Chapter 4
Materials and Methods
4. MATERIALS AND METHODS

4.1 Pharmacognostical analysis

4.1.1 Identification and collection of the raw material

Fresh roots of *Inula cappa* were collected from Nepal in the month of October – November after the flowering season. The authenticity was established by comparing its morphological characters with the available literature and was also authenticated by the taxonomists of the department of Botany, South Gujarat University, Surat. The roots were shade dried and powdered (40 #) and was used for the present work.

4.1.2 Macroscopic Observations

The drug was subjected to macroscopic studies which comprised of study of organoleptic characters of the drugs viz., color, odor, appearance, taste, smell, texture, fracture, etc.

4.1.3 Microscopic Studies

Free hand transverse sections of the roots of *Inula cappa* were taken. They were studied to locate the presence of starch (Iodine solution), cell content (cleared with chloralhydrate) and lignified elements (stained with phloroglucinol and HCl). Powder was also studied separately.

4.1.4 Evaluation of Physical Parameters (Anonymous (c) 1996; Anonymous (d) 1999)

4.1.4.1 Determination of foreign matter

100-500 g of the *Inula cappa* sample to be examined was weighed accurately, and spread out in a thin layer. Foreign matter was detected by inspection with the unaided eye or by the use of lens (6 x). The foreign matter was separated and weighed and percentage foreign matter was calculated.
4.1.5 Determination of Quality Parameters (Anonymous (c), 1996; Anonymous (d), 1999)

4.1.5.1 Determination of the ash value

**Total Ash**

2.0 g of the accurately weighed ground drug was incinerated in a Gooch Crucible at a temperature not exceeding 450°C until free from carbon. It was cooled and weighed. The percentage of the total ash was calculated with reference to the air dried drug.

**Acid insoluble Ash**

The ash obtained above was boiled with 25 ml of 2M HCl for 5 min. The insoluble matter was collected on an ashless filter paper and was washed with hot water and ignited to constant weight. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

4.1.5.2 Determination of the extractive value

**Alcohol soluble extractive value**

5.0 g coarsely powdered air dried drug was macerated with 100 ml of ethanol of the 95% strength in a closed flask for 24 hour, shaking frequently during 6 hour and then allowing to stand for 18 hour. It was filtered rapidly taking precautions against the loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish and dried at 105°C to constant weight. The percentage of the alcohol soluble extractive value was calculated with reference to the air dried drug.

**Water soluble extractive value**

5.0 g coarsely powdered air dried drug was macerated with 100 ml of chloroform water (0.25 % chloroform in water) in a closed flask for 24 hour, shaking frequently
during 6 hour and then allowing to stand for 18 hour. The extract was filtered and 25 ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish and dried at 105°C to constant weight. The percentage of the water soluble extractive value was calculated with reference to the air dried drug.

n-hexane soluble extractive value

5.0 g coarsely powdered air dried drug was macerated with 100 ml of n-hexane in a closed flask for 24 hour, shaking frequently during 6 hour and then allowing to stand for 18 hour. It was filtered rapidly taking precautions against the loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish and dried at 100°C to constant weight. The percentage of the n-hexane soluble extractive value was calculated with reference to the air dried drug.

4.2 Phytochemical analysis

4.2.1. Extraction and Fractionation

Plant Material (1 kg dried powdered root)

\[ \text{Extraction with methanol (4 x 2.5 litres)} \]

\[ \text{Marc extracted with water (4 x 1 litre)} \]

\[ \text{Methanol soluble portion (Extract A)} \]

\[ \text{Methanol Insoluble portion (Extract A1)} \]

Evaporate the solvent till dryness

\[ \text{Suspend the dried extract in water and extract with ethyl acetate (3 x 100 ml)} \]

\[ \text{Ethyl acetate soluble Portion (Extract A2)} \]

\[ \text{Ethyl acetate insoluble Portion (Extract A3)} \]

Fig. 9 Scheme for the preparation of different extracts
One kilogram of shade-dried herb of *Inula cappa* was powdered and extracted three times with 2.5 litre of methanol for 8 hour each. The methanolic extract was concentrated under reduced pressure (Extract A). The marc was further extracted with water to get the methanol insoluble portion (Extract A1). Methanolic extract was further fractionated with ethyl acetate (Extract A2, ethyl acetate soluble portion), the extract remained after the fractionation with ethyl acetate was the residual extract (Extract A3, ethyl acetate insoluble portion). The extracts were concentrated under reduced pressure and air dried to remove the solvent completely (Fig. 9).

### 4.2.2. Preliminary Phytochemical Screening

The methanolic extract prepared above, was subjected to preliminary phytochemical analysis for the detection of the individual components using specific reagents.

#### 4.2.2.1 Test for Alkaloids (Sim, 1969)

5.0 ml of the filtrate was evaporated to dryness. The residue was dissolved in 15 ml H$_2$SO$_4$ (2N) and filtered. After making alkaline with sodium carbonate solution, the filtrate was extracted with chloroform. The residue left after evaporation, was treated with Dragendorff’s reagent. (Orange color precipitates indicates the presence of alkaloids)

#### 4.2.2.2 Test for Sterols (Geissman, 1955)

**Lieberman Burchadt test**

1.0 g powder drug was moistened with 1.0 ml of acetic anhydride on a clean tile and two drops of sulphuric acid was added. The powder was mixed properly and the color gained by the powder was observed. (Formation of green- blue-purple-red color indicates presence of sterols)

#### 4.2.2.3 Test for Flavonoids (Geissman, 1955)

**Shinoda test**
To the alcoholic extract, a small piece of Magnesium ribbon and 3-4 drops of concentrated HCl was added. (Formation of red color indicates the presence of flavonoids).

**Fluorescence test**

1.0 g of the powder was extracted with 15 ml of methanol for 2 min on a boiling water bath, filtered while hot and evaporated to dryness. To the residue, 0.3 ml boric acid solution (3 % w/v) and 1.0 ml oxalic acid solution (10 % w/v) was added. The mixture was evaporated to dryness and the residue was dissolved in 10 ml of ether. (Greenish fluorescence of the ethereal layer under U.V. indicates presence of flavonoids)

4.2.2.4 **Test for Saponins** *(Griffin et al., 1968)*

**Froth test**

0.1 g of the powder was vigorously shaken with 5 ml of distilled water in a test tube for 30 seconds and was left undisturbed for 20 min. (Persistent froth indicates presence of saponins)

**Hemolytic Zone test**

0.5 ml of blood was mixed with gelatin solution (3.0g of gelatin powder dissolved in 100 ml of 0.85 % NaCl solution at 60°C) and taken on a glass slide. A thick section of the drug was placed on it. (A clear hemolytic zone formed around the section indicates the presence of saponins in the drug)

4.2.2.5 **Test for Coumarins** *(Fritz, 1956)*

1.0 g of the powder was extracted with 10 ml of ether. A drop of the ether solution is treated in a porcelain dish with a drop of saturated alcoholic hydroxylamine hydrochloride solution and a drop of saturated alcoholic potassium hydroxide. The mixture was heated over water bath until the reaction began as indicated by a slight
bubbling. After cooling, the mixture was acidified with 0.5N HCl and a drop of 1.0% w/v of FeCl₃ solution was added. (Appearance of violet color indicates the presence of coumarins)

4.2.2.6 Test for Tannins (Robinson, 1964; Clerk et al., 1947; Shah and Quadry, 1995)

Aqueous extract of different parts of the plant was prepared by refluxing 10.0 g of the powder of different parts with 50 ml of water for about 1 hour and was used for the following tests:

Reaction with Lead Acetate

Tannins were precipitated from the aqueous extract by adding 2.0 ml of 10% w/v solution of Lead Acetate. (Precipitates that are partially soluble in 1.0 ml of 10% acetic acid indicates presence of condensed tannins)

Reaction with Ferric chloride

2.0 ml of aqueous extract was treated with 0.5 ml of 5.0% w/v FeCl₃. (A green color formed indicates presence of tannins)

Reaction with Bromine water

To 2.0 ml aqueous extract. 0.5 ml of freshly prepared Bromine water was added. (Precipitates indicate presence of condensed tannins)

Reaction with FeCl₃ and Sodium Bicarbonate

To 2.0 ml of the aqueous extract. 2.0 ml of the 10% w/v FeCl₃ solution and 2.0 ml of sodium bicarbonate was added. (Formation of Red violet color indicates presence of condensed tannins)

Reaction with Vanillin- HCl

\[ \text{Vanillin- HCl} \]
2.0 ml of aqueous extract upon treatment with 1.0 % w/v vanillin in alcohol followed by HCl (Pinkish red color indicates presence of condensed tannins)

**Matchstick Test**

A wood portion of matchstick was dipped into the aqueous extract, dried and moistened with HCl. The matchstick was warmed by bringing near to the flame. (Pink stain developed on wood indicates presence of condensed tannins)

