6.1. Introduction

Plant based medicines are widely used and form an integral part of primary health care in many developing countries across the globe (Gill and Akinwumi, 1986; Liu, 1987; Anesini and Perez, 1993; Nick et al., 1995). The medicinal properties of the plants have been investigated due to their potent pharmacological activities, low toxicity and economic viability (Prashant et al., 2008). Natural products present in higher plants are the important source of therapeutic agents and many research groups are currently screening different biological activities of plants (Kuete and Efferth, 2010). This development could lead to new drug discovery or advance the use of indigenous herbal medicines.

Due to this fact, the present study was aimed at to evaluate the biological properties viz., in vitro antimicrobial, in vitro and in vivo antioxidant, acute oral toxicity and in vivo antiinflammatory activities of the leaf and root parts of the study species, Hypochaeris radicata.

6.2. Materials and methods

6.2.1. Chemicals

For the present study all the chemicals are purchased from HiMedia Pvt. Ltd., Bombay. The chemicals used were of analytical grade.

6.2.2. Plant collection

The fresh plant material was collected from Kattabettu, Nilgiris, the Western Ghats, Tamil Nadu, India (above 2000m above msl) during February, 2012 (Plate I).

6.2.3. Preparation of plant extracts

The dust free leaves and roots of Hypochaeris radicata were shade dried and powdered. About 50g of coarsely powdered plant materials (50g/250mL) were extracted in a soxhlet apparatus for 8 to 10hrs, sequentially with petroleum ether, chloroform, ethyl acetate, methanol and aqueous separately in order to extract non-polar and polar compounds (Elgorashi and Van Staden, 2004). The extracts obtained were then
concentrated and finally dried to a constant weight. Dried extracts were kept at 20°C until further test will be carried out.

6.2.4. In vitro antimicrobial activity

6.2.4.1. Disc diffusion assay

6.2.4.1.1. Sources of microbial strains

In vitro antimicrobial activity was examined for various alcoholic and aqueous extracts of *H. radicata* against 15 bacterial species which include the Gram-positive strains *viz.*, *Streptococcus faecalis*, *S. pyogenes*, *Enterococcus faecalis*, *Bacillus subtilis*, *B. thuringiensis* and *Staphylococcus aureus* and Gram-negative strains *viz.*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *P. mirabilis*, *Salmonella paratyphi*, *S. paratyphi A*, *S. paratyphi B*, *Pseudomonas aeruginosa* and *Escherichia coli* and nine fungal species *viz.*, *Paecilomyces lilacinus*, *Mucor sp.*, *Trichoderma viride*, *Verticillium lecanii*, *Candida albicans*, *Fusarium sp.*, *Penicillium sp.*, *Aspergillus fumigatus* and *A. niger*. All these microbial stains were obtained from the Department of Biotechnology, Hindustan College of Arts and Science, Coimbatore.

6.2.4.1.2. Preparation of media

Freshly prepared nutrient agar medium and potato dextrose agar (PDA) medium were used for the culture of bacteria and fungi respectively.

**Composition of Nutrient agar medium**

- Peptone: 5.0g
- Beef extract: 3.0g
- Sodium chloride: 3.0g
- Agar: 15.0g
- Distilled water: 1000mL
- pH: 6.8±0.2

**Composition of Potato dextrose agar (PDA) medium**

- Potato: 200.0g
- Dextrose: 20.0g
- Agar: 15.0g
- Distilled water: 1000mL
- pH: 5.6±0.2
6.2.4.1.3. Preparation of inoculums

For the preparation of bacterial inoculums, a loop full of cells from the stock cultures were transferred to test tubes of nutrient agar medium and were incubated without agitation for 24hrs at 37°C. The cultures were diluted with fresh nutrient agar broth to achieve optical densities corresponding to \( 2 \times 10^6 \) colony forming units (CFU/mL). For the preparation of fungal inoculums, spores of each fungal species were collected from cultures on agar plates after 7 days (Broekaert et al., 1990). PDA broth prepared by transferring a loop full of cells from the stock cultures was diluted with fresh potato dextrose broth. The sporangial suspension concentration was adjusted to \( 2 \times 10^5 \) (CFU/mL) spores (Abril et al., 2008).

6.2.4.1.4. Method

Plant extracts of *Hypochaeris radicata* which was prepared with different solvents viz., petroleum ether, chloroform, ethyl acetate, methanol and aqueous were used to test their antimicrobial activity by disc diffusion method (Bauer et al., 1966). The culture media were prepared and autoclaved at 121°C at 15 psi for 20min and stored in refrigerator. The media were melted before the process of inoculation. The clean dry sterile Petri dishes were poured with nutrient agar medium (for bacteria) and potato dextrose agar medium (for fungi). The bacterial \( (2 \times 10^6) \) and fungal \( (2 \times 10^5) \) suspensions were streaked on the Mueller–Hinton agar medium (for respective microbes) containing the Petri plates. Discs of 5mm diameter were impregnated with the various alcoholic and aqueous leaf and root extracts of *H. radicata* and placed upon the surface of the inoculated plates separately. Similarly, each plate was placed with a sterile disc containing ampicillin and tetracycline as positive control for antibacterial and antifungal activity respectively. All the plates were incubated at 28°C for 24-48hrs. The plates were incubated for overnight at 37°C. The zones of growth inhibition around the disc were measured after 48hrs. The sensitivity of the microbes to the plant extracts were determined by measuring the sizes of inhibition zones (measured in diameter) on the agar surface around the disc.

6.2.4.2. Determination of Minimum Inhibitory Concentration (MIC)

Determination of minimum inhibitory concentration of leaf and root parts of *H. radicata* was made in methanolic extract. For this purpose, the same microbial strains which have been used for antimicrobial assay were used.
6.2.4.2.1. Method

MIC was determined through the broth dilution method (Koneman et al., 1997). Microbes were first grown in the respective broths for 24-48hrs and then the inoculums were diluted for five times (10\(^{-5}\) dilution) to control its vigorous growth. For the determination of MIC, each test tube with 1800μL of respective broth (for bacteria and fungi separately) were supplemented with eight different concentrations of both leaf and root extracts, 100-800μg/mL separately followed by inoculation of 200μL of respective microbes. The results of the extracts were compared with the standard, positive control (ampicillin 100μg/mL and tetracycline 100μg/mL for bacteria and fungi respectively) and negative control (methanol 100μL). All the test tubes were incubated at 37°C for 24-48hrs. The tubes were examined for visual turbidity. The MIC values were taken as the lowest concentration that inhibited the visual growth of the tested organisms (Janovska et al., 2003).

6.2.4.3. Statistical analysis

The antimicrobial activity of *H. radicata* leaf and root extracts was indicated by clear zones of growth inhibition. Analysis was carried out in triplicates and mean±SD (Standard Deviation) using Duncan’s Multiple Range Test (DMRT) (Duncan, 1955). Statistical significance (\(p<0.05\)) were subjected to one way analysis of variance (ANOVA) by using a statistical Package for Social Science (SPSS) (Version 9, SPSS, Inc., Chicago, USA).

6.2.5. *In vitro* antioxidant activity

6.2.5.1. Reducing power activity

The reducing ability of *H. radicata* was determined by the method of Yildirim et al. (2001). Various concentrations of plant extracts (300-700μg/mL) were mixed with 1.0mL of 0.2M sodium phosphate buffer (pH 6.6) and 1.0mL of freshly prepared 1% potassium ferric cyanide. The mixture was incubated in water bath at 50°C for 20min. Then 1.0mL of 10% trichloro acetic acid (TCA) was added and centrifuged at 3000rpm for 10min. The supernatant (2.0mL) was mixed with 2.0mL of distilled water and 500μL of 1% ferric chloride (freshly prepared). The absorbance was read at 700nm. Higher absorbance of the reaction mixture indicates greater reducing power. The results were compared with that of the standard antioxidants viz., rutin, quercetin, BHA and BHT.
6.2.5.2. DPPH radical scavenging activity

The ability of *H. radicata* extracts to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH’) radicals was assessed by using Blois (1958) method with some modifications. A 0.2mM solution of DPPH’ in methanol was prepared and 500µL of this solution was added to different concentrations of the extracts (50-250µg/mL). The mixture was shaken vigorously and allowed to stand for 30min at room temperature. Control was prepared as above but without the sample extracts and methanol was used for the baseline correction. Then changes in the absorbance of the plant samples were measured at 517nm using spectrophotometer. A lower absorbance value indicates the higher radical scavenging activity. Results were compared with the standard antioxidants (rutin, quercetin, BHA and BHT). The ability of DPPH radical scavenging activity was calculated by using the following formula:

\[
\text{DPPH’ scavenging effect (\% of inhibition) = \left( \frac{A_0 - A_1}{A_0} \right) \times 100}
\]

Where, \(A_0\) is the absorbance of the control, and \(A_1\) is the absorbance of the extracts. The IC\(_{50}\) (the microgram of extract to scavenge 50\% of the radicals) value was calculated using linear regression analysis. Lower IC\(_{50}\) value indicates greater antioxidant activity.

