Materials & Methods
6. MATERIALS AND METHODS

6.1. PHARMACOGNOSTICAL STUDIES

The original and basic approach towards pharmacognosy includes study of morphological system, study of the cell structures and organization and study of tissue system, which still holds a key in the identification of the correct species of the plant and also to help us to differentiate between closely related species of the same genus. It is also the first step to standardize a drug, which becomes the need of the day. A detailed pharmacognostic investigation of the fruit of the plant of *Benincasa hispida* (Thunb.) Cogn was carried out to establish its correct pharmacognostical identity through morphological and microscopic methods.

6.1.1 Procurement and authentication of plant material

The fresh fruit of *Benincasa hispida* (Family: Cucurbitaceae) was collected in the month of September 2004 from the local vegetable market of Ahmedabad, Gujarat. The authentication of the plant was established and voucher specimen was deposited in the Department of Pharmacognosy and Phytochemistry, L. M. College of Pharmacy, Ahmedabad, India. The fruit was studied for its macroscopical and microscopical characters. The macroscopical characters and microscopical characters of the fruit were confirmed by comparing with the available literature. The dried fruit was powdered and then used for the present work.
6.1.2 Microscopical observation

Free hand sections of the fruit pulp with pericarp were taken, cleared with chloral hydrate and studied. The lignified elements were visualized by staining the section with phloroglucinol and hydrochloric acid. Macerates were prepared by the Schulz maceration method (Evans, 1997). Photomicrographs were shot for histological observation.

6.2 PHYTOCHEMICAL STUDIES

Each and every drug has got its own physical and chemical characteristics, which help in separating it from other closely, related drugs or species. To explore the hidden secrets of the plant kingdom such as their complex compounds or active principles. It is necessary to undertake the chemical and analytical research on them. Hence, an attempt is made to analyse the fruit of *Benincasa hispida* physically as well as chemically.

6.2.1 Determination of quality control parameters

*(a) Determination of ash values (anonymous, 1986)*

_Determination of total ash_

2 g of accurately weighed fruit powder was incinerated in crucible at a temperature not exceeding 450°C on a hot plate, till carbon free ash was obtained. It was then cooled, weighed and percentage of ash was calculated with reference to the air-dried drug.

_Determination of acid insoluble ash_

The ash obtained in above was boiled for 5 minutes with 25 ml of 2 M hydrochloric acid and filtered using an ash-less filter paper to collect
insoluble matter. The ash obtained was washed with hot water and filter paper was burnt to a constant weight. The percentage of acid-insoluble ash was calculated with reference to the air-dried drug.

**Determination of water-soluble ash**

Ash was boiled for 5 minutes with 25ml of water, and insoluble matter was collected on an ash-less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°C. Difference in weight of ash and weight of water insoluble matter gave the weight of water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried drug.

**(b) Determination of extractive values (anonymous, 1986)**

**Determination of alcohol soluble extractive**

5 g of the air-dried fruit powered material was macerated with 100 ml of alcohol in a closed flask for 24 h, shaking frequently at an interval of 6 h. It was then allowed to stand for 18 hours and filtered rapidly to prevent any loss during evaporation. 25 ml of the filtrate was evaporated to dryness in a porcelain dish and dried at 105°C to a constant weight. The percentage of alcohol soluble extractive value was calculated with reference to the air-dried drug.

**Determination of water-soluble extractive**

5 g of the air-dried fruit powder was macerated with 100 ml of distilled water in a closed flask for 24 h, shaking in a closed flask at an interval of

6 h. It was then allowed to stand for 18 h and filtered rapidly to prevent loss during evaporation. 25 ml of the extract was evaporated to dryness in a porcelain dish and dried at 105°C to a constant
weight. The percentage of water-soluble extractive value was calculated with reference to the air-dried drug.

**Determination of ether soluble extractive (fixed oil content)**

5 g of accurately weighed fruit powder was extracted with petroleum ether (B.P. 40°C to 60°C) in a soxhlet extractor for 6 hours and filtered. The solvent was evaporated on a water bath and the residue was dried at 105°C to a constant weight. The percentage of ether-soluble extractive value was calculated with reference to the air-dried drug.

### 6.2.2 Preliminary phytochemical screening

The dried fruit powder was subjected to the following tests individually for the presence of various phytoconstituents like alkaloids, flavonoids, saponins, tannins, anthraquinone, and carbohydrates.

**Tests for alkaloids (Sim, 1969)**

1 g of fruit powder was extracted with 20 ml of ethanol (95%) by refluxing for 15 minutes. The extract was filtered and the filtrate was evaporated to dryness. The residue was dissolved in 15 ml of H₂SO₄ (2N) and filtered. After making alkaline the filtrate was extracted with chloroform. The residue was then tested for the presence of alkaloids with dragendorff's reagent.

