants in grape (*Vitis vinifera*). *Journal of Agricultural and Food Chemistry* **45**, 1638–1643

Meyers, K.J., Watkins, C.B., Pritts, M.P. and Liu, R.H. (2003) Antioxidant and antiproliferative activities of strawberries. *Journal of Agricultural and Food Chemistry* **51**, 6887-6892

Mikkola, R,, Kolari, M., Andersson, M.A., Helin, J. and Salkinoja-Salonen, M.S. (2000) Toxic lactonic lipopeptide from food poisoning isolates of *Bacillus licheniformis. European Journal of Biochemistry* **267**, 4068–4074

Mikkola, R., Andersson, M.A., Grigoriev, P., Teplova, V.V., Saris, N.E., Rainey, F.A. and Salkinoja-Salonen, M.S. (2004) *Bacillus amyloliquefaciens* strains isolated from moisture-damaged buildings produced surfactin and a substance toxic to mammalian cells. *Archives of Microbiology* **181**, 314–323

Miller, N.J. and Rice-Evans, C.A. (1997) The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chemistry* **60**, 331–337

Milner, J.A. (1994). Reducing the risk of cancer, pp. 39-70. In: *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals.* I. Goldberg, ed. Chapman & Hall, New York, N.Y.

Minamiyama, Y., Andoshikawa, T., Tanigawa, T., Takahashi, S., Naito, Y., Ichikawa, H. and Kondo, M. (1994) Antioxidative effects of a processed grain food. *Journal of Nutritional Science and Vitaminology* **40**, 467–477

Moerman, D.E. (1996) An analysis of the food plants and drug plants of native North America. *Journal of Ethnopharmacology* **52**, 1–22

Moss, M.O. (1998) Recent studies of mycotoxins. *Journal of Applied Microbiology* **84**, 62S–76S

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: applications to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**, 55-63

Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C. and Yolke, R.H., eds. (1995) *Manual of Clinical Microbiology*, 6th edn. ASM Press, Washington, D.C.

Musarrat, J. and Wani, A.A. (1994) Quantitative immunoanalysis of promutagenic 8-hydroxy-2-deoxyguanosine in oxidized DNA. *Carcinogenesis* **15**, 2037-2043

Nair, R. and Chanda, S. (2006) Activity of some medicinal plants against certain pathogenic bacterial strains. *Indian Journal of Pharmacology* **38**, 142-144

Nandi, R.P. (1997) *Little Known Medicinal Plants of Darjeeling Himalayan Hills*. Indian Council of Alternative Medicines, Kolkata

Nawar, W.W. (1996) Lipids. In: *Food Chemistry*, O.R. Fennema, ed., pp. 225-319. Marcel Dekker, Inc., New York

Negi, S.S. (1993) In: Floral diversity and botanical regions. In: *Biodiversity and Its Conservation in India*. Indus Publishing, New Delhi

Nelson, R. (1982) The comparative clinical pharmacology and pharmacokinetics of vindesine, vincristine and vinblastine in human patients with cancer. *Medical and Pediatric Oncology* **10**, 115–127

Ngwendson, J.N., Bedir, E., Efange, S.M., Okunji, C.O., Iwu, M.M., Schuster, B.G. and Khan, I.A. (2003) Constituents of *Peucedanum zenkeri* seeds and their antimicrobial effects. *Die Pharmazie* **58**, 587–589

Nikaido, H. (1994) Prevention of drug access to bacterial targets - permeability barriers and active efflux. *Science* **264**, 382–388

Nikaido, H. (1996) Outer membrane, pp. 29–47. In: *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, F.C. Neidhardt, ed. ASM Press, Washington, D.C.

Noguchi, N., Komuro, E., Niki, E. and Willson, R.L. (1994) Action of curcumin as an antioxidant against lipid peroxidation. *Journal of Japanese Oil Chemists' Society* **43**, 1045–1051

O'Neill, P. and Fieden, E.M. (1993) Primary free radical processes in DNA, pp. 53–120. In: *Primary Free Radical Processes in DNA: Advances in Radiation Biology*. Academic Press, New York, N.Y.

