## PHARMACOGNOSTICAL EVALUATIONS

### 1. Plant material and authentication

The root tubers of the plant *Leea macrophylla* (Leeaceae) were collected in the month of September-October 2013 from the medicinal plant garden of Department of Dravyaguna, Banaras Hindu University. The plant was authenticated by Prof. V.K. Joshi, Department of Dravyaguna, Institute of Medical Sciences, Banaras Hindu University and the specimen (No.COG/LM/01) of the plant has been submitted in Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi.

### 2. Macroscopic and microscopic evaluation

Macroscopic identification of the root tuber was done by examining its shape, size, colour, surface characteristics, odour and texture on the basis of appearance of the cut surface. Microscopic examination was done as per the method described by Johansen, 1940. The plant specimen was fixed for 24 hours in a mixture of formalin, acetic acid and ethyl alcohol (70%) and then series of different grades of tertiary butyl alcohol was used for dehydration. Melted paraffin wax was used for embedding which was then allowed to cool. Paraffin blocks containing the specimen was prepared and thin sections ranging from 10-12 μm thickness were obtained with the help of rotary microtome followed by dewaxing with xylene. The sections were then stained with Toludine blue (O'Brien et al., 1964) and phloroglucinol & hydrochloric acid mixture. Histochemical analysis was performed using various reagents to identify histochemical components (Evans, 2002). The powdered root tuber was also examined under

microscope. For the study of isolated cells, disintegration of tissue was performed according to the procedure described in WHO guidelines in which, the root tuber was treated with concentrated nitric acid and potassium chlorate, washed with distilled water and was mounted in glycerine for observation (Anonymous, 2002b). Photographs at different magnification were taken using Nikon trinocular microscopic unit, Model E-200, Japan.

**3. Physicochemical evaluations** (WHO, 2002)

The shade-dried root tubers were coarsely powdered and were used for evaluation of various physicochemical parameters. The methods described in WHO guidelines (Anonymous, 2002b) and Indian Herbal Pharmacopoeia (Anonymous, 2002a) were followed.

***3.1. Foreign matter:***

The coarsely powdered air–dried root tuber (100g) was evenly spread on white paper sheet and was precisely monitored with the help of magnifying glass. Foreign matter such as sand, clay and other particles were removed from the plant material and was further weighed. The foreign matter in plant material was estimated as percentage weight/weight (% w/w).

***3.2. Total ash***

Incineration of about 2-4 g air dried coarsely powdered plant material was performed in tarred silica dish at a temperature not exceeding 450 °C until it was free from carbon. The total ash was allowed to cool, weighed and expressed as percentage (w/w) with reference to air dried drug.

### *3.3. Acid–insoluble ash*

Total ash in the crucible obtained as a result of incinerating powdered plant material was weighed and 25 mL of hydrochloric acid (70 g/L) was added followed by gently boiling for 5 min. The solution was then filtered using an ash–less filter paper and was later washed with hot water unless it becomes neutral. The filter paper containing the insoluble matter was dried, ignited and later cooled in a desiccator. The content was then weighed and percentage acid insoluble ash (w/w) was calculated with reference to the air dried plant material.

### *3.4. Water–soluble ash*

25 mL of water was added to the crucible containing the total ash and was boiled for 5 minutes. The insoluble matter was allowed to filter using ash less– filter paper, washed with hot water, and ignited for 15 min at a temperature not exceeding 450°C. The weight of the insoluble matter was subtracted from the weight of the total ash taken and the percentage (w/w) of water–soluble ash was calculated.

### *3.5. Alcohol–soluble extractable matter*

4 g of the coarsely powdered air–dried plant material was macerated for 24 h using 100 mL of ethanol in a glass stopper conical flask. Initially the mixture was frequently shaken for 6 h and then allowed to stand for 18 h. 25 mL of filtrate was transferred to previously weighed flat bottom dish and evaporated to dryness on a water bath. Further it was dried at 105°C for 6 h, cooled in dessicator for 30 minutes and weighed immediately. The percentage (w/w) of ethanol–soluble extractive was calculated with reference to the air–dried drug.

### *3.6. Water–soluble extractable matter*

For the determination of water soluble extractable matter, the same method as above was followed using water as solvent for extraction. The percentage (w/w) of water–soluble extractive was calculated with reference to the air–dried drug.

### *3.7. Loss on Drying*

Previously dried and weighed (1g) plant material was taken in a glass–stoppered, shallow weighing bottle and was evenly distributed by gentle sidewise shaking to a depth not exceeding 10 mm and dried to constant weight for the specified time and temperature not exceeding 105 °C. The bottle was cooled by keeping in desiccators after attaining constant weight. It was then weighed and the percentage (w/w) loss on drying was calculated.

### *3.8. Foaming Index*

The air–dried powdered drug (1 g) was weighed accurately and transferred into a 500 mL conical flask containing 100 mL of boiling water. The mixture was allowed to boil for 30 minutes, cooled and filtered into 100 mL volumetric flask and the volume was made up to the mark with water. The filtrated decoction was then poured into 10 stoppered test–tubes (height 16 cm, diameter × 16 mm width) in successive portions of 1 mL, 2 mL, 3 mL up to 10 mL, and in each tube the volume was adjusted with water up to 10 mL. The tubes were closed using a stopper and were shaken in a lengthwise direction for 15 sec with two shakes per sec and was then allowed to stand for 15 min. The height of the foam was measured and results were estimated as follows:

- If the height of the foam in each tube is less than 1 cm, the foaming index is taken to be less than 100.

- If in any of tube from 1 to 10, the height of foam of 1 cm is measured, then the volume of the decoction in this tube is used to determine the index and is marked as 'a' in the formulae given below.
- If this tube is the first or second tube in a series, then prepare an intermediate dilution in a similar manner to obtain a more precise result.
- If the height of the foam is more than 1 cm in every tube, the foaming index is over 1000. In this case repeat the determination using a new series of dilutions of the decoction in order to obtain a result.

The foaming index was calculated using the following formula:

$$\text{Foaming Index} = 1000/a$$

Where 'a' is the volume in mL of the decoction used for preparing the dilutions in the tube where foaming to a height of 1 cm was observed.

### *3.9. Swelling Index*

"The volume (in mL) taken up by the swelling of 1 g of plant material under specified condition" is defined as swelling index. 1 g of air dried plant material was weighed accurately and was taken in a 25 mL glass–stoppered measuring cylinder (16 mm internal diameter and 125 mm length). 25 mL of water was added and the mixture was thoroughly shaken after every 10 min interval for 1 h. It was allowed to stand for 3 h at room temperature. The volume in mL occupied by the plant material was measured. The mean value of the individual determinations, related to 1 g of plant material was calculated.

### *3.10. Hemolytic activity*

Hemolytic activity was determined using standard diosgenin (saponin) as positive control. To a serial dilution of plant material extract an equal volume of suspension of erythrocytes (Ox blood) was added. The lowest concentration which produced complete haemolysis was taken as the haemolytic index.

While determination of haemolytic index preliminary screening was followed by main haemolytic testing. Sodium citrate (36.5 g/L) was filled in glass-stoppered flask to one–tenth of its volume to prepare erythrocyte suspension. Freshly collected blood of healthy ox was added to the flask and shaken immediately. This citrated blood prepared can be retained and stored at 2-4°C for 8 days. 1 mL of citrated blood was diluted with phosphate buffer (pH 7.4) upto 50 mL in volumetric flask resulting in 2% (v/v) solution. Fresh solution of reference solution was prepared by dissolving 10 mg of saponin R, in 100 mL phosphate buffer pH 7.4 in volumetric flask. Serial dilution of the plant extract was also prepared in phosphate buffer pH 7.4.

$$\text{Heamolytic index} = [(1000) \times (a/b)]$$

Different dilutions of the extract and standard diosgenin were mixed thoroughly with the blood suspension and was allowed to stand at room temperature for 6 h. All the tubes were carefully monitored and examined for haemolysis. The minimum concentration of plant extract and diosgenin that produces heamolysis were noted and the haemolytic activity of the plant material were calculated incorporating the formulae

Where, 1000 is the defined haemolytic activity of saponin (diosgenin) in relation to ox blood, ‘a’ is the quantity of saponin (diosgenin) that produces total haemolysis (g), ‘b’ is the quantity of plant extract that produces total haemolysis (g).

### *3.11. Pesticide content*

Preparation of samples: The pesticide residues determination was done as per the methods prescribed by the WHO guidelines (Anonymous, 2002b). The crude drug was reduced to a fine powder and then extracted with a 35% mixture of water in acetonitrile. The mixture was then extracted with light petroleum where the pesticide residues were likely to be more soluble.

Preparation of the column: The column was prepared using stationary phase Florisil (60/100) which was previously activated at 130°C for two consecutive days prior before packing. The florisil was pre wetted with light petroleum ether and finally 1 cm of anhydrous sodium sulphate was layered over it.

Preparation of sample extract: 50 g of the powdered drug was blended thoroughly with a mixture of 35% acetonitrile and water solution. The solution mixture was filtered under vacuum and the filtrate was shaken vigorously for 1–2 min with 100 mL light petroleum ether. 10g of NaCl and 600 mL water was then added. Separation was allowed and finally the aqueous layer was discarded and again washed with two portions of 100 mL water which was later discarded. Then to the filtrate, 15 g of anhydrous sodium sulphate was added and shaken vigorously and the final volume was reduced to 5–10 mL capacity. The concentrated volume was then transferred directly to a florisil column and eluated with a mixture of petroleum ether and diethyl ether (94:6) to obtained the first eluate (TS1). The second eluate (TS2) was also collected by eluting with a mixture of petroleum ether and diethyl ether (85:15). Finally the third eluate (TS3) was eluted with 50% mixture of petroleum ether and diethyl ether. All the eluates were concentrated and were subjected for combustion.

Combustion of the organic matter: Combustion was done in a one liter conical flask fused with a platinum wire (1mm) attached with a platinum gauze (1.5 × 2 cm) as a means for holding the sample. The sample holder was made up of halide free filter paper (5 cm long and 3 cm wide). The samples eluates were finally concentrated under vacuum, evaporate to dryness, transferred to sample holder and burned in the presence of oxygen till the combustion process was completed.

Combustion of Chlorine and phosphorus containing residues: For chlorine containing residues, aliquots of the eluates (TS1 and TS2) as prepared above were placed separately in a funnel. The eluates were evaporated, dried and the sample holder containing the residues were then folded to form a cone (1$cm^2$ in area) which was finally placed at the centre of the sample holder for combustion. After combustion process was completed, the combustion flask which contained 30 mL of water was then tilted and shaken for 10 min for mixing of the combusted material along with water. Finally the solution was then transferred to a 50 mL volumetric flask and the volume was then making up with water to the mark. The solution was finally incorporated for determination of chlorine containing residues.

In case of Phosphorus containing residues the same procedure was performed as that of chlorine containing residues but sulphuric acid (0.375 mol/L) was used as the solvent to dissolve the phosphate residue which was finally proceeded for spectrophotometric analysis of the samples.

Quantification of Chlorides: The quantification of chloride containing pesticide was done using UV spectrophotometer. A standard solution of sodium chloride containing 5µg/mL was prepared in different aliquots (0, 2, 4, 6, 8 and 10 mL) and dilute up to 15

mL with water. The colour was then developed by mixing the combusted sample and aliquots of standard chloride solution with 1 mL ferric ammonium sulphate (0.25 mol/L) and 3 mL of mercuric thiocyanate followed by swirling and allowed to stand for 10 min. The absorbance was measured spectrophotometrically at 460 nm and the quantity of chloride was calculated.

Quantification of Phosphates: For the quantification of phosphate containing pesticide, 7 mL of the combusted solution and various aliquots of standard phosphate solution were placed in a previously calibrated 10 mL test tube. 2.2 mL of sulphuric acid (3 mol/L) was then added and mixed thoroughly which was followed by the addition of 0.4 mL of ammonium molybdate (40 g/l). The mixture was mixed properly and 0.4 mL of amino naptho lsulfonic acid was finally added to the solution mixture to develop a clear blue colour solution. The absorbance of the final solutions were recorded spectrophotometrically at 820 nm.