**Tests for flavonoids**

(a) *Shinoda test (Geissman, 1955)*

1 g of fruit powder was extracted with 10 ml of ethanol (95%) for 15 minutes on a boiling water bath and filtered. To the filtrate was added a small piece of magnesium ribbon and 3 to 4 drops of concentrated sulphuric acid. Red color formation of indicates the presence of flavonoids.
(b) Fluorescence test (Geissman, 1955)
1 g of fruit powder was extracted with 15 ml methanol for 2 minutes on a boiling water bath, filtered while hot and evaporated to dryness. To the residue was added 0.3 ml boric acid solution (3% w/v) and 1 ml oxalic acid solution (10% w/v). The mixture was evaporated to dryness and the residue was dissolved in 10 ml ether. The ether layer was checked for the fluorescence under UV spectrophotometer.

Tests for saponins (Fischer, 1952)

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

(b) Haemolytic zone
0.5 ml of blood was mixed with gelatin solution (3 g gelatin powder dissolved in 100ml of 0.85% NaCl solution) at 60°C and taken on a glass slide. A thick section, of fruit was placed on it. A clear haemolytic zone formation around the section indicates the presence of saponins in the drug.

Test for sterols (Griffin et al., 1968; Simes et al., 1959)

(a) Liberman burchad test
1 g of fruit powder was moistened with 1.0 ml of acetic anhydride on a clean tile was added 2 drops of sulphuric acid. The powder was mixed well and the color gained by the powder was observed. Formation of green-blue-purple-red color indicates presence of sterols.
Tests for tannins

Aqueous extract of the fruit was prepared by refluxing 10g of fruit with 50ml of water for about 1 hour and was used for the following tests.

(a) Gelatin test (Freudenberg et al., 1962; Robinson, 1964)
To 2-3 ml of aqueous extract, was added 1% gelatin solution containing NaCl. Heavy white precipitate indicates presence of tannins.

(b) Precipitation with alkaloids (Robinson, 1964)
To 2-3 ml of aqueous extract, solution of cinchonine was added. Bulky precipitate indicates presence of tannins.

(c) Reaction with lead acetate (Robinson, 1964)
Tannins were precipitated from the aqueous extract by adding 2 ml of 10% solution of lead acetate. Precipitate obtained was partially soluble in 1 ml of 10% acetic acid indicates presence of condensed tannins.

(d) Reaction with FeCl₃ (Clerk et al., 1947; Geiss et al., 1995)
2 ml of aqueous extract was treated with 0.5 ml of 5% FeCl₃. A green color formation indicates presence of pyrocatechol partial structures (whereas a blue color is characteristics for partial pyrogallol structure)

(e) Reaction with bromine water (Robinson, 1964)
To 2 ml of aqueous extract, 0.5 ml of freshly prepared bromine water was added. Precipitate formed indicates presence of condensed tannins.

(g) Reaction with FeCl₃ and Sodium bicarbonate (Robinson, 1964)
To 2 ml of the aqueous extract, 2 ml of 10% FeCl₃ and 2 ml of sodium bicarbonate were added. Formation of red violet color indicates presence of condensed tannins (flavan 3-ols).
(h) Reaction with formaldehyde (Hillis and Urbach, 1959)
To 2 ml of the aqueous extract, 1 ml of formaldehyde and HCl were added. Formation of red precipitate of phlobaphenes upon heating indicates presence of condensed tannins (flavan 3-ols).

(i) Reaction with vanillin-HCl (Bate-Smith et al., 1954)
2 ml of aqueous extract upon treatment with 1% vanillin in alcohol followed by hydrochloric acid gives pinkish red color suggesting presence of condensed tannins (flavan 3-ols)

(j) Matchstick's test (Shah & Quadry, 1995)
A wood portion of matchstick was dipped into the aqueous extract, dried and it was moistened with HCl. The matchstick was warmed near to the flame. Pink stain development on wood indicates presence of condensed tannins.

(k) Reaction with p-toluene sulphonic acid (Roux, 1957)
When tested on paper using P-toluene sulphonic acid followed by heating, brown spots development indicates presence of flavan 3-ols (Flavan 3,4-diols are detected as rose pink spots).

6.3. PHARMACOLOGICAL STUDIES

6.3.1 In-vitro anti-oxidant activity

6.3.1.1 Antiradical Activity (Free radical scavenger activity) (Navarro et al., 1993)

Antiradical activity was measured by a method based on the reduction of a methanolic solution of the colored free radical DPPH (1, 1 - diphenyl - 2-picrylhydrazyl). A stock solution of DPPH (4.3 mg/ 3.3 ml methanol) was prepared so that 75 µl of it in 3 ml of methanol gave an
initial absorbance of 0.9. The activity was expressed as effective concentrations at 50% (EC50) i.e. decrease in absorbance compared to that of a blank solution. For results, linear regression was applied to the data values between 10 and 80%. Ascorbic acid was used as a standard.

