CHAPTER 3

MATERIALS AND METHODS

3.1 CHEMICALS AND DRUGS

All chemicals used in the present study were of analytical grade. Petroleum ether, methanol, ammonia, sulphuric acid, sulphur powder, acetic acid, nitric acid, mercury and hydrochloric acid were purchased from Rankem, New Delhi, India. Zinc dust, magnesium turnings, potassium iodide, α-naphthol and ferric chloride and resorcinol were procured from Qualikems Fine Chemicals Pvt. Ltd. New Delhi, India. Thiopental sodium, silica gel G for TLC, silica gel (60-120 mesh), n-butanol, gelatin powder, n-hexane and L-hydroxyproline were purchased from the Central Drug House, New Delhi, India. Fehling solution, Sodium nitroprusside, and iodine were procured from E-Merck, Mumbai, India. Sodium chloride, ammonium sulphate, chloroform, pyridine, sodium lauryl sulphate, acetone and sodium hydrogen carbonate were purchased from Fisher Scientific. Soft white paraffin and bismuth carbonate were procured from SD Fine-Chem Ltd. Mumbai, India. Piracetam, 2-thiobarbituric acid and 5’5- Dithiobis (2-nitrobenzoic acid) were purchased from Sigma-Aldrich.

3.2 ANIMALS

Swiss albino mice (30-40 g) and Wister rats (180-220 g) were obtained from Central animal house facility of S.V. Subharti University, Meerut, U.P, India. The animals were housed in polypropylene cage under standard conditions (25 ± 2 °C, 12 h light and dark cycle) and animals were fed on standard chow diet and water ad libitum.

3.3 ETHICAL CONSIDERATION

All the experimental procedures and protocols involving animals were reviewed and approved by the Institutional Animal Ethical Committee (registration number:
1204/PO/ac/2009/CPCSEA) and were in accordance with the guidelines of CPCSEA (Appendix I). All efforts were made to minimize suffering of animals.

3.4 METHODOLOGY

3.4.1 Collection and authentication of plant material

The stem woods of Cedrus deodara Loud. and Pinus roxburghii Sarg. were collected from naturally growing regions of Pauri Garhwal, Uttarakhand, India. The stem wood of Cedrus deodara Loud. was authenticated by taxonomist Dr. H.B. Singh, Head, Raw Materials Herbarium and Museum (RHMD), National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India (Appendix II). A voucher specimen has been kept at the RHMD (NISCAIR/RHMD/Consult/-2011-12/1711/11 dated April 11, 2011) for reference. The stem wood of Pinus roxburghii Sarg. was authenticated by Dr. E. Roshni Nayar, Principal Scientist, National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India (Appendix III). A voucher specimen has been kept at the NBPGR Herbarium (NH CP/NBPG/R/2011-14/7288 dated April 07, 2011) for reference.

3.4.2 Drying and communion of plant material

The woods of Cedrus deodara Loud. and Pinus roxburghii Sarg. were thoroughly washed and then shade dried under 25 ± 2°C for 10 days. The dried plant samples were grounded well into a fine powder in a mixer grinder and sieved to get particle size 50-150 mm.

3.4.3 Isolation of volatile oils

The powder plants material (stem woods of Cedrus deodara Loud. and Pinus roxburghii Sarg.), were subjected to steam distillation by using Clevenger apparatus. On cooling, the volatile oils were separated from the aqueous layer, dried over the anhydrous sodium sulphate and stored in an amber colored glass bottle in cool place (Handa et al., 2008; Shinde et al., 1999).

3.4.4 Preparation of plant extracts

The powder plants material (stem woods of Cedrus deodara Loud. and Pinus roxburghii Sarg.) were extracted successively with chloroform and methanol using Soxhlet apparatus. The solvents were completely removed under reduced pressure till the semi solid mass was obtained. The extracts were kept in amber colored bottle and stored in refrigerator until further use.
3.4.5 Preliminary phytochemical testing

The volatile oils and extracts were subjected to preliminary phytochemical screening for the presence of alkaloids, glycosides, flavonoids, steroids, terpenoids, carbohydrates, proteins, tannins, phenolic compounds, fats and fixed oils (Kandelwal, 2004; Wagner et al., 2004).

3.4.5.1 Chemical tests for alkaloids

To the different extracts added dilute hydrochloric acid, shaken well and then filtered. With the filtrates, performed following test:

Dragendorff’s test

Different filtrates were treated with few drops of Dragendorff’s reagent and formation of orange brown precipitate indicated the presence of alkaloids.

Mayer’s test

Different filtrates were treated with few drops of Mayer’s reagent and formation of cream color precipitate indicated the presence of alkaloids.

Wagner’s test

Different filtrates were treated with few drops of Wagner’s reagent and formation of reddish brown precipitate indicated the presence of alkaloids.

Hager’s test

Different filtrates were treated with few drops of Hager’s reagent and formation of yellow color precipitate indicated the presence of alkaloids.

3.4.5.2 Chemical tests for specific glycosides

i) Chemical tests for anthraquinone glycosides

Borntrager’s test

Different extracts were boiled with 1 ml of sulphuric acid in test tube for 5 minutes and filtered. Then cooled filtrate was shaken with equal volume of chloroform. The organic layer was separated and it was shaken with equal volume of dilute ammonia. Formation of pink or red color in the ammonical layer indicated the presence of anthraquinone glycosides.
Modified Borntrager’s test

Different extracts were heated with equal volume of 5% ferric chloride and diluted hydrochloric acid in a test tube for 5 minutes in boiling water bath and filtered even as hot. The cooled filtrate was shaken with equal volume of chloroform. Then organic layer was separated and it was shaken with equal volume of dilute ammonia. Formation of pinkish red color in the ammonical layer indicated the presence of C-glycosides.

ii) Chemical tests for cardiac glycosides

Keller-Kilianni test (test for deoxy sugars)

Different extracts were treated with glacial acetic acid and one drop of 5% ferric chloride. After transferring to a test tube, 0.5 ml of concentrated sulphuric acid was added by the side of test tube. Appearance of reddish brown color at junction of two liquid and bluish green color of acetic acid layer indicated the presence of cardiac glycosides.