Okhawa, H., Ohishi, N. and Yagi, K. (1979) Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* **95**, 351-358

Ono, K., Nakane, H., Fukishima, M., Chermann, J.C. and Barre-Sinoussi, F. (1989) Inhibition of reverse transcriptase activity by a flavonoid compound, 5,6,7-trihydroxyflavone. *Biochemical and Biophysical Research Communications* **3**, 982– 987

Oyaizu, M. (1986) Studies on product of browning reaction prepared from glucose amine. *Japanese Journal of Nutrition* **44**, 307-315

Pansarasa, O., Bertorelli, L., Vecchiet, J., Felzani, G. and Marzatico, F. (1999) Age-dependent changes of antioxidant activities and markers of free radical damage in human skeletal muscle. *Free Radical Biology & Medicine* **27**, 617-622

Paranjpe, P. and Kulkarni, P.H. (1995) Comparative efficacy of four Ayuredic formulations in the treatment of acne vulgaris: a double-blind randomised placebo-controlled clinical evaluation. *Journal of Ethnopharmacology* **49**, 127–132

Patt, H.M., Turee, E.B., Straube, R.L. and Smith, D.E. (1949) Cysteine protection against X irradiation. *Science* **110**, 213-214

Peres, M.T.L.P., Monache, F.D., Cruz, A.B., Pizzolatti, M.G. and Yunes, R.A. (1997) Chemical composition and antimicrobial activity of *Croton urucurana* Baillon (Euphorbiaceae). *Journal of Ethnopharmacology* **56**, 223–226

Pin-Der-Duh, X. (1998) Antioxidant activity of burdock (*Arctium lappa* Linne): its scavenging effect on free-radical and active oxygen. *Journal of the American Oil Chemists' Society* **75**, 455–461

Porter, N.A. (1984) Chemistry of lipid peroxidation. *Methods in Enzymology* **105**, 273-282

Prior, R.L. and Cao, G. (1999) Antioxidant capacity and polyphenolic components of teas: Implications for altering in vivo antioxidant status. *Proceedings of the Society for Experimental Biology and Medicine* **220**, 255–261

Prior, R.L., Wu, X. and Schaich, K. (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry* **53**, 4290–4302

Pryor, W.A., Cornicelli, J.A., Devall, L.J., Tait, B., Trivedi, B.K., Witiak, D.T. and Wu, M. (1993) A rapid screening test to determine the antioxidants potencies of natural and synthetic antioxidants. *Journal of Organic Chemistry* **58**, 3521–3532

Pryor, W.A. (1966). *Free Radicals*. McGraw-Hill, New York, N.Y., p. 355

Pulido, R., Bravo, L. and Saura-Calixo, F. (2000) Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry* **48**, 3396–3402

Puupponen-Pimiä, R., Nohynek, L., Meier, C., Kähkönen, M., Heinonen, M., Hopia, A. and Oksman-Caldentey, K.M. (2001) Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology* **90**, 494-507

Quick, K.L., Hardt, J.I. and Dugan, L.L. (2000) Rapid microplate assay for superoxide scavenging efficiency. *Journal of Neuroscience Methods* **97**, 138–144

Rahman, M.M., Wahed, M.I.I., Biswas, M.H.U., Sadik, G.M.G. and Haque, M.E. (2001) In vitro antibacterial activity of the compounds of *Trapa bispinosa* Roxb. *Science* **1**, 214-216

Rai, P.C., Sarkar, A., Bhujel, R.B. and Das, A.P. (1998) Ethnomedicinal studies in some fringe areas of Sikkim and Darjeeling Himalaya. *Journal of Hill Research* **11**, 12-21

Rai, S.K. and Bhujel, R.B. (1999) Notes on some less known ethnomedicinal plants from Darjeeling Himalaya. *Journal of Hill Research* **12**, 160-163

