CHAPTER-3
3. MATERIAL AND METHODS

3.1 Plant material

The aerial parts of *Calotropis gigantea* was collected around Jhansi city in 2001 from wild. A voucher specimen has been deposited at the Institute of Pharmacy, Bundelkhand University, Jhansi, Uttar Pradesh, India. The aerial part were dried under shade, each dehydrated plant powdered to a fine texture and 100 g of the dried plant was repeatedly extracted with water:ethanol (50:50). The extract was concentrated under vacuum and the residue was used in the experiments. The dried plant extracts were freshly dissolved in normal saline just before administration. Unless otherwise stated, henceforth, the term ‘extract’ means the water:ethanol (50:50) extract of aerial part of *Calotropis gigantea*.

3.2 Animals

Albino swiss rats of either sex weighing 150-180 g were used for tail-flick method using analgesiometer, carrageenin-induced rat paw oedema, cotton pellet granuloma, Brewer’s yeast-induced pyrexia, antidiarrheal, stress-induced gastric ulcer, pyloric ligation-induced gastric ulcer and chronic toxicity studies. Male swiss albino mice weighing 18-24 g were used for acute toxicity, Eddy’s hot plate, Xylene-induced ear oedema, and acetic acid-induced writhing study. White rabbits of body weight 1.5-2.0 kg were used for TAB vaccine-induced pyrexia. All animals were fed standard animal feed and tap water *ad libitum* before the experiments. Each experimental group consisted of six animals housed in separate cages. All the experiments were carried out with the consent of Institutional Animals Ethics Committee of the Institute (Approved by CPCSEA, Chennai, India Reg. No. 716/02/a/CPCSEA)
3.3 Anti-inflammatory activity

3.3.1 Xylene-induced ear oedema in mice

Male Swiss albino mice of body weight 18-24 g divided into five groups of six each. The extract under testing was administered intraperitoneally 30 min prior to the application of xylene (0.03ml) to the anterior and posterior surfaces of the right ear. Mice were sacrificed 2 h after xylene application and both ears were removed. The circular section of both treated and untreated ears were taken using a 7 mm diameter cork bored and weighed. The oedematous response was measured as weight difference between the two plugs and the anti-inflammatory activity was expressed as percentage of oedema reduction in treated mice with regard to control mice. All experiments were uniformly started between 11:00 and 14:00 h in order to avoid variations in inflammatory response due to circadian fluctuations in the levels of corticosteroids (Tang et al., 1984).

3.3.2 Carrageenin-induced pedal oedema in rats

Carrageenin oedema was induced by injecting 0.1 ml of freshly prepared suspension of carrageenin in normal saline into the sub-plantar tissue of the right hind paw of either sex albino rats weighing 150-180 g. Drugs (or vehical, standard) were administered intraperitoneally 30 min before Carrageenin administration. The paw volume was measured five times at 1 h intervals after injection (Winter et al., 1962) by the volume displacement of a water-mercury column using a plethysmometer (Bhatt et al., 1977). The anti-inflammatory activity in animals that received extract was compared with that in the diclofenac sodium and control groups.

3.3.3 Cotton pellet-induced granuloma in rats

The effect of the extract was examined using the cotton-pellet granuloma method (Sciatti and Toja, 1986). Pellets weighing approximately 20 mg each were made with 5 mm of dental cotton tampon. The pellets were sterilized with an autoclave at 100 C for 30
min and then impregnated with 0.4 ml 5% ampicillin aqueous solution at the moment of implantation. Under pentobarbitone sodium anaesthesia (30 mg/kg ip), the pellets were introduced subcutaneously (sc) through skin incisions at the groin region of the rats, one on each side. The daily intraperitoneal administration of normal saline, 100, 200 and 400 mg/kg of extract and 5 mg/kg of diclofenac sodium as reference standard were started 3 h after cotton pellet implants and continued for 7 days. On the 8th day, the animals were killed with ether overdose and the granulomas were removed and weighed. The difference between the initial weight of cotton pellet and final weight of the granuloma and the cotton were considered to be the weight of granulomatous tissue produced.

3.4 Analgesic activity

3.4.1 Hot-plate test in mice

The method described by Eddy and Leimback (1953) was applied. Groups of 6 mice each were used. Mice were placed in a 2 L glass beaker placed on a hot plate maintained at 55 °C. Latency to exhibit the nociceptive response such as licking paws or jumping was determined for a period of 5 h with an interval of 1 h, after 30 min of the ip injection of the extract. Aspirin was used as the reference standard. Cut-off time of 30 S was selected to avoid tissue damage.