6.3.1.2 Superoxide Radical Scavenging Activity (Beauchamp & Fridovich, 1971)

The assay was based on the capacity of the plant extracts to inhibit Nitro blue tetrazolium (NBT) up to 50% in presence of riboflavin-light-NBT system. The reaction medium contained 50 mM phosphate buffer pH 7.6, 20 μg riboflavin, 12 mM EDTA, NBT 0.1 mg and different concentrations of test solutions with a final volume of 3 ml. The reaction was initiated by illuminating the sample cuvettes at regular intervals of 30 sec and increase in absorbance was measured at 590 nm up to 2.5 min. The superoxide radical scavenging activity was calculated using the formula:

$$\%\text{ Inhibition of superoxide radical} = \frac{\text{OD (control) - OD (extract)}}{\text{OD (control)}} \times 100$$

6.3.1.3 Erythrocyte membrane stabilization (Navarro et al., 1993)

Blood from healthy donors was collected in heparinized tube. Erythrocytes were separated by centrifugation from plasma and buffy coat; then washed three times with saline. During the last washings, the cells were centrifuged at 2000 g for 10 min to obtain a constantly packed cell preparation. Finally at 10% suspension of erythrocytes in isosaline (0.95% w/v NaCl, pH 7.2) solution was prepared. Haemolysis was induced with superoxide radical by a riboflavin-light-NBT system.
as described earlier. The reaction mixture contained the extract (100 μg/ml of reaction medium), 1 ml phosphate buffer (0.15 M, PH 7.4) and 2 ml of distilled water and 0.5 ml of erythrocyte suspension. The assay mixture was incubated at 50°C for 30 min and centrifuged at 1000 g for 10 min. The hemoglobin content in the supernatant solution was estimated by measuring the optical density at 560 nm. The percentage haemolysis was calculated according to the equation described by Miki et al (1986).

\[
\text{O.D. of treated sample} \\
\% \text{Haemolysis} = \frac{\text{O.D. of treated sample}}{\text{O.D. of control}} \times 100
\]

6.3.1.4 Assay for nitric oxide scavenging activity (Marcocci et al., 1994)

The assay is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent (Marcocci et al., 1994). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline was mixed with different concentration of methanolic extract dissolved in methanol and incubated at room temperature for 150 min. The same reaction mixture without the sample but with equivalent amount of solvent served as control. After the incubation period, 0.5 ml of Greiss reagent (1 % sulphanilamide, 2 % H₃PO₄ and 0.1 % naphthylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was recorded at 546 nm.
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\[
\text{% Nitric oxide scavenging activity} = \frac{\text{OD (control)} - \text{OD (extract)}}{\text{OD (control)}} \times 100
\]

6.3.1.5 **In-vitro hydroxyl radical scavenging potential of the test extract** (*Klein et al., 1981*)

The formaldehyde formed during the oxidation of the dimethyl sulphoxide by the Fe$^{3+}$-ascorbic acid system was used to detect hydroxyl radicals (*Klein et al. 1981*). The reaction mixture contained 60 μl EDTA (0.2 mM), 20 μl Fe$^{3+}$ (0.1 mM) (as a 1:2 with EDTA), 167 μl DMSO (33 mM) in 1.5 ml phosphate buffer (50 mM, PH 7.4). The test extract was added at three different concentrations (10, 100 and 1000 μg/ml of the final volume) separately. The reaction was started by the addition of 100 μl ascorbic acid (2 mM). The mixture was incubated at 37°C for 30 min and then the reaction was stopped by the addition of 125 μl trichloroacetic acid (17.5% w/v). The formaldehyde formed was assayed spectro-photometrically by the method of Nash (1953).

6.3.1.6 **In-vitro lipid peroxidation** (*Buege & Aust, 1978*)

Rat liver homogenate (10% w/v) was prepared according to the method of Ohkawa et al., 1979. Briefly, 0.2 ml suspension of fresh liver (pH 7.5), 0.1 ml of 0.15 M KCL and 2 μM adenosine diphosphate with different concentration of the test extract (10 μg/ml, 100 μg/ml, 1000 μg/ml of final volume) separately taken in different test tubes. After 5 min, 0.1 ml each of 10 μM FeSO$_4$ and 0.1 μM ascorbic acid were added and incubated at 37°C for 1 h. The reaction was terminated by addition of 2 ml of thiobarbituric acid reagent. Boiled for 15 min at 95°C, cooled, centrifuged and absorbance was read at 535 nm.
6.3.2 CNS activity

Animals
Swiss albino mice of either sex, weighing between 30-40 g were taken. Animals were fed with standard chow diet. All animals were kept under a controlled light / dark cycle and temperature (22 ± 2 °C) with food and water ad libitum. Animals were divided into different groups. Coprophagy was prevented by fasting the animals in cages with grating on the floor. Throughout the experiment, the animal house was maintained in the same identical conditions. This experiment complied with the guidelines of our laboratory for animal experimentation and conducted after permission of IAEC.

6.3.2.1 Hypnotic Potentiation (Gaitonde et al., 1980)
Five groups of mice were taken containing six animals in each group.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.

Group II: Test-1: Animals received methanolic extract (300 mg/kg, p.o.).

Group III: Test-2: Animals received methanolic extract (1000 mg/kg, p.o.).

Group IV: Standard-1: Animals received amphetamine (1 mg/kg, p.o.) as a reference standard drug.

Group V: Standard-2: Animals received lorazepam (1 mg/kg, p.o.) as a reference standard drug.