Rai, S.K. and Bhujel, R.B. (2002) Ethnic uses of some monocotyledonous plants in the Darjeeling Himalayan region, pp. 635–644. In: *Perspectives of Plant Biodiversity*, A.P. Das, ed. Bishen Singh Mahendra Pal Singh, Dehradun, India

Rampon, V., Lethuaut, L., Mouhous-Riou, N. and Genot, C. (2001) Interface characterization and aging of bovine serum albumin stabilized oil-in-water emulsions as revealed by frontsurface fluorescence. *Journal of Agricultural and Food Chemistry* **49**, 4046-4051

Rapisarda, P., Tomaino, A., Lo Cascio, R., Bonina, F., De Pasquale, A. and Saija, A. (1999) Antioxidant effectiveness as influenced by phenolic content of fresh orange juices. *Journal of Agricultural and Food Chemistry* **47**, 4718–4723

Rauha, J.P., Remes, S., Heinonen, M., Hopia, A., Kähkönen, M., Kujala, T., Pihlaja, K., Vuorela, H. and Vuorela, P. (2000) Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *International Journal of Food Microbiology* **56**, 3-12

Ray, P.G. and Majumdar, S.K. (1976) Antimicrobial activity of some Indian plants. *Economic Botany* **30**, 317-320

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

Recio, M.C., Ríos, J.L. and Villar, A. (1989) A review of some antimicrobial compounds isolated from medicinal plants reported in the literature 1978–88. *Phytotherapy Resarch* **3**, 117–125

Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M. and Pridham, J.B. (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research* **22,** 375-383

Richter, C. (1992) Reactive oxygen and DNA damage in mitochondria. *Mutation Research* **275,** 249-255

Ríos, J.L., Recio, M.C. and Villar, A. (1988) Screening methods for natural products with antimicrobial activity: a review of the literature. *Journal of Ethnopharmacology* **23**, 127–149

Rojas, A., Hernandez, L., Pereda-Miranda, R. and Mata, R. (1992) Screening for antimicrobial activity of crude drug extracts and pure natural products from Mexican medicinal plants. *Journal of Ethnopharmacology* **35**, 275–283

Russell, A.D. (1991) Mechanisms of bacterial-resistance to non-antibiotics — food-additives and food and pharmaceutical preservatives. *Journal of Applied Bacteriology* **71**, 191–201

Sajjadi, S.E. and Noroozi, P. (2007) Isolation and identification of xanthotoxin (8-methoxypsoralen) from the fruits of *Heracleum persicu*m. Desf. ex. Fischer *Research in Pharmaceutical Sciences* **2**, 13-16

Salah, N., Millerm, N.J., Paganga, G., Tijburg, L., Bolwell, G.P, and Rice-Evans, C. (1995) Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain breaking antioxidants. *Archives of Biochemistry and Biophysics* **322**, 339–346

Salkinoja-Salonen, M.S., Vuorio, R., Andersson, M.A., Kämpfer, P., Andersson, M.C., Honkanen-Buzalski, T. and Scoging, A.C. (1999) Toxigenic strains of *Bacillus licheniformis* related to food poisoning. *Applied and Environtmental Microbiology* **65**, 4637–4645

Salvat, A., Antonacci, L., Fortunato, R.H., Su´arez, E.Y. and Godoy, H.M. (2004) Antimicrobial activity in methanolic extracts of several plant species from northern Argentina. *Phytomedicine* **11**, 230–234

Sanchez-Moreno, C. (2002) Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Science and Technology International* **8**, 121–137

Sanchez-Moreno, C., Larrauri, Z.A. and Saura-Calixto, F. (1998) A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture* **76**, 270-276

Sawer, I., Berry, M., Brown, M. and Ford, J. (1995) The effect of cryptolepine on the morphology and survival of *Escherichia coli, Candida albicans* and *Saccharomyces cerevisiae. Journal of Applied Bacteriology* **79**, 314–321

Scalbert, A. (1991) Antimicrobial properties of tannins. *Phytochemistry* **30**, 3875–3883

Schultes, R.E. (1978) The kingdom of plants, p. 208. In: *Medicines from the Earth*, W.A.R. Thomson, ed. McGraw-Hill, New York, N.Y.