**4.2.3. Estimation of total phenolics**

The total phenolic content of the extract was estimated according to the method described by Singleton and Rossi (1965). Briefly the method is as follows: A stock solution (1.0 mg ml\(^{-1}\)) of the methanolic extract of *Inula cappa* was prepared in methanol. From the stock solution, suitable quantity of the extract was taken into 25 ml volumetric flask and 10 ml of water and 1.5 ml of Folin Ciocalteu’s reagent were added to it. The mixture was kept for 5 min, and then 4.0 ml of 20% w/v sodium carbonate solution was added and made up to 25 ml with double distilled water. The mixture was kept for 30 min and the absorbance was recorded at 765 nm in a Shimadzu 2450 spectrophotometer. Percentage of total phenolics was calculated from calibration curve of gallic acid (50 µg – 250 µg) plotted by using the above procedure and total phenolics were expressed as % gallic acid.

\[
C = A \times 282.6 - 8.451 \quad (A= \text{absorbance})
\]

**4.2.4 Estimation of total tannins (William, 1960)**

2.0 g of powdered roots of *Inula cappa* was defatted with petroleum ether (25 ml x 4) and filtered. The residue was boiled for 2 hour with 300 ml of double distilled water, cooled, diluted up to 500 ml and filtered. To 25 ml of this infusion, 20 ml indigo solution and 750 ml double distilled water were added. It was titrated with 0.1N KMnO\(_4\) solution until blue solution changes to golden yellow in color. Similarly, a blank titration was carried out by replacing the extract with distilled water.
Each ml of 0.1 N KMnO₄ ≈ 0.004157 g of total Tannins

4.2.5 Estimation of total flavonoids (Woisky and Salatino, 1998)

1.0 g of powdered roots of *Inula cappa* was extracted with 25 ml of 95% ethanol under 200 rpm shaking for 24 hour. After filtration, the filtrate was adjusted to 25 ml with 80% ethanol. 0.5 ml of ethanol extract was mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm using Shimadzu 2450 spectrophotometer. The amount of 10% w/v of aluminium chloride was substituted by the same amount of distilled water in blank.

4.2.6 Qualitative analysis of Volatile Oil

4.2.6.1 Plant material and isolation procedure

The roots of the *Inula cappa* were air-dried at ambient temperature in the shade. Volatile oil was isolated from 100 g of the dried roots by hydro distillation for 6 hour using a Clevenger-type apparatus. The yield of oil was calculated with reference to the air dried drug and the color of the oil was noted. The oil was dried over anhydrous sodium sulfate and stored at 2-4°C.

4.2.6.2 Identification of the components of oil

The components of the volatile oil were separated with the capillary chromatographic column and the amount of the components from the essential oil was determined by normalization method. Temperature programming was performed from 60-240°C at the rate of 3°C/min, with injector and detector temperature of 240°C and 260°C, respectively. GC/MS was performed on a crossed linked 5% methylphenylsiloxane (HP-5, 30 m x 0.25 mm, 0.25 μm film thickness) with Helium as the carrier gas and split ratio 1:20 and quadruple mass spectrometer (Helwett- Packard 6890) operating at 70 eV ionization energy. Mass spectra were obtained in scan mode in m/e range 0-700.
Retention indices were determined by using retention times of n-alkanes injected after the oil under the same chromatographic conditions. The compounds were identified by comparison of retention indices with those reported in the literature and by comparison of their mass spectra with the published mass spectra.

4.3 Pharmacological evaluation

4.3.1 Study of Free radical scavenging activity of Inula cappa

4.3.1.1 Assay for antiradical activity

Antiradical activity of the different extracts of Inula cappa was determined by measuring the change in absorbance at 516 nm of a methanolic solution of colored \(\alpha,\alpha\)-diphenyl-\(\beta\)-picrylhydrazyl (DPPH) brought about by the extracts (Ravishankara et al., 2002).

**Preparation of DPPH solution**

Accurately weighed 4.3 mg of DPPH was dissolved in 3.3 ml of methanol. Aliquot of 70-75 \(\mu\)l of the above solution was diluted to 3.0 ml with methanol and its absorbance at 516 nm was adjusted to 0.9.

**Preparation of the sample solution**

25 mg of the extracts A, A1, A2 and A3 were dissolved in 25 ml of methanol in a volumetric flask to give a final concentration of 1.0 mg ml\(^{-1}\). Further diluted solutions were prepared using methanol to give a concentration range of 20-300 \(\mu\)g ml\(^{-1}\) and 10-100 \(\mu\)g ml\(^{-1}\) for extracts A and A2 respectively and 100-1000 \(\mu\)g ml\(^{-1}\) for Extracts A1 and A3 respectively from the stock solutions.

**DPPH Assay**

The mixture of 75 \(\mu\)l of the DPPH solution and 25 \(\mu\)l of the different concentrations of extracts A, A1, A2 and A3 was taken in a test tube and the final volume was made...
to 3 ml and incubated at room temperature for 15 minutes and the absorbance was measured at 516 nm using Shimadzu 2450 spectrophotometer. Decrease in the absorbance in the presence of extracts A, A1, A2 and A3 of *Inula cappa* root powder at different concentrations was noted after 15 min. EC$_{50}$ was determined from the graph plotted between % inhibition and concentration. Pyrogallol was used as positive control.

4.3.1.2 Assay for superoxide radical scavenging activity

The assay was based on the capacity of the extracts of *Inula cappa* root to inhibit formazon formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system (*Beuchamp and Fridovich, 1971*).

Preparation of the reagents

- Phosphate buffer (50 mM, pH 7.6): Accurately weighed 2.32 g of Na$_2$HPO$_4$ and 303 mg of NaH$_2$PO$_4$ was added to a 100 ml volumetric flask and the final volume was made up with water and stored till further use.

- EDTA (12 mM) solution: 1.338 g of accurately weighed EDTA was dissolved in 20 ml of water in a 50 ml volumetric flask and the final volume made up to 50 ml with water.

- Riboflavin solution: 2.0 mg accurately weighed Riboflavin was dissolved in 50 ml of water in a volumetric flask. The solution is prepared fresh before the experiment.

- Nitro Blue Tetrazolium (NBT) solution: 1mg ml$^{-1}$ solution of NBT was prepared fresh by dissolving 10 mg in 10 ml of methanol.

Preparation of sample solutions
Extracts A and A2 were dissolved in methanol to give a final concentration of 1.0 mg ml\(^{-1}\) by dissolving 25 mg of the extracts in 25 ml of methanol in 25 ml volumetric flask. Further diluted solutions were prepared using methanol to give a concentration range of 10-1000 µg ml\(^{-1}\) and 10-300 µg ml\(^{-1}\) for extracts A and A2 respectively from the stock solutions.

**Assay**

The reaction mixture contained 1.0 ml of 50 mM phosphate buffer pH 7.6, 500 µl riboflavin solution, 500 µl of 12 mM EDTA solution, 100 µl NBT solution, 100 µl of different concentrations of extracts and water to make up the volume to 3 ml, added in that sequence. Reaction was started by illuminating the reaction mixture with white light for 90 seconds. Immediately after illumination, the optical density (OD) was measured at 590 nm using Shimadzu 2450 spectrophotometer. Ascorbic acid was used as positive control.

\[
\% \text{ inhibition of superoxide radical} = \frac{\text{OD (control)} - \text{OD (extract)} \times 100}{\text{OD (control)}}
\]

4.3.1.3 *Measurement of effect on lipid peroxidation*

Extent of lipid peroxidation was measured by estimation of malondialdehyde (MDA) formation (Ravishankara *et al.*, 2002). The change in the absorbance is directly proportional to malondialdehyde formation.

**Preparation of reagents**

- Rat liver homogenate (10 % w/v) was prepared according to the procedure described by Tripathi *et al.* (1996) in phosphate buffered saline.
- Phosphate buffered saline (pH 7.4): 2.38 g Na\(_2\)HPO\(_4\), 0.19 g KH\(_2\)PO\(_4\), 8.0 g of sodium chloride was dissolved in 1000 ml of water in a volumetric flask and pH was adjusted to 7.4.
• Sodium dodecyl sulphate (SDS, 8.0 % w/v) solution: 8.0 g of sodium dodecyl sulphate was dissolved in 100 ml water to make 8.0 % w/v solution in a 100 ml volumetric flask.
• Thiobarbituric acid (1.0% w/v) solution: 100 mg of thiobarbituric acid was dissolved in 10 ml hot water to give a solution of 1.0 % w/v.
• Acetic acid (20% v/v) solution: 20 % v/v solution of acetic acid was prepared by diluting 10 ml acetic acid with 40 ml water.
• Trichloroacetic acid (10% w/v) solution: weigh 2.0 g of trichloroacetic acid and dissolve in 20 ml of water in a 20 ml volumetric flask to give a 10 % w/v solution.
• Ferric chloride (100 µM) solution: 8.1 mg of ferric chloride was dissolved in 500 ml of water in a 500 ml volumetric flask to prepare 100 µM ferric chloride solution.
• Adenosine diphosphate (1.7 mM) solution (ADP): accurately weighed 7.26 mg of ADP was dissolved in 10 ml of water in a volumetric flask to prepare a 1.7 mM ADP solution.
• Ascorbic acid (500 µM) solution: accurately weighed 8.806 mg of ascorbic acid was dissolved in 100 ml of water in a volumetric flask to prepare a 500 µM ascorbic acid solution.