### *3.12. Heavy metals analysis*

In the present investigation four important heavy metals were estimated viz. Arsenic (As), Lead (Pb), Cadmium (Cd) and Mercury (Hg) in the plant material. Digestion of 2 g powdered drug was carried out using10 mL conc. HNO3 followed by heating in a hot plate for 15 min at 95°C. Two more cycle of similar procedure was repeated for another 30 min until the red fumes of nitric acid disappeared. Peroxide reaction was initiated by adding 2 mL of deionised water and 3 mL hydrogen peroxide (30% v/v) to 2 mL of above cooled solution. After completion of the reaction, 5 mL of conc. HCl and 10 mL deionised water was added and the samples were allowed to heat for another 15 minutes. The sample was cooled, filtered and make up to 50 mL volume. The

estimation of heavy metals were analyzed by using atomic absorption spectroscopy (Shimadzu–AA6300) (Gomez et al., 2007). The results obtained were expressed in terms of parts per million (PPM) per gram of drug.

### *3.13. Determination of crude fiber content (Dutch Method)*

Crude fiber content of the plant material was determined as per method described in Khandelwal, 2007. 2 g of air dried powdered drug was treated with 50 mL of 10% nitric acid followed by boiling with constant stirring. The matter was strained using a muslin cloth. The residue was washed with boiling water and was further treated with 50 mL 2.5% w/v sodium hydroxide solution followed by boiling. The remaining matter was further strained, dried and the percentage (w/w) of crude fiber was calculated.

### *3.14. Fluorescence powder drug analysis*

Detection and differentiation of one powdered drug from another can be done with the help of Fluorescence analysis of powdered drug like for examples detection of ergot in flour, cocoa shells in powder cocoa and vice versa.

The air dried plant material is treated with various chemical reagents, mixed vigorously followed by filtration. The resulting filtrate is subjected to screening by visualization using long and short UV (254 and 365 nm) as well as in daylight. The colour of the fluorescence produced was observed by comparing with the reference standard colour available at http://trac.dojotoolkit.org (Chase and Pratt, 1949).

### 4. DNA Fingerprinting

DNA fingerprinting was performed at the Science Foundation for Tribal and Rural Resource Development, Bhuwaneshwar, India using the Random Amplified

Polymorphic DNA (RAPD) technique. The plant was subjected to RAPD assay of their genomic DNA using six different primers.

*Isolation of genomic DNA of the plant sample*

Plant samples were collected and subsequently stored at –20°C for isolation of genomic DNA. The genomic DNA was extracted from young plant using N-Cetyl-N, N, N-trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990) with modifications. Accurately about 2 g of fresh plant material were washed in distilled water and subsequently rinsed with 80% (v/v) ethanol and grounded in liquid nitrogen. 10 mL of preheated extraction buffer [4 % (w/v) CTAB, 0.2% ß-mercaptoethanol (v/v), 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0 and 1.4 M NaCl] were added per 2 g of plant powder and incubated for 2 h at 65°C. The treated sample was purified with chloroform: isoamylalcohol (24:1). The DNA pellet was re-suspended in 200 µL of Tris-EDTA buffer (10 mM Tris –HCl, 1 mM EDTA, pH = 8.0). DNA was re-precipitated by adding 80% ethanol in the presence of 0.3 M sodium acetate, and precipitated by centrifugation. The pellets were lyophilized and re-suspended in TE buffer. The RNA was removed by RNase treatment at 37°C for 1 h. For further purification, the DNA sample was treated with equal volume of phenol and chloroform: isoamyl alcohol (24:1:1) followed by two extractions with chloroform: isoamyl alcohol (24:1). The upper aqueous phase was separated after centrifugation and mixed with $1/10^{th}$ volume of 3 M sodium acetate. DNA was precipitated by adding two volumes of chilled absolute alcohol, precipitated and dried in lyophilizer and dissolved in TE buffer. Quantification of DNA was accomplished by analysing the purified DNA on 0.8% (w/v) agarose gel electrophoresis alongside diluted uncut lambda DNA as

standard. DNA was further diluted with TE to a concentration of 20 ng/µL for use in PCR analysis (Doyle and Doyle, 1990).

*PCR amplification and electrophoresis*

A set of RAPD decamer primers were used for the analysis of plant genotype. These primers were purchased from commercially available primer kits (Merck Bioscience, India). Individual PCR amplifications for each primer were performed in programmable thermal cycler (Peqlab, Germany). The PCR protocol involved a total volume of 25 µL reaction mixture containing 20 ng of genomic DNA, 1X PCR buffer (pH 8.3), 200 µM dNTP mix, 10 pmol of primers, 2 mM of MgCl and 1 U of *Taq* DNA polymerase (Merck Bioscience, India). The basic PCR program to amplify DNA was as follows: an initial denaturation at 94°C for 5 min followed by 40 cycles (1 min denaturation at 94°C, 1 min annealing at 37°C temperature and 1 min primer elongation at 72°C). Thereafter a final extension step at 72°C for 10 min was performed.

*Gel electrophoresis*

A 25 µL aliquot of the PCR sample was mixed with 2 µL of a loading buffer (0.4% Bromophenol blue, 0.4% Xylene Cyanole and 5 mL of Glycerol) and was loaded directly on 1 % agarose gels in 0.5 X TBE buffer. Electrophoresis was done for about 2 h at 60 V. Low range DNA ruler (Merck Bioscience) was used to compare the molecular weights of amplified products. Visualization of the amplified bands was done by staining with Ethidium Bromide for 20 min and de-staining with double distilled water for 20 min. The photographs of the gel were taken under Gel Documentation System (UVITEC, UK).

## 5. Extraction, Qualitative and Quantitative Estimations & Standardization of Extract

The shade-dried root tubers (600 g) were coarsely powdered and subjected to Soxhlet extraction using ethanol (1.5 L) until the whole plant material was exhausted. The obtained ethanolic extract of *Leea macrophylla* (ELM) (22.0% w/w) was concentrated and dried in a rotary evaporator which was then stored in desiccator until use.

### *5.1 Optimization of Extract*

#### *Working with BBD towards response surface*

The efficiency of soxhlet extraction depends upon factors such as degree of sample homogenization, nature of solvent used for extraction and length of time invested in completion of extraction process (Gullberg et al, 2004). The critical factors were identified by preliminary experimentation and data mining as being capable of influencing extraction. Powdered drug was screened for its extractive value in different solvent including petroleum ether, hexane, chloroform, ethyl acetate and ethanol. It was observed that maximum yield was obtained in ethanol, thus it was selected as extracting solvent for the experiment. Design Expert (Version 6.0.0 Trial, Stat-Ease Inc., MN) was used to determine the boundary of experiment, for evaluating response and for finally optimizing the process. The level of design factors have been shown in Table 5. The actual extractive experiments with values of independent variables and obtained results are shown in Table 6. A total of 15 extractive cycles were performed with three repeat runs to establish random errors. A sixteenth run was performed according to optimized run suggested by the software.

**Table 5:** Input factors with their ascribed values and coded levels, along with the expected outcomes of the response.

| Factor | Levels used, Actual (coded) | | |
|---|---|---|---|
| | **Low(-1)** | **Medium(0)** | **High (+1)** |
| $X_1$=Mesh Size (sieve number) | 10 | 20 | 40 |
| $X_2$=Solvent Blend (ethanol:water) | 85 | 92.5 | 100 |
| $X_3$=Time (h) | 12 | 18 | 24 |
| **Dependent Variables** | | **Constraints** | |
| $Y_1$=Extraction Efficiency (%) | | Maximize | |
| $Y_2$=Phenolic content(mg/g) | | Maximize | |

**Table 6:** Actual extractive runs for obtainment of best results

| Batch | Independent variables | | | Dependent variables | |
|---|---|---|---|---|---|
| | $X_1$ (Mesh Size) | $X_2$:Solvent Blend (ethanol:water) | $X_3$ (Time h) | $Y_1$ (%) | $Y_2$ (mg/g) |
| 1 | 10 | 85 | 18 | 14 | 113.24 |
| 2 | 40 | 85 | 18 | 14.6 | 117.53 |
| 3 | 10 | 100 | 18 | 17 | 138.18 |
| 4 | 40 | 100 | 18 | 16.5 | 135.34 |
| 5 | 10 | 92.5 | 12 | 15.6 | 128.93 |
| 6 | 40 | 92.5 | 12 | 15 | 123.43 |
| 7 | 10 | 92.5 | 24 | 13 | 103.55 |
| 8 | 40 | 92.5 | 24 | 17 | 140.47 |
| 9 | 20 | 85 | 12 | 16 | 130.43 |
| 10 | 20 | 100 | 12 | 18 | 145.05 |
| 11 | 20 | 85 | 24 | 20 | 143.47 |
| 12 | 20 | 100 | 24 | 21 | 142.74 |
| 13* | 20 | 92.5 | 18 | 27.5 | 175.32 |
| 14* | 20 | 92.5 | 18 | 29 | 181.54 |
| 15* | 20 | 92.5 | 18 | 30 | 185.82 |
| **16** | **20** | **93.5** | **18** | **28.9** | **184.9** |

*Marks the replicate runs at design center to determine the random error. The bold values represent the final optimized experiment.

The % yield of extraction is indicative of extraction efficiency while phenolic yield of extract was estimated via standard methods.

### *5.2 Preparation of fractions*

The dried reddish brown powder of ethanolic extract of *Leea macrophylla* so obtained was then fractionated by suspending in aqueous layer and partitioning between solvents of increasing polarity to obtain hexane fraction (LMH), chloroform fraction (LMC), ethyl acetate fraction (LMEA), n-butanol fraction (LMBU) and aqueous fraction (LMAQ).

### *5.3 Phytochemical evaluations*

The optimized ethanolic extract and its successive fractions were subjected to preliminary phytochemical evaluation for the presence of different phytoconstituents using following methods (Evans, 2002; Khandelwal, 2007).

#### *5.3.1. Test for alkaloids*

*Mayer's test*

Test solution when treated with Mayer's reagent results in formation of cream coloured precipitate ensuring the presence of alkaloids.

*Dragendroff's test*

Required volume of the test solution when treated with Dragendroff's reagent leads to formation of orange to reddish coloured precipitate confirming the presence of alkaloids.

*Wagner's test*

Test solution (extracts) when treated with Wagner's reagent, formation of reddish brown coloured precipitate ensures the presence of alkaloids

### *5.3.2. Test for phytosterols/steroids*

*Libermann Burchard test*

To the test solution (extract), 3 mL of acetic anhydride was added. To this solution, two drops of concentrated sulphuric acid was added slowly along the side of the test tube. Appearance of bluish green colour confirms the presence of phytosterols/steroids.

### *5.3.3. Test for glycosides*

#### *5.3.3.1. Test for anthraquinones glycosides:*

*Borntrager's test*

In this test, the extract was first shaken gently with equal volume of chloroform and then the chloroform layer was separated. To this layer dilute ammonium solution was added and if ammonia layer acquires pink colour, it confirms the presence of anthraquinone glycosides.

*Modified Borntrager's test*

To the extract, 5% ferric chloride solution and dilute hydrochloric acid was added, heated on boiling water bath for 5 min to bring about oxidative hydrolysis and was shaken gently by addition of benzene. Benzene layer was further separated and to this, dilute ammonia solution was added. Formation of pink colour indicates the presence of anthraquinone glycosides.

#### *5.3.3.2. Test for cardiac glycoside*

*Legal's test*

To the test solution, 1 mL of pyridine and a few drops of sodium nitroprusside were added and were made alkaline with NaOH solution. Appearance of pink colour is an indicative of presence of cardiac glycosides.

#### *5.3.3.3. Test for coumarins glycoside*

To the test solution (extract), 10% dilute NaOH was added and the mixture was monitored under UV light (366 nm). Appearance of blue colour fluorescence confirms the presence of coumarin glycoside.

#### *5.3.3.4. Test for cynogenetic glycosides*

*Sodium picrate test*

Sodium picrate paper turns brick red in colour when comes in contact with the extract containing cynogenetic glycosides. Sodium picrate paper is prepared by soaking filter paper first with 10% picric acid and then with 10% sodium carbonate.

### *5.3.4. Test for Phenols*

To the test solution (extract dissolved in methanol), few drops of freshly prepared solution of 5% ferric chloride was added. Formation of blue colour indicates the presence of phenol.