Schultz, J.C. (1988) Tannin-insect interactions, p. 553. In: *Chemistry and Significance of Condensed Tannins*, R.W. Hemingway and J.J. Karchesy, eds. Plenum Press, New York, N.Y.

Schwartsmann, G., Ratain, M.J., Cragg, G.M., Wong, J.E., Saijo, N., Parkinson, D.R., Fujiwara, Y., Pazdur, R., Newman, D.J., Dagher, R. and Di Leone, L. (2002) Anticancer drug discovery and development throughout the world. *Journal of Clinical Oncology* **20**, 47S–59S

Shah, L., Patil, S.P. and Patil, J. (1997) Observations on clinical evaluation of indigenous herbal drugs in the treatment of mental illnesses. *Indian Journal of Pharmacology* **29**, S347–S349

Shigenaga, M.K., Hagen, T.M. and Ames, B.N. (1994) Oxidative damage and mitochondrial decay in aging. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 10771-10778

Shyu, Y.-S. and Hwang, L.S. (2002) Antioxidant activity of the crude extract of lignan glycosides from unroasted Burma black sesame meal. *Food Research International* **35**, 357-365

Sies, H., ed. (1991) *Oxidative Stress: Oxidants and Antioxidants.* Academic Press, New York, N.Y.

Silva, F.A.M., Borges, F., Guimaraes, C., Lima, J.L.F.C., Matos, C. and Reis, S. (2000) Phenolic acids and derivatives: studies on the relationship among structure, radical scavenging activity, and physicochemical parameters. *Journal of Agricultural and Food Chemistry* **48**, 2122–2126

Silva, O., Duarte, A., Cabrita, J., Pimentel, M., Diniz, A. and Gomes, E. (1996) Antimicrobial activity of Guinea-Bissau traditional remedies. *Journal of Ethnopharmacology* **50**, 55–59

Singleton, V.L., Orthofer, R., Lamuela-Ravenos, R.M. (1999) Analysis of total phenols and other oxdiation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology* **299**, 15–27

Singleton, V.L. and Rossi J.A. Jr., (1965) Colorimetry of total phenolics with phosophomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* **16**, 144-158

Skerget, M., Kotnik, P., Hadolin, M., Hras, A.R., Simonic, M. and Knez, Z. (2005) Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chemistry* **89**, 191–198

Skinner, D. and Keefer, C.S. (1941) Significance of bacteremia caused by *Staphylococcus aureus. Archives of Internal Medicine* **68**, 851–875

Sohn, H.Y., Son, K.H., Kwon, C.S., Kwon, G.S. and Kang, S.S. (2004) Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L., *Morus mongolica* Schneider, *Broussnetia papyrifera* (L.) Vent, *Sophora flavescens* Ait and *Echinosophora koreensis* Nakai. *Phytomedicine* **11**, 666–672

Stadtman, E.R. (1992) Protein oxidation and ageing. *Science* **257**, 1220-1224

Steinmetz, K.A. and Potter, J.D. (1991) Vegetables, fruit, and cancer. II. Mechanisms. *Cancer Causes Control* **2**, 427-442

Stich, H.F. and Rosin, M.P. (1984) Naturally occurring phenolics as antimutagenic and anticarcinogenic agents. *Advances in Experimental Medicine and Biology* **177**, 1-29

Subramanian, M. (2005) Studies on the redox and radiomodifying properties of some biologically active compounds. University of Mumbai, Mumbai