Legal’s test

Different extracts were treated with pyride and then alkaline sodium nitroprusside solution was added. Appearance of pink to red color indicated the presence of cardiac glycosides.

Baljet’s test

Different extract samples were treated with sodium picrate. Appearance of yellow to orange color indicated the presence of cardiac glycosides.

iii) Chemical tests for coumarin glycosides

Test tubes contain different extracts were covered with filtered paper moistened with dilute sodium hydroxide solution. Covered test tubes were then placed on the water bath for several minutes. Paper covering the test tubes was then removed and exposed to ultra violet light, it showed yellowish-green fluorescence.

iv) Chemical tests for cyanogenetic glycosides

Grignard reaction

Different extracts were placed in a conical flask and moistened with few drops of water. Soak a filter paper strip first in 10% picric acid and then in 10% sodium carbonate solution. The dry filter paper was suspended in the neck of flask by means of cork. It was warmed gently at about 37°C. Change in color was observed. Formation of brick red or maroon color on filter paper indicated the presence of cyanogenetic glycosides.
v) Chemical tests for saponin glycosides

Froth formation test

Different extracts were placed with water in a test tube and shaken well. Formation of stable froth (foam) indicated the presence of saponin glycosides.

3.4.5.3 Chemical tests for flavonoids

Shinoda test

Different extracts were treated with few magnesium turnings, ethanol and then drop wise concentrated hydrochloric acid. After few minutes, appearance of orange, pink, red or occasionally purple color indicated the presence of flavonoids.

Alkaline reagent test

Different extracts were treated with few drops of sodium hydroxide solution. Appearance of deep yellow color which turned colorless after addition of few drops of dilute acid indicated the presence of flavonoids.

Zinc hydrochloride test

Different extracts were heated with mixture of zinc dust and concentrated hydrochloric acid. After few minutes appearance of pink to red color indicated the presence of flavonoids.

3.4.5.4 Chemical tests for steroids

Salkowski test

Different extracts were treated with few drops of chloroform and concentrated sulphuric acid through the side of the test tube and shaken well. Chloroform layer appears red and acid layer showed greenish yellow color.

Libermann-Burchard reaction

Different extracts were treated with few drops of chloroform, acetic anhydride and concentrated sulphuric acid from the side of the test tube and shaken well. Appearance of first red then blue and finally green color indicated the presence of steroids.

Libermann reaction

Different extracts were heated with equal volume of acetic anhydride. Then few drops of concentrated sulphuric acid were added to the cool mixture. Appearance of blue color indicated the presence of steroids.
3.4.5.5 Chemical tests for triterpenoids

Salkowski test

Different extracts were treated with few drops of concentrated sulphuric acid and shaken well. On standing, appearance of golden yellow color to the lower layer indicated the presence of triterpenoids.

Libermann-Burchard’s test

Different extracts were boiled with acetic anhydride and then few drops of concentrated sulphuric acid were added to the cool mixture from the side of the test tube and shaken well. Appearance of pink color at the junction of the liquids indicated the presence of triterpenoids.

3.4.5.6 Chemical tests for carbohydrates

Molish test

Different extracts were treated with few drops of $\alpha$-naphthol solution in alcohol and then concentrated sulphuric acid was added from side of test tube. Formation of violet color ring at junction indicated presence of carbohydrates.

Fehling solution test

One ml of each Fehling’s A and Fehling’s B solutions were boiled for one minute and then added equal volume of different extracts. The mixtures were heat for 5-10 minutes on boiling water bath. Appearance of first yellow, then red precipitate indicated the presence of reducing sugar.

3.4.5.7 Chemical tests for protein

Biuret’s test

Different extracts were treated with few drops of 1% copper sulphate solution and 4% potassium hydroxide. Emergence of violet or pink color showed the presence of proteins.

Ninhydrine test

Different extracts were heated with few drops of ninhydrin reagent in boiling water bath for ten minutes. Emergence of purple or bluish color showed the presence of amino acids.
3.4.5.8 Chemical tests for fats and fixed oils

**Spot test**

Spot test of extracts were performed by using filter paper. Filter paper gets permanently stained with oils.

**Solubility test**

Solubility of different extracts was performed with different polar and non polar solvent. Oils were soluble in ether, chloroform and benzene, but insoluble in water and ethanol.

3.4.5.9 Chemical test for tannins and phenolic compounds

**Ferric chloride test**

Different extracts were treated with 5% ferric chloride solution. Appearance of blue-black color indicated the presence of tannins and phenolic compounds.

**Dilute iodine solution test**

Different extracts were treated with dilute iodine solution. Appearance of red color indicated the presence of tannins and phenolic compounds.

3.4.6 Thin layer chromatography profiling

The samples were subjected to TLC to find out the number of compounds present in it (Stahl et al., 1969).