### *5.3.5. Test for tannins*

To the test solution (extract dissolved in methanol), 0.5 mL of 10% lead acetate was added. Appearance of white precipitate indicates the presence of tannins.

### *5.3.6. Test for flavonoids*

To the test solution (extract dissolved in methanol), required amount of magnesium turnings and a few drops of concentrated hydrochloric acid were added. Formation of pink colour indicates the presence of flavonoids.

### *5.3.7. Test for saponins*

To the extract, 1 mL of distilled water was added and shaken vigorously. Formation of persistent foam/froth up to 10 min or more indicates the presence of saponins.

### *5.3.8. Test for carbohydrates*

*Molisch's test*

In this test, the extract was made to react with 0.5 mL of α–naphthol solution. The mixture was vortexed and two drops of concentrated sulphuric acid was added from the side of the test tube. Formation of purple ring at the junction of two liquid showed the presence of carbohydrate.

### *5.3.9. Test for proteins*

*Biuret test*

Test solution (extract) was treated with 40% sodium hydroxide and dilute copper sulphate solution. Appearance of blue colour indicates presence of proteins.

*Xanthoproteic test*

The test solution was treated with conc. $HNO_3$ and boiled for few minutes. Appearance of yellow precipitate indicates presence of proteins.

### *5.3.10. Test for amino acids*

To the known volume of test solution, 2 mL of ninhydrin solution was added and the solution was heated. Formation of violet colour indicates the presence of amino acids.

**5.4. Quantitative evaluation**

After preliminary phytochemical screening the extract/fraction(s) were further subjected to quantification of phytoconstituents which were found to be present in them.

***5.4.1. Estimation of total phenolic and tannin content***

Folin–Ciocalteu method (Hagerman el al., 2000) was followed to determine total phenolic and tannin content. 200 mg of extract/successive fraction(s) was subjected to extraction using10 mL of 50% aqueous methanol in an ultrasonic water bath for 20 min. The resulting extract was cooled centrifuged (3000 × g) for 10 min at 4°C and the clear supernatant was collected. The remaining residue was again extracted with 5 mL of 50% aqueous methanol for 20 min and finally both the supernatant were mixed and made up to the volume of 10 mL. A serial dilution of tannic acid solutions were prepared concentration range of 0.02–0.10 mg/mL.

Analysis of total phenols (TP)

0.05 mL of aliquots of standard tannic acid (0.02–0.10 mg/mL) and extract/fractions (0.05 mL) were diluted with triple distilled water (TWD) and volume was made upto 0.5 mL. 0.25 mL of Folin–Ciocalteu reagent (2 N) and 1.25 mL sodium carbonate (20%) were mixed vigorously to this solution and allowed to stand for 40 min at room temperature. The absorbance of all the tested samples were measured at 725 nm and the amount of total phenols as tannic acid equivalent was calculated from the standard curve of tannic acid.

**Analysis of total tannins**

To the extract/fraction(s) 1 mL of triple distilled water (TWD) and 100 mg polyvinyl pyrollidone (PVP) was added. The mixture was mixed thoroughly by vortexing and centrifuged at 3000 × g for 10 min. The supernatant was collected and finally estimated for total simple phenolics as per the Folin-Ciocaltea methods. The difference in total phenolic content and total simple phenolics was expressed as total tannin content (equivalent to tannic acid).

### *5.4.2. Estimation of total flavonoid content*

Total flavonoid content was determined as per method described by Kumaran and Karunakaran, 2006 using aluminum chloride method where rutin is used as reference standard. In this method a flavonoid–aluminum complex having the absorption maxima at 415 nm is formed which can be detected by spectroscopic technique.100 µL of plant extract/fraction(s) in methanol (10 mg/mL) was mixed with 100 µL of 20% aluminum trichloride (20%) in methanol, to which a drop of acetic acid was added and the mixture was diluted with methanol up to 5 mL. The resulting solution was allowed to stand for 40 min after and the absorbance was measured spectrophotometrically at 415 nm. 100 µL of methanol in place of plant extract were treated as blank.

The absorbance of standard rutin solution (0.5 mg/mL) in methanol was also measured under the same experimental conditions and the total flavonoid content (mg rutin equivalent/mg plant extract) was calculated using the following equation:

$X = (A. m_o)/(A_o . m)$

Where X is the flavonoid content, mg/mg sample in RE, A is the absorbance of sample solution, Ao is the absorbance of standard rutin solution, m is the weight of sample in mg and $m_a$ is the weight of rutin in the solution in mg.

### *5.4.3. Estimation of total flavonol contents*

1 mL of plant extrat/fraction(s) (10 mg/mL) was measured accurately and was mixed with 1 mL 20% aluminum trichloride and 3 mL 5% sodium acetate solution. The absorbance was taken after 2.5 h using methanol as blank. The absorbance of standard rutin solution (0.5 mg/mL) in methanol was also measured under similar experimental conditions. The total flavonol content (mg rutin equivalent/mg plant extract) was calculated using the following equation:

Total flavonol content = (A × mo) / (Ao × m)

Where 'A' is the absorbance of plant extract solution, 'Ao' is the absorbance of standard rutin solution, 'm' is the weight of plant extract, and 'mo' is the weight od mixed rutin in the solution.

### *5.4.4. Estimation of total alkaloid content*

Gravimetric method (Wagner and Bladt, 1996) was followed for determination of total alkaloid content. Extraction of 5 g of the powdered drug was conducted repeatedly in an ultrasonic bath using (3 x 50 mL) 0.1 N $H_2SO_4$. The final solution was filtered; the mixed acid solution was washed with 4 successive quantities of 25 mL chloroform (washing each chloroform solution with 20 mL of acid). Rejecting the chloroform washings, dilute ammonia solution was added to the acid solution in order to basify it, which was then further extracted with (20 mL x 5) diethyl ether. The combined diethyl ether extracts were washed with 5 mL of distilled water and the ether was evaporated to

dryness in a weighed beaker on a water bath, which was further dried to constant weight at 105°C and the % w/w of alkaloid was calculated.

### *5.4.5. Estimation of total saponin content*

The total saponin content was determined as per the methods described by Baccou et al, (1977). The plant material was first defatted with petroleum ether and 0.5 g of the defatted material was accurately weighed and kept in plastic centrifuge tubes containing 10 mL of absolute methanol. The mixture was kept for overnight in a magnetic stirrer and the content was centrifuged. The supernatant was collected in a 25 mL capacity measuring flask. The remaining residue was again washed out twice with 5 mL of fresh methanol and again centrifuged. The overall supernatant was collected and the final volume was made up to 25 mL with methanol. Standard diosgenin solution was used as reference and was freshly prepared by dissolving 10 mg diosgenin in 20 mL methanol and its standard curve was prepared using different dilutions in concentration ranging between 25–125 µg/mL.

The solution was transferred to the test tubes placed in ice–water bath and vanillin reagent (0.25 mL) was added to all the test tube and was kept on a magnetic stirrer. To this 2.5 mL (75 % v/v) of sulphuric acid with proper stirring was added slowly and the whole mixture was warmed in a water bath at 60°C for 10 min which was later cooled in ice cold water. The absorbance of the solution mixture after cooling was measured at 544 nm. A blank solution was prepared using methanol as solvent. For determination of total saponin in the plant extract 0.25 mL of the extract was used. The results were calculated using the standard regression equation of saponins and the results were expressed as mg/g diosgenin equivalent.

### *5.4.6. Estimation of total carbohydrate content*

The total carbohydrate content was determined as per the calorimetric method as described by Yemm and Willis (1954) using anthrone reagent which was freshly prepared by dissolving 0.2 g of anthrone in 100 mL of water. The plant material was exhaustively extracted with 70% (v/v) ethanol and was used for this estimation. The anthrone reagent (5 mL) was pipetted out into a thick walled pyrex tube (150 × 25 mm) and which was chilled in ice cold water. The test solution (1 mL) was layered on the acid medium, cooled for further 5 min and was finally mixed thoroughly in cooling condition. The tubes were then loosely fitted with corks, heated in boiling water bath and were later cooled for 5 min. Finally the absorbance of the solution was measured spectrophotometrically at 600 nm, where the blank was prepared and read against water. Fructose at different dilutions was used as reference standard for the above estimation.

## *6. Quantification of chlorogenic acid in ethanolic extract of Leea macrophylla by HPLC*

The plant *Leea macrophylla* has not been explored in terms of chemistry till date. The extract of *Leea macrophylla* was first subjected to phytochemical screening followed by quantification of phytoconstituents. The above study revealed that the extract was highly rich in phenolics. As per literature review (Kubitzki, 200) it was found that the family Leeaceae (Vitaceae) to which plant belongs have shown presence of Chlorogenic acid (CGA) which has been implicated with wound healing potential in several studies. Considering the above probability TLC was performed to substantiate

the lead. TLC confirmed the presence of CGA and hence it was selected out of all the possible active constituents for quantification with help of HPLC.

A validated HPLC method (Yuan et al., 2005) was followed for standardizing ethanolic extract of *Leea macrophylla.* Commercially procured chlorogenic acid (Sigma-Aldrich [purity: 95%]) was employed as standard. HPLC system (Waters) equipped with gradient pumps was used for analysis. A Cosmosil C18 column (150mm× 4.6mm, 5 µm particle) was used for chromatographic separation of sample. The injection volume of sample was 10 µL. An aqueous phase consisting of 0.4% acetic acid and 4.5% tetrahydrofuranin triple distilled water was modified with acetonitrile in a variable pattern to form the mobile phase flowing at 1 mL/min. Gradient elution began with an aqueous phase to acetonitrile phase ratio of 5:95 and changed up to 25:75 during the initial 15min. Further, ratio of the mobile phase was altered from 25:75 to 60:40 for next 35 minutes. An equilibration period of 10 min was allowed using the initial mobile phase composition before injecting the next sample. The entire operation was carried out in ambient conditions. The peak area of extracted data was calculated at 326 nm using class VP series software. The identity of peak was affirmed by cross checking retention time of the standard chlorogenic acid sample.

**7. *In vitro* antioxidant activity**

***7.1. Total antioxidant capacity***

Phosphomolybdenum method described by Prieto et al. (1999) was used to describe the total antioxidant capacity of plant extracts and fractions. The assay was based on the reduction of Mo (VI) to Mo (V) by the extract/fractions and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. In this method, 0.3 mL of

extract/fractions in methanol (1 mg/mL) and ascorbic acid (50-300 µg/mL) were combined with 3 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) separately. A blank solution consisted of 3 mL of reagent solution and the appropriate volume of the same solvent was used for the extract/fractions. All tubes were capped and incubated in a boiling water bath at 95 $^{o}$C for 90 min. After cooling to room temperature, the absorbance of each samples were measured at 695 nm against the blank. The antioxidant activity was expressed as the number of equivalent of ascorbic acid.

### *7.2. Assay of reducing power*

Assay of reducing power was carried out by implementing potassium ferricynide method (Yildrim et al., 2001). The plant extract/fractions (1 mL) was mixed with 2.5 mL phosphate buffer ( 0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [ $K_3Fe(CN_6)$] (10 g/l). Then the mixture was incubated at 50 $^{0}$C for 20 minutes. Further, 2.5 mL of trichloroacetic acid (100 g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of the supernatant solution was mixed with 2.5 mL of distilled water and 0.5 mL $Fecl_3$ (1 g/l) and absorbance was measured at 700 nm. Ascorbic acid was used as standard and phosphate buffer was used as blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power.

### *7.3. Free radical scavenging activity*

The free radical scavenging activity of plant extract/fractions was measured by the 1, 1-diphenyl-2-picryl-hydrazil (DPPH) method (Blois, 1958). In this method, 100 µM/mL solution of DPPH in methanol was prepared and 5 mL of this solution was added to 1 mL of sample in different concentrations (25–200 µg/mL). The absorbance was than

measured 30 min later at 517 nm. The free radical scavenging activity was calculated using the following equation: DPPH scavenging effect = $[(1-A_1/A_0)] \times 100$. Where $A_0$ is the absorbance of the blank and $A_1$ is the absorbance of test sample. Further, % inhibition was plotted against respective concentrations used and $IC_{50}$ was calculated by using ascorbic acid as positive control.