Suffredini, J.B., Sader, H.S., Goncalves A.G., Reis, A.O., Gale,s A.C., Varella, A.D. and Younes, R.N. (2004) Screening of antimicrobial extracts from plants native to the Brazilian Amazon rainforest and Atlantic forest. *Brazilian Journal of Medical and Biological Research* **37**, 379-384

Suominen, I., Andersson, M.A., Andersson, M.C., Hallaksela, A.M., Kämpfer, P., Rainey, F.A. and Salkinoja-Salonen, M. (2001) Toxic *Bacillus pumilus* from indoor air, recycled paper pulp, Norway spruce, food poisoning outbreaks and clinical samples. *Systematic and Applied Microbiology* **24**, 267–276

Tadolini, B., Juliano, C., Piu, L., Franconi, F. and Cabrini, L. (2000) Resveratrol inhibition of lipid peroxidation. *Free Radical Research* **33**, 105–114

Tanaka, M., Kuie, C.W., Nagashima, Y. and Taguchi, T. (1988) Applications of antioxidative Maillard reaction products from histidine and glucose to sardine products. *Nippon Suisan Gakkaishi* **54**, 1409–1414

Taniguchi, M. and Kubo, I. (1993) Ethnobotanical drug discovery based on medicine men's trials in the African savanna: screening of east African plants for antimicrobial activity II. *Journal of Natural Products* **56**, 1539–1546

Tian, B. and Hua, Y. (2005) Concentration-dependence of prooxidant and antioxidant effects of aloin and aloe-emodin on DNA. *Food Chemistry* **91**, 413-418

Tirosh, O., Katzhendler, Y., Barenholz, Y., Ginsburg, I. and Kohen, R. (1996) Antioxidant properties of amidothionophosphates: novel antioxidant molecules. *Free Radical. Biology & Medicine* **20**, 421-432

Tosun, F., Kİzilay, Ç.A,, Erol, K., Kiliç, F.S., Kürkçüoglu, M. and Can Baser, K.H. (2008) Anticonvulsant activity of furanocoumarins and the essential oil obtained from the fruits of *Heracleum crenatifolium. Food Chemistry* **107**, 990-993

Trease, G. and Evans, W. (1972) *Pharmacognosy*. Univ. Press, Aberdeen, Great Britain

Tsarong, T.J. (1986) In: *Handbook of Traditional Tibetan drugs*. Tibetan Medical Publications, Kalimpong, India

Turano, A., Scura, G., Caruso, A., Bonfanti, C. and Luzati, R. (1989) Inhibitory effect of papaverine on HIV replication in vitro. *AIDS Research and Human Retroviruses* **5**, 183-192

Uzun, E., Sariyar, G., Adsersen, A., Karakoc, B., Otuk, G., Oktayoglu, E. and Pirildar, S. (2004) Traditional medicine in Sakarya province (Turkey) and antimicrobial activities of selected species. *Journal of Ethnopharmacology* **95**, 287–296

Van den Akker, E., Lutgerink, J.T., Lafleur, M.V.M., Joenje, H. and Retel, J. (1994) The formation of one-G deletions as a consequence of single-oxygen-induced DNA damage. *Mutation Research* **309**, 45-52

Valero, M. and Salmeron, M.C. (2003) Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. *International Journal of Food Microbiology* **85**, 73–81

Venkatachalam, S.R. and Chattopadhyay, S. (2003) Natural radioprotectors: An Overview. *Current Organic Chemistry* **9**, 389-404

Viscoli, C. (2002) Management of infection in cancer patients. Studies of the EORTC International Antimicrobial Therapy Group (IATG). *European Journal of Cancer* **38**, S82– S87

Visioli, F., Bordone, R., Perugini, C., Bagnati, M., Cau, C. and Bellomo, G. (2000) The kinetics of copper-induced LDL oxidation depend upon its lipid composition and antioxidant content. *Biochemical and Biophysical Research Communications* **268**, 818-822