Preparation of sample solution

25 mg of extracts A and A2 were dissolved in 25 ml methanol to give a final concentration of 1.0 mg ml$^{-1}$. Further diluted solutions were prepared using methanol to give a concentration range of 5 -100 µg ml$^{-1}$ and 5 - 200 µg ml$^{-1}$ for extracts A and A2 respectively from the stock solution.

Assay

Peroxidation was induced in rat liver homogenate by Iron- ADP complex in the presence of ascorbic acid. The reaction mixture constituted of 0.5 ml of liver homogenate (10% w/v), 0.3 ml of 100 µM FeCl$_3$, 0.3 ml of 1.7 µM ADP, 0.3 ml of 500 µM of ascorbic acid, 100 µl of different concentrations of extracts and the final
volume was made up to 2 ml with phosphate buffered saline. The mixture was incubated for 20 min at 37°C. After 20 min, 0.2 ml of SDS, 1.5 ml of thiobarbituric acid solution and 1.5 ml of acetic acid solution was added and again incubated for 30 min at 95°C and cooled at room temperature. 2.0 ml of the above mixture was treated with 1.0 ml of trichloroacetic acid solution and centrifuged at 1000 g for 10 min. The absorbance of the supernatant was measured at 532 nm spectrophotometrically. α-tocopherol acetate was used as positive control.

4.3.1.4 Measurement of reducing power (Oyaizu, 1986)

It is believed that antioxidant activity and reducing power are related (Duh, 1998; Duh et al, 1999; Tanaka et al., 1988). Reductones inhibit LPO by donating a hydrogen atom and thereby terminating the free radical chain reaction (Yen and Chen, 1995).

Preparation of the reagents

- Phosphate buffer pH 6.6: Phosphate buffer was prepared by mixing 100 ml 0.1 M KH₂PO₄ and 32.8 ml of 0.1 M NaOH.
- Potassium ferricyanide solution (1% w/v): Dissolve accurately weighed 1.0 g of potassium ferricyanide in 25 ml of water in a 100 ml volumetric flask and make up the volume with water to 100 ml.
- Trichloroacetic acid (10% w/v) solution: weigh 10 g of trichloroacetic acid and dissolve in 100 ml of water in a 100 ml volumetric flask to give a 10% w/v solution.
- Ferric chloride (FeCl₃, 0.1% w/v) solution: accurately weigh 100 mg of FeCl₃ and transfer in a 100 ml volumetric flask. Dissolve in 25 ml of water and make up the volume to 100 ml using water.

Preparation of sample solution

25 mg of the extracts A and A2 were dissolved in 25 ml of methanol to give a final concentration of 1.0 mg ml⁻¹. Further diluted solutions were prepared using methanol
to give a concentration range of 20-1000 µg ml\(^{-1}\) and 20-400 µg ml\(^{-1}\) for Extracts A and A2 respectively from the stock solution.

**Assay**

The reducing capability of the sample extracts was measured by determining the change in absorbance due to the transformation of Fe\(^{3+}\) to Fe\(^{2+}\) in the presence of the extracts at 700 nm. Different concentrations of extracts in 1.0 ml of water were mixed with 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% w/v potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (2.5 ml, 10% w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The supernatant was decanted and 2.5 ml of supernatant was mixed with 2.5 ml distilled water and 0.5 ml of FeCl\(_3\) solution and the absorbance was measured at 700 nm. The change in the absorbance of the reaction mixture is directly proportional to reducing power capacity. Gallic acid and tannic acid were used as positive controls.

**4.3.2 Anti-inflammatory activity of Inula cappa**

**4.3.2.1 Animals**

Wistar rats (200–250 g) and albino mice (18-20 g) of either sex, obtained from Zydus research centre, Ahmedabad, Gujarat, India were used in this investigation. Animals were maintained under standard environmental conditions of 12 hour light and dark cycle at 22 ± 2°C and had free access to feed and water *ad libitum*. Experiments on animals were performed based on animal ethics guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals). Eight hours before the animal experiment only tap water was available to the animals. The animals were divided into three main groups and each group was consisting of 6 animals: Control group, Standard group and Treated group. The treated group was further divided into four groups:

- **Group I**: treated with 300 mg kg\(^{-1}\) Extract A (Referred as Extract A)
- **Group II**: treated with 300 mg kg\(^{-1}\) Extract A1 (Referred as Extract A1)
- **Group III**: treated with 300 mg kg\(^{-1}\) Extract A2 (Referred as Extract A2)
Group IV: treated with 300 mg kg\(^{-1}\) Extract A3 (Referred as Extract A3)

Extract A group was further tested at lower doses and hence was further divided into three groups:

Group A: treated with 75 mg kg\(^{-1}\) Extract A (Referred as 75)
Group B: treated with 150 mg kg\(^{-1}\) Extract A (Referred as 150)
Group C: treated with 300 mg kg\(^{-1}\) Extract A (Referred as 300)

Treatment regime varied according to each experiment protocol. The studies for different groups were carried out at different time and each time separate control groups were used. During the study, standard food and water were provided *ad libitum*. Changes in body weight, food intake and water intake were recorded.

4.3.2.2 *Carrageenan* -induced rat paw oedema (*Yesilada and Kupeli, 2002*)

The rats were divided into six groups, each group consisting of six animals. The rat paw oedema was induced by subplantar injection of 100 μl of 0.1 % w/v of freshly prepared carrageenan suspension in sterile saline into the right hind paw of each rat. Animals were orally treated with extracts A, A1, A2 and A3 at a dose of 300 mg kg\(^{-1}\) 1 hour before the injection of carrageenan. The control group of rats received normal saline orally at a dose of 5 ml kg\(^{-1}\) and the standard group received indomethacin (10 mg kg\(^{-1}\), orally) respectively. The oedema was measured at 1 hour interval up to 6 hours after the injection of carrageenan. Extract A was further tested at lower doses of 75 and 150 mg kg\(^{-1}\) orally in oedema induced rats. The percentage inhibition in the oedema was calculated according to the formula given below:

\[
\text{% inhibition} = \frac{(C_i - C_0) \text{ control} - (C_i - C_0) \text{ treated}}{(C_i - C_0) \text{ control}} \times 100
\]

4.3.2.3 *Histamine* induced paw oedema (*Ghosh and Singh, 1974*)

The mice paw oedema was induced by subcutaneous injection of 50 μl of 1% w/v of freshly prepared solutions of histamine (20 μg in 0.05 ml) into the hind paws of the mice after 30 min of oral administration of extract A, A1, A2 and A3 at a dose of 300
mg kg$^{-1}$. The volume of injected paws and contra-lateral paws were measured at 5 minutes interval up to 30 minutes using a pair of Vernier callipers. The remaining groups of animals received normal saline (control, 5 ml kg$^{-1}$) and chlorpheniramine (standard, 5 mg kg$^{-1}$ orally) respectively for assessing comparative pharmacological significance.

4.3.2.4 Cotton pellet-induced granuloma in rats (Winter and Porter, 1957)

Sliced cotton rolls (Brisk Ltd, India) were boiled in culture-grade water for 5 min and dried till constant weight at 80°C (Willoughby and Ryan, 1970; Winter et al., 1963). For implantation, the pellets weighing 20 ± 2 mg were autoclaved at 2 bar pressure for 50 minutes prior to use. A small incision made in the dorsal region, near axila in ether-anaesthetized rats, the cotton pellet was implanted subcutaneously and the wound was then closed by suturing. Extracts A and A2 (300 mg kg$^{-1}$ per day), vehicle (control, 5 ml kg$^{-1}$ per day) and Indomethacin (standard, 5 mg kg$^{-1}$ per day) were given orally for 7 days. The animals were killed by cervical dislocation, the granuloma dissected out and weighed (wet weight) on 8th day. The granulomas were dried at 80°C till constant weight (dry weight). The mean weights for different groups were determined and compared to the control group.