### *7.4. Scavenging of hydrogen peroxide*

Scavenging activity of plant extract/fractions was evaluated by the method described by Jayaprakasha et.al. (2004). A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Here, 1 mL of various concentrations of the extract/fractions and standards in methanol were added to 2 mL of hydrogen peroxide solution in PBS. Finally, the absorbance was measured at 230 nm after 10 min. All readings were performed in triplicates and the percentage inhibition was calculated using above equation.

### *7.5. Nitric oxide scavenging assay*

Nitric oxide scavenging assay was carried out as described by Sreejayan and Rao, (1997). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions. This can be determined by the use of the Griess Illosvoy reaction. In this method, 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of extract at various concentrations and the mixture was incubated at 25 $^0$C for 150 min. From the incubated mixture 0.5 mL was taken out and added into 1.0 mL sulfanilic acid reagent (33% in 20% glacial acetic acid), which was incubated at room temperature for 5 min. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1%

w/v) was mixed and incubated at room temperature for 30 min. The absorbance was measured at 546 nm and the nitric oxide radical scavenging activity was calculated according to the following equation:

% Inhibition = $((A_0-A_1) / A_0 \times 100)$

Where $A_0$ is the absorbance of the control (blank without extract) and $A_1$ is the absorbance of the extract. Further, % inhibition was plotted against the respective concentrations used and $IC_{50}$ was calculated by using rutin, as positive control.

### *7.6. Scavenging of hydroxyl radical by deoxyribose method*

Hydroxyl radical scavenging activity of plant extract/fractions were measured according to the method of Halliwell et al. (1987). To 1 mL of the final reaction solution consisting of aliquots (500 µL) of various concentrations of the extract/fractions, 1 mM $FeCl_3$, 1mM EDTA, 20 mM $H_2O_2$, 1 mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4) were added and incubated for 1 h at 37 ºC. This was further heated in a boiling water bath for 15 min after addition of 1 mL of 2.8% (w/v) trichloroacetic acid and 1 mL of 1% (w/w) 2-thiobarbituric acid. The colour development was measured at 532 nm against a blank containing phosphate buffer.

### 8. Antibacterial activity

Antibacterial activity of the extracts and fractions of *Leea macrophylla* was determined on four reference bacterial strains i.e. *Escherichia coli* (ATCC 25922), *Shigella flexneri* (ATCC 12022), *Pseudomonas aeruginosa* (ATCC 27893), *Staphylococcus aureus* (ATCC 25323) and four clinical bacterial isolates- *Salmonella typhi*, *Klebsiella*

*pneumonia, Shigella boydii* and *Enterococcus faecalis* were obtained from the American Type Culture Collection (ATCC), MTCC and clinical strains preserved at Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

Disc diffusion method was used for determining the efficacy of extract and its successive fractions against different bacterial strains. Fresh bacterial strains were suspended in sterile saline and the suspension was spread on the surface of Muller Hinton agar (MHA) plates. Further, the plates were allowed to dry for 5 min. The test sample (extract and its successive fractions) at different concentrations (50 and 100 mg/mL) was then applied on 6 mm sterile disc of Whatman filter paper no. 1. These discs were then placed on the surface of the nutrient medium and the extract was allowed to diffuse for 5 min. The plates were then incubated for 24 h at 37 °C and inhibition zones around the discs were recorded in triplicate. The guideline proposed by National Committee for Clinical Laboratory Standards (NCCLS, 2000) was adopted for determining the MIC (Minimum Inhibitory Concentration) of the extract and its successive fractions using micro-dilution method. The test sample was first diluted with equal volume of nutrient broth which was further mixed in wells of microtiter plate. 0.1 mL of standardized inoculums was then added in each tube and the plates were incubated aerobically at 37 °C for 18-24 h. The lowest concentration at which there was no visible bacterial growth observed, as conclusive through no turbidity compared to the control was referred as MIC (Teke et al., 2007).

# PHARMACOLOGICAL EVALUATION

## 1. Experimental Animals

Young albino rats (Charles foster) 150–200 g bred in the Institutional animal facility, were obtained from Central Animal House (Reg. No. 542/02/ab/CPCSEA), Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. The animals were kept in polypropylene cages and maintained under standard conditions (12 h light and dark cycle at an ambient temperature of 22 ± 3 °C and 45-55% Relative humidity) and were granted free access to standard commercially available rat feed (Hindustan Lever Ltd., Mumbai, India) and water. The animals were afforded with sufficient time for acclimatization before experiment initiation. Care and non-experimental handling of animals was performed by dedicated animal house staff. All experimental protocols were approved (No. Dean 10-11/60 dated 07/01/2011) and conducted as per accepted standard guidelines of Central Animal Ethical Committee of Banaras Hindu University.

## 2. Doses

### *2.1. Oral Dose*

For oral administration the ethanolic extract of *Leea macrophylla* was suspended in carboxy methyl cellulose (CMC) at 250, 500 and 750 mg/kg p.o. Vitamin E (Sigma-Aldrich, [purity 95%]) was selected as oral standard drug to evaluate wound healing activity in experimental animals as reported earlier (Joshi et al., 2013). Ketamine (100 mg/kg i.p.) was used as anaesthetizing agent.

### *2.2. Topical Dose*

***Topical Formulation and its characterization***

***Preparation of Bioadhesive gel of ethanolic extract of Leea macrophylla***

Sodium carboxy methyl cellulose (SCMC; 2.5 or 4%, w/v) was initially dispersed in triple distilled water overnight using a magnetic stirrer. Following complete solublization, polyvinylpyrrolidone (PVP; 2.5 or 4%, w/v) was added to swelled SCMC dispersion. To the SCMC/PVP containing gel, polycarbophil (PC; 2%, w/v) followed by differing concentration of ethanolic solution of extract (2.5, 5.0 and 7.5 g per mL ethanol) were added and mixed, which yielded 2.5%, 5% and 7.5% w/v of active extract containing bioadhesive gel. All samples were tapped vigorously to remove entrapped air, and kept in a desiccator. For comparative reference blank gels were also prepared by following the above described steps with the exception of adding extract. Experimental Design for the manufacture of bioadhesive gel is given in Table 3. The optimized biogel was lyophilized using lyophilizer (Lypholizer, Decibel, India) for 36 hrs at -40°C for further analysis (Singh et al., 2015).

**Table 7:** Experimental design for manufacture of bioadhesive gels with their codes

| Batch No. | SCMC (%w/v) | PVP (%w/v) | PC (gm) (%w/v) |
|---|---|---|---|
| BG 1 | 2.5 | 2.5 | 2 |
| BG 2 | 4 | 2.5 | 2 |
| BG 3 | 4 | 4 | 2 |
| BG 4 | 2.5 | 4 | 2 |

Mechanical behaviour of extract containing bioadhesive gel was evaluated using a texture analyser (BrookField, CT3) working in TPA mode. TPA was calibrated for probe, force and frame stiffness using Texture Pro CT 64 bit software package which drives the CT3 Texture analyser. The biogels were packed into sample holder and a polymeric probe (20 mm across) was compressed twice into them at a rate of 2.0 mm/s to a defined depth (30 mm) allowing delay period (10 s) between two consecutive

compression cycles. From the resultant force–time plot obtained for three replicate samples, hardness, compressibility and adhesiveness were calculated. On the basis of these parameters, best topical formulation was selected and carried forward for further biological evaluation (Singh et al., 2015).

The surface morphology of optimized gel and blank gel was captured using scanning electron microscope (EVO-50, ZEISS, United Kingdom). The samples were prepared by mounting lyophilized gels on a double adhesive tape attached to an aluminium stub and sputter coated with a gold palladium alloy under an argon atmosphere using a high vacuum evaporator (SC7640 Polaron Sputter Coater). Gels were imaged using a 5 kV accelerating voltage, at a working distance of 10 mm by randomly scanning several imaging fields at appropriate magnifications.

**3. Acute Toxicity Studies**

***3.1. Acute oral toxicity study***

The study was conducted on fasted regular nulliparous and non-pregnant female rats by orally administering ethanolic extract of *Leea macrophylla*. The treated animals were then observed vigilantly for 48 hrs for signs of acute toxicity (OECD-425) by noticing their para-sympathetic and communicative changes. More precisely occurrence of convulsions, excessive salivation, lacrimation, defecation, tremors, shivers and any alteration in sleep feeding pattern were observed.

***3.2. Acute skin irritation test***

The method of Gfeller et al., 1985 was used on rats to determine acute skin irritation of *Leea macrophylla* extract bioadhesive gel. 500 $mm^2$ area of dorsal hair of animals were shaved and cleaned. The bioadhesive gel formulations (2.5%, 5% and 7.5% w/v) were

then applied to different groups of animals. The skin of each animal was checked for any sign and symptom of inflammation following 4 hrs of application.

**4. Wound healing activity**

***4.1. Incision wound model***

Each group of animals for this study included six rats. The control group (Group 1) remained untreated whereas animals in Group 2 were treated with blank gel. The animals in Group 3-5 received oral treatment with ethanolic extract of *Leea macrophylla* at 250, 500 and 750 mg/kg p.o. while Group 6-8 animals were treated topically with bioadhesive gel of ethanolic extract of *Leea macrophylla* at concentration of 2.5%, 5% and 7.5%. Group 9 served as oral standard and was given Vitamin E (200 mg/kg p.o.) and Group 10 animals were treated with *Aloe vera* cream as topical standard (Chitra et al., 1998). Each animal was kept separately in cages. The rats were anaesthetized universally across vertebral column and two paravertebral incisions (6 cm long) were created along the full thickness of the skin (Figure 5A). The wounds were closed with interrupted sutures at a distance of 1cm (Figure 5B). Oral and topical treatments were continued up to 10 days with sutures being removed after the $6^{th}$ day. Method described by Lee, 1968 was used to determine wound breaking strength (WBS) on the $10^{th}$ post-wounding day (Figure 6).

**INCISION WOUND IN RAT**

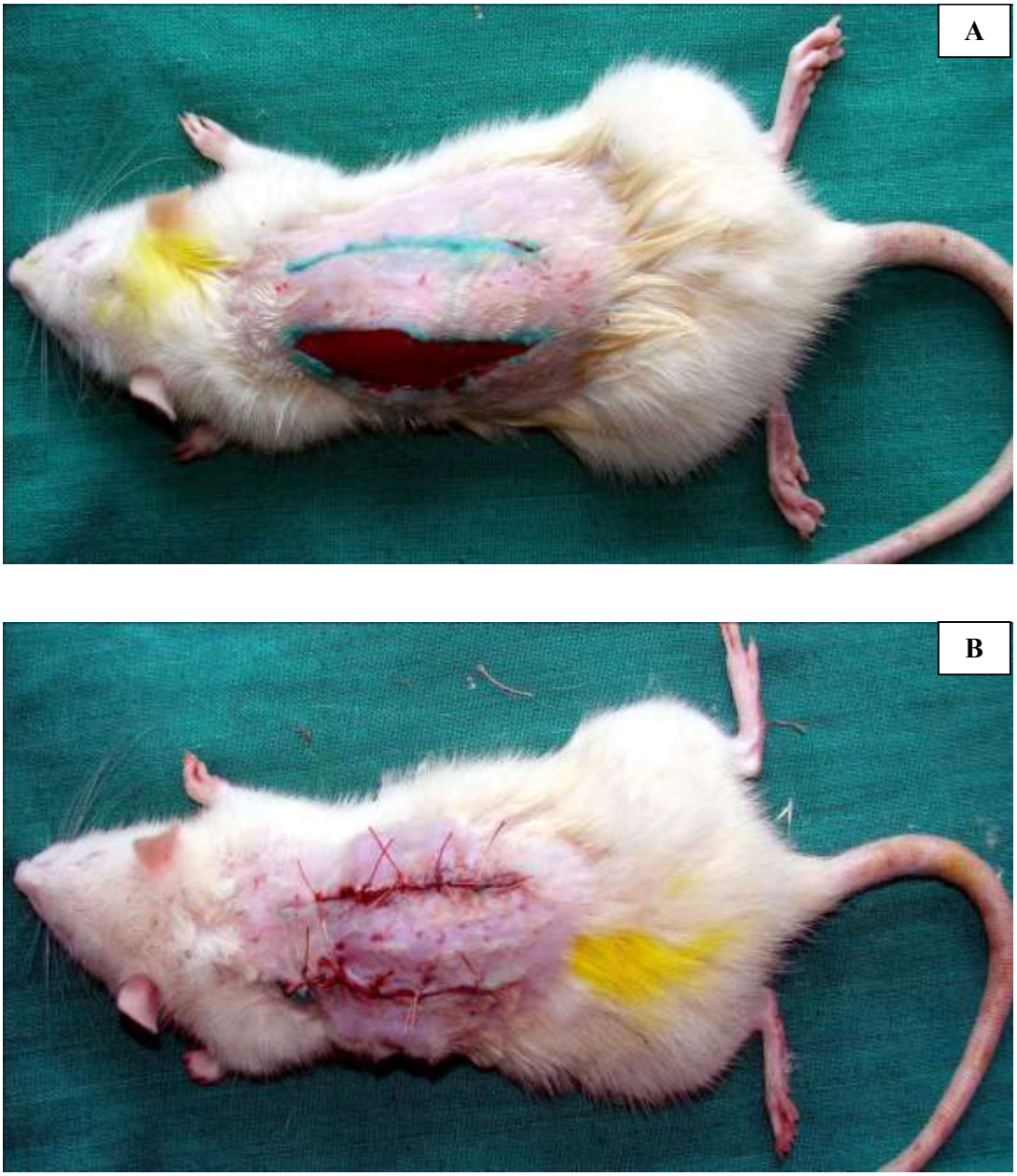


**Figure 5:** (A) Rat showing full thickness paravertebral incisions of the skin on either side of the vertebral column. (B) Incision wound closer by the interrupted suture