6.2.5.3. Nitric oxide (NO’) radical scavenging activity

Nitric oxide radical scavenging activity was assessed by the method of Sreejayan and Rao (1997). Nitric oxide radicals were produced from sodium nitroprusside solution and measured by the Griess reagent. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide radicals which interfere with oxygen to produce nitrite ions. Scavengers of nitric oxide act against oxygen, leading to reduce production of nitrite ions. 3.0mL of sodium nitro prusside (10mM) in phosphate buffer saline (PBS, 0.2mM, pH 7.4) was added with various concentrations of the extracts (250-450µg/mL) and it was incubated at 25°C for 150min. Then 500µL of Griess reagent (1% sulphailamide, 2% orthophosphoric acide, 0.1% N-1-napthylethylenediamine dihydrochloride) was added. The absorbance values were measured at 546nm and percentage of inhibition was calculated using the same formula as followed for DPPH’. The decreasing of OD (optical density) values shows high nitric oxide radical scavenging activity. The IC\(_{50}\) value was calculated and it was compared with the standard antioxidants, rutin, quercetin, BHA and BHT.
6.2.5.4. Total antioxidant activity by ABTS radical cation decolorization assay

The total antioxidant activity of *H. radicata* was measured by decolorization of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS⁺⁺) radical cation using the method of Siddhuraju and Manian (2007). ABTS radical cation was generated by oxidation of ABTS⁺⁺ (7mmol/L) with potassium persulfate (2.45mmol/L) which was dissolved in 5.0mL of distilled water. After incubation for 12-16hrs at room temperature in dark condition blue/green ABTS⁺⁺ chromophore was produced. The ABTS⁺⁺ solution was diluted with ethanol (1:89v/v) and adjusted to equilibrate the absorbance of 0.700±0.001 at 734nm. The generated ABTS⁺⁺ solution (2.0mL) was mixed with 20µL of sample extracts or trolox standards (0-15µM). The absorbance values were read at 734nm exactly after 30min. The total antioxidant activity unit was defined as the concentration of trolox having the equivalent antioxidant activity expressed as µmole/g extract.

6.2.5.5. Inhibition of β-carotene bleaching assay

The antioxidant activity of *H. radicata* was evaluated by the β-carotene linoleate model system (Taga et al., 1984). A solution of β-carotene-linoleic acid mixture was prepared by dissolving 1mg of β-carotene in 10mL of chloroform. To this, 20µL of linoleic acid and 200mg of Tween-40 emulsifier were added. Then the mixture containing chloroform solvent was completely removed by vacuum using a rotary vacuum evaporator at 45°C. For the formation of emulsion, 50mL of oxygenated distilled water was slowly added to this semi-solid residue and shaken vigorously. Aliquots (5.0mL) of this emulsion were transferred into different test tubes containing 100µg/mL of the sample extracts. An initial (0 time) absorbance at 470nm was immediately recorded. Subsequent absorbance readings were recorded at 15min intervals by keeping the sample tubes in a water bath at 50°C until the visual of β-carotene in the control sample disappeared (about 120min). Control samples (devoid of β-carotene) were used for the subtraction. Rutin, quercetin, BHA and BHT were used as standards. The antioxidant activity (AA) was expressed as per cent relative inhibition compared to control samples after 120min and calculated as:

\[
(\%) \text{ AA} = [1-(A_0-A_1)/(Z_0-Z_1))] \times 100
\]
Where, $A_0$ is the absorbance of sample at 0 minute, $A_1$ is the absorbance of sample at 120min, $Z_0$ is the absorbance of control at 0 min and $Z_1$ is the absorbance of control at 120min.

6.2.5.6. Antihaemolytic activity

The inhibition of cow erythrocyte haemolysis by the extract was evaluated according to the method described by Naim et al. (1976). The cow erythrocyte was performed with the help of $H_2O_2$ (hydrogen peroxide) as free radical initiator. The erythrocytes from cow blood were separated by centrifugation at 2000rpm for 10min and washed with saline phosphate buffer (0.9g of sodium chloride dissolved in 100mL of 0.2M phosphate buffer, pH 7.4) until the supernatant become colourless. The erythrocytes were then diluted with saline phosphate buffer to give 4% (v/v) suspension. 500µg samples and 1.0mL saline phosphate buffer were added to 2.0mL of 4% erythrocyte suspension and the volume was made up to 5.0mL with saline phosphate buffer. This mixture was pre-incubated for 5min and the 500µL of $H_2O_2$ in the reaction mixture was adjusted as to bring about 90% haemolysis of blood cells after 240min. After incubation, the mixture was centrifuged at 1500rpm for 10min and measured at 540nm. Natural (rutin and quercetin) and synthetic (BHA and BHT) standards at the same concentrations as sample extracts were used for comparison. The per cent haemolysis inhibition was calculated by using the same formula employed in DPPH’ assay.

6.2.5.7. Statistical analysis

Analysis was carried out in triplicates and mean±SD (Standard Deviation) using Duncan’s Multiple Range Test (DMRT) (Duncan, 1955). Statistical significance ($p<0.05$) were subjected to one way analysis of variance (ANOVA) by using a statistical Package for Social Science (SPSS) (Version 9, SPSS, Inc., Chicago, USA).

6.2.6. Pharmacological activity

6.2.6.1. Experimental Animals

For acute oral toxicity study, Swiss albino female mice (15-20g) and for in vivo antiinflammatory activity, the healthy female Sprague Dawley (SD) rats, weighing 150-200g, were obtained from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Kerala Agricultural University, Manuthy, Thrissur, Kerala, India. The rats were housed in polyethylene cages allowed for one week for acclimatization prior to
experiment. The animals were maintained under standard environmental conditions such as temperature and humidity (25±0.50°C) and light (14hrs light and 10hrs dark period). They were fed with standard pellet diet (M/S Hindustan Unilever Ltd., Mumbai) and water *ad-libitum*. The experiments were performed in the institute with due permission from Institutional Animal Ethical Committee (Approval No. 659/02/a/CPCSEA).

6.2.6.2. Acute oral toxicity

Swiss albino mice were divided into one control group and five treated groups, each group consisting of six animals (Lorke, 1983). The methanolic leaf and root extracts were administered orally as a single dose at a different concentration of 100, 500, 1000, 2000 and 3000mg/kg b.w. (body weight) dissolved in distilled water. After administration, the animals were observed individually for 4hrs and thereafter 14days. They were observed for their any change in general behaviour or other physiological activities along with mortality (Ghosh, 2007) if any as per Organisation for Economic Cooperation and Development (OECD) guidelines (OECD, 2001).

6.2.6.3. *In vivo* antiinflammatory activity

6.2.6.3.1. Preparation of test samples for bioassay

For the antiinflammatory test model, samples were given orally to test animals after suspending in distilled water. The control group animals received the same experimental handling as those for the test groups except the drug treatment which was replaced with appropriate volumes of the dosing vehicle. Indomethacin (10mg/mL) in distilled water was used as a non-steroidal reference drug.

6.2.6.3.2. Carrageenan induced hind paw oedema

Carrageenan induced hind paw oedema model of acute inflammation was used for the determination of *in vivo* antiinflammatory activity by the method of Winter *et al.* (1962). After adaptation, the female Sprague-Dawley rats were randomly divided into seven groups of six animals each. Group I (normal rats), Group II (negative control-induced), Groups III and IV, and V and VI (test groups) received 150 and 300mg/kg b.w. of methanolic leaf and root extracts respectively, Group VII (positive control-standard) received 10mg/kg b.w. of indomethacin orally. The animals were deprived of food prior to the experiment but they have given free access for water *ad-libitum*. After 1hr of drug administration, acute inflammation was induced in Groups II to VII by sub plantar injection of 0.1mL of 1% (w/v) carrageenan in the left paw of the rats. The paw volume
was measured using vernier caliper at every 30min after the injection of carrageenan upto 4hrs. Group I served as reference to non inflamed paw for comparison. The reduction in the paw volume was measured and the percentage inhibition of inflammation was calculated using the formula:

\[
\text{% inhibition of inflammation} = \frac{P_c - P_t}{P_c} \times 100
\]

Where,

\(P_c\) = the average inflammation (left hind paw oedema) of the negative control group at a given time period.

\(P_t\) = the average inflammation (left hind paw oedema) of the treated group at a given time period.

**6.2.6.3.3. Sacrification, sample collection and tissue homogenate preparation**

After the completion of the treatment, all animals were anaesthetized with light chloroform (using desiccator) and sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture from the animal using the vacutainer into EDTA anticoagulated for haematological studies and normal tubes for blood coagulation. They were subsequently dissected and the organs (spleen, thymus and left hind paw) were excised and washed in ice cold saline (0.9%). 10% homogenate of the organs were prepared using tissue homogenizer with 0.1M Tris-HCl buffer (pH-7.4). The homogenates were centrifuged at 3000rpm for 15min at 4°C for cytosolic separation. For separation of serum, the coagulated blood was centrifuged at 3000rpm for 20min and was stored at 4°C for further analysis. The clear supernatant was used for NO\(^-\), protein, LPO, HPO and various antioxidant assays. A section of hind paw was preserved in 10% formalin for histopathological studies.