4.3.2.5 Evans blue-carrageenan-induced pleural effusion model

Pleurisy was induced by intra-thoracic injection of carrageenan (0.05 % w/v) in a final volume of 0.1 ml under light ether anesthesia. Injection was given to mice between the third and fifth ribs on the right side of the chest through a 26 gauge needle, with penetration restricted to 6 mm by inserting it through a rubber disc (Vinegar et al., 1973; Sancilio and Rodriguez, 1966). Animals were administered with Indomethacin (10 mg kg$^{-1}$, standard), Extract A (300 mg kg$^{-1}$, suspension in normal saline) and normal saline (5 ml kg$^{-1}$, control) orally 1 hour before injecting carrageenan. The animals were sacrificed with an overdose of ether. The pleural cavity was opened and rinsed with cold saline containing 0.1 % EDTA. The pleural fluid was removed by mild suction and evaluated for leukocyte count and protein levels. All parameters
were compared with those of mice administered with intra-thoracic injection of 0.1 ml normal saline.

**Leukocyte count in pleural fluid**

The pleural fluid was diluted with Türk's solution and total leukocyte count (TLC) was measured using a Neubauer's chamber under a light microscope. Smears of pleural fluid were stained with Giemsa stain and differential count of 200 cells for polymorphonuclear (PMN) and mononuclear cells was performed under an oil immersion objective (100 x).

**Measurement of total protein in pleural fluid**

The fluid collected from the pleural cavity was centrifuged (800 g) for 10 min and protein concentration in the supernatant was measured by the Bradford's method (Bradford, 1976). Briefly, 20 μl of pleural fluid was mixed with 1 ml of Coomassie brilliant blue reagent (610-2, Sigma-Aldrich). After 10 min, the absorbance of the assay mixture was measured at 595 nm using a Shimadzu 2450 spectrophotometer zeroed with a reagent blank.

**Evaluation of capillary permeability** (Peters et al., 1999)

In a separate experiment to evaluate the exudate leakage into the pleural cavity, the animals were intravenously injected with a solution of Evans Blue dye (25 mg kg\(^{-1}\), 0.2 ml, i.v.) 2 hour prior to the injection of carrageenan. The pleural fluid (500 μl) was separated for determination of the concentration of Evans blue dye. The concentration of dye in the pleural fluid was determined by colorimetry using Shimadzu 2450 spectrophotometer at 600 nm wavelength by interpolating the readings to a standard curve of Evans Blue in the range of 0.01 – 50 μg ml\(^{-1}\) Vs absorbance.

**4.3.2.6 Plasma membrane stabilizing activity**
Whole blood was obtained through retro orbital plexus from rats. The blood was washed three times with isotonic buffered solution (154 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4). The blood was centrifuged each time at 3000 g for 10 minutes at 4°C temperature and finally the sedimented erythrocytes were suspended in isotonic buffered solution and stored at 4°C till further use. Plasma membrane stabilizing activity of the extracts A and A2 was assessed using heat induced rat erythrocyte haemolysis method (Caprino, 1974). The reaction mixture (the erythrocyte suspension and the drug solution in a proportion to make a final concentration of drug of 50, 100 and 150 mg ml$^{-1}$ in the vial) was pre-incubated at 37°C for 15 min and finally incubated at 54°C for 25 min. After spinning down the precipitate, the $A_{540}$ (absorbance at 540 nm) of the supernatant was determined spectrophotometrically. Aspirin was used as the standard control. The % inhibition was calculated using the formula given below:

\[
\% \text{ Inhibition} = \left(1 - \frac{A_{540 \text{ sample}} - A_{540 \text{ blank}}}{A_{540 \text{ control}} - A_{540 \text{ blank}}} \right) \times 100.
\]

4.3.2.7 Evaluation of prostaglandin synthesis inhibition activity

This activity study was done as described by Dharmasiri et al. (2003). Briefly, the female rats in diestrus stage were selected by microscopic examination of vaginal smears. They were sacrificed with an overdose of ether; their uterine horns were removed and cut into approximately one centimetre pieces. These uteri were individually placed in 35 ml organ bath containing Krebs Henseleit solution having the following composition (mmol l$^{-1}$): Na$^+$ 143, K$^+$ 5.8, Ca$^{2+}$ 2.6, Mg$^{2+}$ 1.2, Cl$^-$ 128, H$_2$PO$_4^{-}$ 1.2, HCO$_3^{−}$ 25, SO$_4^{2−}$ 1.2 and glucose 11.1 at a pH of 7.4. The organ bath was maintained at 37°C and aerated with a gas mixture of 95 % O$_2$ and 5% CO$_2$. Contractions of uteri were recorded isometrically under a resting tension of 1 g until it became regular (usually within 10 min). The normal activity of the uteri was recorded for further 10 min. Following this, Extract A was added till the final concentration became 1 mg ml$^{-1}$ in the organ bath (n=3/dose). After each treatment, the spontaneous activity of the uteri was further recorded for 15 min. The percentage reduction in the amplitude and frequency of contractions with respect to the normal contractions was calculated.
4.3.2.8 Evaluation of antihistaminic activity (Spector, 1956)

Fur on the left lateral side of the posterior region of 30 rats was shaved under aseptic conditions. Twenty four hours later, these rats were randomly divided into five equal groups (n=6/group). These rats were orally treated in the following manner. Control group with 5 ml kg\(^{-1}\) of normal saline; standard group with 0.5 mg kg\(^{-1}\) chlorpheniramine and extract treated groups with 75 mg kg\(^{-1}\), 150 mg kg\(^{-1}\) and 300 mg kg\(^{-1}\) of Extract A orally. After 1 hour, these rats were subcutaneously injected with 50 µl of 500 µg ml\(^{-1}\) of histamine dihydrochloride in the shaved region and 2 min later the area of the wheal formed was measured.

4.3.3 Immunomodulatory activity of Inula cappa

4.3.3.1 Animals

Male Swiss albino mice weighing 18-20 g and Balb/c mice weighing 18-20 g were obtained from Zydus research centre, Ahmedabad, Gujarat, India. All the animals were fed standard laboratory chow and water ad libitum. The environment was maintained at 22 ± 2°C with 12 hour light and dark cycle. Animal experiments were conducted in accordance with the CPCSEA norms on animal research and all animals used in the experiments received humane care. The treatment of the animals varied as described in the section 4.3.2.1.

4.3.3.2 Phagocytic activity (DiCarlo et al., 1964; Dua et al., 1989)

Male Swiss albino mice were divided into two main groups, namely control and treated. Group I (control), consisted of six animals, was treated with normal saline (5 ml kg\(^{-1}\), orally) for 5 days. Group II (treated) was further divided into four other groups with six animals in each group. Treated groups were administered with extract A, A1, A2 and A3 (300 mg kg\(^{-1}\)) orally for 5 days. After 48 hour of the fifth day, mice were injected intravenously via the tail vein with carbon ink suspension (10 µl gm\(^{-1}\) body wt., Pelican AG, Germany). Blood samples were withdrawn (in EDTA solution 5 µl) from the retro-orbital vein at 0 and 12 min. 25 µl of blood sample was mixed
with 0.1 % w/v sodium carbonate solution (2 ml) and its optical density was measured at 650 nm spectrophotometrically. The phagocytic index \( K \) was calculated using the following equation:

\[
K = \frac{(\log_{e} O_{D_1} - \log_{e} O_{D_2})}{12}
\]

where \( O_{D_1} \) and \( O_{D_2} \) are the optical densities at 0 and 12 min, respectively. Results were expressed as the arithmetic mean ± S.E.M. of six mice.

4.3.3.3 Neutrophil adhesion test (Wilkinson, 1978)

The mice were divided into four groups and each group was consisting of six animals. The Extract A of *Inula cappa* at dose of 75, 150 and 300 mg kg\(^{-1}\) per day was administered orally to three groups of mice. The fourth group of mice received 5 ml kg\(^{-1}\) normal saline orally as vehicle control. The drug treatment was continued for 14 days. On the 14\(^{th}\) day of drug treatment, blood samples were collected by puncturing the retro orbital plexus in to heparinised vials and analysed for total leukocyte count (TLC) and differential leukocyte counts (DLC). After initial counts, blood samples were incubated with 80 mg ml\(^{-1}\) of nylon fibres for 15 min at 37°C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil gives Neutrophil Index (NI) of blood sample. % Neutrophil adhesion was calculated as shown below:

\[
\text{Neutrophil adhesion (\%) } = \frac{NI_t - NI_u}{NI_u} \times 100
\]

where \( NI_u \) = Neutrophil index of untreated blood sample; \( NI_t \) = Neutrophil index of treated blood sample

4.3.3.4 HA titre (Nelson and Mildenhall, 1967)

The control group was treated with normal saline (5 ml kg\(^{-1}\)) during the entire experimental protocol of 7 days and the treatment group was further divided into four
groups of six animals each, treated with 300 mg kg\(^{-1}\) of extract A, A1, A2 and A3 (orally) respectively. The animals were immunized by injecting 0.2 ml of fresh Sheep Red Blood Cells (SRBC) suspension (\(10^8\) cells ml\(^{-1}\) prepared in normal saline) intraperitonially on day 8. Blood samples were collected in heparinized microcentrifuge tubes from individual animals by retro-orbital plexus on day 11 for antibody titre. Serum was separated and antibody levels were determined by the haemagglutination technique. Briefly, equal volumes of individual serum samples of each group were pooled. Two fold dilutions of pooled serum samples were made in 25 µl volumes of normal saline in a microtitration plate to which 25 µl of 1 % suspension of SRBC in saline was added. After mixing, the plates were incubated at room temperature for 1 hour and examined for haemagglutination under the microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

![Diagram for haemagglutination assay]

**Fig. 10** Schematic representation of the assay of Haemagglutination titre
4.3.3.5 Delayed type hypersensitivity (Doherty, 1981)

In a separate experiment the mice treated as above (section 4.3.3.4) were challenged by injecting 20 µl of $5 \times 10^9$ SRBC ml\(^{-1}\) suspension intradermally into the left hind footpad on the 11\(^{th}\) day. The thickness of the left hind footpad was measured with Vernier calipers before the injection and was considered as a control. The foot thickness was measured again after 24 hour of the injection of SRBC.