**TENSOMETER TEST (WOUND BREAKING STRENGTH)**

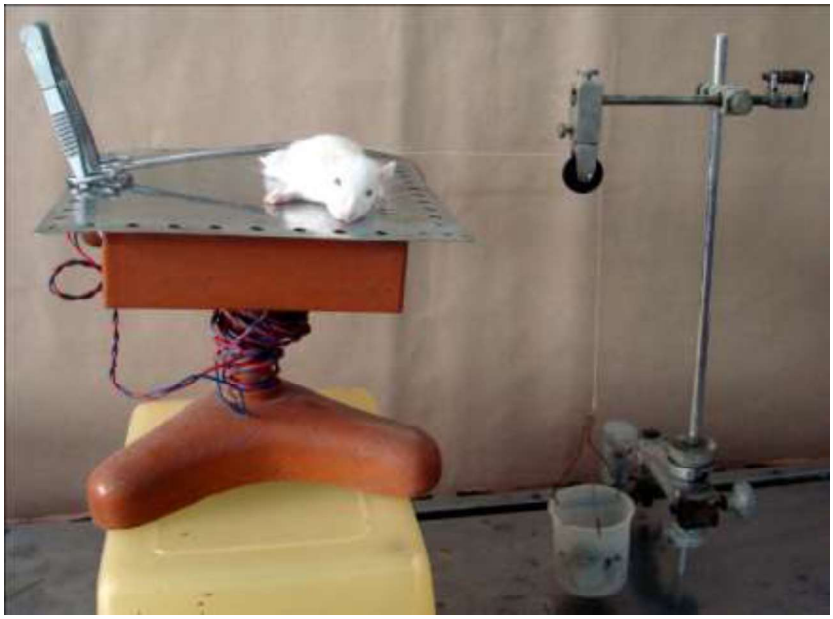

**Figure 6:** Measurement of wound breaking strength on 10$^{th}$ day post incision wound by self-made tensometer

***4.2. Excision wound model***

A total of thirty animals were divided into five groups of six animals each. The control (Group 1) remained untreated whilst Group 2 was administered 500 mg/kg p.o. ethanolic extract of *Leea macrophylla* (equivalent to optimum dose in incision model) and Group 3 animals were treated with bioadhesive gel of ethanolic extract of *Leea macrophylla* at concentration of 5% (dose optimised in incision model), Group 4 received standard oral dose of 200 mg/kg Vitamin E, and Group 5 animals received *Aloe vera* cream as topical standard. The excision wounds were created as per the protocol prescribed by Morton & Malon (Figure 7). The wound was traced on 1 mm$^2$ graph paper on the wounding day and subsequently on passage of each 4 day interval till the 12$^{th}$ day. The tracing process was then accelerated and performed on each alternate day until completion of healing to accurately determine scar area (Morton and Malon, 1972). Following formula was used for calculating change in wound area.

% wound contraction = (HA / TWA) x 100.

Where healed area (HA) is the difference between the total wound area (TWA) and current wound area. Epithelisation period is the number of days required for falling of the scar without any residual raw wound.

**EXCISION WOUND IN RAT**

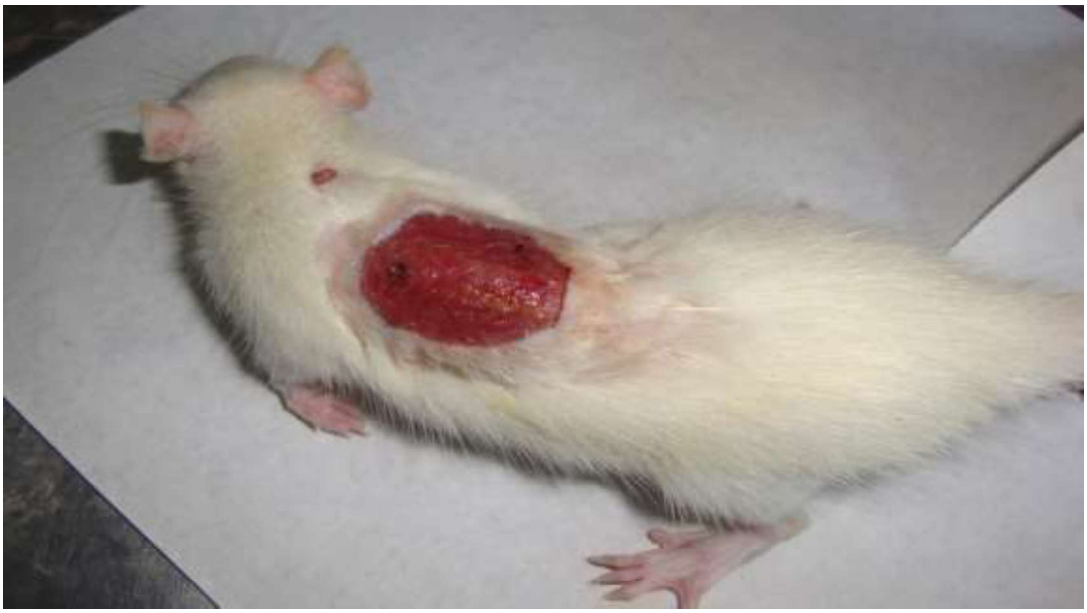

**Figure 7:** Excision wound created in rat

### *4.3. Biochemical estimations*

Excision wound was created in a parallel group of animals and similar treatment was given as mentioned earlier. On $10^{th}$ post-wounding day, the treated animals from the parallel groups were sacrificed and the granulation tissue was removed from the respective wound patches, weighed and were subjected for estimation of free radicals, antioxidants, collagen tissue parameters and inflammatory marker. The granulation tissue was divided into two parts - (a) Wet tissue (≈220 mg) and (b) dried tissue

{remaining wet tissue (>200 mg) dried to have at least 50 mg of dried tissue in weight}. Throughout the tissue preparation, tissue was stored at freezing temperature.

### *4.3.1. Estimation in wet granulation tissue*

Wet tissue (220 mg approx. made into 100 mg/mL homogenate) was used for the estimation of protein, free radicals lipid peroxidise and nitric oxide (LPO, NO), antioxidants i.e. catalase, superoxide dismutase and reduced glutathione. (CAT, GSH, SOD) and acute inflammatory marker myeloperoxidase (MPO). The granulation tissue was homogenised at 4°C in Phosphate buffered saline {PBS: 1.761 gm $KH_2PO_4$ + 3.634 gm $Na_2HPO_4.2H_2O$ in 50 mL DW (pH = 7.0)}.

#### *4.3.1.1. Estimations of Wet Protein*

The protein content of the granulation tissue was estimated using the method of Lowry *et al*., (1951).

**Principle**

Peptide bonds in protein is allowed to react with $Cu^{2+}$ under alkaline conditions to produce $Cu^{+}$, which reacts with phosphomolybdotungstate of Folin and Ciocalteu's phenol reagent to generate hetero polymolybdenum blue by the copper catalysed oxidation of aromatic amino acids i.e., tryptophan and tyrosine to produce dark blue colour, which is measured spectrophotometrically.

**Reagents**

a) Alkaline reagent - 2 g of Sodium carbonate (Analar) added to 100 mL of 0.1N NaOH.

b) 0.1N Sodium hydroxide (Merck) - 400 mg NaOH in 100 mL distilled water (1M NaOH is 40 g in 1000 mL DW).

c) 4 % Sodium potassium tartarate (Merck) - 40 mg SPT in 1 mL distilled water.

d) 2 % Copper sulphate (Analar) - 20 mg $CuSO_4$ in 1 mL distilled water.

e) Alkaline mixture - To 100 mL of alkaline reagent, 1 mL of 4 % aqueous SPT and 1 mL of 2% aqueous $CuSO_4$ were added. This was prepared fresh before use.

f) Phenol reagent - 5 mL folin-ciocalteu's phenol reagent mixed in 5 mL distilled water.

g) Absolute alcohol.

h) Bovine albumin was used as standard for protein estimation, 1 mg/mL stock solution.

**Procedure**

0.9 mL Absolute alcohol was added to 0.1 mL homogenate (equivalent to 10 mg tissue) and was centrifuged at 3000 rpm for 5 minutes. The precipitate so obtained was dissolved in 1 mL of 0.1N NaOH. Out of this 1 mL solution, 0.4 mL was taken into another test tube and to this 4 mL of alkaline reagent was added and kept for 10 minutes. To the obtained solution 0.4 mL of the phenol reagent was added and was allowed to stand again for 10 minutes for colour development. Readings were taken, against the blank prepared with water, at 610 nm. The protein content was calculated from the standard curve prepared with bovine albumin and expressed in terms of mg/g wet tissue.

### *4.3.1.2. Estimation of Free radical parameters*

#### *4.3.1.2.1 Estimation of Lipid peroxidation (LPO)*

Lipid peroxidation in granulation tissue was estimated by method described by Ohkawa *et al*., 1979.

**Principle**

Malondialdehyde (MDA), a secondary product of LPO reacts with thiobarbituric acid in order to form a pink chromogen (Thiobarbituric acid-2 malondialdehyde adduct), which is measured spectrometrically.

**Reagents**

a) 8.1% Sodium dodecyl sulphate (Sigma) - 810 mg SDS dissolved in 10 mL distilled water.

b) 20% Acetic acid (Merck) - 20 mL of Glacial acetic acid added to 80 mL of distilled water (freshly prepared). The pH of the solution was adjusted to 3.5 by adding NaOH.

c) 0.8% Thio-barbituric acid (Sigma) - 800 mg TBA dissolved in 100 mL of distilled water.

d) 15:1 Butanol and Pyridine mixture (Merck) - 15 mL of n-butanol was mixed with 1 mL of Pyridine.

e) 1, 1, 3, 3-tetramethoxypropane (Sigma) was used as standard for LPO estimation.

**Procedure**

LPO level was estimated in terms of malondialdehyde (MDA). To 0.2 mL of 100 mg/mL tissue homogenate 0.1 mL of 8.1 % SDS, 0.75 mL of 20 % acetic acid solution (pH 3.5) and 0.75 mL of 0.8 % aqueous solution of TBA was added in stoppered tubes. The mixture was made up to 2 mL with distilled water, and then heated in an oil bath at 95°C for 60 minutes. After cooling with tap water, 0.5 mL of distilled water and 2.5 mL of mixture of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 3000 rpm for 10 min the organic layer was taken and its absorbance at 532 nm was measured against blank containing 0.2 mL of distilled water in place of sample. 1, 1, 3, 3- tetra-methoxypropane was used as external standard and the level of LPO was expressed as nmol MDA/g wet tissue.