**6.2.6.3.4. Haematological studies**

Haematological parameters such as complete blood cell count i.e. haemoglobin (Hg), packed cell volume (PCV) or hematocrit (HCT), white blood cell count (WBC), red blood cell count (RBC), platelet count (PLC), neutrophils (Neu), lymphocyte (LYM), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) were performed at Sysmex XS800i automated haematology analyzer (Sysmex Corporation, Japan) using standard methods (Dacie and Lewis, 1994).
6.2.6.3.5. Estimation of nitric oxide (NO•)

500μL of Griess reagent (1% sulfanilamide, 2% orthophosphoric acid and 0.1% N-1-naphyl ethylene diamine dihydrochloride) was added to 100μL of serum samples. Pink colour was developed and it was measured at 540nm. Nitrite concentration was calculated using a standard curve for sodium nitrite and expressed as mg/dL (Green et al., 1982).

6.2.6.3.6. Estimation of protein

Estimation of protein by Folin’s reaction (Lowry et al., 1951) has been most widely used method to estimate the amount of total proteins present in serum samples. Briefly, 0.1mL of the serum sample was made up to 1mL with distilled water and then treated with 5mL of alkaline copper reagent. This solution was vortexed and allowed to stand for 10min. After, 500μL of Folin’s ciocalteaus reagent (1:2 dilution with water) was added and incubated at room temperature for 30min. The aromatic aminoacids in copper solution reduce the phosphomolybdic-phosphotungstic acid present in the Folin’s reagent. The end of this reaction blue colour was produced; it was measured at 660nm using bovine serum albumin as a standard. The total protein content was expressed as g/dL.

6.2.6.3.7. Enzymic antioxidant activity

6.2.6.3.7.1. Estimation of superoxide dismutase (SOD)

Estimation of SOD was done by according to the procedure suggested by Das et al. (2000). A 0.1ml of tissue homogenate was added to 1.4mL of reaction mixture comprised of 1.1mL of 50mM phosphate buffer (pH-7.4), 75μL of 20mM L-methionine, 40μL of 1% (v/v) Triton X-100, 75μL of 10mM hydroxylamine hydrochloride and 100μL of 50mM EDTA followed by a brief incubation at 37ºC for 5min. After that 80μL of 50mM riboflavin was added and then this mixture was kept under 20W fluorescent light. After the exposure, 1mL of Griess reagent was added and absorbance of the pink colour developed was measured at 543nm. One unit of enzyme activity is defined as the amount of SOD inhibiting 50% of nitrite formation under assay conditions.

6.2.6.3.7.2. Estimation of catalase (CAT)

The activity of CAT in the tissue homogenate was determined by the method of Sinha (1972). To 0.9mL of 0.01M phosphate buffer (pH-7.4), 0.1mL of tissue
homogenate and 0.4mL of 0.2M hydrogen peroxide were added at 0 seconds and after 60 seconds. The reaction was terminated by the addition of 2mL of dichromate–acetic acid reagent which was prepared by mixing of 5% of potassium dichromate with glacial acetic acid (1:3 by volume). Then the tubes were kept in boiling water bath for 10min. Dichromate in acetic acid is reduced to chromic acetate when heated in presence of H₂O₂ resulting in formation of per chromic acid as an unstable intermediate. The colour of the chromic acetate was measured at 620nm. The standard in the range of 0.2M H₂O₂ were taken and processed as test and blank containing reagent alone. The activity was expressed as μmole of H₂O₂ decomposed/min/mg protein.

6.2.6.3.7.3. Estimation of glutathione peroxidase (GPx)

GPx activity was measured by the method ascribed by Ellman (1959). Briefly, the reaction mixture contained 0.4mL of 0.4M phosphate buffer (pH-7.0), 0.2mL of 4mM EDTA, 0.1mL of 10mM sodium azide, 0.2mL of 4mM reduced glutathione, 0.1mL of 2.5mM H₂O₂ together with 0.2mL of tissue homogenate was added and the contents were mixed well. Then these contents were incubated at 37°C for 10min. After incubation, the reaction was terminated with 0.5mL of 10% ice cold trichloro acetic acid solution. To determine the glutathione content, 1.0mL of supernatant was removed by centrifugation. To that added, 3.0mL of buffer and 0.5mL of Ellman’s reagent (19.8mg 5,5′-dithiobis-(2-nitro benzoic acid) (DTNB) in 1% sodium citrate). The colour developed was read at 412nm. Standards in the range of 4-200μg of reduced glutathione was taken and treated in the similar manner. The activity was expressed in term of μmole of glutathione consumed/min/mg protein.

6.2.6.3.7.4. Estimation of glutathione-s-transferase (GST)

GST assay was determined according to the method of Habig et al. (1974). A 0.1mL of tissue homogenate was added to the reaction mixture containing 1mL of 0.5M phosphate buffer (pH-6.5), 1.7mL of distilled water and 0.1mL of 30mM 1-chloro-2,4-dinitrobenzene (CDNB). The phosphate buffer-CDNB mixture was preincubated at 37°C for 10min and the reaction was started by adding 0.1mL of reduced glutathione (GSH). The rate of increase in absorbance was measured at 340nm. This enzyme activity was expressed as μmole of CDNB-GSH conjugate formed/min/mg protein.
6.2.6.3.7.5. Estimation of glucose-6-phosphate dehydrogenase (G6PD)

The activity of G6PD in the tissue homogenate was done by the method of Balinsky and Bernstein (1963). This solution containing, tissue homogenate 0.2mL each of 0.4mL of 1M Tris-HCl buffer (pH-8.2), 0.2mL of 0.2mM nicotinamide adinine dinucleotide phosphate (NADP), 0.2mL of 1M magnesium chloride and 1mL of distilled water. After the addition of 0.2mL of 6mM glucose-6-phosphate the reaction was initiated and the increase in the absorbance was measured at 340nm. The activity of the enzyme is expressed in terms of units/mg protein, in which one unit is equal to the amount of the enzyme that brought about an increase in OD of 0.01/min.

6.2.6.3.8. Non-enzymic antioxidant activity

6.2.6.3.8.1. Estimation of total reduced glutathione (GSH)

GSH was estimated as described by the method of Moron et al. (1979). For the estimation, 0.5mL of tissue homogenate was immediately precipitated with adding 2mL of 5% ice cold trichloro acetic acid (TCA) and the precipitate was removed after centrifugation (5000rpm for 5min). Free endogenous-SH was assayed in a total volume of 3mL by the addition of 0.5mL of Ellman’s reagent [19.8mg 5, 5’ dithio-bis-(2-nitrobenzoic acid) in 1% sodium citrate] and 3mL of 0.2M phosphate buffer (pH-8.0) to 1mL of supernatant. The intensity of the yellow colour formed was measured at 412nm with 4min. Reduced glutathione was taken at different concentrations (20mg in 100mL distilled water) and the standard graphs were plotted. The levels of GSH were expressed as μg of GSH/mg protein.

6.2.6.3.8.2. Estimation of ascorbic acid (Vitamin C)

The content of ascorbic acid in tissue homogenate was determined by Omaye et al. (1979). To 1mL of tissue homogenate 0.5mL of 5% ice cold trichloro acetic acid was added and centrifuged at 3500rpm for 20min. To 1mL of supernatant, 0.2mL of DTC reagent [3g of 2, 4-dinitrophenyl hydrazine (DNPH), 0.4g thiourea and 0.05g of copper sulphate were dissolved in 9N H2SO4] was added. This mixture containing solution was incubated at 3hrs for 37°C. After incubation, 1.5mL of ice cold 65% sulfuric acid was added and mixed well. Then after 30min of incubation colour was developed and it was read at 530nm. Ascorbic acid content was expressed in terms of μg/mg protein. Standard solution was prepared at different concentrations (4-20μg/mL in 5% oxalic acid) and standard graphs were plotted.
6.2.6.3.9. Estimation of lipid peroxides (LPO)

LPO in the tissue homogenate and serum samples were evaluated by thiobarbituric acid reactive substances and measuring Malondialdehyde (MDA) according to the method of Niehaus and Samuelsson (1968). 0.5mL of the samples was treated with 2mL of TBA-TCA-HCl reagent (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25N hydrochloric acid in 1:1:1 ratio). The mixture was placed in boiling water bath for 15min, cooled and centrifuged at 1000rpm for 10min to remove the precipitate. The absorbance was read at 535nm, and the contents were expressed as μmole of MDA formed/mg protein. Standards in the range of 20-100μg MDA/100mL of water were treated similarly.

6.2.6.3.10. Estimation of hydroperoxides (HPO)

The estimation was carried out by the method of Jiang et al. (1992). The tissue homogenate and serum samples were treated with 0.9mL of Fox reagent (88mg of butylated hydroxyl toluene, 7.6mg of xylenol orange and 98mg of ammonium ferrous sulphate were added to 90mL of methanol and 10mL of 250mM H2SO4). This mixture was incubated at 37°C for 30min. Then the colour produced was read at 560nm. The contents were expressed mmole/g protein. H2O2 (0.1M H2O2 diluted to 100mL of distilled water) was used as reference standard.