Vulto, A.G. and Smet, P.A.G.M. (1988) Drugs used in non-orthodox medicine, pp. 999–1005. In: *Meyler's Side Effects of Drugs*, M.M.G. Dukes, ed., 11th edn. Elsevier, Amsterdam

Waling, C. (1975). Fenton's reagent revisited. *Accounts of Chemical Research* **8**, 125-131

Wang, H., Cao, G. and Prior, R.L. (1996) Total antioxidant capacity of fruits. *Journal of Agricultural and Food. Chemistry* **44**, 701-705

Wang, M., Li, J., Rangarajan, M., Shao, Y., LaVois, E.J., Huang, T.C. and Ho, C.T. (1998) Antioxidative phenolic compounds from Sage (*Salvia officinalis*). *Journal of Agricultural and Food Chemistry* **46**, 4869–4873

Wang, S.Y. and Lin, H.S. (2000) Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *Journal of Agricultural and Food Chemistry* **48**, 140-146

Wang, S.Y. and Zheng, W. (2001) Effect of plant growth temperature on antioxidant capacity in strawberry. *Journal of Agricultural and Food Chemistry* **49**, 4977-4982

Wannissorn, B., Jarikasem, S., Siriwangchai, T. and Thubthimthed, S. (2005) Antibacterial properties of essential oils from Thai medicinal plants. *Fitoterapia* **76**, 233–236

Wedge, D.E., Meepagala, K.M., Magee, J.B., Smith, S.H., Huang, G. and Larcom, L.L. (2001) Anticarcinogenic activity of strawberry, blueberry, and raspberry extracts to breast and cervical cancer cells. *Journal of Medicinal Food* **4**, 49-51

Weiss, J.F. and Landauer, M.R. (2003) Protection against ionizing radiation by antioxidant nutrients and phytochemicals. *Toxicology* **189**, 1-20

Weitzman, S.A., Turk, P.W., Howard-Milkowski, D. and Kozlowski, K. (1994) Free radical adducts induce alterations in DNA cytosine methylation. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 1261-1264

Wilcox, A.L. and Marnett, L.J. (1993) Polyunsaturated fatty acid alkoxyl radicals exist as carbon-centered epoxyallylic radicals: a key step in hydroperoxide-amplified lipid peroxidation. *Chemical Research in Toxicology* **6**, 413-416

Willett, W.C. (1995) Diet, nutrition, and avoidable cancer. *Environmental Health Perspectives* **103** (Suppl. 8), 165-170

von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology.* Taylor & Francis, London

Yen, G. and Chen, H.Y. (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of Agricultural and Food Chemistry* **43**, 27-32

Yokozawa, T., Chen, C.P., Dong, E., Tanaka, T., Nonaka, G.I. and Nishioka, I. (1998) Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochemical Pharmacology* **56**, 213–222

Yonzone, G.S., Yonzone, D.K.N. and Tamang, K.K. (1984) Medicinal plants of Darjeeling district. *Journal of Economic and Taxonomic Botany* **5**, 605-616

Yoshida, T., Mori, K., Hatano, T., Okumura, T., Uehara, I., Komagoe, K., Fujita, Y. and Okuda, T. (1989) *Studies on inhibition mechanism of autoxidation by tannins and flavonoids.* V. Radical-Scavenging effects of tannins and related polyphenols on 1,1-diphenyl-2-picrylhydrazyl radical. *Chemical & Pharmaceutical Bulletin* **37**, 1919–1921

Zaika, L.L. (1988) Spices and herbs - their antimicrobial activity and its determination. *Journal of Food Safety* **9**, 97–118

Zafrilla, P., Ferreres, F. and Tomás-Barberán, F.A. (2001) Effect of processing and storage on the antioxidant ellagic acid derivatives and flavonoids of red raspberry (*Rubus idaeus*) jams. *Journal of Agricultural and Food Chemistry* **49**, 3651–3655

Zhang, Y. and Lewis, K. (1997) Fabatins: New antimicrobial plant peptides. *FEMS Microbiology Letters* **149**, 59–64