***4.3.1.2.2 Estimation of Nitric oxide (NO)***

As nitrite and nitrate are formed as end products of the reactive nitrogen intermediates, the measurement of nitrite by using the Griess reagent (Green *et al*., 1982; Krol *et al*., 1995) is generally employed as a marker for formation of NO.

**Principle**

In this method, nitrite is first treated with a diazotizing reagent, e.g., sulfanilamide (SA), in acidic media to form a transient diazonium salt. This intermediate is then allowed to react with a coupling reagent, N-naphthyl-ethylenediamine (NED), to form a stable azo compound. The intense purple color of the product allows nitrite assay with high sensitivity and is used to measure nitrite concentration as low as ~0.5 mM level. Vanadium (III) chloride is used as a reductant in the reaction. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample.

**Reagents**

a) Griess reagent (Sigma)

b) 1M HCl - 10 mL of concentrated HCl (12N) added to 110 mL distilled water.

c) Vanadium (III) chloride (Merck) - 400 mg $VCl_3$ in 506 mL of 1M HCl.

d) Sodium Nitrite (Merck) ($NaNO_2$) was used as standard for NO estimation.

**Procedure**

0.4 mL of granulation tissue homogenate (100 mg/mL) is mixed with 0.4 mL of absolute alcohol and then centrifuged at 4°C at 14000 rpm for 1 hr. 0.5mL of supernatant was taken, mixed with 0.5 mL of vanadium (III) chloride and 0.5 mL of freshly prepared Griess reagent, and incubated at 37°C for 30 min. The absorbance was measured at 540 nm spectro-photometrically, against blank prepared by using distilled water. Nitrite content was determined from standard curve prepared by using sodium nitrite and expressed as nmol/g wet tissue (Miranda *et al*., 2001).

***4.3.1.3. Estimation of Anti-oxidants***

***4.3.1.3.1. Estimation of Catalase (CAT)***

CAT activity in the granulation tissue by the method described by Sinha (1972).

**Principle**

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide ($H_2O_2$), resulting in the formation of perchromic acid as an unstable intermediate. The chromic acetate, thus produced is measured spectrophotometrically.

**Reagents**

a) Phosphate buffer (0.01M; pH-7.0)

b) Disodium hydrogen phosphate

c) potassium dihydrogen orthophosphate

d) Potassium dichromate ($K_2Cr_2O_7.7H_2O$-5%)

e) Glacial acetic acid

f) Hydrogen peroxide ($H_2O_2$-0.2M)

**Procedure**

The tissue homogenate was mixed with phosphate buffer (1.0 mL) followed by addition of $H_2O_2$ for initiation of the reaction. The reaction was arrested immediately by addition of 2.0 mL dichromate- acetic acid reagent at 0, 30 and 60 seconds intervals. The reagent blank was prepared by addition of 1.6 mL of buffer and 2.0 mL of dichromate acetic acid reagent taken in separate tubes. The test and blank tubes were then heated in boiling water bath for 10 min to develop green colour. The tubes were cooled to room temperature and their intensity was measured at 570 nm using spectrophotometer against the blank. Results obtained were expressed as μmol $H_2O_2$ consumed/min/mg of protein.

***4.3.1.3.2. Estimation of Reduced Glutathione (GSH)***

Sedlak and Lindsay, (1968) method was followed to estimate GSH in granulation tissue.

**Principle**

DTNB is a disulphide compound which is reduced by sulfhydryl group of reduced glutathione (GSH) and can form yellow colour. The yellow colour is measured by spectrophotometer at 412 nm.

**Reagents**

a) 50 % Trichloro acetic acid - 10 g TCA added to 20 mL distilled water.

b) 0.4M Tris buffer (Sigma) - Prepared by dissolving 4.84 g of Tris in small amount of distilled water and by adding 10 mL of 0.2M EDTA and make up to 100 mL with distilled water. Adjust the pH to 8.9 with 1N HCl.

c) 0.2M Ethylene diamine tetra acetic acid (Merck) - 7.4 g of sodium salt of EDTA is dissolved in distilled water to make it a volume of 100 mL.

d) 0.02M EDTA - Take 10 mL of 0.2M EDTA and make up to 100 mL with distilled water.

e) 0.1M Di-thio bis nitro benzoic acid (Ellman's reagent) (Hi Media) - Dissolve 9.9 mg of DTNB ($C_{14}H_8N_2O_8S_2$) in 25 mL of absolute methanol.

f) Reduced glutathione ($C_{10}H_{17}N_3O_6S$) was used as standard for GSH estimation.

**Procedure**

1 mL of tissue homogenate (100 mg/mL) was mixed in 15 mL test tube with 0.8 mL of distilled water and 0.2 mL of 50 % TCA. The tubes were shaken intermittently for 10-15 min and centrifuged for 15 min at 3000 rpm for 10 min. 0.6 mL of supernatant was mixed with 0.8 mL of 0.4M Tris buffer (pH 8.9) and 20 µL of 0.1M DTNB in absolute methanol, and the sample was shaken. The absorbance was read

within 5 min of the addition of 40 µL DTNB at 412 nm against a reagent blank with no homogenate. The results were expressed as nmol/g wet tissue and were calculated from the standard curve prepared by using standard glutathione.

#### *4.3.1.3.3. Estimation of Superoxide dismutase (SOD)*

Superoxide dismutase in granulation tissue was estimated by method described by Kakkar *et al*., 1984.

**Principle**

The assay of SOD is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction is extracted into butanol and measured at 560nm.

**Reagents**

a) 0.052M Sodium pyrophosphate (Sigma) - 1.16 g of SPP added in 50 mL of distilled water and the pH was adjusted to 8.3.

b) 186 µM Phenazine methosulphate (Sigma) - 18 µL of 100mM of PMS added to 10 mL of distilled water. 100mM of PMS prepared by adding 306 mg in 10 mL of distilled water.

c) 300 µM Nitroblue Tetrazolium (Sigma) - 2.754 mg of NBT dissolved in 10 mL of distilled water.

d) 780 µM Nicotineamide adenine dinucleotide (Sigma) - 11.1 mg of NADH dissolved in 20 mL of distilled water.

e) Glacial Acetic acid.

f) n-Butanol.

**Procedure**

The inhibition of reduction of nitro-blue tetrazolium (NBT) to blue colour formozan in presence of phenazine metha sulphate (PMS) and Nicotineamide adenine dinucleotide (NADH) was measured at 560 nm using n-butanol as blank. To 0.2 mL of tissue homogenate was added 0.6 mL of 0.052 M sodium pyrophosphate buffer (pH 8.3), 50 µL of 186 µM of PMS, 150 µL of 300 µM NBT and 0.4 mL of distilled water to make up the volume up to 1.5 mL including with 0.1 mL of 780 µM NADH. Reaction was started by the addition of NADH. After incubation at 30°C for 60 sec, the reaction was stopped by the addition of 0.5 mL of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 2 mL of n-Butanol. The mixture was allowed to stand for 10 min, centrifuged at 3000 rpm for 10 min and butanol layer was taken out. Colour intensity of the chromogen in the butanol was measured at 560 nm in spectrophotometer against n-Butanol, a system devoid of enzyme served as control. One unit of enzyme activity is defined as enzyme concentration required inhibiting the optical density at 560 nm of chromogen protection by 50% in one min under the assay conditions, and the results have been expressed as units (IU) of SOD activity/g wet tissue.

#### *4.3.1.4. Estimation of inflammatory marker*

##### *4.3.1.4.1. Estimation of Myeloperoxidase (MPO)*

Myeloperoxidase (MPO) was estimated by method described by Bradley *et al*., 1982.

**Principle**

Myeloperoxidase (MPO) extracted from cells by hexadecyltrimethylammonium bromide (HTAB) then reacts with o-dianisidine dihydrochloride and hydrogen peroxide which is measured by spectrophotometer.

**Reagents**

(a) 0.5% Hexadecyltrimethylammonium bromide (HTAB) (Sigma)

(b) Potassium phosphate buffer, pH 6.0

(c) o-dianisidine dihydrochloride (Sigma)

(d) Hydrogen peroxide ($H_2O_2$) (Mallinckrodt)

**Procedure**

MPO was extracted from cells by suspending the tissue in 2.0 mL 0.5% hexadecyl trimethyl ammonium bromide (HTAB) in 50 mM potassium phosphate buffer, pH 6.0, to solubilize MPO.8 For MPO measurement in cell-free medium or plasma, aliquots were mixed with an equal volume of 1.0% HTAB in 100 mM buffer, pH 6.0. Specimens were subjected to sonication in an ice bath for 10 sec. 40 W (Heat Systems Ultrasonics, Plainview, N.Y.), after which they were freeze-thawed 3 times. Sonication was repeated, and the specimens were centrifuged at 40,000 g for 15 mm; the resulting supernate was assayed. MPO was assayed spectrophotometrically: 0. 1 mL of the material to be measured was mixed with 50 mM phosphate buffer, pH 6.0, containing 0. 1 67 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide in a final volume of 3 mL. Then the absorbance change at 460 nm was measured with a Beckman DU spectrophotometer (Beckman Instruments Lab., Fullerton, Calif.) with a

recording attachment (Gilford Instrument Labs., Oberlin, Ohio). A Cary spectrophotometer Model 1 18 (Varian, Palo Alto, Calif.) was employed for turbid suspensions. One unit of activity was defined as that degrading 1 μmole of peroxide/mm at 25° C (Bradley *et al*., 1982).

### *4.3.2. Estimation in dry granulation tissue*

Approximately 250 mg of wet tissue was dried at 50°C for 24 h. Dried tissue was weighed and kept in glass stoppered test tubes. Dried tissue was subsequently used for the estimation of protein and connective tissue parameters like hydroxyproline, hexosamine and hexuronic acid.

**Distribution of tissue -**

(i) Protein - 5 mg dry tissue dissolved in 2.5 mL of 0.1N NaOH and 0.4 mL was taken for protein estimation.

(ii) To each tube containing 40 mg of the dried granulation tissue, 1 mL of 6N HCl was added. The tubes were kept on boiling water bath for 24 hours (12 hours each day for two days or 8 hours each day for 3 days) for hydrolysis. The hydrolysate was then cooled and excess of acid was neutralised by 10N NaOH using phenolphthalein as indicator. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/mL of dried granulation tissue in the final hydrolysate with distilled water. The hydrolysate was used for the estimation of hydroxyproline, hexosamine and hexuronic acid following the standard procedures. Following is the volume taken from the hydrolysate for the estimation of above parameters.

**Estimations**

***4.3.2.1. Estimation of Dry Protein***

The protein content of the granulation tissue was estimated using the method of Lowry *et al*., (1951).

**Principle**

Peptide bonds in protein is allowed to react with $Cu^{2+}$ under alkaline conditions to produce $Cu^{+}$, which reacts with phosphomolybdotungstate of Folin and Ciocalteu's phenol reagent to generate hetero polymolybdenum blue by the copper catalysed oxidation of aromatic amino acids i.e., tryptophan and tyrosine to produce dark blue colour, which is measured spectrophotometrically.

**Reagents**

a) Alkaline reagent - 2 g of Sodium carbonate (Analar) added to 100 mL of 0.1N NaOH.

b) 0.1N Sodium hydroxide (Merck) - 400 mg NaOH in 100 mL distilled water (1M NaOH is 40 g in 1000 mL DW).

c) 4 % Sodium potassium tartarate (Merck) - 40 mg SPT in 1 mL distilled water.

d) 2 % Copper sulphate (Analar) - 20 mg $CuSO_4$ in 1 mL distilled water.

e) Alkaline mixture - To 100 mL of alkaline reagent, 1 mL of 4 % aqueous SPT and 1 mL of 2% aqueous $CuSO_4$ were added. This was prepared fresh before use.

f) Phenol reagent - 5 mL folin-ciocalteu's phenol reagent mixed in 5 mL distilled water.

g) Absolute alcohol.

h) Bovine albumin was used as standard for protein estimation, 1 mg/mL stock solution.