6.2.6.3.11. Histopathological studies

After scarification of rats, one left hind paw from each group was taken, washed under ice cold saline and a small portion of tissue from inflamed region were quickly fixed in 10% formalin.

6.2.6.3.11.1. Tissue processing

The tissues were placed in 10% formal saline (10% formalin in 9% sodium chloride) for one hour to rectify shrinkage due to higher concentration of formalin. The tissues were dehydrated by ascending grades of isopropyl alcohol by immersing in 80% overnight, 100% for one hour and second change of 100% isopropyl alcohol for one hour. The dehydrated tissues were cleared in two changes of xylene, one hour each. Then the tissues were impregnated with histology grade paraffin wax at 60°C for 2 changes of one hour each. The wax impregnated tissues were embedded in paraffin blocks using the same grade wax. The paraffin blocks were mounted and cut with rotary microtome at 3 micron thickness. The sections were floated on a tissue floatation bath at 40°C and taken
on glass slides and smeared with equal parts of egg albumin and glycerol. The sections were then melted in a incubator at 60°C and after 5min, the sections were allowed to cool.

6.2.6.3.11.2. Tissue staining

The sections were deparaffinised by immersing in xylene for 10min in horizontal staining jar. The deparaffinised sections were washed in 100% isopropyl alcohol and stained in Ehrlich’s hematoxylin for eight minutes in horizontal staining jar. After staining in hematoxylin, the sections were washed in tap water and dipped in acid alcohol to remove excess stain (8.3% HCl in 70% alcohol). The sections were then placed in running tap water for 10min for slow alkalinization. The sections were counter stained in 1% aqueous eosin (1.0g in 100mL water) for one minute and the excess stain were washed in tap water and the sections were allowed to dry. Complete dehydration of stained sections was ensured by placing the sections in the incubator at 60°C for five minutes. When the sections were cooled, they were mounted in DPX mount having the optical index of glass (the sections were wetted in xylene and inverted on to the mountant placed on cover slip). The architecture was observed at low power objective. The tissue injury and other aspects were observed under high power dry objective (Allen, 1992).

6.2.6.3.12. Statistical analysis

All the experimental results were presented as the mean±SD (Standard Deviation) (n=6) using Duncan Multiples Range Test (DMRT) (Duncan, 1955). Statistical significance between the control and experimental groups were determined using one-way analysis of variance (ANOVA) by Tukey’s multiple comparison range test (Graph pad prism 4.0 software) statistical significance were expressed as (a)p<0.001, (b)p<0.01, (c)p<0.05, (d)p<0.001, (e)p<0.01 and (f)p<0.05. Here, p-value of 0.001 (p<0.001) was determined to be awfully significant.

6.3. Results

6.3.1. In vitro antimicrobial activity

6.3.1.1. In vitro antibacterial activity

Tables 19 and 20, Figs. 30 and 31 and Plate X exhibit the data on antibacterial activity of leaf and root extracts of the study species, H. radicata. The results of the
study revealed that higher antibacterial activity was produced by methanol extract of both leaf and root parts than that of the other solvent extracts attempted viz., chloroform, ethyl acetate, petroleum ether and aqueous. Further, it was observed that the inhibitory activity of the extracts on both bacterial strains were solvent specific. Among the solvents tried, greater zone of inhibition was produced by methanolic leaf extract against the bacterium, *Enterococcus faecalis* (11.86 mm) while the petroleum ether extract showed lower inhibitory activity for all bacterial strains tested which was ranging between 5.80 and 7.36 mm. The methanolic root extract showed higher zone of inhibition against the bacteria, *Bacillus subtilis* (17.10 mm) and *Staphylococcus aureus* (16.06 mm), while the petroleum ether and water extracts showed lower antibacterial activity of 5.66 and 7.20, and 5.93 and 6.20 mm respectively. The bacteria viz., *Streptococcus faecalis*, *S. pyogenes*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *P. mirabilis*, *Salmonella parathypi* A, *S. paratyphi* B and *Pseudomonas aeruginosa* were found to be most resistant to water extract showing no inhibition. It was further observed that the inhibitory activity of methanolic leaf extract against the bacteria viz., *Bacillus subtilis*, *Klebsiella pneumoniae* and *Proteus mirabilis*, and inhibitory activity of root extract against the bacteria viz., *Bacillus subtilis*, *Staphylococcus aureus*, *Serratia marcescens*, *Proteus mirabilis* and *Salmonella paratyphi* B were significantly greater than that of the standard drug, ampicillin.

**6.3.1.2. In vitro antifungal activity**

Exploitation of the evaluation of antifungal activity of the present study revealed that the *Hypochaeris radicata* possess potential antifungal activity against nine pathogenic fungal species (Tables 21 and 22, Figs. 32 and 33 and Plate XI). From the evaluation it was found that ethyl acetate extract inhibited the growth of the colonies of large number of fungal species than the other solvent extracts studied. Among the five solvents attempted, the ethyl acetate extract showed higher inhibitory activity (24.83 mm) followed by methanol extract (19.96 mm) and chloroform extract (12.96 mm) against the fungus, *Aspergillus niger*. The chloroform and methanol extracts showed significant activity against all the tested fungal species which was ranging between 7.86 mm and 13.50 mm, and 7.60 mm and 19.96 mm respectively. However, the petroleum ether and water extracts showed moderate activity against the fungal species viz., *Mucor* sp., *Aspergillus fumigatus*, *A. niger* and *Candida albicans* and *A. niger* respectively. The
greater zone of inhibition was produced by ethyl acetate and chloroform extracts of root of *Hypochaeris radicata* against the fungi, *Mucor* sp. and *Trichoderma viride* (20.96mm) followed by methanol, aqueous and petroleum ether extracts. Methanol extract showed highest activity against *Trichoderma viride* (14.83) and *Aspergillus niger* (14.65mm) and exerted minimum activity against the fungus, *Fusarium* sp. The fungal growth in the petroleum ether and aqueous extracts was very less and not noteworthy.

**6.3.1.3. Minimum Inhibitory Concentration (MIC)**

As the methanolic extracts are more promising and effective, determination of MIC was made only for this alcoholic extract of both leaf and root parts of *Hypochaeris radicata* against 15 pathogenic bacteria of both Gram-positive and Gram-negative types (Table 23 and Fig. 34). The MIC of the methanolic leaf and root extracts of the study species was ranging between 300 and 500µg/mL, and 300 and 700µg/mL respectively. MIC of methanolic leaf and root extracts of *H. radicata* against the 9 pathogenic fungi was shown in Table 24 and Fig. 35. The leaf and root extracts exhibited remarkable antifungal activity which was ranging between 200 and 500µg/mL and 200 and 600µg/mL respectively. Further, the results were compared with positive and negative controls. The positive control (ampicillin for bacteria and tetracycline for fungi) inhibits the growth all the microbes and the negative control (methanol) did not showed any inhibitory activity against the microbes.

**6.3.2. In vitro antioxidant activity**

**6.3.2.1. Reducing power activity**

The reducing capacity of leaf and root parts of *H. radicata* was much varied across the solvents used for extraction (Table 25 and Fig. 36). The reducing power activity increased with the increase in concentration of extracts. In the present study, ethyl acetate leaf extract showed highest reducing ability (absorbance 2.142 at 700nm). In addition, chloroform, ethyl acetate and methanol extracts of both leaf and root parts exhibited high reducing power activity than that of the petroleum ether and aqueous extracts and it was comparable to that of the standard antioxidants (Table 26 and Fig. 37).

**6.3.2.2. DPPH radical scavenging activity**

The data on DPPH radical scavenging activity of leaf and root parts of *H. radicata* along with the best known natural and synthetic antioxidant standards, viz.,
rutin, quercetin, BHA and BHT are presented in Table 27 and Fig. 38. The mean IC\textsubscript{50} values of ethyl acetate leaf and root extracts were lower which showed that the radical scavenging activity was effective in this solvent extracts (125.94 and 122.54µg/mL respectively). Further, the IC\textsubscript{50} values of the leaf and root extracts were comparable to that of the natural and synthetic standard antioxidants. Next to methanolic leaf (130.54µg/mL) and root (134.40µg/mL) extracts, root chloroform extract (135.87µg/mL) showed significant activity. Among the all extracts, aqueous leaf extract had higher IC\textsubscript{50} value (595.23µg/mL) which indicated its poor scavenging property of DPPH radical.

6.3.2.3. Nitric oxide radical scavenging activity

The nitric oxide scavenging activity of the leaf and root extracts of \textit{H. radicata} was found to be strong which has been indicated by the IC\textsubscript{50} values of 114.94µg/mL for chloroform leaf extract and 91.07µg/mL for methanolic root extract (Table 27 and Fig. 39). The IC\textsubscript{50} values of the extract were comparable to that of the standards used.

