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).