3.4.6.1 Preparation of plates

The absorbent used for TLC was silica gel G, mesh size 100-200. About 25 g of silica gel G and 35 ml of distilled water were stirred in mortar with glass rod until it becomes homogeneous mixture. This mixture was then allowed to swell for about fifteen minutes. Then additional water was added to it with continuous stirring. After that the suspension was transferred to a 150 ml flask and shaken vigorously for two minutes. Then suspension was spread instantaneously on TLC plates to form a coat.

3.4.6.2 Drying and storage of the plates

The freshly coated plates were then air dried until the transparence of the layer had missing. The plates set in a drying rack and were heated in an oven for 30 minutes at 110°C for activation. The activated plates were kept in desiccators for further use.
3.4.6.3 Application of the samples

The test samples were applied on TLC plates with the help of fine capillaries. The samples in particular solvent were applied as a single spot along one side of plate, about 2 cm from the edge.

3.4.6.4 Chromatographic chamber and the development of TLC plates

Chromatographic rectangular glass chamber was used for TLC development. For appropriate solvent system, firstly elutropic series of different solvents were tried by running on the TLC plate. The TLC plate having the sample spot was placed at 45° angle in the development chamber casing the bottom of the plate by the solvent up to just about one cm. The solvent border was marked and the plate was allowed to dry.

3.4.6.5 Spraying equipment

Compressed air sprayer with a fine nozzle was used to detect the different constituents present on TLC plates. The glass sprayer was attached to air compressor and filled with about 50 ml of the detecting reagent and then used. The components were detected by using visualizing agent, anisaldehyde sulphuric acid at 120°C (Wagner et al., 1984). After each spray, the sprayer was washed separately with water, chromic acid and distilled and then with acetone.

3.4.7 Dose Selection

The doses of Cedrus deodara Loud. were selected on the basis of the previous literature (Shinde et al., 1999a; Shinde et al., 1999b; Shinde et al., 1999c; Patil et al., 2011), pilot study and LD50 value (Dhar et al., 1968; Viswanatha et al., 2009; Perveen et al., 2008). The doses of Pinus roxburghii Sarg. were selected on the basis of the previous literature (Puri et al., 2011), pilot study and LD50 value calculations (Kaushik et al., 2012a; Kaushik et al., 2012b; www.mdidea.com).

3.4.8 Antimicrobial screening: Agar dilution method

Dilution methods were used to determine the MIC of antimicrobial agents. In agar dilution tests, microorganisms were tested for their ability to produce visible growth on a series of agar plates containing dilutions of the antimicrobial agents (Mazumder et al., 2004). The lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism is known as MICs (Barry et al., 1996).

3.4.8.1 Test organisms

The pure cultures of the gram negative bacteria (Escherichia coli and Pseudomonas aeruginosa), gram positive bacteria (Bacillus subtilis, Streptococcus pyogenes
and *Staphylococcus aureus* and fungi (*Aspergillus niger*, *Candida albicans* and *Aspergillus clavatus*) were used for the antimicrobial study.

### 3.4.8.2 Procedure

**a) Preparation of inoculums**

Procedure for preparation of inoculums was adopted as described by Hanlon *et al.*, 2007:

- Pure culture was reserved as stock strains.
- New culture strains were prepared from stock strains and were used as inoculums.
- The saline dilutions of each culture used were made appear in turbidity equivalent to 0.5 McFarland standards.
- Fresh subculture of the each microorganism were picked up with sterilized wire loop and introduced to 5 ml saline water.
- It was shaken thoroughly and homogenized to prepare a suspension that was then incubated until its turbidity was visually equivalent to 0.5 McFarland standards.
- From this bacterial suspension 1 ml was taken and mixed with 9 ml of saline water gave inoculums concentration of 107 CFU/ml.

**b) Preparation of drug dilution**

Dilution of extracts, volatile oils and standard drugs in agar dilution susceptibility tests were given in Table 3.1.

**c) Pouring the plates**

1 Liter of media for bacteria and fungi were prepared as per instructions given on the pack (24.8 g in 1000 ml distilled water). The media was cooled and the pH of the medium was maintained at 7.2 to 7.4. The sample dilutions were added in the liquid agar media. The flask was swirled to mix the content carefully. The liquid agar media was poured into round petri plates on a level surface to a depth of 2-3 mm (approximately 9-10 ml per plates) and plates were allowed to solidify at room temperature.

**d) Preparation of control plates**

In contrast to antibiotic dilution plates, control plates consist of only agar based media with no antibiotic.

**e) Assay method**

Antimicrobial activity of oils and extracts were determined using agar dilution method. For the study equipment were cleaned and sterilized by autoclave method at 12 psi for 30 min. Nutrient agar medium was used for growth of bacteria, while potato dextrose agar
was used for growth of fungi. The procedure was carried out to observe strictly sterile conditions.

**Table 3.1**: Different dilution of extracts, volatile oils and standard drugs in agar dilution susceptibility tests.

<table>
<thead>
<tr>
<th>S. N</th>
<th>Antimicrobial concentration (µg/ml) in stock</th>
<th>Volume of stock solution in ml</th>
<th>Volume of distilled water in ml</th>
<th>Antimicrobial concentration obtained (µg/ml)</th>
<th>Final concentration in medium after addition of 9 ml agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10000</td>
<td>1.024</td>
<td>0</td>
<td>10240</td>
<td>1024</td>
</tr>
<tr>
<td>2</td>
<td>10000</td>
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<td>10000</td>
<td>1000</td>
</tr>
<tr>
<td>3</td>
<td>10000</td>
<td>0.500</td>
<td>0.500</td>
<td>5000</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>10000</td>
<td>0.250</td>
<td>0.750</td>
<td>2500</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>10000</td>
<td>0.200</td>
<td>0.800</td>
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<td>0.975</td>
<td>250</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>1000</td>
<td>0.0125</td>
<td>0.9875</td>
<td>125</td>
<td>12.5</td>
</tr>
<tr>
<td>10</td>
<td>1000</td>
<td>0.00625</td>
<td>0.99375</td>
<td>62.5</td>
<td>6.25</td>
</tr>
</tbody>
</table>

**f) Incubation of plates**

All the plates were incubated at 37°C for 48 h for bacteria and 25°C for 72 h for fungi in incubator.