One hour after the administration of the test drug, pentobarbitone (45 mg/kg, i.p.) was injected in all the animals. The duration of sleep was measured as the duration between loss and regaining of righting reflex. Effect on onset of latency of sleep, duration of sleep and mortality, if any, were noted down.
6.3.2.2 Antidepressant activity

6.3.2.2.1. Behavioral despair test in Mice (Porsolt et al., 1977)
Three groups of mice were taken containing six animals in each group.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.
Group II: Test-1: Animals received methanolic extract (300 mg/kg, p.o.).
Group III: Test-2: Animals received methanolic extract (1000 mg/kg, p.o.).

Each mouse was put into a glass cylinder, of 20 cm height and 9 cm in diameter, filled up to 16 cm with water. Observations were made for 6 minutes after 1 hour of drug administration. First two minutes were not considered for reading and were taken as stabilizing time. The limb movements and the effort of the mice to get out of the cylinder in the next 4 minutes was noted and subtracted later from total time (4 min) to find the time of immobility. This was considered as an index of depression.

6.3.2.3 Anxiolytic activity
6.3.2.3.1. Open field Behaviour (Bhattacharya et al., 1993)
Three groups of mice were taken containing six animals in each group.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.
Group II: Test-1: Animals received methanolic extract (300 mg/kg, p.o.).
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Group III: Test-2: Animals received methanolic extract (1000 mg/kg, p.o.).

This test was carried out in mice using the open field apparatus, which is a square box of 96 X 96 cm and about 15 cm high sidewall. The floor is divided into 16 equal squares. It was kept in a dimly lit and quite area during the experiment. Each mouse was placed at the same corner an hour after drug administration and allowed to explore the area for 5 minutes. The following parameters were noted:

- The number of rearing.
- Number of squares crossed.

6.3.2.3.2. Elevated Plus Maze Test (Lister R. G. 1987)

Three groups of mice were taken containing six animals in each group.

Group I: Control: The animals received only aqueous suspension of 1% w/v Sodium CMC as a vehicle.
Group II: Test: Animals received methanolic extract (300 mg/kg, p.o.).
Group III: Standard: Animals received lorazepam (1 mg/kg, p.o.) as a reference standard drug.

The apparatus used was a wooden 'plus' shaped maze with two open arms and two closed tunnel like arms 25 X 5 cm and 36 cm high for mice and 50 X 10 cm and 40 cm high for rats. The maze elevated from the floor (25 cm for mice and 50 cm for rats). Test drugs were administered to the mice and after an hour they were placed individually on the centre square of the maze. For the next five minutes, the number of entries the animal makes to both open and closed arms and time spent there; the latency of first entry into the
open tunnel and the entry preference were recorded. Each animal was used only once and the experiment was carried out at a fixed time of the day.

6.3.2.4 Test for muscle tone and balance by using Rotating Rod

(Janssen, 1960a; Dunham and Mija, 1957)

Three groups of mice were taken containing six animals in each group.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.

Group II: Test: Animals received methanolic extract (300 mg/kg, p.o.).

Group III: Standard: Animals received lorazepam (1 mg/kg, p.o.) as a reference standard drug.

In this method the albino mice of either sex were used. Untreated mice were placed on a horizontal rotating iron rod having a diameter of 32 mm and rotating at the rate of 24 revolutions per minute. Animals that remain on the rod for 2 or more minutes in four trials, carried out in two days divided into morning and evening two sessions, were selected and divided into different groups. Vehicle and test drugs were administered by oral route and placed on rod at intervals for 4 hours after the administration. The following parameters were noted down.

(i) Time of fall (in seconds)
(ii) % Decrease in time of rotation
6.3.2.5 Anticonvulsant activity

Supramaximal Electric Shock induced convulsions (Goodmen et al., 1953)

Four groups of rats were taken containing six animals in each group.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.

Group II: Test-1: Animals received methanolic extract (300 mg/kg, p.o.).

Group III: Test-2: Animals received methanolic extract (1000 mg/kg, p.o.).

Group IV: Standard: Animals received phenytoin (100 mg/kg, p.o.) as a reference standard drug.

The test was carried out in four groups of rats. They were first tested for sensitivity to electric shock of 150 mA. Only those rats, which exhibited full seizure components, were used for further experiment. The selected rats were given the electric shocks through corneal electrode wetted with normal saline. The current strength was 180 mA for 0.2 sec, delivered using INSIF electro convulsometer. On next day again the drugs were given to all animals according to their groups and then after five hour, the shock of same intensity and duration stated above was given.

The following parameters were recorded.

- Tonic flexion
- Tonic Extension
- Clonic seizure
- Regaining of righting reflex.
- Mortality, if any
The electric shock may produce the threshold discharge of neurotransmitters in the brain areas (Exact site unknown), which may lead to tonic and clonic convulsions.

6.3.2.6 **Antipsychotic activities**

6.3.2.6.1 *D-amphetamine induced stereotypy in mice (Valame & Gupta 1981)*

Three groups of mice were taken containing six animals in each group.

Group I: Control: The animals received only aqueous suspension of 1% w/v Sodium CMC as a vehicle.

Group II: Test-1: Animals received methanolic extract (300 mg/kg, p.o.).

