3
Materials and Methods

3.1. Materials

3.1.1. Culture media

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

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

3.1.2. Reagents

Acetate buffer

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

Dragendorff reagent

(Fluka 44578)

Fenton reagent (Tian and Hua, 2005)

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

Folin-Ciocalteau reagent

(SRL 62015)

Hepes buffer, pH 7.4

(Sigma 49897)

Krebs buffer (Lucas and Szweda, 1998)

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

Phosphate buffer (pH 6.6)

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

Phosphate buffer (pH 7.4)

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

3.1.3. Test microorganisms

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

3.2. Experimental

3.2.1. Survey

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

Table 3. Areas under survey

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

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

Table 4. Questionnaire used for survey

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

3.2.2. Sampling

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

3.2.3. Preparation of methanolic extracts of samples

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

3.2.4. Assay of antioxidant activities of crude methanolic extracts

3.2.4.1. Total phenolics

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

3.2.4.2. Total flavonoids

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

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

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

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

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

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

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

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

3.2.4.5. •OH-scavenging

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

3.2.4.6. Oxygen radical absorbance capacity (ORAC)

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

3.2.4.7. Reducing power (RP)

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

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

3.2.4.8. Metal-chelating (MC) power

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

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

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

3.2.5. Evaluation of protection to biological targets/whole cells

3.2.5.1. Preparation of mice liver homogenate

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

3.2.5.2. Anti-LPO assay

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

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

3.2.5.3. DNA protection assay

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

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

3.2.5.4. Protein oxidation assay

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

3.2.6. Antimicrobial activity assay

3.2.6.1. Disc diffusion protocol

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

3.2.6.2. Broth dilution protocol

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

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

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

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

3.2.8. Antiproliferative potential of the extracts

3.2.8.1. Cell Culture

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

3.2.8.2. Cytotoxic activity of FNR extract

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

3.2.9. Isolation of furanocoumarins from *Heracleum nepalense* fruits

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

3.2.10. Isolation of alkaloids from *Stephania hernandifolia* roots

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

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

3.2.11. Statistical analysis

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