**g) Determination of MIC**

Effectiveness of the chemotherapeutic agent against a pathogen can be obtained from the MIC. Antibiotic plates were read for end points on a dark nonreflecting plane to find MIC, the first antibiotic concentration that inhibits the growth of the organism completely. The MIC (µg/ml) was considered to be the lowest concentration that completely inhibited growth on agar plates (Hanlon et al., 2007).

**3.4.9 Memory enhancing activity: Morris water maze test**

To test the effects of volatile oils and chloroform extracts of plant *Cedrus deodara* Loud. and *Pinus roxburghii* Sarg. on memory, MWM paradigms test were employed. The mice were divided in ten groups. In the set the animals were treated as shown in Table 3.2:
Table 3.2: The experimental protocol for memory enhancing activity.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Treatment, Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>Vehicle, 10 ml/kg of body weight</td>
</tr>
<tr>
<td>Group 2 (Piracetam)</td>
<td>Piracetam, 100 mg/kg of body weight</td>
</tr>
<tr>
<td>Group 3 (Cd O 50)</td>
<td>Volatile oils of Cedrus deodara Loud., 50 mg/kg of body weight</td>
</tr>
<tr>
<td>Group 4 (Cd O 100)</td>
<td>Volatile oils of Cedrus deodara Loud., 100 mg/kg of body weight</td>
</tr>
<tr>
<td>Group 5 (Pr O 50)</td>
<td>Volatile oils of Pinus roxburghii Sarg., 50 mg/kg of body weight</td>
</tr>
<tr>
<td>Group 6 (Pr O 100)</td>
<td>Volatile oils of Pinus roxburghii Sarg., 100 mg/kg of body weight</td>
</tr>
<tr>
<td>Group 7 (Cd C 50)</td>
<td>Chloroform extract of Cedrus deodara Loud., 50 mg/kg of body weight</td>
</tr>
<tr>
<td>Group 8 (Cd C 100)</td>
<td>Chloroform extract of Cedrus deodara Loud., 100 mg/kg of body weight</td>
</tr>
<tr>
<td>Group 9 (Pr C 50)</td>
<td>Chloroform extract of Pinus roxburghii Sarg., 50 mg/kg of body weight</td>
</tr>
<tr>
<td>Group10 (Pr C 100)</td>
<td>Chloroform extract of Pinus roxburghii Sarg., 100 mg/kg of body weight</td>
</tr>
</tbody>
</table>

Treatment schedules

Two week prior to testing, the mice were randomly assigned to ten groups that received once daily as follows: Group 1 (Control) animals received vehicle at 10 ml/kg of body weight. Group 2 (Piracetam) received piracetam in dose of 100 mg/kg, i.p. Group 3 (Cd O 50) and 4 (Cd O 100) received volatile oils of Cedrus deodara Loud. in doses of 50 and 100 mg/kg respectively. Group 5 (Pr 0 50) and 6 (Pr O 100) received volatile oils of Pinus roxburghii Sarg. in doses of 50 and 100 mg/kg respectively. Group 7 (Cd C 50) and 8 (Cd C 100) received chloroform extract of Cedrus deodara Loud. in doses of 50 and 100 mg/kg respectively. Group 9 (Pr C 50) and 10 (Pr C 100) received chloroform extract of Pinus roxburghii Sarg. in doses of 50 and 100 mg/kg respectively. The treatments were performed until the last day of the behavioral procedures.

3.4.9.1 Behavioral testing

The MWM task has been used extensively to investigate spatial learning and memory in rodents (Morris R, 1984; Davoodi et al., 2009). Maze was a semi spherical pool (65 cm in diameter, 25 cm deep) filled with water to a depth of approximately 20 cm. The pool was divided into four equal quadrants and a platform (10.5 cm²) was submerged 1 cm below the opaque surface in the centre of one of the quadrants. The pool was located in a test room and many cues external to the maze were visible from the pool (e.g., pictures, fan, chairs etc.), which could be used by the mice for spatial orientation. The position of the cues was kept constant throughout the task. Escape latency and time spent in target quadrant (probe trial) was recorded by live scoring.
Twenty-four hours prior to the start of training, all animals used in the study were habituated to the pool by allowing them to perform a 60 s swim without the platform. In the water maze experiments, the first day of the experiment was dedicated to swimming training for 60 s in the absence of the platform. In the following days, the mice were given two trial sessions each day for seven consecutive days. In all testing procedures, the swimming paths were recorded on a map of the pool by a single investigator who was blind as to the experimental conditions. During each trial, the escape latencies of mice, as measured with a stop-watch, were recorded by the same experimenter. This parameter was averaged for each session of trials and for each mouse. Once the mouse located the platform, it was permitted to remain on it for 10 s. If the mouse did not locate the platform within 90 s, it was placed on the platform for 10 s and then removed from the pool. The mouse was given two daily trials for 7 days with an inter-trial interval of 20 min. The point of entry of the mouse into the pool and the location of the platform for escape remained unchanged between trials 1 and 2 but was changed on each day. The decrease in escape latency from day to day in trial 1 represents long-term memory or reference memory while that from trial 1 to trial 2, represents short term memory or working memory. All mice were tested for spatial memory 30 min after the treatments. After completion of seven days of training (1 to 7), the mice were returned to their home cages until the retention testing (probe trial) 24 h later on day 8. The probe trial consisted of a 90 s free swim period without a platform in which the time spent in the target quadrant was recorded (Rubio et al., 2007).