Group III: Test-2: Animals received methanolic extract (1000 mg/kg, p.o.).

The test drugs were administered one hour prior to d-amphetamine injection (5 mg/kg, i.p.) and stereotype behavior was assessed at time intervals of 20 minutes, 40 minutes, 60 minutes, 120 minutes, 180 minutes, and 240 minutes after the injection of d-amphetamine. A score of 0 to 3 was given according to the intensity of stereotypy.

The following behavioral patterns were noted:

- Rearing
- Licking
- Grooming
- Sneezing and sniffing
- Gnawing
- Any other particular behavior
6.3.2.6.2 Condition Avoidance Response test (Cook’s pole Climbing) (Piala et al., 1959)

Three groups of rats were taken containing six animals in each group.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.

Group II: Test: Animals received methanolic extract (300 mg/kg, p.o.).

Group III: Standard: Animals received chlorpromazine (1 mg/kg, p.o.) as a reference standard drug.

In this method the stainless steel grid floor in a wooden box was the source of electric shock to rats which can escape the noxious stimulus by climbing a centrally located wooden pole. The stimulus is approximately 0.1 mA of 60-cycle altering current at 40 volts for 2.5 sec duration. The conditioning stimulus was a buzzer attached to the chamber.

The rats were trained for 7 days with two sessions i.e. morning and evening sessions. Each session consisted of three shock treatments that consist of buzzer for 1 sec duration followed by three consecutive shocks for duration of 2.5 seconds each. The rats were trained to climb a pole after the conditional stimulus (Buzzer).

The trained albino rats of either sex weighing 150 to 200g were given the vehicle and drug by oral route according to their grouping. At every one hour intervals and thereafter for 6 hours, the response of each animal to the buzzer was determined by three successive trails. The inhibition of the conditioned response is considered as a measure of the tranquilization.
6.3.2.7 Evaluation for Nootropic effect (Memory enhancing) (Kulkarni, 2003)

Four groups of mice were taken containing six animals in each group.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.

Group II: Test-1: Animals received methanolic extract (300 mg/kg, p.o.).

Group III: Test-2: Animals received methanolic extract (1000 mg/kg, p.o.).

Group IV: Standard: Animals received piracetam (150 mg/kg, p.o.) as a reference standard drug.

In this test, the effect of test drugs on transfer latency that is the time elapsed between the time at which the animal is placed in the open arm and the time at which all its legs have crossed the line into the enclosed arm. This test was carried out using an elevated plus maze for mice. The detail of instrument set up was already provided above under anxiolytic activity evaluation. However, there was a change in procedure. In the anti-anxiety test mice were initially placed in the center square and the latency of entry into the closed tunnel was noted but in this test the mice were kept at the end of the open tunnel and the time taken by the animal to enter the closed tunnel was noted and considered as transfer latency.

Here three different protocols related to memory and learning were assessed.

(a) Acquisition (learning)
(b) Retention (memory)
(c) Drug discrimination model (learning and memory)
The total experimental procedure was carried out for three consecutive days. Daily the drugs and vehicle were administered to the animals according to the groups, one hour prior to exposure to elevated plus maze. The fresh animals were selected for this experiment.

On the first day, the animals were placed individually at the end of one of the open arms. If the animals do not enter an enclosed arm within 180 seconds, it was pushed on the back into one enclosed arm and the transfer latency was given as 180 seconds. The animal can be allowed to move freely to explore the apparatus for some time. The transfer latency was again recorded 24 hour after the first exposure (on 2nd day). If again animal does not enter an enclosed arm within 180 seconds, it was pushed on the back into one enclosed arm and transfer latency was given as 180 seconds. The transfer latency on the first day trial served as acquisition (learning) and the retention consolidation (memory) was examined on 2nd day, 24 hour after fist exposure.

Further on third, day, clozapine (an amnestic agent) (5 mg/kg, i.p.) was administered, one hour after drug administration, then after 1 hour the animals were exposed to the elevated plus maze and the transfer latency was recorded. The difference between this transfer latency and previous day's transfer latency may serve as the "index of cognition."

6.3.3 Anti-ulcer activity

**Animals**

Wistar albino rats of either sex, weighing between 150-250 g were taken. Animals were fed with standard chow diet. All animals were kept under a controlled light / dark cycle and temperature (22 ± 2 °C) with food and water ad libitum. Animals were divided into different
groups. Coprophagy was prevented by fasting the animals in cages with grating on the floor. Throughout the experiment, the animal house was maintained in the same identical conditions. This experiment complied with the guidelines of our laboratory for animal experimentation and conducted after permission of IAEC.

**Drug Treatments**

The animals were divided into following groups of six animals each.

Group I: Control: Animals received only aqueous suspension of 1% w/v sodium CMC as vehicle.

Group II: Test-1: Animals received petroleum ether extract (300 mg/kg, p.o.).

Group III: Test-2: Animals received methanolic extract (300 mg/kg, p.o.).

Group IV: Standard: Animals received omeprazole as a reference standard drug (20 mg/kg, p.o.).