6.3.2.4. Total antioxidant activity by ABTS radical cation decolorization assay

The ethyl acetate extracts of root and leaf parts registered better activity (3675.4 and 2639.2 µmole Trolox equivalent/g extract respectively) than the other solvents performed. Similarly, the root chloroform extract had more pronounced ABTS radical cation scavenging activity (3766.5 µmole Trolox equivalent/g extract) (Table 27 and Fig. 40).

6.3.2.5. Inhibition of β-carotene bleaching assay

Table 27 and Fig. 41 depict the inhibition of β-carotene bleached by the leaf and root parts of various alcoholic and aqueous extracts of \textit{H. radicata}. In this β-carotene bleaching system, the highest antioxidant activity was exhibited by leaf methanol extract (90.43%) and root aqueous extracts (90.34%). These values were comparably higher than that of the natural and synthetic antioxidants used.

6.3.2.6 Antihaemolytic activity

Table 27 and Fig. 42 showed the antihaemolytic activity of various alcoholic and aqueous extracts of \textit{H. radicata}. Highest antihaemolytic activity was found in water root extract (68.45%). It is nearly equal to that of the rutin (69.00%) and quercetin (70.00%) of natural antioxidant standards. The antihaemolytic activity of petroleum ether root
extract and methanolic leaf extract (46.25 and 34.98% respectively) was significantly higher than the other solvent extracts.

6.3.3. Pharmacological activity

6.3.3.1. Acute oral toxicity

In acute toxicity study, oral administration of graded doses (100–3000mg/kg b.w.) of the methanolic leaf and root extracts did not repeat any significant changes in behaviours during the observation period and no mortality or any toxicity reaction was recorded in any group after 72hrs of administering the extract to the animals (Table 28). It did not exhibit any signs of toxicity up to 14 days and no animals died. The results showed methanolic leaf and root extracts of *H. radicata* was safe up to a dose level of 3000mg/kg b.w. in mice. Based on the acute toxicity data, two different dosages, 150 and 300mg/kg b.w. were selected for *in vivo* antiinflammatory studies.

6.3.3.2. *In vivo* antiinflammatory activity

The *in vivo* antiinflammatory effect of methanolic extracts of leaf and root parts of *H. radicata* using carrageenan induced oedema tests is expressed in Table 29 and Plate XII. In this test, the positive control-Group VII (94.25%) significantly ($^{\text{d}}p<0.001$) decreased the paw oedema at 4hrs after carrageenan injection compared to Group I-normal rats (Fig. 43). A maximum oedema paw volume of 6.37±0.19mm was observed in the negative control rats, 4hrs after the carrageenan injection. Rats administered with the root extract at 300mg/kg b.w. significantly decreased ($^{\text{d}}p<0.001$) the carrageenan intoxicated paw oedema from 2 to 4hrs and the results were comparable to that of standard drug, indomethacin (10mg/kg b.w.).

6.3.3.2.1. Haematological studies

The data in Table 30 showed haematological modifications associated with orally administered test drugs of *H. radicata* in experimental rats. The carrageenan induced rats showed significant impact on WBC (Fig. 46), Neu and LMP (Fig. 49), MCV (Fig. 50), MCH (Fig. 51) and MCHC (Fig. 52) levels while Hb content (Fig. 44), PCV (Fig. 45), RBC (Fig. 47) and PLC (Fig. 48) were decreased significantly ($^{\text{a}}p<0.001$) in comparison to normal rats. Treatment with the methanolic leaf and root extracts (150 and 300mg/kg b.w.) and standard drug, indomethacin caused retrieval of these changes towards normal level.
6.3.3.2.2. Estimation of nitric oxide (NO')

Carrageenan intoxicated group of rats evidenced a significant ($^a p<0.001$) scale of raise (1.21mg/dL) in serum nitric oxide levels when compared to normal rats. Among the test groups, the higher dose of the methanolic root extract (300mg/kg b.w.) exhibited significant reduction ($^d p<0.001$) than the leaf extract and it was comparable to that of the standard group treated with indomethacin (Table 31 and Fig. 53).

6.3.3.2.3. Estimation of protein

The carrageenan induced rats exhibited significant ($^a p<0.001$) decline in serum protein content when compared to normal rats. A significant restoration ($^d p<0.001$) of protein levels was noticed in oral administration of the methanolic root extract at the high dose (300mg/kg b.w.) than the leaf extract which was comparable to the Group VII as shown in Table 31 and Fig. 54.

6.3.3.2.4. Enzymic antioxidant activity

6.3.3.2.4.1. Estimation of superoxide dismutase (SOD)

The levels of SOD were analyzed to confirm the antioxidant status in experimental animals in tissue homogenates of spleen, thymus and hind paw. Upon induction with carrageenan, the SOD levels decreased significantly ($^a p<0.001$). The test groups showed an increase in levels of SOD to certain extent. Interestingly, the higher dose of the methanolic root extract (300mg/kg b.w.) offered better protection ($^d p<0.001$), similar to the Group VII standard drug, indomethacin administered rats (Table 32 and Fig. 55).

6.3.3.2.4.2. Estimation of catalase (CAT)

CAT levels in spleen, thymus and hind paw were depressed significantly ($^a p<0.001$) upon carrageenan induction (Table 32 and Fig. 56). Consequently, treatment with the oral administration of methanolic extract of leaf and root parts of *H. radicata* caused significant restoration of their levels to near-normal control values ($^d p<0.001$). The most active was noted in higher dose of the root extract (300mg/kg b.w.) elevated the enzyme activity more efficiently ($^d p<0.001$) than the leaf extract, similar to the Group VII reference standard.
6.3.3.2.4.3. Estimation of glutathione peroxidase (GPx)

The values obtained in the treated groups showed notable rising of antioxidant status on spleen, thymus and hind paw of the tissue homogenate samples. Carrageenan induced animals showed decline in GPx activity ($^ap<0.001$). The methanolic higher dose of leaf and root extracts (300mg/kg b.w.) had better renovation ($^dp<0.001$) attenuated towards normal rats that was comparable to that of standard group, indomethacin (Table 32 and Fig. 57).

6.3.3.2.4.4. Estimation of glutathione-s-transferase (GST)

The antioxidant potential of the methanolic leaf and root extracts of $H. radicata$ was evaluated using antioxidant enzyme, GST on the samples of spleen, thymus and hind paw. The Group II animals showed decrease in GST levels ($^ap<0.001$) upon carrageenan induction. The enzyme levels were found to increase in experimental groups studied. The spleen and thymus tissue homogenate values were restored well ($^dp<0.001$) in the root high dose methanolic extract (300mg/kg b.w.) than the leaf extract. However, the high dose of leaf extract (300mg/kg b.w) exhibited better elevation ($^adp<0.001$) in hind paw samples. These data were comparable to the standard, indomethacin (Table 32 and Fig. 58).

6.3.3.2.4.5. Estimation of glucose-6-phosphate dehydrogenase (G6PD)

The enzyme levels of G6PD were analyzed to determine the antioxidant status in tissue homogenates of spleen, thymus and hind paw tissues. Upon induction with carrageenan, the G6PD levels were decreased significantly ($^p<0.001$) (Table 32 and Fig. 59). Among the two extracts analyzed, the root high dose showed increased level of G6PD ($^dp<0.001$) to the normal rats similar to Group VII.

6.3.3.2.5. Non-enzymic antioxidant activity

6.3.3.2.5.1. Estimation of total reduced glutathione (GSH)

Results on the total reduced glutathione level in tissue homogenates of spleen, thymus and hind paw are reported in Table 33 and Fig. 60. Carrageenan intoxicated group of rats evidenced a significant ($^p<0.001$) reduction in GSH level, when compared to the normal rats. Treatment with the test groups attenuated these changes towards normal levels. The effect was more pronounced only in the group of rats treated with higher dose of both leaf and root parts ($^p<0.001$). However, in thymus and hind paw
tissues, the increased level of antioxidants was noted by the administration of root high dose methanolic extract ($d_p<0.001$).

6.3.3.2.5.2. Estimation of ascorbic acid (Vitamin C)

The carrageenan induced rats exhibited significant ($d_p<0.001$) decline in ascorbic acid content when compared to Group I. A significant restoration ($d_p<0.001$) of ascorbic acid content was noticed in oral administration of the methanolic leaf and root extracts at high dose (300mg/kg b.w.) which was comparable to the Group VII (Table 33 and Fig. 61).

6.3.3.2.6. Estimation of lipid peroxides (LPO)

The level of LPO was found to be higher in carrageenan induced rats ($d_p<0.001$). Conversely, the increased levels of LPO are the indicative of the oxidative stress which was minimized by treatment with the test groups. Upon treatment with the methanolic extract of root high dose prominent reduction ($d_p<0.001$) was noticed in LPO levels in serum and spleen samples. On the other hand, the thymus and hindpaw samples had significant impact in reduction level ($d_p<0.001$) in both leaf and root high doses. The results were tabulated in Table 34 and Fig. 62.