3.4.2 Tail-flick latent period

The technique described by Davies and co-workers (1946) was adopted, using an analgesiometer. The rat was placed in a rat holder, with its tail coming out through a slot in the lid. The tail was kept on the bridge of the analgesiometer, called jacket with an electrically heated nichrome wire underneath. The tail received radiant heat from the wire, heated by passing current of 6 mA. The time taken for the withdrawal of the tail after switching on the current, was taken as the latent period, in S of ‘tail-flicking’ response and was considered as the index of nociception. The cut-off time for
determination of latent period was taken as 30 S to avoid tissue injury (Bhattacharya, et al., 1971). The drugs to be tested (Extract and Aspirin) were given 30 min prior to the test.

3.4.3 Acetic acid-induced writhing response in mice

The acetic acid-induced abdominal constriction test was performed as described by Collier et al., (1968) and Fontenele et al., (1996). Acetic acid (0.7% v/v) was administered intraperitoneally in a volume of 0.1 ml/10g. Vehicle (saline), aspirin (150 mg/kg) and extract (100, 200 and 400 mg/kg) were administered intraperitoneally 30 min before acetic acid injection. The number of abdominal constrictions produced in each group for the succeeding 15 min was counted and compared to the response in the control group as described by Koster et al., (1959). Analgesia was calculated as the percentage inhibition of abdominal constriction. Vehicle-treated control group mean subtracted from treated group (Extract or Aspirin) mean and multiplied by 100.

3.5 Antipyretic

3.5.1 T.A.B. vaccine-induced pyrexia

The anti-pyretic activity was assessed by the method of T.A.B. vaccine-induced pyrexia (Saxena, 1979) with some modification. In this method the rabbits were divided into groups, each group was consists of 5 animals. Control group was treated with 2 ml/kg of saline. Other groups were considered as under test. The normal rectal temperature of a group of rabbits were considered as under test. The normal rectal temperature of a group of rabbits were recorded by telethermometer at hly intervals for a period of 4 h. T.A.B. vaccine was administered intravenously into the marginal ear vein of rabbit in a dose of 0.5 ml/rabbit. The temperature was recorded every 30 m until it approached the normal. The extract to be tested were administered 30 m prior to the administration of the T.A.B. vaccine.
3.5.2 Brewer's yeast-induced pyrexia in rats

Antipyretic property of *Calotropis gigantea* was tested in rats in which hyperthermia had been induced following the method of Teotino et al., (1963). Initial rectal temperatures of the rats were recorded using a six channel electric thermometer connected with probes. Rats were made hyperthermic by subcutaneous injection of 20% yeast suspension in 0.9% saline at a dose of 1 ml/100g body weight. When the temperature was at peak (18 h after yeast injection) the rectal temperature were again recorded. Those animals that showed a rise in rectal temperature of more than 1.2°C were used. Test substances and control vehicle were given intraperitoneally and the rectal temperature of animals were recorded at 1 h intervals for 4 h following the administration of drug or plant extract.

3.6 Gastroprotective activity

3.6.1 Pylorus ligation-induced gastric ulceration

Rats were fasted for 24 h, care being taken to avoid coprophagy. Under light-ether anaesthesia pylorus ligation was carried out. After 7 h of pylorous ligation rats were sacrificed with high dose of anaesthetic ether. The stomachs were then taken out and cut open along the greater curvature and gastric lesions were observed using a 6.4 binocular magnifier then ulcer index was determined (Ganguli and Bhatnagar, 1973). The gastric contents were collected, measured, centrifuged for 20 min at 1000 rpm and subjected to biochemical analysis. Total acid output, acid concentration, pH, total gastric content, were estimated. Drugs were administered 30 min prior to pylorous ligation.

Ulcer Index =\( \frac{10}{X} \) where,

\( X = \frac{\text{Total area of stomach mucosa}}{\text{Total ulcerated area}} \)

Gastric contents were assayed for total acidity by titration against 0.01 N NaOH to pH 8.0 using phenolphthalein as an indicator. The amount of HCl was calculated and
expressed as mEq/L (Hawk, 1965). The volume of the gastric content was measured and the total acid output was estimated. The pH was determined by using pH meter.

3.6.2 Stress-induced acute gastric lesion

In this study, restraint stress was utilized for the induction of gastric ulceration according to the method of Nagura (1972) modified by Basile et al., (1990). The experimental rats were fasted for 24 h with water ad libitum. The animals were randomly grouped into 5 groups of 6 rats per group. The first group received normal saline (2 ml/kg, ip), the second group received ranitidine (40 mg/kg, ip). The remaining three groups received doses of the extract (100, 200 and 400 mg/kg, ip) respectively. Thirty min later, the rats were then put in restraining cages and placed in the presence of intense light for 12 h. After this time the animals were sacrificed with ether. Their stomachs were excised and opened along the greater curvature, washed and stretched on cork plates and the inner surface was examined for the presence of lesions with a binocular stereomicroscope, magnification 10X.