**6.3.3.1 Ethanol-induced gastric mucosal damage (Robert et al, 1979)**

In this model, ethyl acetate extract (300 mg/kg, p.o.) as well as aqueous extract (300 mg/kg, p.o.) were also tested.

Animals were fasted for 24h before an experiment but were allowed free access to water. 1 ml of absolute ethanol was administered orally to rats. In treatment group, drug was administered orally 1h before the administration of ethanol. After 2h of ethanol treatment, animals were sacrificed: stomachs were removed, opened along the greater curvature and examined for lesion severity. Lesion severity was determined by measuring ulcer index (Goswami et al., 1997).
Ulcer Index (UI):
Each lesion of the stomach was measured along its greatest length and breath. For circular lesions, diameter was measured and finally area was calculated. In case of petechies, five of them considered to be equivalent to 1 mm\(^2\) of ulcerated area. The total area of the stomach mucosa and that of ulcerated mucosa were calculated.

$$\text{Ulcer Index} = \frac{10}{X}$$
Where, \(X = \text{Total mucosal area}/\text{Total ulcerated area}\)

The mean ulcer index values obtained for each group were compared with standard treatment group. ED\(_{50}\) was calculated on the basis of the reduction in this index.

**6.3.3.2 Pylorus ligated (Shay) ulcer model (Shay et al, 1945)**

Rats were anaesthetized with light ether anesthesia. The portion of abdomen was opened by a small incision below the xiphoid process. Pyloric portion of the stomach was slightly lifted out avoiding traction to the pylorus or damage to its blood supply. The stomach was replaced carefully and the abdominal wall was closed by interrupted sutures. The drug was administered subcutaneously immediately after pyloric ligation. The animals were deprived of both food and water during the postoperative period and were sacrificed at the end of 19 h after the operation. Stomach was dissected out, contents were drained into tubes and subjected to analysis for pH and free and total acidity. The stomach was then cut open along the greater curvature and the inner surface was examined for ulceration.

The parameters studied in this model includes,

(i) Physical parameters
(1) Ulcer index (Goswami et al., 1997)
(2) Volume of gastric secretion
(3) pH of gastric juice

(II) Biochemical parameters

(a) Acid secretory parameters

(1) Total acidity (Hawk et al., 1965)
(2) Total acid output (Goel et al., 1985)
(3) Pepsin activity (Debnath et al., 1974)

(b) Mucoprotective parameters

(1) Total carbohydrates (TC) (Nair et al., 1976)
(2) Protein content (PR) (Lowry et al., 1951)
(3) Mucin activity (TC : PR ratio)
(4) Vascular Permeability (Szabo et al., 1985)

6.3.3.3 Cold and restraint stress (CRS) - induced gastric ulcer model (Vincent et al, 1977) (Hypothermic restraint ulcer model)

In this method, wistar rats were deprived of food for 12 h. They were then immobilized in a restrainer (stress cage) and forced to remain in refrigerator (4° - 6 °C) for 3 h. The animals were sacrificed by a blow on the head and ulcer-index was calculated (Goswami et al., 1997).

The test drug was administered 30 minutes before immobilizing the animals. In this model additionally free radical levels were also tested. Following tests were performed:

(1) Measurement of lipid-peroxidation (Kiso et al., 1979)
(2) Superoxide dismutase (Misra et al., 1972)
(3) Catalase (Aebi, 1974)
6.3.3.4 Ethanol-induced gastric mucosal damage in indomethacin pretreated rats (Robert, 1981)

Indomethacin was used to block the formation of prostaglandins by inhibition of cyclooxygenase. Animals received indomethacin (10 mg/kg, s.c.) thirty minutes before the administration of the methanolic and petroleum ether extract (300 mg/kg, p.o.). After thirty minutes, the animals were given 1 ml of 50% ethanol orally and were sacrificed after two hour of ethanol administration. Gastric lesions were measured and ulcer index was calculated (Goswami et al., 1997).

6.3.3.5 Ethanol-induced gastric mucosal damage in N-ethyl maleimide (NEM) pretreated rats (Arrieta et al., 2003)

N-ethyl maleimide (NEM) was used to block the sulfhydryl group, which is one of the defensive parameter. Animals received NEM (10 mg/kg, s.c.) thirty minutes before the administration of the methanolic and petroleum ether extract (300 mg/kg, p.o.). After thirty minutes, the animals were given 1 ml of 95% ethanol orally and were sacrificed after two hour of ethanol administration. Gastric lesions were measured and ulcer index was calculated.

6.3.3.6. Ethanol-induced gastric mucosal damage in L-NAME (N-nitro-L-arginine methyl ester) pretreated rats (Arrieta et al., 2003)

L-NAME (N-nitro-L-arginine methyl ester) was used to block the formation of nitric oxide by inhibition of nitric-oxide-synthetase enzyme. Animals received L-NAME (70 mg/kg, s.c.) thirty minutes before the administration of the methanolic and petroleum ether extract (300 mg/kg, p.o.). After thirty minutes, the animals were given 1 ml of 95% ethanol orally and were sacrificed after two hour of ethanol administration. Gastric lesions were measured and ulcer index was calculated.
6.3.4 Anti-inflammatory activity

6.3.4.1 Carrageenan-induced paw edema in rats (Winter et al., 1962)

Thirty six wistar rats of either sex were selected and randomly divided into six groups.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.

