

# 2

## Review of literature

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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 seasmum 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; Yonzon *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 ( $^{\bullet}\text{O}-\text{O}^{\bullet}$ ) with a triplet ground state. Hence, the sequence of one electron reductions of dioxygen yields (i) superoxide anion radical ( $\text{O}_2^{\bullet-}$ ), a base with its conjugate acid hydroperoxyl radical, (ii) the peroxide ion, a very strong base, and (iii) a hypothetical ( $\text{O}_2^{3-}$ ), precursor of oxide and oxene. Oxide is a conjugate base of water, while oxene is a conjugate base of the highly reactive hydroxyl radical ( $^{\bullet}\text{OH}$ ). In addition, in the presence of various photo-sensitizers, dioxygen can undergo easy excitation to the singlet state to generate the singlet oxygen ( $^1\text{O}_2$ ). 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  $\text{O}_2^{\bullet-}$ , 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 peroxyntirite. 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- $\beta$ -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).

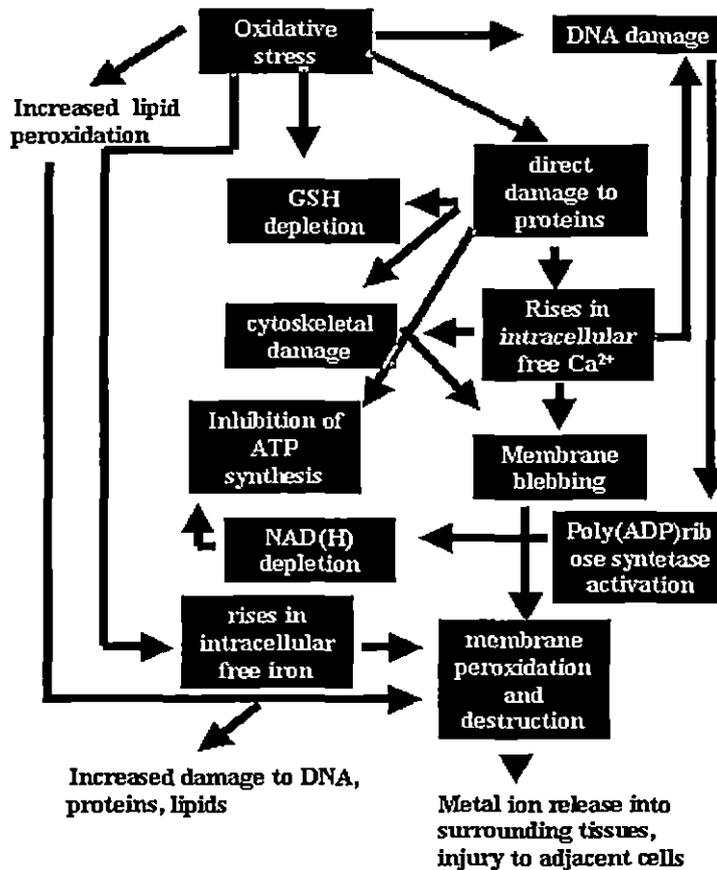


Fig. 1. Cellular events occurring under oxidative stress (Subramanian, 2005)

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 peroxy, alkoxy, 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  $\cdot OH$  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 (Dempfle 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 ( $L\cdot$ ). These radicals can subsequently isomerize to produce conjugated radicals, which can furnish conjugated dienes (by loss of a H atom) or react with

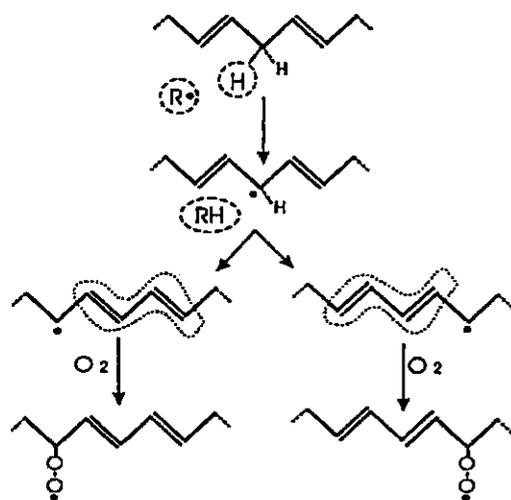


Fig. 2. Reactions involved in LPO (Hogg and Kalyanaraman, 1999)

molecular oxygen forming the lipid peroxy radicals ( $LOO\cdot$ ) or lipid peroxides ( $LOOH$ ) (Wilcox and Marnett, 1993) in a set of chain reactions. The  $LOO\cdot$  can also get degraded (by transition metal ions) to produce alkoxy radicals ( $LO\cdot$ ) (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.