3.4.9.2 Biochemical estimation of markers of oxidative stress

Morris water maze behavior experienced mice of ten different groups were further tested for biochemical estimation of MDA and GSH in frontal cortex and hippocampus of brain (Jainkang et al., 1990; Ellman, 1959). On 8th day following the behavioral testing mice were decapitated under anesthesia and the brain was exposed from its dorsal side by incising the skull. The whole brain was then carefully removed from each mouse, chilled for 15 min and thereafter, dissected into frontal cortex (FC) and hippocampus (HP) over ice according to the Glowinski and Iversen method (Glowinski and Iversen, 1966).

Tissue preparation

Frontal cortex and hippocampus were thawed and homogenized with 10 times (w/v) by homogenizer in ice cold 0.1 M phosphate buffers (pH 7.4). Aliquots of homogenates from each sample were separated and used to determine protein, lipid peroxidation and glutathione.

i) Estimation of Protein

Protein concentration in frontal cortex and hippocampus were determined according to Lowry et al., 1951, using purified bovine serum albumin as standard. Briefly to 990 µl of distilled water, 10 µl of sample and 1 ml of Lowry’s reagent (2% sodium bicarbonate in 0.1N sodium hydroxide, 1% cuprous sulfate, 2% Na+ K+ tartarate) was added
and incubated for another 5 min at room temperature. Further 100 µl of Folin and Ceocalteus reagent was then added and incubated at for 30 min and absorbance was read at 750 nm using UV-Visible spectrophotometer against a reagent blank.

**ii) Estimation of malondialdehyde**

Malondialdehyde a measure of lipid peroxidation was measured as described by Jainkang *et al.*, 1990. Reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulphate (8.1%) were added to 0.1 ml of processed tissue samples, then heated at 100°C for 60 min. Mixture was cooled under tap water and 5 ml of *n*-butanol-pyridine (15:1), 1 ml of distilled water was added and vortexed vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was separated and absorbance was measured at 532 nm using a spectrophotometer and concentration of MDA was expressed as nM/mg of protein.

**iii) Estimation of glutathione**

Glutathione was measured according to the method of Ellman, 1959. The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5-dithiobis (2-nitrobenzoic acid), and 0.4 ml of double distilled water was added. Mixture was vortexed and the absorbance read at 412 nm within 15 min. The concentration of GSH was expressed as nM/mg of protein.

**3.4.10 Wound healing activity**

**Ointment formulation**

The ointment (1% and 2.5% w/w) were formulated using soft white paraffin. White soft paraffin was melted on hot plate at 60°C and mixed with oils and chloroform extracts of *Cedrus deodara* Loud. and *Pinus roxburghii* Sarg. with continuous stirring until samples and soft paraffin completely miscible (Cooper, 1987).

**3.4.10.1 Acute dermal toxicity**

The acute dermal toxicity testing was done by applying the ointment containing oil and extract of the highest concentration 2.5% (w/w) on the shaved back of the mice. The OECD guidelines with no. 402 were followed for the study (OECD guidelines, 1987).

**3.4.10.2 In-vivo healing evaluation: “Burn wound model”**

Partial thickness burn wounds were inflicted on overnight starved animals under thiopental sodium anesthesia, by placing hot cylindrical metal rod (10 mm diameter) on the shaven back of the animals (Rozaini *et al.*, 2004; Sanwal and Chaudhary, 2011). Immediately after the injury and on subsequent days ringer lactate (1 ml/kg) was administered *i.p* daily for
resuscitation. Apart from the drugs under investigation, no local or systemic chemotherapeutic cover was provided to animals. Animals showing the signs of infection were excluded from the study and replaced with fresh animals.

**Experimental protocol:**

Animals bearing the partial thickness burn wounds were randomly divided into ten groups. Group-I (control) was treated with empty ointment base. Group-II (SSD) received the standard drug (silver sulphadiazine and chlorhexidine gluconate cream; 1.0% w/w). Groups-III (Cd O 1%) and IV (Cd O 2.5%) were treated with ointment containing 1% and 2.5% oils of *Cedrus deodara* Loud. respectively. Groups-V (Pr O 1%) and VI (Pr O 2.5%) were treated with ointment containing 1% and 2.5% oils of *Pinus roxburghii* Sarg. respectively. Groups-VII (Cd C 1%) and VIII (Cd C 2.5%) were treated with ointment containing 1% and 2.5% chloroform extracts of *Cedrus deodara* Loud. respectively. Groups-IX (Pr C 1%) and X (Pr C 2.5%) were treated with ointment containing 1% and 2.5% chloroform extracts of *Pinus roxburghii* Sarg. respectively. The treatments (100 mg/mouse) were applied topically once a day, starting from the wound induction until complete healing.

**Assessment of burn healing:**

Animals were inspected daily and the healing was assessed on the basis of parameters namely; rate of wound contraction, epithelization time and hydroxyproline content (Gurung and Skalko-Basnet, 2009).