6.3.3.2.7. Estimation of hydroperoxides (HPO)

Table 38 and Fig. 48 showed the effect of HPO level in serum, spleen, thymus and hind paw samples. The level of HPO increased dramatically in the carrageenan induced rats ($d_p<0.001$). The test groups elicited greater restoration of HPO levels near to normal rats. Surprisingly, the HPO levels were restored ($d_p<0.001$) in the high dose of root extract indicating the effect of the extract which was comparable to the standard group (Table 34 and Fig. 63).

6.3.3.2.8. Histopathological studies

Data obtained from histopathological sections of hind paw tissues of the experimental rats are shown in Plate XIII. It revealed that the normal skin and subcutaneous soft tissue showed is no evidence of inflammation in Group I (normal rats). On the other hand, it has been observed in Group II (negative control), which were signs of acute inflammation of the subcutaneous deeper fibrous tissue with evidence of early granulation tissue formation such as destruction of muscle and plenty of presence of more number of neutrophils. Groups III (leaf-low dose) and V (root-low dose) showed
the subcutaneous fibrous tissue, the muscle bundles and the bone showed features of acute inflammation with evidence of destruction of muscle. However, inflammation was less intense when compared to Group II. The presence of macrophages indicating better antigenic tackling and early healing response also. Group IV (leaf-high dose) showed features of acute inflammation of the subcutaneous deeper fibrous tissue with evidence of granulation. However, infiltration by neutrophils was less in fibrous tissue and the presence of macrophages indicates much better antigenic tackling and healing response. Most interesting, pre-treated with methanolic extract of root at 300mg/kg b.w. (Group VI) showed mild inflammation with very few neutrophils and macrophages which indicated complete healing of inflammation against carrageenan induction. Group VII showed features of acute inflammation of the subcutaneous deeper fibrous tissue. However, inflammation is less intense (very mild) when compared to Group II and there is no granulation.

6.4. Discussion

6.4.1. In vitro antimicrobial activity

6.4.1.1. In vitro antibacterial activity

In plants, secondary metabolites attract beneficial organisms and repel harmful organisms, serve as phytoprotectants and respond to environmental changes. The present study describes the assessment of antibacterial effects of the study species, *H. radicata* using disc diffusion assay against 15 pathogenic bacterial strains. Each of the extract tested in the present study displayed antibacterial activity on all bacterial strains with different degree. These differences could be due to the differences in the chemical composition of these extracts as the secondary metabolites of plants have many effects including antibacterial properties (Cowan, 1999; Noumedem *et al*., 2013).

From the overall performance of extracts, it is known that the methanolic leaf and root extracts of *H. radicata* showed broad spectrum of antibacterial activity in comparison to that of the other solvent extracts. One of the major targets for antimicrobial agents is the bacterial cell envelope, which is a complex, multiple macromolecular structures that undergoes highly ordered cycles of synthesis and hydrolysis, facilitating cell division while maintaining a protective barrier against environmental stress. There are several different classes of antibiotics that target specific cell envelope structures or enzymatic steps of cell wall synthesis (Jordan *et al*., 2008).
The biological membrane is a highly dynamic, complicated system which is composed of weakly interacting protein molecules and lipids (Esteban-Martin and Salgado, 2007).

Results of the present study revealed that the methanolic extracts of both parts were effective against Gram-positive than Gram-negative bacteria, which might be related to the difference in cell envelope structure. Cell wall of bacteria comprises a complex structure that is fundamentally different between Gram-positive and Gram-negative bacteria. The Gram-positive bacteria consist of a polymer of disaccharides cross-linked by short chain peptides, forming a type of peptidoglycan. Cell wall in Gram-positive bacteria is thick (15–80nm), consisting of several layers of peptidoglycans and molecules of teichoic acids. In contrast, cell wall of Gram-negative bacteria is relatively thin (10nm) and is composed of a single layer of peptidoglycan surrounded by a membranous structure (the outer membrane) which may invariably contain lipopolysaccharides. Thus, the outer membrane is more hydrophobic in Gram-negative than in Gram-positive type and constitutes a target for being attacked by hydrophobic agents and other antibiotic agents (Schwarz and Reiter, 2001; Singleton, 2004).

Among the two parts attempted, the methanolic extract of root showed higher inhibition activity. This may be explained due to the presence of rich quantity and variety of bioactive compounds like flavonoids and phenolic acids, the more required bioactive compounds for antibacterial activity in the root part of H. radicata than that of the leaves (Zidorn et al., 2005). The synergetic activity of those rich constituents may be the possible factor for the inhibition of the growth of the bacterial colonies (Pavithra et al., 2010). Most prominent antibacterial activity for many other Asteraceae members has been well documented already (Thorat et al., 2010; Kasim et al., 2011; Vamanu et al., 2011; Wijaya et al., 2011; Chethan et al., 2012). To address the rapid emergence of resistance to the classical antibiotics, naturally occurring antibacterial agents are promising candidates in the search for novel therapeutic agents (Zasloff, 2002).

6.4.1.2. In vitro antifungal activity

The results obtained from the present investigation revealed that the highest antifungal activity was exhibited by the ethyl acetate extract and the least by the petroleum ether and aqueous extracts. The basis of varying degree of sensitivity of test organisms of fungi may be due to the intrinsic tolerance of microorganisms and the nature and combinations of phytocompounds presents in the crude extracts.
The antifungal activity of ethyl acetate extract of *H. radicata* leaf showed highest inhibitory activity against the fungus, *Aspergillus niger* (Plate XIc). It is a filamentous ascomycete fungus that is ubiquitous in the environment and has been implicated in opportunistic infections of humans (Perfect *et al.*, 2001). It causes various diseases in plants and animals. In plants, it causes black mould and rot diseases and in human beings it causes aspergillosis by which pulmonary allergy, bronchopulmonary aspergillosis and pulmonary aspergilloma are made (Young *et al.*, 1970; Londero and Guadalupe, 1990). Varaprasad *et al.* (2009) reported that water extracts of Asteraceae members showed strongest effect on reduction in growth of *A. niger* than the species of certain other families. They explained that certain specific compounds of unknown functional group may present in Asteraceae members which may play role in the inhibition of fungal colonies. This fact perhaps be a reason for the fungal inhibitory property of the study species of Asteraceae family, *H. radicata*. On par with the present study, Duraipandiyan and Ignacimuthu (2011) reported that in majority of the species, out of 45 plants studied, ethyl acetate extract exhibited more pronounced antifungal activity than the other solvents. Similarly, Saheb *et al.* (2011) assayed various extracts like aqueous, alcoholic and ethyl acetate solvents in leaves of five *Terminalia* species against five plant pathogenic fungi *viz.*, *Aspergillus flavus*, *A. niger*, *Alternaria brassicicola*, *A. alternate* and *Helminthosporium tetramera* and found that the ethyl acetate extract showed better inhibitory effect against all the fungi tested. In the present study, chloroform and methanol extracts of leaf showed significant antifungal activity against seven fungi *viz.*, *Mucor* sp., *Trichoderma viride*, *Verticillium lecanii*, *Candida albicans*, *Penicillium* sp., *Aspergillus fumigatus* and *A. niger* and instead of *Penicillium* sp. the *Fusarium* sp. was inhibited by methanol extract. Aqueous and petroleum ether extracts of leaf inhibited less number of two to three fungi only (*Candida albicans* and *Aspergillus niger*, and *Mucor* sp., *Aspergillus fumigatus* and *A. niger*). This could be due to the lack of specific active compounds in the extracts. However, all the extracts of *H. radicata* leaf inhibited the growth of *A. niger* colonies.

The ethyl acetate and chloroform extracts of root of *H. radicata* exhibited greater zone of inhibition against the fungi, *Mucor* sp. (Plate XIId) and *Trichoderma viride* (Plate XIe) which are causing many infectious diseases in human beings. On the other hand, the methanol extract generally inhibited the growth of all fungal species except *Paecilomyces lilacinus*. Petroleum ether and water extracts inhibited only few fungal
species (Mucor sp., Trichoderma viride, Verticillium lecanii Penicillium sp., Aspergillus fumigatus and A. niger and Trichoderma viride, Candida albicans, Aspergillus fumigatus and A. niger respectively). The most susceptible organisms to root extracts of the study species were determined to be Aspergillus fumigatus, A. niger and Trichoderma viride, and the most resistant organism was Paecilomyces lilacinus.

The present results showed that the ethyl acetate extracts of leaf and root parts were more effective than the other extracts tested. In another Asteraceae member, Stevia rebundiana also the ethyl acetate extract has reported to have higher antifungal activity than that of the other alcoholic solvent extracts (Sathishkumar et al., 2008). Among the two parts studied, the root part showed higher antifungal activity than the leaf part. In earlier report also it has been reported that the root part of Hypochaeris radicata displayed prodigious antibacterial activity (Jamuna et al., 2013a). This may be attributed to the presence of variety of flavonoids and phenolic compounds in the roots (Zidorn et al., 2005) which may have the capacity to rupture the cytoplasmic membrane of the fungal cells and damage the intracellular compounds (Chen et al., 2003) or they may interact with lipid bilayers or inhibit the protein and nucleic acid synthesis of the fungal cell (Adetumbe et al., 1986). Many publications have been documented for the effective antifungal activity of Asteraceae members (Prajakta et al., 2012; Sarmad et al., 2012; Malarkodi and Manoharan, 2013; Munmi et al., 2013). It was further observed that the inhibitory activities of ethyl acetate extract of leaf against Fusarium sp. and ethyl acetate extract of root against Mucor sp. and Fusarium sp. were significantly greater than that of the standard drug, tetracycline which indicates the effectiveness and specific inhibitory function of ethyl acetate solvent by deriving specific compounds against these fungi.