4.3.3.6 Plaque forming cell (PFC) assay

The assay was done according to technique of Jerne and Nordin (1963). Swiss albino mice were taken, divided into two groups; Group I served as control; received normal saline solution (5 ml kg\(^{-1}\), orally) and Group II served as treated group; received Extract A (300 mg kg\(^{-1}\) body weight, orally) for 7 days. On 8\(^{th}\) day, spleen cells were separated in RPMI-1640 medium; washed with the same and suspended in the same to a concentration of $1 \times 10^6$ cells ml\(^{-1}\). A bottom layer was prepared with 1.2 % w/v agarose in 0.15 M NaCl in glass petridishes. A mixture of 2.0 ml 0.6 % w/v agarose in RPMI-1640 medium (at 42°C), 0.1 ml suspension of 20 % SRBC (in normal saline) and $1 \times 10^5$ spleen cells were poured over the bottom layer and petridishes were incubated at 37°C for 90 min. 2.0 ml of 1:10 diluted fresh Guinea pig serum in PBS was added as a source of complement in each petridish and further incubated for 60 min. The plaques were counted immediately thereafter and the values are expressed as count per spleen cells.

4.3.3.7 Evaluation of immunoprophylactic effect

Immunoprophylactic effect was determined by a method reported by Pallabi de et al. (1998). The strength of E.coli was standardized to induce 100% mortality. Swiss albino mice were divided into two groups: Group I served as control and received normal saline (5 ml kg\(^{-1}\), orally) and Group II served as control and received Extract A (300 mg kg\(^{-1}\) body weight) orally for 15 days.
On 15th day, *E. coli* (2.5 x 10^8 cells suspended in normal saline) were injected intraperitoneally to both groups of mice, 3 hour after the last dose of Extract A. Percentage mortality was observed for 24 hour.

4.3.3.8 Concanavallin (Con) A induced Splenocyte proliferation (*Yim and Ko, 2001*)

**Animals and Treatment**

Balb/c mice of 18-20 g were selected for the experiments and were divided into five groups. Group I served as control and received normal saline (5 ml kg\(^{-1}\), orally). Group II, III, IV and V received 300 mg kg\(^{-1}\) body weight of extract A, A1, A2 and A3 orally respectively for five days.

**Preparation of splenocytes**

After 5 days treatment mice were sacrificed by cervical dislocation and spleens were removed aseptically. Spleens were placed in RPMI-1640 (Hi media) medium and teased apart with a pair of forceps and needle. The suspension was filtered through a stainless steel filter and the spleen cells were separated. The cells were washed three times with the same medium and suspended in RPMI-1640, supplemented with 10 % heat-inactivated fetal calf serum and 1.0 % w/v antibiotic solution, at a concentration of 3.5 x 10^5 cells ml\(^{-1}\). The viability of the isolated splenocytes, as determined by tryptan blue dye exclusion test was found to be larger than 95 %.

**Ex vivo test**

For ex vivo experiments, the splenocytes obtained from the groups pretreated with extract A, A1, A2 and A3 were cultured in medium with or without Con A (Sigma) added at concentrations varying from suboptimal (1 μg ml\(^{-1}\)), intermediate (2 μg ml\(^{-1}\)) to optimal (4 μg ml\(^{-1}\)) in a final volume of 100 μl in a 96-well flatbottomed microplate at 37°C in a 5% CO\(_2\) humidified atmosphere.

**In vitro test**
Murine splenocytes obtained from the control group were cultured in medium with or without different concentrations of extract A, A1, A2 and A3 in a 96-well flat bottomed microplate. Con A solution was added at varying concentrations (1 μg ml⁻¹, 2 μg ml⁻¹ and 4 μg ml⁻¹) and incubated at 37°C in a 5% CO₂ humidified atmosphere for 72 hours. 10 μl of 0.25 % DMSO solution was used as control.

**Measurement of splenocyte proliferation**

To evaluate the effect of the extracts A, A1, A2 and A3 on the proliferation of splenic lymphocytes, after 72 hour, 10 μl of 3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg ml⁻¹) were added to each well under dark conditions and incubated for 4 hour. The plates were centrifuged (1400 g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well, 100 μl of a DMSO was added, mixed and the absorbance was measured in an ELISA reader at 540 nm. Stimulation index (SI) was calculated using the equation:

\[
SI = \frac{\text{mean absorbance of the cells stimulated with Con A}}{\text{mean absorbance of the cells not stimulated with Con A}}
\]

The extent of Con A stimulated proliferation (Proliferative Index) of isolated splenocytes was estimated by computing the area under curve (AUC) of the graph plotted between the stimulatory index and the Con A concentrations in μg ml⁻¹.

4.3.3.9 *Con A induced splenocyte proliferation in HCA compromised cells*

In a separate experiment, the effect of the Extract A was evaluated on the proliferation of splenocytes obtained from the mice immunocompromised with hydrocortisone acetate (HCA) (*Gillis et al.*, 1979). The animals were divided into three groups: Control, HCA control and Treated group. All the groups except the control group received HCA (0.1 ml, 5 mg ml⁻¹, intra peritoneally). The control and HCA control received only sterile saline and treated group received Extract A at a dose of 300 mg kg⁻¹ orally. The treatment was continued for 5 days. After five days of treatment the mice were sacrificed and the spleens were removed. Splenocytes were isolated and the assay was also carried as described earlier (Section 4.3.3.8). The results were compared with that of the control and HCA control.
### Fig. 11 Pattern of extracts added in 96 well flat bottom ELISA plate for ex-vivo assay

<table>
<thead>
<tr>
<th>Different dilutions of the extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A (µg ml⁻¹)</td>
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</tbody>
</table>

A: Extract A; A₁: Extract A₁; A₂: Extract A₂; A₃: Extract A₃; C: Control and D: DMSO

### Fig. 12 Pattern of extracts added in 96 well flat bottom ELISA plate for in-vitro assay

<table>
<thead>
<tr>
<th>Different dilutions of the extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A (µg ml⁻¹)</td>
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</tbody>
</table>

A: Extract A; A₁: Extract A₁; A₂: Extract A₂; A₃: Extract A₃; C: Control and D: DMSO

Fig. 12 Pattern of extracts added in 96 well flat bottom ELISA plate for in-vitro assay
4.3.3.10 Con A induced thymocyte proliferation

Animals and Treatment

For the experiments, Balb/c mice of 18-20 g were selected and were divided into two groups. Control group received normal saline (5 ml kg⁻¹, orally). Treated group was given 300 mg kg⁻¹ body weight of Extract A orally respectively for five days.

Preparation of lymphocytes

The mice were sacrificed by cervical dislocation after 5 days treatment and thymus glands were removed aseptically. Thymus glands were placed in RPMI-1640 medium and teased apart with a pair of forceps and needle and filtered. Cells were washed three times and suspended in RPMI-1640, supplemented with 10 % heat-inactivated fetal calf serum and 1.0 % w/v antibiotic solution, at a concentration of 5 × 10⁶ cells ml⁻¹. The viability of the isolated thymocytes, was determined by tryptan blue dye exclusion test.

Ex vivo test

For ex vivo experiments, the lymphocytes obtained from the pretreated groups were cultured in medium with or without Con A (Sigma) added at concentrations varying from suboptimal (1 μg ml⁻¹), intermediate (2 μg ml⁻¹) to optimal (4 μg ml⁻¹) in a final volume of 100 μl in a 96-well flatbottomed microplate at 37°C in a 5% CO₂ humidified atmosphere.