### 3.4.10.2.1 Rate of wound contraction

The wound contraction rate was measured as percentage reduction in wound size at every 4 days interval. Progressive decrease in the wound size was monitored periodically by tracing the boundary and the wound area was assessed graphically (Eqn. 3.1). Wound contraction was expressed as reduction in percentage of the original wound size (Eqn. 3.2).

Area of wound in mm\(^2\) (wound closure rate) was calculated by the following formula:

\[
\text{Area of wound} = \text{square of radius of wound in mm} \times \frac{22}{7}
\]  
(3.1)

On the day zero, circular 10 mm wound was made over the animals. Therefore, on day zero the wound area was 78.5 mm\(^2\).

\[
\text{Percentage of wound healing} = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100
\]  
(3.2)
3.4.10.2.2 Epithelialization time

Falling of eschar leaving no raw wound area was considered as end point of complete reepithelization and the days required for this was taken as period of epithelialization.

3.4.10.2.3 Determination of the hydroxyproline content

On the 11\textsuperscript{th} day, the animals from each group were used to determine hydroxyproline content using the techniques described by Neumark and Logan, 1950. The wound tissue was excised and its weight was recorded. The tissue was dried in oven at 60\textdegree C for 12 h and the dry weight was again noted. It was hydrolyzed in 6 N hydrochloric acid for 24 h at 110\textdegree C in sealed glass tubes. The hydrolysate was neutralized to pH 7. The samples (200 \mu l) were mixed with 1ml of 0.01 M copper sulphate followed by the addition of 1ml of 2.5 N sodium hydroxides and then 1 ml of 6% hydrogen peroxide. The solution was mixed and shaken occasionally for 5 min. All the tubes were incubated at 80\textdegree C for 5 min with shaking. Upon cooling, 4 ml of 3 N sulfuric acid was added with agitation. Finally, 2 ml of 5% p-dimethylaminobenzaldehyde was added. The samples were incubated at 70\textdegree C for 16 min, cooled by placing the tubes in water at 20\textdegree C, and the absorbance was measured at 500 nm using colorimeter. The amount of hydroxyproline in the samples was calculated using a standard curve prepared with pure L-hydroxyproline (Gurung and Skalko-Basnet, 2009).

3.4.11 Determination of \textit{in-vitro} antioxidant activity of volatile oils and extracts

3.4.11.1 Total antioxidant capacity

The total antioxidant capacity (TAC) of volatile oils and chloroform extracts of \textit{Cedrus deodara} Loud. and \textit{Pinus roxburghii} Sarg. were measured using phosphomolybdenum method (Prieto et al., 1999). The volatile oils and extracts were dissolved in methanol (2 mg/ml) and 0.3 ml of each sample was added to 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate mixture). The tubes were incubated for 90 min at 95\textdegree C. The mixture was cooled to room temperature and the absorbance was read at 695 nm against blank. Ascorbic acid equivalents were calculated using the standard graph of ascorbic acid. The experiment was conducted in triplicates and values were expressed as equivalents of ascorbic acid in mg per g of sample.

3.4.11.2 Reducing power assay

This method was based on the reduction of the Fe(III)/ferricyanide complex to the ferrous form by one-electron-donating antioxidant. The ferrous ion (Fe\textsuperscript{2+}) is monitored by measuring the formation of Perl's Prussian blue at a wavelength of 700 nm. The ferric ion (Fe\textsuperscript{3+}) reducing power of the volatile oils and chloroform extracts of \textit{Cedrus deodara} Loud. and \textit{Pinus roxburghii} Sarg. were determined by the previously described method (Bajpai \textit{et
Materials & Methods

3.4.12 Antiulcer activity

Experimental protocol

To test the effect of chloroform extracts of plants Cedrus deodara Loud. and Pinus roxburghii Sarg. on ulcer, two paradigms such as pylorus induced and ethanol induced gastric ulceration test in rats were employed. The rats were divided in two sets of six groups each. In both the set, the animals were treated as follows: Group 1 (Control) received vehicle (mixture of acacia and tragacanth in distilled water), 10 ml/kg of body weight. Group 2 (Standard) received famotidine, 20 mg/kg of body weight. Group 3 (Cd C 50) and 4 (Cd C 100) received chloroform extract of Cedrus deodara Loud. in doses of 50 and 100 mg/kg. Group 5 (Pr C 50) and 6 (Pr C 100) received chloroform extract of Pinus roxburghii Loud. in doses of 50 and 100 mg/kg.

3.4.12.1 Pylorus ligation induced gastric ulceration

Gastric secretion content, pH, total acidity, free acidity, number of ulcer, ulcer score and ulcer index were measured according to the method of Shay et al., 1945. Animals were fasted for 36 h before the study, but had free access to water. One hour after oral administration of chloroform extracts of Cedrus deodara Loud. and Pinus roxburghii Sarg. (50 and 100 mg/kg) or famotidine (20 mg/kg) or vehicle, the animals were subjected to pylorus ligation under thiopental sodium anaesthesia. The animals were sacrificed with over dose of thiopental sodium after 4 h of pyloric ligation. The abdomen was opened, cardiac end of the stomach was dissected out, and the contents were drained into a glass tube. The volume of the gastric juice was measured and centrifuged at 2000 rpm for 10 min. From the supernatant, aliquots (1 ml of each) were taken for the determination of pH, total and free acidity. Total acidity and free acidity were determined using titrimetry. The inner surface of free stomach was examined for gastric lesions.
i) Determination of pH

An aliquot of 1 ml gastric juice was diluted with 1ml of distilled water and pH of the solution was measured using pH meter.

ii) Determination of total acidity

An aliquot of 1 ml gastric juice diluted with 1 ml of distilled water was taken into a 50 ml conical flask and two drops of phenolphthalein indicator was added to it and titrated with 0.01N sodium hydroxide solution until a permanent pink color was observed. The volume of 0.01N sodium hydroxide solution consumed was noted.