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 peroxy 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 sulfhydryl (SH) groups, formation of carbonyls, disulphide crosslinks, methionine sulfoxide, dityrosine crosslinks, 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

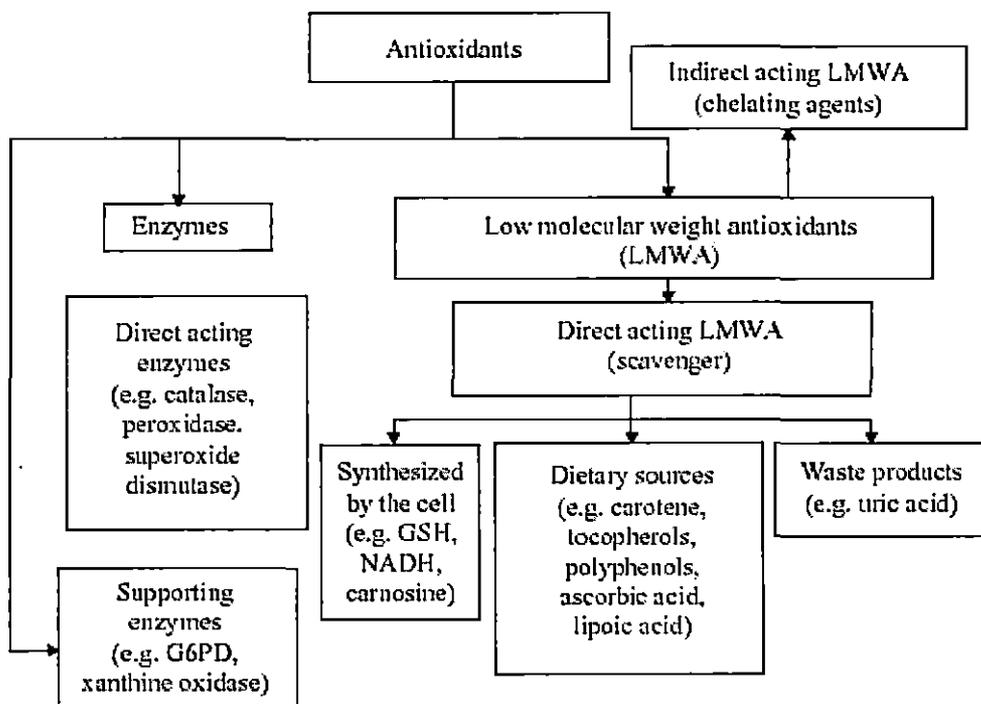


Fig. 3. Overview of cellular antioxidant defense system (Subramanian, 2005)

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  $\beta$ -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.

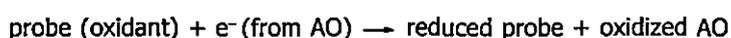
- (A) Monophenols and phenolics acids: These compounds participate mainly via hydrogen donation and radical-scavenging to exert their antioxidant action. To serve as an active antioxidant, the *ortho*- or *para*-position of the phenol moiety must be substituted with electron donating groups to increase 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- $\beta$ -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:



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 ( $\sim 0.7$  V) is similar to ABTS<sup>•+</sup>, and therefore the FRAP and TEAC assays differ in the operating pH values, *viz.* neutral pH (TEAC) and acidic pH,  $\sim 3.6$  (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<sup>•</sup>)-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<sup>•</sup>-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* peroxy radicals may react slowly with DPPH<sup>•</sup>. The reaction kinetics between DPPH<sup>•</sup> and antioxidants is not linear to DPPH<sup>•</sup> concentration; therefore, measurement of the EC<sub>50</sub> 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 peroxy 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 peroxy radical absorbance capacity) of botanical and other biological samples. Initially,  $\beta$ -phycoerythrin ( $\beta$ -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).  $\beta$ -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  $\beta$ -PE leads to its bleaching, even in the absence of any antioxidant. Subsequent replacement of  $\beta$ -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  $\lambda_{\max}$  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<sub>2</sub> consumption or the rate of conjugated diene peroxide formation in order to interpret 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<sub>2</sub> 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 $O_2^{\cdot-}$ -scavenging

The  $O_2^{\cdot-}$  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  $O_2^{\cdot-}$  and  $H_2O_2$ . Thus, XO plus hypoxanthine (or xanthine) at pH 7.4 can be used to generate the  $O_2^{\cdot-}$ , 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  $O_2^{\cdot-}$ . 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 $\cdot OH$ -scavenging

Pulse radiolysis (Bielski, 1985) is one of the often-used techniques for measuring the reaction of the  $\cdot OH$  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- $\cdot OH$  ESR signal by the  $\cdot OH$  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  $\cdot OH$ , 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 peroxyxynitrite 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 *Xylopiya 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

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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, xanthenes 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, galactagogue 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 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*, *Pseudomonas 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 leishmania.

*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 saponins. 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 gonorrhoea 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 *Emblia 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 aeruginosa*. 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.