Group II to V: Treatments: Animals received following treatments:
- Petroleum ether extract, Methanolic extract, Ethyl acetate extract and Aqueous extracts (300 mg/kg, p.o.).

Group VI: Standard: Animals received Valdecoxib as a reference standard drug (5 mg/kg, p.o.).

After 1 h of drug administration, 0.05 ml of 1% w/v carrageenan (Sigma Chemical Company, St. Louis, MO, USA) suspension was injected sub-planterly to the left hind paw as described by Winter et al. (1962). The volume of the injected paw of each of these rats was measured using a plethysmometer at 1 h prior to the injection of carrageenan and 1, 2, 3, 4, and 5 h after the injection. The average foot swelling in test as well as standard groups was compared with that of control and the percentage inhibition of edema was determined, as shown under

\[
\% \text{ Inhibition} = \left[1 - \left(\frac{V_d - V_p}{V_c - V_p}\right)\right] \times 100
\]

Where,

\[V_d - V_p = \text{Difference in paw volume after carrageenan and initial paw volume for drug treated animals.}\]

\[V_c - V_p = \text{Difference in paw volume after carrageenan and initial paw volume for control animals.}\]
6.3.4.2 Histamine-induced paw oedema in mice

Twenty four swiss mice of either sex were selected and randomly divided into four groups.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.
Group II: Test-1: Animals received petroleum ether extract (300 mg/kg, p.o.).
Group III: Test-2: Animals received methanolic extract (300 mg/kg, p.o.).
Group IV: Standard: Animals received cetrizine (20 mg/kg, p.o.) as a reference standard drug.

After 1 h of drug administration, Histamine (0.1 % w/v in normal saline) was injected sub-planterly into the left hind paw of each mice at a dose of 0.05 ml to induce oedema. The paw volume was measured at 0 min, 10 min, 20 min, 30 min and 40 min respectively. The anti inflammatory effect was expressed as percent inhibition of edema (As above formula).

6.3.4.3 Cotton pellet-induced granuloma formation in rats (Winter et al., 1957; Goldstein et al., 1976)

Twenty four wistar rats of either sex were selected and randomly divided into four groups.

Group I: Control group: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle for seven days.
Group II: Test-1: Animals received petroleum ether extract (300 mg/kg, p.o.).
Group III: Test-2: Animals received methanolic extract (300 mg/kg, p.o.).
Group IV: Standard: Animals received Diclofenac sodium (5 mg/kg, p.o.) as a reference standard drug for seven days.

Autoclaved cotton pellets (50±1 mg), soaked in 0.2 ml of distilled water containing ciprofloxacin, were implanted subcutaneously, one on each side above the scapula region, under ether anesthesia using aseptic precautions. Drugs or vehicle were administrated orally for 7 consecutive days starting from the day of surgery. On day 7th, animals were killed and the pellets along with granuloma were removed and dried in oven in 60°C until a constant weight was obtained. The weight of cotton pellet before implantation was subtracted from the weight of the dried dissected pellets. The mean weight was calculated for pellets from the groups of rats receiving drugs and compared with the mean values for the control.

6.3.5 Analgesic activity

6.3.5.1 Acetic acid-induced writhing test in mice (Koster et al., 1959; Turner, 1965)

Twenty four swiss mice of either sex were selected and randomly divided into four groups.

Group I: Control: The animals received only aqueous suspension of 1% w/v Sodium CMC as a vehicle.

Group II: Test-1: Animals received Petroleum ether extract (300 mg/kg, p.o.).

Group III: Test-2: Animals received Methanolic extract (300 mg/kg, p.o.).

Group IV: Standard: Animals received Aspirin (25 mg/kg, p.o.) as a reference standard drug.
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Writhing was induced 1 h after drug administration by intraperitoneal injection of 10 ml/kg of 0.6% acetic acid in distilled water. The number of writhes was counted for 30 min immediately after the acetic acid injection. The percentage inhibition was calculated.

\[
\text{% Inhibition} = \frac{(Wc - Wd)/Wc} \times 100
\]

Where,

- \(Wd\) = No. of writhing in drug treatment group
- \(Wc\) = No. of writhing in control group

6.3.5.2 Formalin -induced Licking (Shibata et al., 1989)

Twenty four swiss mice of either sex were selected and randomly divided into four groups.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.

Group II: Test-1: Animals received petroleum ether extract (300 mg/kg, p.o.).

Group III: Test-2: Animals received methanolic extract (300 mg/kg, p.o.).

Group IV: Standard: Animals received Aspirin (25 mg/kg, p.o.) as a reference standard drug.