The total acidity is expressed as meq/l by the following formula:

\[
\text{[Total acidity} = n \times 0.01 \times 40.00 \times 1000] 
\]

(3.3)

Where n is volume of sodium hydroxide solution consumed, 40.00 is molecular weight of sodium hydroxide, 0.01 is normality of sodium hydroxide solution, and 1000 is the factor (to be represented in liter).

iii) Determination of free acidity

Instead of phenolphthalein indicator, the Topfer’s reagent was used. Aliquot of gastric juice was titrated with 0.01N sodium hydroxide solution until canary yellow color was observed. The volume of 0.01N sodium hydroxide solution consumed was noted. The free acidity was calculated by the same formula for the determination of total acidity.

iv) Macroscopic evaluation of stomach

The stomachs were opened along the greater curvature, rinsed with saline to remove gastric contents and blood clots and examined by a ×5 magnifier lens to assess the formation of ulcers. The number of ulcers was counted. Ulcer scoring was undertaken according to Vogel and Vogel, 1997.

The scores were: 0 = no ulcer, 1 = superficial ulcer, 2 = deep ulcer, 3 = perforation.

Ulcer index was measured by using following formula according to Vogel and Vogel, 1997.

\[
\text{[UI} = U_N + U_S + U_P \times 10^{-1}] 
\]

(3.4)

\[
\text{UI} = \text{Ulcer Index} \\
U_N = \text{Average number of ulcers per animal} \\
U_S = \text{Average number of severity score} \\
U_P = \text{Percentage of animals with ulcers}
\]

Percentage inhibition of ulceration was calculated as below:
[\% \text{Inhibition of ulceration} = (\text{Ulcer index Control} - \text{Ulcer index Test}) \times 100/\text{Ulcer index Control}]

(3.5)

### 3.4.12.2 Ethanol induced gastric ulcers

Lesions were induced according to the method of Vogel and Vogel, 1997. Rats, fasted for 18 h but had free access to water were used. One hour after the treatments (Cedrus deodara Loud. and Pinus roxburghii Sarg.: 50 and 100 mg/kg or Famotidine: 20 mg/kg or vehicle: 10 ml/kg), 1 ml absolute ethanol were administered orally. After 1 h of ethanol treatment, the animals were sacrificed under high dose of anesthesia. The stomach of each animal was excised and opened along the greater curvature. The numbers of ulcer, ulcer score, ulcer index and percentage inhibition of ulcer were determined.

### 3.4.12.3 Histopathological evaluation

The stomach samples from the pylorus ligated and ethanol treated groups were preserved in 10% buffered formalin and processed for routine paraffin block preparation. Using a rotary microtome, sections of thickness of about 5 µm was cut and stained with haematoxylin and eosin. These were examined under the microscope for histopathological changes such as degeneration, hemorrhage, edematous appearance, erosion and necrosis.

### 3.4.13 Isolation and characterization of phytoconstituents from most bioactive extract

#### 3.4.13.1 Column chromatography of chloroform extract of Cedrus deodara Loud.

**Requirements:**

- Stationary phase: Silica gel G (100-200 mesh); 260 g
- Mobile phase: n-hexane/ethyl acetate combinations and finally ethyl acetate
- Charged material: Chloroform extracts (10 g)
- TLC solvent system: n-hexane: ethyl acetate
- Volume of each fraction: 50 ml

**Procedure:**

The dry chloroform extract (10 g) was combined with a minimum amount of chloroform and mixed thoroughly with silica gel (100 to 200 mesh size) in ratio of 1:1.5 (drug: silica) to make slurry. The slurry was dried under reduced pressure in rotavapor so that extract gets adsorbed on silica. Column was packed using silica gel 100 to 200 mesh size in n-hexane by wet packing method. Adsorbed silica was loaded to the prepared column. The column was eluted initially in n-hexane 1.0 liter (100% n-hexane), followed by n-hexane: ethyl acetate gradient system (98:2, 96:4, 94:6, 92:8, 90:10, 88:12, 86:14, 84:16, 82:18 and so
on) and finally exhausted using 100% ethyl acetate (Rajput and Rajput, 2012). Each fraction of 50 ml volume was collected and fractions were pooled on the basis of TLC profile to make different fractions.

3.4.13.2 Processing of the isolated fractions

Pooled fractions were collected on the basis of TLC profile. The further processing were as follows:

3.4.13.2.1 Fraction 16-21 (92% n-hexane in ethyl acetate)

The fractions collected were dried in rotary evaporator and weighed. A light yellowish gum was obtained from the fraction 16-21 and was named as CdC-B (320 mg). This was dried in vacuum, weighed, and stored in suitably labeled container and then compound was subjected for characterization by FT-IR, DART-MS and 1H NMR.

TLC analysis of compound CdC-B with chloroform extract:

Stationary phase: Silica gel G  
Mobile phase : n-hexane: ethyl acetate (90:10)  
Detection : Anisaldehyde sulphuric acid  
On charring at 120°C gives blue color

3.4.13.2.2 Fraction 27-31 (90% n-hexane)

The fractions collected were dried in rotary evaporator and weighed. A light yellowish gum compound was obtained from the fraction 27-31 and was named as CdC-C (90 mg). This was dried in vacuum, weighed, and stored in suitably labeled container and then compound was subjected for characterization by FT-IR, DART-MS and 1H NMR.