**Procedure**

To 0.4 mL (0.8 mg dry tissue) of hydrolysate was added 4 mL of alkaline reagent and kept for 10 minutes. Then 0.4 mL of the phenol reagent was added and again 10 minutes were allowed for colour development (because its time bound reaction). Readings were taken immediately, against the blank prepared with water, at 610 nm. The protein content was calculated from the standard curve prepared with bovine albumin and expressed in terms of mg/g dry tissue.

***4.3.2.2. Estimation of connective tissue parameters***

***4.3.2.2.1. Estimation of Hydroxyproline (HP)***

The hydroxyproline content of the granulation tissue was estimated as per method described by Newman and Logan, 1950.

**Principle**

Calorimetric method for hydroxyproline detection involving sodium peroxide oxidation and colour formation with p-dimethylaminobenzaldehyde.

**Reagents**

a) 6N Hydrochloric acid - Concentrated HCl (12N) diluted to two times with distilled water.

b) 10N Sodium hydroxide - 40 g of NaOH is dissolved in distilled water to make it total volume of 100 mL.

c) 2.5N Sodium hydroxide - 10 g of NaOH dissolved in distilled water to make volume of 100 mL.

d) 0.01M Copper sulphate - 25 mg of $CuSO_4$ dissolved in 10 mL distilled water.

e) 6% Hydrogen peroxide - 6 mL of 50% $H_2O_2$ added to 44 mL distilled water.

f) 5% Paradimethyl amino-benzaldehyde - 5 g of PDNB dissolved in 100 mL of n-Propanol (freshly prepared).

g) 3N Sulphuric acid - 10 mL of concentrated $H_2SO_4$ (36N) is added slowly to 110 mL of distilled water with constant stirring.

h) Standard Hydroxyproline solution (Hi Media) - Hydroxy-L-proline ($C_5H_9NO_3$), 1 mg/mL stock solution.

**Procedure**

0.3 mL of hydrolysate homogenate (6 mg of dry granulation tissue) was taken in separate stoppered tubes. To the each tube 0.3 mL each of 2.5N NaOH, 0.01M $CuSO_4$ and 6 % $H_2O_2$ was added, shaken vigorously and were immediately placed in water bath at 80°C. After 15 minutes tubes were removed and cooled for 5 minutes in cold water. To 1.2 mL of above sample was added 0.6 mL of freshly prepared 5 % solution of paradimethyl amino-benzaldehyde in n-Propanol and 1.2 mL of 3N $H_2SO_4$. The test tubes were once again placed in a hot water bath at 75°C for 15 minutes and then cooled for 5 minutes under running stream of water. Colour intensity (pink colour) was measured at 540 nm against the blank prepared by using water. Hydroxyproline content in the tissue was estimated as per standard curve prepared using standard L-hydroxyproline. The Hydroxyproline content was expressed as mg/g of dry granulation tissue.

#### *4.3.2.2.2. Estimation of Hexosamine (HA)*

The hexosamine content in the granulation tissue was evaluated using the method of Dische and Borenfreund, 1950.

**Principle**

Hexosamine reacts with acetyl acetone (2,4-pentane diox) in the presence of alkali to form cyclic oxazole or pyrole that is coupled with Ehrlich's reagent to form a stable chromophore which is measured at 530 nm.

**Reagents**

a) 1.5N sodium carbonate (Analar) - 795 mg $Na_2CO_3$ anhydrous dissolved in 10 mL distilled water (1N $Na_2CO_3$ is 53 g in 1000 mL DW; 1M $Na_2CO_3$ is 106 g in 1000 mL DW).

b) Acetyl acetone reagent (Merck) - 0.3 mL Acetyl acetone and 9.7 mL of 1.5N $Na_2CO_3$ anhydrous were mixed just before use.

c) 95 % Ethanol was dehydrated with lime and distilled water before use.

d) Ehrlich's reagent - 1.6 g of Para dimethyl amino benzaldehyde (Merck) was dissolved in 30 mL of 95% ethanol and then to it 30 mL of concentrated HCl (12N) was added. Reagent freshly prepared at 15 days interval and stored in the refrigerator in between.

e) Standard Hexosamine solution (Hi Media) - D (+) Glucosamine (Chitosamine, $C_6H_{14}Cl\ NO_5$) hydrochloride, 1 mg/mL stock solution.

**Procedure**

0.05 mL of hydrolyzed fraction (1 mg of dry granulation tissue) was diluted to 0.5 mL with distilled water. To this was added 0.5 mL of acetyl acetone reagent. The mixture was heated in boiling water bath for 20 minutes then cooled under tap water. To this 1.5 mL of 95% alcohol was added, followed by an addition of 0.5 mL of Ehrlich's reagent. The reaction was allowed for 30 minutes to complete. Colour intensity was measured at 530 nm against the blank prepared by using distilled water. Hexosamine contents of the samples were determined from the standard curve prepared by using D (+) glucosamine hydrochloride. The Hexosamine content was expressed as mg/g of dry granulation tissue.

***4.3.2.2.3. Estimation of Hexuronic acid (HUA)***

The hexuronic acid content of the granulation tissue was estimated using the method of Bitter and Muir, 1953.

**Principle**

Hexuronic acid reacts with carbazole in the presence of borate (in conc. $H_2SO_4$) to develop a pink colour which is read photometrically at 530 nm.

**Reagents**

a) 0.025M of Sodium tetraborate, 10 $H_2O$ (Hi Media) in concentrated $H_2SO_4$ (Sp. gr. 1.84, analytical grade) - 950 mg Borax dissolved in 100 mL of concentrated sulphuric acid (36N).

b) 0.125% Carbazole (Hi Media) in Absolute Ethanol - 125 mg carbazole dissolved in 100 mL Absolute alcohol.

c) Standard Hexuronic acid solution (Hi Media) - D (+) Glucurono-6, 3-lactone ($C_6H_8O_6$), 1 mg/mL (stock solution).

**Procedure**

2.5 mL of 0.025M Borax in concentrated sulphuric acid was placed in stoppered tubes, fixed in a rack and cooled to 4°C. 0.125 mL of sample hydrolysate (2.5 mg of dry granulation tissue) made to volume 0.5 mL by adding distilled water in separate glass stoppered test tubes. Now to this 0.5 mL of hydrolysate was layered carefully on Borax kept in rack at 4°C. The tubes were closed with ground glass stoppers and then rack shaken, first slowly then vigorously, with constant cooling by placing tubes in ice container. At no time should the temperature of the mixture exceed room temperature. The tubes were then heated for 10 min in a vigorously boiling water bath and cooled to room temperature thereafter. 0.1 mL of 0.125 % carbazole reagent in absolute alcohol was then added to each tube then shaken and again heated in the boiling water bath for further 15 min and cooled to room temperature. The optical density (OD) was then read at 530 nm against the blank prepared by using water instead of the hydrolysate. Hexuronic acid contents of the samples were determined from the standard curve prepared by using glucuronolactone. The Hexuronic acid content was expressed as mg/g of dry granulation tissue.

**4.4. Estimation of pro-inflammatory cytokines and growth factor**

The pro-inflammatory cytokines IL-1β, IL-6, TNF-α and growth factor VEGF were assessed by enzyme linked immunosorbent assay (ELISA) using IL-1β, IL-6, TNF-α (Komabiotech) and VEGF (RayBiotech) ELISA kits on granulation tissue homogenate

obtained on 10th post-wounding day from excision wound model. The ELISA protocol was followed as per manufacturer guideline.

### *4.4.1. Estimation of Interleukin - 1 beta (IL-1β)*

**Reagents**

(a) Pre-Coated 96 well ELISA microplate: Antigen-affinity purified Goat anti-Rat IL-1 beta pre-coated 96 well plate.

(b) Detection Antibody (Lyophilized): Biotinylated antigen-affinity purified Goat anti- Rat IL-1 beta.

(c) Standard Protein (Lyophilized): Recombinant Rat IL-1 beta.

(d) Colour Development Enzyme: Streptavidin-HRP conjugate.

(e) Assay Diluent: 1% BSA in PBS.

(f) Colour Development Reagent: TMB solution.

(g) Stop Solution: 2M $H_2SO_4$.

(h) Wash Buffer (20X): Concentrated PBST for 1L.

(i) Plate Sealer.

**Procedure:**

**Reagent Preparations**

All preparations were mixed thoroughly and warmed up at room temperature prior to use.

1. Washing Solution (PBST): Wash Buffer was diluted as 1:20 in sterile water and mixed well.

2. Pre-coated ELISA 96 well plate: The number of wells required as per number of the samples were taken for the assay, while the remaining wells were placed in the resealable pouch with a desiccant. The pouch must be resealed to prevent it from moisture.

3. Standard: The standard was diluted to a proper concentration in Assay Diluent.

**Table 8:** Serial dilutions of standards in Assay Diluent at 1:2 for estimation of Interleukin - 1 beta (IL-1β)

| Step | Dilution Method | Standard conc. |
|---|---|---|
| Step A | 20 µL of Standard +0.98 mL of Assay Diluent | 4000 pg/mL |
| Step B | 0.5 mL of Step A + 0.5 mL of Assay Diluent | 2000 pg/mL |
| Step C | 0.5 mL of Step B + 0.5 mL of Assay Diluent | 1000 pg/mL |
| Step D | 0.5 mL of Step C + 0.5 mL of Assay Diluent | 500 pg/mL |
| Step E | 0.5 mL of Step D + 0.5 mL of Assay Diluent | 250 pg/mL |
| Step F | 0.5 mL of Step E + 0.5 mL of Assay Diluent | 125 pg/mL |
| Step G | 0.5 mL of Step F + 0.5 mL of Assay Diluent | 62.5 pg/mL |

Note: 100 µL of Assay Diluent was transferred to empty well as Standard Blank.

4. Sample dilution: The samples were diluted to a proper concentration in Assay Diluent.

5. Detection Antibody: The reconstituted detection antibody was diluted in Assay Diluent to a concentration of 0.35 µg/mL (1:20 dilution).

6. Colour Development Enzyme: The Streptavidin-HRP conjugate was diluted as 1:20 in Assay Diluent.

**ELISA Protocol**

200 µL of washing Solution was added to the each well. The wells were aspirated to remove liquid and the plate was then washed three times using 300 µL of washing

solution per well. After the last wash, the plate was inverted to allow to drain off the residual solution and blotted on paper towel.100 µL of standard or sample(s) was added to the each well in duplicate and covered with the plate sealer. The plate was allowed to incubate at room temperature for at least 2 hours. The wells were allowed to aspirate to remove liquid and the plate was washed 4 times with washing solution (300 µL/well). 100 µL of the diluted detection antibody (0.35 µg/mL) was added into each well. The plate was covered with plate sealer and allowed to incubate at room temperature for 2 hours. The plate was aspirated and washed 4 times with washing solution (300 µL/well) again. 100 µL of the diluted Colour Development Enzyme (1:20 dilute) was added per well covered with plate sealer and incubated for 30 minutes at room temperature (or 37°C for 30 minutes) followed by aspiration and washing again. Finally 100 µL of colour development solution was added to each well and incubated at room temperature for a proper colour development. (22-32 minutes). The colour reaction was stopped by adding 100 µL of the stop solution to each well and reading were recorded using a microtiter plate reader at 450 nm wavelength. Results were expressed in pg/mL.

### *4.4.2. Estimation of Interleukin - 6 (IL-6)*

***Reagents***

(a) Pre-Coated 96 well ELISA microplate: Antigen-affinity purified Mouse anti-Rat IL-6 pre-coated 96 well plate.

(b) Detection Antibody (Lyophilized): Biotinylated antigen-affinity purified Goat anti- Rat IL-6.

(c) Standard Protein (Lyophilized): Recombinant Rat IL-6.

(d) Colour Development Enzyme: Streptavidin-HRP conjugate.

(e) Assay Diluent 1% BSA in PBS.

(f) Colour Development Reagent: TMB solution.

(g) Stop Solution: 2M $H_2SO_4$.

(h) Wash Buffer (20X): Concentrated PBST for 1L.

(i) Plate Sealer.

**Procedure:**

**Reagent Preparations**

All preparations were mixed thoroughly and warmed up at room temperature prior to use.