In vitro test

Lymphocytes obtained from the control group were cultured in medium with or without different concentrations of Extract A and 1, 2 and 4 μg ml⁻¹ concentration of Con A in a 96-well flat bottomed microplate at 37°C in a 5% CO₂ humidified atmosphere for 72 hours. Control wells contained 10 μl of DMSO solution.
Measurement of thymocyte proliferation

To evaluate the effect of the Extract A on the proliferation of lymphocytes, the cell suspension was cultured as described for that of the splenocyte proliferation assay. After 72 hours, 10 μl of 3-[4, 5- dimethylthiazol-2-y1]-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg ml⁻¹) were added to each well under dark conditions and incubated for 4 hours. The untransformed MTT was removed carefully by pipetting after centrifugation. To each well, 100 μl of DMSO was added, and the absorbance was measured in ELISA reader at 540 nm. Stimulation index (SI) and proliferation index (PI) were calculated as explained earlier. The pattern of extracts added in 96 well flat bottomed ELISA plate for in-vitro assay and ex-vivo assays remained the same as mentioned earlier (Fig. 11 and 12).

4.3.3.11 Con A induced thymocyte proliferation in HCA compromised cells

As described earlier, hydrocortisone acetate (HCA) was used as a suppressant of the immune response of mice. It is reported that HCA does not inhibit thymic lymphocytes to a greater extent compared to the other lymphocytes (Warner, 1964; Blomgren and Andersson, 1969; Ishidate and Metcalf, 1963). Hence the effect of HCA on the proliferation rate of the lymphocytes obtained from the thymus gland was also studied. HCA was given intra-peritoneally to the control mice as well as the mice treated with Extract A for 5 days as mentioned earlier. After five days of treatment the mice were sacrificed and the thymus glands were removed. Lymphocytes were isolated and the assay was also carried as described earlier. The results were compared with that of the control and HCA treated control.

4.3.3.12 Con A induced production of IL-2 and other mediators

The protocol involved three groups namely control, HCA control and treated group. Control group and HCA control group received normal saline and treated group was given 300 mg kg⁻¹ body weight orally of Extract A for the five day treatment. HCA was given by i.p route as described earlier (4.3.3.9). Production of IL-2 by the spleen cells was analyzed by the thymocyte assay method with minor modifications (Xiu et al., 1991; Osserman and Lawlor, 1966). The
spleen cell suspension from the mice of different groups were aseptically prepared as described above and adjusted to $1 \times 10^6$ cells ml$^{-1}$. 1.0 ml of each cell suspension was mixed with 1.0 ml of Con A (final concentration 5 μg ml$^{-1}$) in a 10 ml culture flask. The mixtures were first incubated at 37°C in an atmosphere of 5% CO$_2$ and saturated humidity for 40 hours and then centrifuged at 3000 g for 10 min. The supernatants were collected as crude IL-2 preparations and kept frozen at -70°C for the IL-2 assay. For the determination of IL-2, a thymocyte suspension was prepared from aseptically collected thymus glands from euthanized mice and adjusted to $5 \times 10^6$ cells ml$^{-1}$. 100 μl of the thymocyte suspension, 100 μl of the crude IL-2 supernatant and 20 μl of Con A (final concentration 1.5 μg ml$^{-1}$) were added to the wells of a microplate. The samples were incubated for 80 hours (37°C, 5% CO$_2$ and saturated humidity). After addition of 10 μl of MTT to the sample wells, the mixtures were reincubated for 16 hours. The crystals were dissolved in DMSO and the absorbance was measured at 540 nm.

4.3.3.13 Cyclophosphamide-induced myelosuppression assay (Wagner and Jurcic, 1991)

Swiss albino mice were divided into three groups of 6 animals each. Animals in the treated group received the Extract A in a daily dose of 75, 150 and 300 mg kg$^{-1}$ body weight for 13 days. Positive control and negative control group animals received normal saline (0.3 ml/mouse, orally, daily for 13 days). On days 11, 12 and 13, all the animals except in the negative control group were given cyclophosphamide intraperitonially, 1 hour after the administration of the extract. Blood samples were collected on 14th day by retro orbital puncture and the white blood cell (WBC) levels of the whole blood were analyzed by an automatic multi-parameter blood cell counter (Sysmex KX-21N). Data are presented as the mean ± S.E.M. The one way analysis of variance (ANOVA) followed by Dunnets test was used for comparing the WBC levels among the positive control, negative control and treated groups.

4.3.4 Toxicity Studies

Acute Toxicity Study
Swiss albino mice of either sex were fasted overnight but water was allowed *ad libitum*. A pilot study was carried out before the main experiment and based on the findings, doses of 100, 500, 1000 and 1500 mg kg\(^{-1}\) were selected for the final study. Animals were divided into four groups of 8 animals in each. The Extract A in different doses was administered orally as a single dose. The mortality was observed after a period of 24 hours.

**Sub-acute Toxicity Study**

Twenty male Wistar strain albino rats were divided into two groups. The first group (control) received vehicle (normal saline) and other group (test) received Extract A in a dose of 300 mg kg\(^{-1}\) orally daily. Both groups of rats were kept in similar laboratory conditions and were allowed to take usual food and water. The animals were observed for their general condition, gross behavior, body weights etc. All the animals were sacrificed on the 15\(^{th}\) day, the viscera were removed and examined for histopathological changes including gastric erosions in the stomach. Tissue sections were prepared, stained with haematoxylin and eosin, and examined microscopically for evidence of any abnormality.

**4.3.5 Statistical analysis**

Values are expressed as mean ± S.E.M. of six observations. One-way ANOVA followed by Dunnet’s test was used for the comparison of data among groups unless otherwise specified. A value of \( p \leq 0.05 \) and \( p \leq 0.01 \) was taken as statistically significant.
4.4 Chromatographic analysis

4.4.1 TLC Fingerprinting

TLC fingerprint profiles of methanolic extract of *Inula cappa* roots were established using HPTLC in solvent systems of varying polarity. Suitably diluted stock solution of methanolic extracts were spotted on pre-coated silica gel 60 F254 TLC plates (E. Merck) using CAMAG Linomat IV Automatic Sample Spotter (Switzerland) and the plate were developed in the following solvent systems:

- Solvent system I: Hexane: ethyl acetate (9:1, v/v)
- Solvent system II: Toluene: methanol (9:1, v/v)
- Solvent system III: Toluene: ethyl acetate: formic acid (7:3:0.5, v/v/v)
- Solvent system IV: Chloroform: methanol: water (7:3:0.5, v/v/v)
- Solvent system V: Toluene: ethyl formate: formic acid: water (3:3:0.8:0.2, v/v/v/v)
- Solvent system VI: Ethyl acetate: formic acid: glacial acetic acid: water (10:1.1:1.1:2.6, v/v/v/v)

The plates were dried at room temperature and the number of bands and their \( R_f \) was documented under UV 254 and 366 nm. Further, the plates were derivatised by spraying with anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 5 minutes and the \( R_f \) and colours of the bands resolved were recorded.

4.4.2 Isolation and characterisation

4.4.2.1 Isolation using column chromatography

1 kg dried powdered root of *Inula cappa* (40 #) was extracted exhaustively with methanol (3 × 1 liter). The solvent was evaporated and the dried methanolic extract was suspended in the water and successively partitioned between petroleum ether (300 ml × 3) and water. The dried petroleum ether extract (1 g) was chromatographed on 100 g silica gel (230-400 #) and eluted with petroleum ether (60-80 °C) containing increasing concentrations of ethyl acetate (0-5 %). Fractions of 25 ml were collected.
Different fractions were monitored by TLC for the presence of the compounds using the solvent system of hexane:ethyl acetate (9.8:0.2, v/v). Fractions yielding similar bands on TLC were pooled and concentrated to dryness. Compounds were purified by recrystallisation from methanol.

4.4.2.2 Characterization and purity check by M.P., TLC, UV, IR, EIMS, and NMR

Melting Point analysis

Melting point (M.P.) of the isolated compounds were determined using Toshniwal melting point apparatus.

Thin-Layer Chromatography (TLC)

For TLC experiments, precoated plates of silica gel 60 F$_{254}$ (E. Merck) were used and spotting was done on CAMAG LINOMAT V Automatic TLC spotter. For purity assessment of the isolated compounds, the plates were developed in different solvent systems and checked for single band. UV absorption spectrum of compound separated on TLC was recorded at start, middle and end position of the band using CAMAG TLC Scanner 3.

UV spectrum of isolated compounds

UV spectra of isolated compounds were recorded on the Shimadzu 2450 spectrophotometer by preparing the solution of isolated compound in methanol and also on the CAMAG TLC Scanner 3 by scanning the TLC plate.

IR spectra of the isolated compounds

IR spectra was recorded on BUCK SCIENTIFIC IR Spectrophotometer (model 500). A KBr disc was prepared by mixing the 2 mg drug with anhydrous KBr.

EIMS
EIMS of the isolated compounds were taken on Hewlett-Packard model 5973.