6.4.1.3 Determination of Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of the extract inhibiting the visible growth of each microorganism (Jennifer, 2001). Available information through literature showed that MIC values between 50-500μg/mL exhibit strong activity, 600-1500μg/mL exhibit moderate activity and above 1500μg/mL exhibit weak activity (Muhamed et al., 2011). The MIC of the methanolic leaf and root extracts of Hypochaeris radicata was ranging between 300 and 500μg/mL, and 300 and 700μg/mL respectively. For the methanolic leaf extract, the most susceptible bacteria identified were Enterococcus faecalis, Bacillus subtilis, Proteus vulgaris, P. mirabilis, Salmonella paratyphi B and Pseudomonas aeruginosa. The most resistant bacteria were Streptococcus faecalis and
Salmonella paratyphi. For the methanolic root extract, the most susceptible and resistant bacteria were Serratia marcescens and Bacillus thuringiensis respectively.

MIC of methanolic leaf and root extracts of H. radicata against the 9 pathogenic fungi was ranging between 200 and 500µg/mL and 200 and 600µg/mL respectively. The most susceptible species for leaf extracts were Fusarium sp. and Aspergillus fumigatus (200µg/mL) and most resistant species was Mucor sp. (500µg/mL). MIC of methanolic root extract of the study species was ranging between 200 (against Fusarium sp. and Aspergillus niger) and 600µg/mL (against Penicillium sp.). From the above results it is known that Fusarium sp. and Aspergillus sp. were susceptible to both the leaf and root extracts. Based on the overall performance, it is known that the methanolic leaf and root extracts of Hypochaeris radicata may be a solution for infectious diseases. The present study on the in vitro antimicrobial activity forms a primary platform for further investigations on pharmacological studies in this species.

6.4.2. In vitro antioxidant activity

The biological property, in vitro antioxidant activity was determined to be effective through various assays for the leaf and root parts of H. radicata.

The reducing power of the extracts may serve as a significant indicator of its potential antioxidant activity (Oliveira et al., 2008). In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of test specimen. The presence of reductones (i.e. antioxidants) in the sample extracts might cause the reduction of Fe$^{3+}$/Ferric cyanide complex to ferrous form which can be monitored by spectrophotometrically at 700nm. The reducing power increased with the increase in the extract concentrations. This may be served as significant indicator of its potential antioxidant activity. Hence, this study presumed that the ethyl acetate extracts of leaf and root parts of H. radicata may have high amount of reductones and hence the antioxidant property.

The concentration of an antioxidant compounds needed to decrease the DPPH radicals by 50% [IC$_{50}$] is a parameter widely used to estimate the antioxidant activity (Kouri et al., 2007). DPPH$^-$ is a stable free radical and that can accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). A freshly prepared DPPH$^-$ solution is of deep purple colour with absorption maximum at 517nm and in the presence of antioxidant, this colour disappears due to quenching of
DPPH free radicals and convert them into a colourless product 2,2-diphenyl-1-picrylhydrazine (Ferreira et al., 2007). Hence, DPPH• is usually used as a substance to evaluate the antioxidant activity (Shah et al., 2010). In the present study, the extracts had significant scavenging effects on the DPPH radical which was increasing with the increase in the concentration of the sample from 50-250µg/mL. Similar trend of DPPH free radical scavenging activity was well documented already (Thambiraj and Paulsamy, 2012a; Vishnu et al., 2013). Among the various solvent extracts tested, the ethyl acetate extracts of leaf and root parts of H. radicata exhibited higher DPPH radical scavenging activity. This might be due to the presence of higher flavonoids content, the most required biocompounds for scavenging activity in this extracts. Next to ethyl acetate, the methanolic extract of both parts of H. radicata showed effective DPPH• scavenging activity which may be attributed to the presence of greater amounts of phenolics and tannins in this solvent extracts.

Nitric oxide (NO•) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological process (Saurabh et al., 2012). Nitric oxides formed during their reduction with oxygen or with superoxides such as NO2, N2O4 and N3O4 are very reactive. The excess concentration of NO• will cause diseases in human beings which can alter the structural and functional behavior of many cellular components. Nitrite ions react with Griess reagent and form a purple azo dye. The decrease in the formation of purple azo dye reflects the presence of scavengers in the test compounds. The methanolic extracts of leaf and root parts of H. radicata are found to be efficient scavenger of nitric oxide radicals in sodium nitroprusside. It clearly indicates that this plant extract has a noticeable effect as scavenging nitric oxide radicals. Among the samples of two parts evaluated, the methanolic root extract of H. radicata exhibited more activity which may be due to the presence of water soluble compounds like phenolics with potent free radical scavenging effects.

The reduction of 2,2’azinobis [3-ethylbenzothiazoline 6-sulphoic acid] radical cation [ABTS•+] has been widely used to measure the antioxidant capacity of natural extracts (Cai et al., 2006). ABTS•+, stable free radical with the characteristic absorbance at 734nm was used to study the radical scavenging effect of extracts of H. radicata. The presence of bioactive chemical compounds in the tested extracts that inhibit the potassium persulfate activity may reduce the production of ABTS•+. The study revealed
that the chloroform and ethyl acetate extracts of root part of \textit{H. radicata} exhibited higher ABTS$^{\cdot+}$ radical scavenging activity. On the other hand, in the leaf extract, the activity was decreasing in the order of ethyl acetate $>$ water $>$ chloroform $>$ methanol $>$ petroleum ether. This assay was calibrated with the water soluble trolox.

In the $\beta$-carotene bleaching assay, linoleic acid produces hydperoxides as free radicals during incubation at 50°C. The presence of antioxidants in the extract will minimize the oxidation of $\beta$-carotene by hydperoxides. Hydperoxides formed in this system will be neutralized by the antioxidants present in the extracts (Govindappa \textit{et al.}, 2011). Therefore, the degradation of $\beta$-carotene linolate depends on the antioxidant activity of the extracts. In the present study, significant positive correlation between degradation rate of $\beta$-carotene linolate and phenolics, flavonoids and tannins has obtained which exhibited the highest antioxidant activity due to these secondary metabolites. Among the samples evaluated, leaf methanol and root water extracts have more pronounced activity (90.43% for leaf methanol and 90.34% for root water extracts). Further, the activity of all the extracts was comparable to that of natural and synthetic antioxidant standards used.

Erythrocytes are considered as prime targets for free radical attack owing to the presence of both membrane concentration of polyunsaturated fatty acids (PUFA) and the O$_2$ transport associated with redox active haemoglobin molecules, which are potent promoters of reactive O$_2$ species. H$_2$O$_2$ mediated oxidation of lipid in cow blood erythrocytes membrane induces membrane damage and subsequently it leads to haemolysis. It is a peroxyl radical initiator that generates free radicals by its thermal decomposition and will attack erythrocytes to induce the chain oxidation of lipid and protein, disturbing the membrane organization and eventually leading to haemolysis. The root water extracts of \textit{H. radicata} possessed effective antihaemolytic activity (68.45%). The more pronounced antioxidant activity of high polar solvent extracts may be involved in the activation of lipid free radicals or prevented decomposition of hydperoxides into free radicals (Karuppusamy \textit{et al.}, 2011).

On the basis of response in terms of scavenging radicals, reducing power, reduction of radical cation, $\beta$-carotene bleaching and antihaemolytic activity, it is concluded that the species, \textit{H. radicata} possessed potential antioxidant activity. It may be due to the presence of respective secondary metabolites like phenolics, flavonoids, tannins \textit{etc.}, in the plant species. Therefore, this species can be attempted to derive the
drugs of antioxidant properties. However, further studies by in vivo models are needed still to confirm this property.

6.4.3. Pharmacological activity

6.4.3.1. Acute oral toxicity

Phytomedicine is indeed worldwide accepted therapeutic approach for chronic diseases and practically it can not be avoided in the health care systems. In developing (low and middle income) countries, it often plays a heart of medicine in traditional therapy, because it is beloved that phytomedicines are harmless (WHO, 2007). There has been enormous rise in the number of uses of traditional medicine and new scientific evidences are coming up regarding the safety of the medicinal plants. Therefore, this creates a warning regarding the toxicity and therapeutic effect of drugs from plant origin.