1. Washing Solution (PBST): Wash Buffer was diluted as 1:20 in sterile water and mixed well.
2. Pre-coated ELISA 96 well plate: The number of wells required as per number of the samples were taken for the assay, while the remaining wells were placed in the resealable pouch with a desiccant. The pouch must be resealed to prevent it from moisture.
3. Standards: The standard was diluted to a proper concentration in Assay Diluent.
4. Sample dilution: The samples were diluted to a proper concentration in Assay Diluent.
5. Detection Antibody: Dilute the reconstituted detection antibody in Assay Diluent to a concentration of 0.4 ug/mL (1:20 dilution).

6. Colour Development Enzyme: The Streptavidin-HRP conjugate was diluted as 1:20 in Assay Diluent.

**Table 9:** Serial dilutions of standards in Assay Diluent at 1:2 for estimation of Interleukin - 6 (IL-6)

| Step | Dilution Method | Standard conc. |
|---|---|---|
| Step A | 20 µL of Standard + 0.48 mL of Assay Diluent | 8000 pg/mL |
| Step B | 0.25 mL of Step A + 0.25 mL of Assay Diluent | 4000 pg/mL |
| Step C | 0.25 mL of Step B + 0.25 mL of Assay Diluent | 2000 pg/mL |
| Step D | 0.25 mL of Step C + 0.25 mL of Assay Diluent | 1000 pg/mL |
| Step E | 0.25 mL of Step D + 0.25 mL of Assay Diluent | 500 pg/mL |
| Step F | 0.25 mL of Step E + 0.25 mL of Assay Diluent | 250 pg/mL |
| Step G | 0.25 mL of Step F + 0.25 mL of Assay Diluent | 125 pg/mL |

Note: 100 µL of Assay Diluent was transferred to empty well as Standard Blank.

**ELISA Protocol**

200 µL of washing Solution was added to the each well. The wells were aspirated to remove liquid and the plate was then washed three times using 300 µL of Washing Solution per well. After the last wash, the plate was inverted to allow to drain off the residual solution and blotted on paper towel.100 µL of standard or sample(s) was added to the each well in duplicate and covered with the plate sealer. The plate was allowed to incubate at room temperature for at least 2 hours. The wells were allowed to aspirate to remove liquid and the plate was washed 4 times with washing solution (300 µL/well). 100 µL of the diluted detection antibody (0.4 µg/mL) was added into each well. The plate was covered with plate sealer and allowed to incubate at room temperature for 2 hours. The plate was aspirated and washed 4 times with washing solution (300 µL/well) again. 100 µL of the diluted Colour Development Enzyme (1:20 dilute) was added per

well covered with plate sealer and incubated for 30 minutes at room temperature (or 37°C for 30 minutes) followed by aspiration and washing again. Finally 100 µL of colour development solution was added to each well and incubated at room temperature for a proper colour development. (20-30 minutes). The colour reaction was stopped by adding 100 µL of the stop solution to each well and reading were recorded using a microtiter plate reader at 450 nm wavelength. Results were expressed in pg/mL.

### *4.4.3. Estimation of Tumor Necrosis Factor-α (TNF-α)*

**Reagents:**

(a) Pre-Coated 96 well ELISA microplate: Antigen-affinity purified Rabbit anti-Rat TNF- α pre-coated 96 well plate.

(b) Detection Antibody (Lyophilized): Biotinylated antigen-affinity purified Rabbit anti- Rat TNF- α.

(c) Standard Protein (Lyophilized): Recombinant Rat TNF- α.

(d) Colour Development Enzyme: Streptavidin-HRP conjugate.

(e) Assay Diluent: 0.1% BSA in PBS.

(f) Colour Development Reagent: TMB solution.

(g) Stop Solution: 2M $H_2SO_4$.

(h) Wash Buffer (20X): Concentrated PBST for 1L.

(i) Plate Sealer.

**Reagent Preparations**

All preparations were mixed thoroughly and warmed up at room temperature prior to use.

1. Washing Solution (PBST): Wash Buffer was diluted as 1:20 in sterile water and mixed well.

2. Pre-coated ELISA 96 well plate: The number of wells required as per number of the samples were taken for the assay, while the remaining wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to prevent it from moisture.

3. Standards: The standard was diluted to a proper concentration in Assay Diluent.

**Table 10:** Serial dilutions of standards in Assay Diluent at 1:2 for estimation of (TNF-α)

| Step | Dilution Method | Standard conc. |
|---|---|---|
| Step A | 3 µL of Standard + 1 mL of Assay Diluent | 3000 pg/mL |
| Step B | 0.5 mL of Step A + 0.5 mL of Assay Diluent | 1500 pg/mL |
| Step C | 0.5 mL of Step B + 0.5 mL of Assay Diluent | 750 pg/mL |
| Step D | 0.5 mL of Step C + 0.5 mL of Assay Diluent | 375 pg/mL |
| Step E | 0.5 mL of Step D + 0.5 mL of Assay Diluent | 187.5 pg/mL |
| Step F | 0.5 mL of Step E + 0.5 mL of Assay Diluent | 93.75 pg/mL |
| Step G | 0.5 mL of Step F + 0.5 mL of Assay Diluent | 46.87 pg/mL |

Note: 100 µL of Assay Diluent was transferred to empty well as Standard Blank.

4. Sample dilution: The samples were diluted to a proper concentration in Assay Diluent.

5. Detection Antibody: The reconstituted detection antibody was diluted in Assay Diluent to a concentration of 0.5 ug/mL (1:20 dilution).

6. Colour Development Enzyme: The Streptavidin-HRP conjugate was diluted as 1:20 in Assay Diluent.

**ELISA Protocol**

200 µL of washing Solution was added to the each well. The wells were aspirated to remove liquid and the plate was then washed three times using 300 µL of Washing Solution per well. After the last wash, the plate was inverted to allow to drain off the residual solution and blotted on paper towel.100 µL of standard or sample(s) was added to the each well in duplicate and covered with the plate sealer. The plate was allowed to incubate at room temperature for at least 2 hours. The wells were allowed to aspirate to remove liquid and the plate was washed 4 times with washing solution (300 µL/well). 100 µL of the diluted detection antibody (0.5 µg/mL) was added into each well. The plate was covered with plate sealer and allowed to incubate at room temperature for 2 hours. The plate was aspirated and washed 4 times with washing solution (300 µL/well) again. 100 µL of the diluted Colour Development Enzyme (1:20 dilute) was added per well covered with plate sealer and incubated for 30 minutes at room temperature (or 37°C for 30 minutes) followed by aspiration and washing again. Finally 100 µL of colour development solution was added to each well and incubated at room temperature for a proper colour development. (1-11 minutes). The colour reaction was stopped by adding 100 µL of the stop solution to each well and reading were recorded using a microtiter plate reader at 450 nm wavelength. Results were expressed in pg/mL.

### *4.4.4. Estimation of Vascular Endothelial Growth Factor (VEGF)*

(a) Pre-Coated 96 well ELISA microplate: Antigen-affinity purified Mouse anti-Rat VEGF pre-coated 96 well plate.

(b) Detection Antibody (Lyophilized): Biotinylated anti-rat VEGF.

(c) Standard Protein (Lyophilized): Recombinant Rat VEGF.

(d) Colour Development Enzyme: Streptavidin-HRP conjugate.

(e) Assay Diluent: 1% BSA in PBS.

(f) Colour Development Reagent: 3, 3', 5, 5'-tetramethylbenzidine (TMB) in buffered solution.

(g) Stop Solution 0.2M Sulfuric Acid.

(h) Wash Buffer (20X) Concentrated PBST.

(i) Plate Sealer.

**Reagent Preparation**

All preparations were mixed thoroughly and warmed up at room temperature prior to use.

1. Washing Solution (PBST): Wash Buffer was diluted as 1:20 in sterile water and mixed well.
2. Pre-coated ELISA 96 well plate: The number of wells required as per number of the samples were taken for the assay, while the remaining wells were placed in the resealable pouch with a desiccant. The pouch must be resealed to prevent it from moisture.

3. Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water before use.

4. Standards: The standard was diluted to a proper concentration in Assay Diluent.

**Table 11:** Serial dilutions of standards in Assay Diluent at 1:2 for estimation of Vascular Endothelial Growth Factor (VEGF)

| Step | Dilution Method | Standard conc. |
|---|---|---|
| Step A | 4 µL of Standard + 996 µL of Assay Diluent | 200 pg/mL |
| Step B | 200 µL of Step A + 300 µL of Assay Diluent | 80 pg/mL |
| Step C | 200 µL of Step B + 300 µL of Assay Diluent | 32 pg/mL |
| Step D | 200 µL of Step C + 300 µL of Assay Diluent | 12.8 pg/mL |
| Step E | 200 µL of Step D + 300 µL of Assay Diluent | 5.12 pg/mL |
| Step F | 200 µL of Step E + 300 µL of Assay Diluent | 2.05 pg/mL |
| Step G | 200 µL of Step F + 300 µL of Assay Diluent | 0.82 pg/mL |

5. Sample dilution: The samples were diluted to a proper concentration in Assay Diluent.

6. Detection Antibody: The reconstituted detection antibody was diluted in Assay Diluent to a concentration of 0.1 µg/mL (1:20 dilution).

7. Colour Development Enzyme: The Streptavidin-HRP conjugate was diluted as 1:20 in Assay Diluent.

**ELISA Protocol**

200 µL of washing Solution was added to the each well. The wells were aspirated to remove liquid and the plate was then washed three times using 300 µL of Washing Solution per well. After the last wash, the plate was inverted to allow to drain off the residual solution and blotted on paper towel.100 µL of standard or sample(s) was added

to the each well in duplicate and covered with the plate sealer. The plate was allowed to incubate at room temperature for at least 2.5 hours. The wells were allowed to aspirate to remove liquid and the plate was washed 4 times with washing solution (300 ul/well). 100 µL of the diluted detection antibody (0.1 µg/mL) was added into each well. The plate was covered with plate sealer and allowed to incubate at room temperature for 1 hours. The plate was aspirated and washed 4 times with washing solution (300 µL/well) again. 100 µL of the diluted Colour Development Enzyme (1:20 dilute) was added per well covered with plate sealer and incubated for 45 minutes at room temperature followed by aspiration and washing again. Finally 100 µL of colour development solution was added to each well and incubated at room temperature for a proper colour development. (30 minutes). The colour reaction was stopped by adding 50 µL of the stop solution to each well and reading were recorded using a microtiter plate reader at 450 nm wavelength. Results were expressed in pg/mL.

### *4.5 Western Blot analysis for proliferation marker Ki-67*

#### *Procedure for Western Blot Analysis*

Effect of *Leea macrophylla* on cellular proliferation was evaluated using western blot analysis of proliferation marker Ki67. The rats were divided into three groups including control, ELMO and ELMT treated groups with 9 animals of either sex in each. An excision wound was created as described earlier and skin wound specimens were collected on 1$^{st}$, 7$^{th}$ and 14$^{th}$ day post- wounding. About 100 mg of excised wound tissue was subjected to homogenization in 1 mL RIPA buffer followed by centrifugation at 14000xg for 15 minutes discarding the supernatant. Separation of protein from aliquots was processed on sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane followed by incubating the membrane with primary antibody against ki67 (Sigma, St. Louis, MO) . Anti-rabbit antibodies were used as secondary antibody. Visualization of protein was performed by enhanced chemiluminescence employing luminescent image analyser (Xiao et al., 2015)

### *4.6 Histological studies*

The animals from excision wound model were anesthetized on $10^{th}$ post-operative day of the experiment and cross-sectional full thickness of the wound tissue were collected followed by immediate blotting, drying and fixing in 10% formalin. The treated tissues were parched in dehydrant acetone and embedded in paraffin wax blocks only to be cut in very thin sections (5 μm) using a microtome cutter. Haematoxylin-eosin stained sections were then examined with the help of a microscope (Nikon Trinocular Microscope, Model E-200, Japan) (Piskin et al., 2012)

## 5. Statistical analysis

The results of experiment are indicated as mean ± S.E.M., with six animals in each group followed by one-way analysis of variance (ANOVA). Newman–Keuls multiple comparison test was selected to compare different groups and to evaluate the statistical significance between them. Also, two-way ANOVA followed by Bonferroni post hoc test was used to find the significant level in excision model and cellular proliferation study. The statistical analysis was performed using GraphPad Prism (version 4; San Diego, CA, USA) software. $P < 0.05$ was deemed as significant.