TLC analysis of compound CdC-C with chloroform extract:

Stationary phase: Silica gel G  
Mobile phase : n-hexane: ethyl acetate (88:12)  
Detection : Anisaldehyde sulphuric acid  
On charring at 120°C gives blue color

3.4.13.2.3 Fraction 42-46 (84% n-hexane)

The fractions collected were dried in rotary evaporator and weighed. A brown color oily compound was obtained from the fraction 42-46 and was named as CdC-F (118 mg). This was dried in vacuum, weighed, and stored in suitably labeled container and then compound was subjected for characterization by FT-IR, DART-MS and 1H NMR.
TLC analysis of compound CdC-F with chloroform extract:

Stationary phase: Silica gel G
Mobile phase : n-hexane: ethyl acetate (82:18)
Detection : Anisaldehyde sulphuric acid
On charring at 120°C gives blue color

3.4.13.2.4 Fraction 55-60 (80% n-hexane)

The fractions collected were dried in rotary evaporator and weighed. A brownish gum compound was obtained from the fraction 55-60 and was named as CdC-G (100 mg). This was dried in vacuum, weighed, and stored in suitably labeled container and then compound was subjected for characterization by FT-IR, DART-MS and $^1H$ NMR.

TLC analysis of compound CdC-G with chloroform extract:

Stationary phase: Silica gel G
Mobile phase : n-hexane: ethyl acetate (78:22)
Detection : Anisaldehyde sulphuric acid
On charring at 120°C gives blue color

3.4.13.2.5 Fraction 85-88 (60% n-hexane)

The fractions collected were dried in rotary evaporator and weighed. A light brown color gum compound was obtained from the fraction 85-88 and was named as CdC-H (85 mg). This was dried in vacuum, weighed, and stored in suitably labeled container and then compound was subjected for characterization by FT-IR, DART-MS and $^1H$ NMR.

TLC analysis of compound CdC-H with chloroform extract:

Stationary phase: Silica gel G
Mobile phase : n-hexane: ethyl acetate (58:42)
Detection : Anisaldehyde sulphuric acid
On charring at 120°C gives blue color

3.4.13.3 Characterization of isolated compounds

The isolated compounds namely CdC-B, CdC-C, CdC-F, CdC-G and CdC-H were subjected to spectroscopic analysis. The analyses were done at Sophisticated Analytical Instrument Facility (SAIF), Central Drug Research Institute, Lucknow, UP.

FT-IR spectral studies:

FT-IR spectra were recorded on the PEService (Excalibur HE 3600, PERKIN ELMER Spectrum Version 10.03.06A).
Nuclear Magnetic resonance spectroscopy (NMR):

$^1$H NMR spectra were recorded on Bruker DRX-300; SWITZERLAND spectrometer. Samples were dissolved in CDCl$_3$ based on the solubility of the sample.

Mass spectroscopy (DART-MS):

Mass spectra were recorded on DART-MS (JMS-T100LC, Accu TOF).

3.4.14 Determination of *in-vitro* antioxidant activity of isolated compounds

3.4.14.1 Total antioxidant capacity

The total antioxidant capacity was measured using phosphomolybdenum method (Prieto *et al.*, 1999). The chloroform extract and five isolated compounds (CdC-B, CdC-C, CdC-D, CdC-G and CdC-H) from *Cedrus deodara* Loud. were dissolved in methanol (2 mg/ml) individually and 0.3 ml of each samples were added to 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate mixture). The tubes were incubated for 90 min at 95°C. The mixture was cooled to room temperature and then absorbance was read at 695 nm against blank sample. Standard graph of ascorbic acid was used for calculation of ascorbic acid equivalents and the testing was performed in triplicates. The values were expressed as equivalents of ascorbic acid in mg per g of sample.

3.4.14.2 Reducing power assay

This method was based on the reduction of the Fe (III)/ferricyanide complex to the ferrous form by one-electron-donating antioxidant. The ferrous ion is monitored by measuring the formation of Perl’s Prussian blue at a wavelength of 700 nm. The ferric ion reducing power of the chloroform extract and five isolated compounds (CdC-B, CdC-C, CdC-D, CdC-G and CdC-H) were determined by the previously described method (Bajpai *et al.*, 2013). Aliquots (50 μl) of different concentrations of samples (5–25 μg/ml) were mixed with 50 μl phosphate buffer (0.2 M, pH = 6.6) and 50 μL potassium ferricyanide (1%), followed by incubation at 50°C for 20 min in dark. After incubation, 50 μl of trichloro acetic acid (10%) was added to terminate the reaction and the mixture was subjected to centrifugation at 3000 rpm for 10 min. For final reaction mixture, the supernatant (50 μl) was mixed with 50 μl distilled water and 10 μl ferric chloride solution (0.1%). The reaction mixture was incubated for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank sample. A elevated absorbance of the reaction mixture indicated greater reducing power ability, confirming quantitative increase in the reduction of Fe$^{3+}$ to Fe$^{2+}$ in the reaction mixture and vice versa. All tests were run in triplicate in this assay. Ascorbic acid as positive control was also tested for the reducing power assay.
3.5 STATISTICAL ANALYSIS

All values were expressed as mean ± SD. The data obtained from the various groups were statistically analyzed using One-way ANOVA followed by multiple comparisons test. The Morris water maze latencies were analyzed by two-way ANOVA with the day as one variable and the treatment as a second. "*" denotes $p < 0.05$, "**" denotes $p < 0.01$, "***" denotes $p < 0.001$, "ns" denotes $p > 0.05$ significant of difference vs control group.