**NMR spectra of the isolated compounds**

The isolated compounds were dissolved in CDCl₃ and Proton wideline measurements were conducted on a Bruker DSX spectrometer operating at a ¹H resonance frequency of 300MHz on a standard Bruker double resonance magic angle spinning (MAS) probe head.

The data were compared with the literature (Hong-Gang et al., 2007; Tada et al., 1993; Goswami et al., 1984) to elucidate the structures of the isolated compounds.

**4.4.3 Quantification of bioactive compounds using HPTLC**

**4.4.3.1 Development of Sensitive HPTLC Method for the Estimation of β-sitosterol from Inula cappa**

**Preparation of standard solutions**

A stock solution of β-sitosterol (50 μg ml⁻¹) was prepared by dissolving 5 mg of accurately weighed β-sitosterol in methanol and making up the volume of the solution to 100 ml with methanol in a volumetric flask. The aliquots (1.6 to 9.6 ml) of stock solutions were transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol to obtain standard solutions containing 8 μg ml⁻¹, 16 μg ml⁻¹, 24 μg ml⁻¹, 32 μg ml⁻¹, 40 μg ml⁻¹ and 48 μg ml⁻¹ of β-sitosterol respectively.

**Preparation of sample solutions**

Accurately weighed 1.0 g quantity of powdered roots of *Inula cappa* was extracted with methanol (4 × 25 ml) under reflux on a water bath. The methanolic extract was filtered through Whatman I filter paper (Nair Industries, Mumbai), filtrates were combined, concentrated and transferred to 25 ml volumetric flask and the volume was made up with methanol. This extract was used for the estimation of β-sitosterol.
Calibration curve for β-sitosterol

10 μl each of the standard solutions of β-sitosterol (8 to 48 μg ml⁻¹) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicate on a HPTLC plate using Camag Linomat automatic sample spotter V (Camag, Switzerland). The plates were developed in a twin trough chamber (20 × 10 cm) up to a distance of 8 cm using a mobile phase of Toluene: Methanol (10 ml) (9.4 : 0.6, v/v) at 25 ± 2°C temperature and 40 % relative humidity. After development, the plates were dried at room temperature in air, derivatized with anisaldehyde-sulphuric acid reagent, heated at 105°C till coloured bands appeared and scanned densitometrically at 525 nm in absorbance mode using tungsten lamp using Camag TLC Scanner 3 (Camag, Switzerland). The peak areas were recorded. Calibration curve of β-sitosterol was obtained by plotting a graph of peak areas vs concentrations of β-sitosterol (8 to 48 μg ml⁻¹).

Estimation of β-sitosterol

10 μl of suitably diluted sample solutions was applied in triplicate on a precoated HPTLC plate. The plate was developed and scanned as mentioned above. The peak areas and absorption spectra were recorded and the amount of β-sitosterol was calculated using its calibration curve.

4.4.3.2 Development of Sensitive HPTLC Method for the Estimation of lupeol from Inula cappa

Preparation of standard solutions

A stock solution of lupeol (150 μg ml⁻¹) was prepared by dissolving 7.5 mg of accurately weighed lupeol in methanol and making up the volume of the solution to 50 ml with methanol in a volumetric flask. The aliquots (1 to 6 ml) of stock solutions were transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol to obtain standard solutions containing 15 μg ml⁻¹, 30 μg ml⁻¹, 45 μg ml⁻¹, 60 μg ml⁻¹, 75 μg ml⁻¹ and 90 μg ml⁻¹ of lupeol respectively.

Preparation of sample solutions
Accurately weighed 1.0 g quantity of powdered roots of *Inula cappa* was extracted with methanol (4 × 25 ml) under reflux on a water bath. The methanolic extract was filtered through Whatman I filter paper, filtrates were combined, concentrated and transferred to 25 ml volumetric flask and the volume was made up with methanol. This extract was used for the estimation of lupeol.

**Calibration curve for Lupeol**

10 μl each of the standard solutions of lupeol (15 to 90 μg ml⁻¹) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicate on a HPTLC plate using Camag Linomat automatic sample spotter V (Camag, Switzerland). The plates were developed in a twin trough chamber (20 × 10 cm) up to a distance of 8 cm using a mobile phase of Toluene: Methanol (10 ml) (9.4 : 0.6, v/v) at 25 ± 2°C temperature and 40 % relative humidity. After development, the plates were dried at room temperature in air, derivatized with anisaldehyde-sulphuric acid reagent, heated at 105°C till coloured bands appeared and scanned densitometrically at 525 nm in absorbance mode using tungsten lamp using Camag TLC Scanner 3 (Camag, Switzerland). The peak areas were recorded. Calibration curve of lupeol was obtained by plotting a graph of peak areas vs concentrations of lupeol (15 to 90 μg ml⁻¹).

**Estimation of Lupeol**

10 μl of suitably diluted sample solutions was applied in triplicate on a precoated HPTLC plate. The plate was developed and scanned as mentioned above. The peak areas and absorption spectra were recorded and the amount of lupeol was calculated using its calibration curve.

4.4.3.3 Development of Sensitive HPTLC Method for the Estimation of α-amyrin from *Inula cappa*

**Preparation of standard solutions**

A stock solution of α-amyrin (80 μg ml⁻¹) was prepared by dissolving 4.0 mg of accurately weighed α-amyrin in methanol and making up the volume of the solution
with methanol to 50 ml in a volumetric flask. The aliquots (2 to 7 ml) of stock solutions were transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol to obtain standard solutions containing 16 µg ml$^{-1}$, 24 µg ml$^{-1}$, 32 µg ml$^{-1}$, 40 µg ml$^{-1}$, 48 µg ml$^{-1}$ and 56 µg ml$^{-1}$ of α-amyrin respectively.

**Preparation of sample solutions**

Accurately weighed 1.0 g quantity of powdered roots of *Inula cappa* was extracted with methanol (4 × 25 ml) under reflux on a water bath. The methanolic extract was filtered through Whatman I filter paper, filtrates were combined, concentrated and transferred to 25 ml volumetric flask and the volume was made up with methanol. This extract was used for the estimation of α-amyrin.

**Calibration curve for α-amyrin**

10 µl each of the standard solutions of α-amyrin (16 to 56 µg ml$^{-1}$) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicate on a HPTLC plate using Camag Linomat automatic sample spotter V (Camag, Switzerland). The plates were developed in a twin trough chamber (20 × 10 cm) up to a distance of 8 cm using a mobile phase of toluene : ethyl acetate (10 ml) (9.8 : 0.2, v/v) at 25 ± 2°C temperature and 40 % relative humidity. After development, the plates were dried at room temperature in air, derivatized with anisaldehyde-sulphuric acid reagent, heated at 105°C till coloured bands appeared and scanned densitometrically at 525 nm in absorbance mode using tungsten lamp Camag TLC Scanner 3 (Camag, Switzerland). The peak areas were recorded. Calibration curve of α-amyrin was obtained by plotting a graph of peak areas vs applied concentrations of α-amyrin (16 to 56 µg ml$^{-1}$).

**Estimation of α-amyrin**

10 µl of suitably diluted sample solutions was applied in triplicate on a precoated HPTLC plate. The plate was developed and scanned as mentioned above. The peak areas and absorption spectra were recorded and the amount of α-amyrin was calculated using its calibration curve.
4.4.3.4 Development of Sensitive HPTLC Method for the Estimation of chlorogenic acid from Inula cappa

Preparation of standard solutions

A stock solution of chlorogenic acid (80 μg ml⁻¹) was prepared in methanol by dissolving 2 mg of accurately weighed chlorogenic acid in methanol and making up the volume of the solution to 25 ml with methanol in a volumetric flask. The aliquots (2 to 3.5 ml) of stock solutions were transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol to obtain standard solutions containing 16 μg ml⁻¹, 18 μg ml⁻¹, 20 μg ml⁻¹, 22 μg ml⁻¹, 24 μg ml⁻¹, 26 μg ml⁻¹ and 28 μg ml⁻¹ of chlorogenic acid respectively.

Preparation of sample solutions

1.0 g of root powder of Inula cappa was extracted exhaustively with methanol (4 × 25 ml) and the extract was filtered, pooled and the combined extract was concentrated under vacuum and volume was made up to 25 ml in a volumetric flask.

Calibration curve for chlorogenic acid

10 μl each of the standard solutions containing 16 to 28 μg ml⁻¹ chlorogenic acid were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicate on a HPTLC plate using Camag Linomat automatic sample spotter V (Camag, Switzerland). The plate was developed in a twin trough chamber (20 × 10 cm) up to a distance of 8 cm using mobile phase, ethyl acetate: glacial acetic acid: formic acid: water (10: 1.1: 1.1: 2.6, v/v) (Wagner and Bladt, 1996) at 25 ± 2°C temperature and 40 % relative humidity. After development, the plates were dried at room temperature in air and scanned at 330 nm using Camag TLC Scanner 3 (Camag, Switzerland). Calibration curve of chlorogenic acid was prepared by plotting a graph of peak areas vs. concentration of chlorogenic acid.