The number of stress-induced acute lesions was counted for each animal, and the ulcer index was calculated as above.

3.7 Antidiarrheal activity

3.7.1 Castor oil-induced diarrhea

Rats were divided into five groups of six animals each, diarrhea was induced by administering 1 ml of castor oil orally to rats. Group 1 served as control (2 ml/kg, ip saline), group 2 received atropine (3 mg/kg, ip) served as standard and group 3, 4, and 5 received extract (100, 200 and 400 mg/kg, ip) 1 h before castor oil administration. The number of both wet and dry diarrheal droppings were counted every h for a period of 4 h mean of the stools passed by the treated groups were compared with that of the control (Awouters, et al., 1978).
3.7.2 Small intestinal transit

Rats were fasted for 18 h divided into six groups of six animals each. Group 1 received 2 ml normal saline orally, group 2 received 2 ml of castor oil orally with saline 2 ml/kg intraperitoneally, group 3 received atropine (3 mg/kg, ip), group 4, 5 and 6 received 100, 200 and 400 mg/kg intraperitoneally of the plant extract respectively. 1 h before administration of castor oil. One ml of marker (10% charcoal suspension in 5% gum acacia) was administered orally 1 h after castor oil treatment. The rats were sacrificed after 1h and the distance traveled by charcoal meal from the pylorus was measured and expressed as percentage of the total length of the intestine from the pylorus to caecum (Mascolo, et al., 1994).

3.7.3 Castor oil-induced enteropooling

Intraluminal fluid accumulation was determined by the method of Robert et al., (1976). Overnight fasted rats were divided into five groups of six animals each. Group 1 received normal saline intraperitoneally (2 ml/kg, ip) served as a control, group 2 received atropine (3 mg/kg, ip) and groups 3, 4 and 5 received the extract of 100, 200 and 400 mg/kg intraperitoneally respectively 1h before the oral administration of castor oil. Two h later the rats were sacrificed, the small intestine was removed after tying the ends with thread and weighed. The intestinal contents were collected by milking into a graduated tube and their volume was measured. The intestine was reweighed and the difference between full and empty intestines was calculated.

3.8 Toxicity study

3.8.1 Acute toxicity study (LD₅₀)

The intraperitoneal (ip) acute toxicity (LD₅₀) profile of the extract was evaluated in swiss albino mice according to the method of Lorke (1983) with some modification, briefly, this method was carried out in two steps; the initial investigation in which nine
animals were used, three animals per treatment group and widely differing dose ranges. 
1:10:100 mg/kg respectively of the extract per body weight administered and was 
observed for 24 h. Based on the results of the first step, the second step was initiated in 
which specific dose ranges of the extract was administered to four treatment groups of ten 
animals each. The (LD₅₀) was then calculated based on the pattern of death observed in 
the second step.

3.8.2 Chronic Toxicity

Adult healthy swiss albino rats of either sex of body weight 180-240 g were used 
in the present study. These animals were kept in polypropylene cages (five rats per cage) 
under identical animal house conditions and provided with pelleted 'Gold mohaur' rat 
feed (manufactured by Hindustan lever ltd. India), food and water were given ad libitum. 
The rats were equally divided into the following groups, i (control), the rats of this group, 
were fed standard rat feed with intraperitoneal administration of normal saline (2 ml/kg); 
group-ii (extract treated 100 mg/kg, ip); group iii (extract treated 200 mg/kg, ip); group 
III (extract treated 400 mg/kg, ip); the animals were administered intraperitoneally per 
day for 30 days.

Histopathology

At the end of 30 days of treatment, the animals were autopsied and the vital organ, viz. 
liver, kidney, heart lung and pancreas were excised, these organs were fixed in buffered 
10% formalin and 3 µm thick paraffin sections were stained with haematoxylin and eosin 
according to Lillie, (1965) and microscopic examinations were carried out.

Haematology

Blood samples collected out of 6 rats from each group were examined for 
haemoglobin concentration by using haemometer, the peripheral count of red blood cells
(RBCs), total white blood cells (WBCs) and differential count of WBCs were carried out according to the method of Bharucha et al (1976).

3.9 Statistical analysis

The experimental results are represented as mean ± SE (Standard error of the mean). Student's unpaired t-test was used for the evaluation of data and P<0.05 accepted as significant.