The results of the present study indicate that methanolic leaf and root extracts were well tolerated in mice upto oral dose of 3000mg/kg b.w. According to classification of Loomis and Hayes (1996) LD₅₀ (50% lethal dosage) value within the range of 5000 to 15000mg/kg b.w. is considered as practically non-toxic (Miller and Tainter, 1944). The LD₅₀ value of this extract in our study suggested that the methanolic extract of H. radicata could be regarded as practically non-toxic in acute ingestion that also showed no significant changes in the general behaviour, as well as mortality of rats. This implicate that the long oral administration of this extract could be safely used for the chronic ailments. Acute toxicity studies are designed to determine the dose that will produce either mortality or serious toxicological effects or high level safety when give once or over a few administrations. They can also give an early indication of the possible target organs toxicity (Arunachalam and Singh, 2012). Therefore, this extract can be said to possess high index of safety and its continued use among the rural and urban population can still be greatly encouraged.

6.4.3.2. In vivo antiinflammatory activity

The antiinflammatory effect of methanolic leaf and root extracts of H. radicata was evaluated in carrageenan induced paw oedema which is widely used for the screening of antiinflammatory compounds and has been frequently used to assess the antiinflammatory effect of medicinal plants (Abbas and Lichtman, 2009). Carrageenan induced inflammation has been well established as a valid model to study free radical generation in paw tissue after inflammatory states (Kumar and Kuttan, 2009).
Macrophages and neutrophils have been found to play an important role, and inflict acute and chronic inflammation. These cells were triggered at the site of inflammation and release many inflammatory mediators. An oedema induced by carrageenan is a biphasic response. In the first phase (90-180min), the release of histamine, serotonin and bradykinin, cause early inflammation. Then in the second phase (270-360min), prostaglandins, proteases and lysosome are associated to late inflammation (Orhan et al., 2007). In the delayed phase, also noticed neutrophil infiltration and the production of neutrophil derived free radicals, such as hydrogen peroxide, superoxide and hydroxide radicals, as well as to the release of neutrophil-derived mediators such as tumor necrosis factor alpha and interleukin-1 beta (TNF α and 1L-1β). However, a reduction in paw swelling size is a good index in determining the protective action of antiinflammatory agents.

In the present work, pre-treated with of methanolic leaf and root extracts were effective in reducing the oedematogenic response evoked by carrageenan induced rats between the 2nd and 4th hrs after the injection, compared with Group I (normal rats) and Group VII (positive control-indomethacine). However, the maximum effect was (dp<0.001) observed during the late hrs of drug administration of methanolic root high dose (85.16%). This evidence suggests that the antiinflammatory actions of the extract were related to the inhibition of one or more intracellular signaling pathways involved in the effect of several inflammatory mediators.

Study of haematological status is one of the important ways for the diagnosis of root cause of diseases (Olson et al., 2000). From the observed values of Group II, all the haematological parameters such as total white blood cell count (WBC), neutrophils (Neu) lymphocytes (LYM) etc., it is clear that an increase in the number of these cells were normal reaction of rats to foreign substances, which alter their normal physiological processes. Administration of the methanolic leaf and root extracts at two different doses produce significant changes near normal levels in all the haematological parameters tested.

Nitric oxide is a free radical, is an important cellular signaling molecule involved in many physiological and pathological processes (Hou et al., 1999). It is a powerful vasodilator with a short half-life of a few seconds in the blood. Despite being a simple molecule, NO’ is an important biological regulator and is therefore a fundamental component in the fields of neuroscience, physiology and immunology. Carrageenan
induced rats showed increase in the level of serum NO\(^{-}\) when compared with normal rats (Group I). However, the elevated NO\(^{-}\) levels were restored to normalcy (\(^{d}p<0.001\)) on treatment with root high dose of methanolic extracts of *H. radicata* (Group VI) when compared to the other treated groups (Groups III, IV and V).

Proteins are essential parts of organisms and participate in virtually every process within cells. Many proteins are enzymes that catalyze biochemical reactions and are vital to metabolism. They also have structural or mechanical functions, such as actin and myosin in muscle and the proteins in the cytoskeleton, which form a system of scaffolding that maintains cell shape. Other proteins are important in cell signaling, immune response, cell adhesion and the cell cycle. The current study showed retrieval of serum protein levels (\(^{d}p<0.001\)) in the methanolic root extract of *H. radicata* (Group VI) by inhibiting the production of free radicals. It is clearly indicates the improvement of functional integrity of the cell.

The spleen is the organ that is responsible for purifying the blood as well as storing blood cells. It is positioned in the superior abdomen, and is the largest lymphatic organ in the body. The spleen serves a valuable role in immune function because it purifies the blood and helps the immune system with recognize and attack foreign antibodies and disease. The spleen is composed of the red and white pulp. The white pulp produces and grows immune cell as well as blood cells. On the other hand, the red pulp is responsible for purifying the blood and removing dead or old blood cells. The thymus is a specialized organ of the immune system. Within the thymus, T-cells mature. Each T cell attacks a specific foreign substance which identifies with its receptor. In our present investigation, the selected two organs were analyzed to determine the level of antioxidant enzymes that contributing antiinflammatory activity which can be treated by methanolic extract of *H. radicata*.

Antioxidant enzymes are capable of eliminating ROS (reactive oxygen species) thereby protecting cells, tissues and organs against oxidative damage. Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide (\(\text{O}_2^\cdot\)) into oxygen and hydrogen peroxide. In higher plants, superoxide dismutase enzymes (SODs) act as antioxidants and protect cellular components from being oxidized by ROS (Alscher *et al.*, 2002). Catalase is a common enzyme found in nearly all living organisms exposed to oxygen (vegetables, fruits and animals). It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). Likewise,
catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second (Goodsell, 2004). Glutathione peroxidase (GPx) is a selenium enzyme, plays a major role in regulating the concentration of \( \text{H}_2\text{O}_2 \) and a wide variety of organic peroxides (Sies, 1993). GSTs catalyses the conjugation of GSH-via a sulphydryl group-to electrophilic centers on a wide variety of substrates in order to make the compounds more soluble (Douglas, 1987; Oakley, 2011). This activity detoxifies endogenous compounds such as peroxidised lipids and enables the breakdown of xenobiotics. GSTs may also bind toxins and function as transport proteins, which gave rise to the early term for GSTs, *ligandin* (Leaver and George, 1998; Litwack et al., 1971). Glucose-6-phosphate dehydrogenase (G6PD or G6PDH) is a cytosolic enzyme. It is the first and key enzyme of pentose phosphate metabolic pathway and it is wide spread in all tissues and blood cells. In this study, there was a significant increase in enzymic antioxidants such as SOD, CAT, GPx, GST and G6PD in the tissues of spleen, thymus and hind paw while treated with the methanolic root extracts of *H. radicata* (Group VI) than the other treated Groups such as III, VI and V which was compared with Group VII (positive control). These activities indicate the effective free radical scavenging potential of methanolic extract administrated groups.

Non-enzymic antioxidants such as reduced glutathione and ascorbic acid play an excellent role in protecting the cells from oxidative damage (Kavitha et al., 2012). Glutathione (GSH) is an important antioxidant in plants, animals, fungi and some bacteria and archaea, preventing damage to important cellular components caused by ROS such as free radicals and peroxides (Pompella et al., 2003). Thiol group of GSH participates in the protection against deleterious effects of ROS evolved during biological imbalance. The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity (Pastore et al., 2003). Ascorbic acid is an essential nutrient for humans and certain other animal species also. The biological role of ascorbate is to act as a reducing agent, donating electrons to various enzymatic and a few non-enzymatic reactions. In the present investigation, methanolic root extract (Group VI) treated rats showed high levels of reduced glutathione and ascorbic acid contents in spleen, thymus and hind paw tissues which were near normal level than the other treated groups (Groups III, IV and V).
Lipid peroxidation is a free radical mediated process which has been implicated in a variety of disease states. It involves in the formation and propagation of lipid radicals, the uptake of oxygen and rearrangement of double bonds in unsaturated lipids which eventually results in destruction of membrane lipids (Cheesman and Slater, 1993). It serves as marker of tissue damage and also recognized to be the inducer of inflammatory processes. It indicates the changes in membrane fluidity and permeability and increases in rates of protein degradation which will eventually lead to cell lysis. LPO and HPO are used as a biomarker to show the index of oxidative stress. In the present study, the negative control groups reported to have increased LPO and HPO levels in serum, spleen, thymus and hind paw samples. Administration of methanolic extract to carrageenan induced rats significantly decreased the LPO and HPO levels. However, treatment with root high dose methanolic extract showed significant decrease ($dp<0.001$) in LPO and HPO levels.

The histopathological studies are direct means for assessing the protective effect of the drug from the tissue injuries. The inhibition of the increasing hind paw volume was associated with inhibition of neutrophil infiltration and by significantly lesser tissue destruction. The present investigation revealed that the protective nature of the methanolic extract of Hyporchaeris radicata against carrageenan induction.

Concerning the crude nature of the extract, the results allow concluding that it exhibited significant bioactivity and properties that support its uses in the folk medicine. The results presented here should encourage the use of this plant species, H. radicata for medicinal health, and nutraceutical applications due to their biological properties.