Estimation of chlorogenic acid
10 µl of the sample solution was applied in triplicate on a pre-coated HPTLC plate. The plate was developed in the solvent system of ethyl acetate: glacial acetic acid: formic acid: water (10 : 1.1 : 1.1: 2.6, v/v) and scanned at 330 nm as mentioned above. The peak areas and absorption spectra were recorded. The amount of chlorogenic acid in the sample was calculated using the calibration curve.

4.4.3.5 Development of Sensitive HPTLC Method for the Estimation of Thymol Isobutyrate from Inula cappa

Preparation of standard solutions

A stock solution of thymol isobutyrate (80 µg ml⁻¹) was prepared in methanol by dissolving 4 mg of accurately weighed thymol isobutyrate in methanol and making up the volume of the solution to 50 ml with methanol in a volumetric flask. The aliquots (1 to 6 ml) of stock solutions were transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol to obtain standard solutions containing 8 µg ml⁻¹, 16 µg ml⁻¹, 24 µg ml⁻¹, 32 µg ml⁻¹, 40 µg ml⁻¹ and 48 µg ml⁻¹ of thymol isobutyrate respectively.

Preparation of sample solutions

1.0 g of root powder of Inula cappa was extracted exhaustively with methanol (4 × 25 ml) and the extract was filtered, pooled and the combined extract was concentrated under vacuum and volume was made up to 25 ml in a volumetric flask.

Calibration curve for Thymol Isobutyrate

10 µl each of the standard solutions containing 8 to 48 µg ml⁻¹ thymol isobutyrate were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicate on a HPTLC plate using Camag Linomat automatic sample spotter V (Camag, Switzerland). The plate was developed in a mobile phase of toluene : ethyl acetate (9.3 : 0.7, v/v) at 25 ± 2°C temperature and 40 % relative humidity. After development, the plates were dried at room temperature in air and scanned at 530 nm after derivatisation with anisaldehyde sulphuric acid followed by heating at 110°C for
5 min using Camag TLC Scanner 3 (Camag, Switzerland). Calibration curve of thymol isobutyrate was prepared by plotting graph of peak areas vs. concentration of thymol isobutyrate.

**Estimation of Thymol Isobutyrate**

10 µl of the sample solution was applied in triplicate on a pre-coated HPTLC plate. The plate was developed in the solvent system of toluene : ethyl acetate (9.3 : 0.7, v/v) and scanned at 530 nm after derivatisation with anisaldehyde sulphuric acid followed by heating at 110°C for 5 min as mentioned above. The peak areas and absorption spectra were recorded. The amount of thymol isobutyrate present in the sample was calculated using the calibration curve.

4.4.3.6 Development of Sensitive HPTLC Method for the Estimation of Isoalantolactone from *Inula cappa*

**Preparation of standard solutions**

A stock solution of isoalantolactone (100 µg ml⁻¹) was prepared in methanol by dissolving 5 mg of accurately weighed isoalantolactone in methanol and making up the volume of the solution to 50 ml with methanol in a volumetric flask. The aliquots (1 to 7 ml) of stock solutions were transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol to obtain standard solutions containing 10 µg ml⁻¹, 20 µg ml⁻¹, 30 µg ml⁻¹, 40 µg ml⁻¹, 50 µg ml⁻¹, 60 µg ml⁻¹ and 70 µg ml⁻¹ of isoalantolactone respectively.

**Preparation of sample solutions**

1.0 g of root powder of *Inula cappa* was extracted exhaustively with methanol (4 × 25 ml) and the extract was filtered, pooled and the combined extract was concentrated under vacuum and volume was made up to 25 ml in a volumetric flask with methanol.

**Calibration curve for isoalantolactone**
10 μl each of the standard solutions containing 100 to 700 μg ml\(^{-1}\) isoalantolactone were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicate on a HPTLC plate using Camag Linomat automatic sample spotter V (Camag, Switzerland). The plate was developed in a mobile phase, toluene : methanol (9.4 : 0.6, v/v) at 25 ± 2°C temperature and 40 % relative humidity. After development, the plates were dried at room temperature in air and scanned at 530 nm after derivatisation with anisaldehyde sulphuric acid followed by heating at 110°C for 5 min using Camag TLC Scanner 3 (Camag, Switzerland). Calibration curve of isoalantolactone was prepared by plotting graph of peak areas vs. concentration of isoalantolactone.

**Estimation of isoalantolactone**

15 μl of the sample solution was applied in triplicate on a pre-coated HPTLC plate. The plate was developed in the solvent system of Toluene : Methanol (9.4 : 0.6, v/v) and scanned at 530 nm after derivatisation with anisaldehyde sulphuric acid followed by heating at 110°C for 5 min as mentioned above. The peak areas and absorption spectra were recorded. The amount of isoalantolactone in the sample was calculated using the calibration curve.

4.4.3.7 Development of Sensitive HPTLC Method for the Estimation of germacrolide from *Inula cappa*

**Preparation of standard solutions**

A stock solution of germacrolide (100 μg ml\(^{-1}\)) was prepared in methanol by dissolving 5 mg of accurately weighed germacrolide in methanol and making up the volume of the solution to 50 ml with methanol in a volumetric flask. The aliquots (1 to 7 ml) of stock solutions were transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol to obtain standard solutions containing 10 μg ml\(^{-1}\), 20 μg ml\(^{-1}\), 30 μg ml\(^{-1}\), 40 μg ml\(^{-1}\), 50 μg ml\(^{-1}\) and 60 μg ml\(^{-1}\) of germacrolide respectively.
Preparation of sample solutions

1.0 g of root powder of *Inula cappa* was extracted exhaustively with methanol (4 × 25 ml) and the extract was filtered, pooled and the combined extract was concentrated under vacuum and volume was made up to 25 ml in a volumetric flask with methanol.

Calibration curve for germacrolide

10 µl each of the standard solutions containing 10 to 60 µg ml⁻¹ IC-3 were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicate on a HPTLC plate using Camag Linomat automatic sample spotter V (Camag, Switzerland). The plate was developed in a mobile phase, toluene : methanol (9.4 : 0.6, v/v) at 25 ± 2°C temperature and 40 % relative humidity. After development, the plates were dried at room temperature in air and scanned at 530 nm after derivatisation with anisaldehyde sulphuric acid followed by heating at 110°C for 5 min using Camag TLC Scanner 3 (Camag, Switzerland). Calibration curve of germacrolide was prepared by plotting graph of peak areas vs. concentration of germacrolide.

Estimation of germacrolide

15 µl of the sample solution was applied in triplicate on a pre-coated HPTLC plate. The plate was developed in the solvent system of Toluene : Methanol (9.4 : 0.6, v/v) and scanned at 530 nm after derivatisation with anisaldehyde sulphuric acid followed by heating at 110°C for 5 min as mentioned above. The peak areas and absorption spectra were recorded. The amount of germacrolide in the sample was calculated using the calibration curve.

4.4.4 Validation *(Anonymous (a), 1994; Anonymous (b), 1996; Anonymous (e), 2002)*

The methods were validated for the linearity, accuracy, repeatability, specificity, limit of detection and limit of quantification.
4.4.4.1 Linearity

The ranges of the concentration of the standard compounds were determined for the linearity. The obtained test results were in direct proportion to the concentration of the analyte in the sample calibration curve of the analyte. The results were expressed as correlation coefficient.

4.4.4.2 Accuracy

Accuracy of the method was tested by performing the recovery studies at three levels by addition of 50%, 100% and 125% of standard to the *Inula cappa* root powder. The percentage recovery as well as the average percentage recovery was calculated.

4.4.4.3 Repeatability

Precision of the instrument was checked by repeated scanning of the same spot seven times and the coefficient of variation (CV) was calculated. All other conditions were as per the proposed methods. Area of the peak was measured 7 times without changing plate position. Variability of the method was studied by analyzing aliquots of different concentrations of standard solutions on same day (intra-day precision) and on different days (inter-day precision) and relative standard deviation (RSD) was calculated.

4.4.4.4 Specificity

It is an ability of the developed analytical method to detect the analyte quantitatively in presence of other compounds which are expected to be present in the sample matrix. The proposed method was developed for estimating the different compounds in the roots of *Inula cappa* such that the impurities present do not interfere.

4.4.4.5 Limit of Detection

Lowest amount of the analyte which can be detected but not necessarily quantitated as an exact value is called limit of detection. Determination of the signal-to-noise ratio
was performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio of 3:1 was considered acceptable for estimating the detection limit.

4.4.4.6 Limit of Quantification

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Determination of the signal-to-noise ratio was performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio was 10:1.
4.4 References


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