The nociceptive response was induced by injecting 0.05 ml of formalin (1%v/v) subcutaneously into the plantar surface of one rear paw using a 30 G needle. Animals were pretreated orally with the extracts one hour prior to the formalin challenge. Control animals received only the vehicle. Pain response was observed as paw licking from 0 to 40 min after the challenge. The results were expressed as percentage inhibition of the pain responses (number of paw lickings in the duration of test) when compared with the control group.
% Inhibition = \[(\text{Lc} - \text{Ld})/\text{Lc}\] × 100

Where,

\( \text{Ld} = \text{No. of licking in drug treatment group} \)
\( \text{Lc} = \text{No. of licking in control group} \)

6.3.5.3 Tail flick method (D' Amour & Smith, 1941)

The prescreened animals (reaction time: 3-4 sec) were divided into following four groups containing six animals in each.

Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.

Group II: Test-1: Animals received petroleum ether extract (300 mg/kg, p.o.).

Group III: Test-2: Animals received methanolic extract (300 mg/kg, p.o.).

Group IV: Standard: Animals received pentazocin (10 mg/kg, i.p.) as a reference standard drug.

The tail flick latency was assessed by the analgesiometer (Inco, India). The strength of the current passing through the naked nichrome wire was kept constant at 6 Amps. The time taken for the withdrawal of the tail after switching on the current, was taken as the latent period, in sec, of "tail flicking" response and was considered as the index of nociception. The cut-off reaction was fixed at 10 sec to avoid tissue damage. Three tail flick latencies were measured per rat at each time interval and the means of the tail-flick latencies were used for statistical analysis.
6.3.6 Immunomodulatory activity

ANTIGEN
Sheep erythrocytes (SRBC) collected in Alsever's Solution, were washed three times in large volumes of pyrogen free 0.9% Normal Saline and adjusted to a concentration of 0.5x10⁹ cells/ml for immunization and challenge.

6.3.6.1 Haemagglutinating antibody (H. A.) titre (Tiwari et al., 2004)
Eighteen wistar rats of either sex were selected and randomly divided into three groups.
Group I: Control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle.
Group II: Test-1: Animals received methanolic extract (100 mg/kg, p.o.).
Group III: Test-2: Animals received methanolic extract (300 mg/kg, p.o.).
Group IV: Test-3: Animals received petroleum ether extract (100 mg/kg, p.o.).

Drugs were administered on the day 0 and administration was continued once daily up to seven days. On day 7, all the rats were immunized with SRBC (0.5 X 10⁹ cells/ml/100g) intraperitoneally. Five days later, on 13th day, blood samples were collected from the retro-orbital plexus and antibody level was determined by haemagglutination techniques (Puri et al., 1994).

The H. A. titre was determined on sera obtained from all three groups of immunized animals. In brief, a twofold dilution (Range: 1 in 2 to 1 in 2048) of sera was prepared by adding 25 μl of normal saline in
**Materials & methods**

Microtitre plates. A 25 μl of a fresh 1% SRBC suspension in normal saline was dispensed into each well and mixed thoroughly. After mixing, the plates were incubated at 37°C for 1 h and examined for haemagglutination. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

6.3.6.2 Delayed type Hypersensitivity (DTH) response (Mitra et al., 1999)

In addition to above procedure, on 13th day, three groups of SRBC immunized rats were injected 0.2 ml of 2 x 10^7 SRBC in right hind foot pad. Normal saline was similarly injected into left hind foot pad as control. After 24h, the DTH response was measured using vernier calipers, in terms of increase in thickness of footpad due to swelling caused by the hypersensitivity reaction. The foot pad reaction was expressed as the difference in thickness (mm) between the right foot pad injected with SRBC and the left injected normal saline.

6.3.6.3 Haematological Profile (Davis & Kuttan, 2000)

Thirty swiss mice of either sex were selected and randomly divided into five groups.

Group I: Control: The animals received only aqueous suspension of 1% w/v Sodium CMC as a vehicle.

Group II & III: Test-1 & 2: Animals received Methanolic extract (100 mg/kg and 300 mg/kg, p.o.) for 15 days.

Group IV & V: Test-3 & 4: Animals received petroleum ether extract (100 mg/kg and 300 mg/kg, p.o.) for 15 days.
After 15 days of administration of the extracts blood was collected from the retro orbital plexus of each animal. The hematological parameters such as i) Haemoglobin content ii) haematocrit value iii) Leukocytes and iv) Erythrocytes were determined by using ERMA-PC-607 cell counter.

### 6.3.6.4 Cyclophosphamide induced Myelo-suppression in Mice (Bafna & Mishra, 2004)

Thirty swiss mice of either sex were selected and randomly divided into three groups.

**Group I:** Normal control: The animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle for 13 days.

**Group II:** Control: Animals received only aqueous suspension of 1% w/v sodium CMC as a vehicle for 13 days & exposure to cyclophosphamide.

**Group III & IV: Test1 & 2:** Animals received methanolic extract (100 mg/kg and 300 mg/kg, p.o.) for 13 days.

On days 11, 12, 13 all the animals, expected in group I, were injected with cyclophosphamide (30 mg/kg, i.p.) 1 h after administration of the extracts. Blood was collected from retro orbital plexus of each animal on day 14 and erythrocytes, haematocrit, Mean Corpuscles Volume, Leukocytes and Haemoglobin values were determined by using ERMA-PC-607 cell counter.