A. Plant collection and authentication

Clitoria ternatea (CT) is available in two varieties – blue flowered and white flowered. The blue flowered variety was used for the present investigation. The plant was collected during summer (April–May, 2007) from the fields and road side of the Charotar region of the Gujarat state, India. The plant was botanically identified by Dr. G. C. Jadeja, Professor and Head, Department of Agricultural Botany, B. A. College of Agriculture, Anand, India. The specimens of the sample were stored in the Department of Pharmacology, L. M. College of Pharmacy, Ahmedabad.

B. Primary quality standards

The quality of seeds and roots was ascertained as specified in the Ayurvedic Pharmacopoeia of India by determining foreign matters, total ash, acid insoluble ash, alcohol soluble extractive and water soluble extractive values (Anonymous, 2003).

C. Preparation of extracts

The dried powdered (mesh # 40) seeds of plant (2 kg) were defatted with petroleum ether and then extracted successively with Ethyl acetate, acetone, and with 50% v/v alcohol and finally marc was acid hydrolyzed and extracted with 50% v/v ethanol. The solvents were evaporated at 60°C to have pasty mass. The CT roots (1 kg) were directly extracted with 50% v/v alcohol by maceration and evaporated to have hydroalcoholic extract of roots. The 50 % v/v alcohol extract of seed and root were referred as hydroalcoholic extracts or simply CT seed and root extracts respectively.

D. Phytochemical analysis

The hydroalcoholic extracts of seed and root were used for the preliminary phytochemical screening, estimation of total phenolics and total flavonoids, and fingerprinting.
1. **Preliminary phytochemical screening**

   The hydroalcoholic extracts of seed and root were subjected to preliminary phytochemical screening following the methodology of Kokate (2003).

2. **Estimation of total phenolic compounds**

   The amount of total phenolics in the extracts was determined by the method of Rathee *et al.* (2006). Each test extract (100 μL) was mixed with 1:10 Folin–Ciocalteau’s reagent (500 μL) followed by addition of aqueous Na₂CO₃ (400 μL, 7.5%). After incubating the reaction mixture at 24°C for 2 h, the absorbance at 765 nm was recorded. Gallic acid monohydrate was used as the standard. The total phenolic contents of CT seed and root were expressed in terms of mg gallic acid equivalent (GAE)/g dry weight of the plant extract.

3. **Estimation of total flavonoids**

   The amount of total Flavonoids in the extracts was determined by the reported method of Rathee *et al.* (2006). Each extracts (100 μg) was added to 0.4 mL distilled water followed by NaN0₂ (0.03 ml, 5%). After 5 min at 25°C, AlCl₃ • 6H₂O (0.03 mL, 10%) was added followed by aqueous NaOH (0.2 mL, 1 M) after 6 min. The mixture was diluted with water to 1 mL and the absorbance at 510 nm was read. Catechin was used as the standard and the total flavonoid contents of CT seed and root extracts were expressed as mg catechin equivalents (CE)/g dry weight of the extract.

4. **High performance thin layer chromatography (HPTLC) fingerprinting**

   CT seed and root extracts were dissolved separately in alcohol to have 1 mg/mL solutions. 10 μL of solution was applied in duplicate on the TLC aluminium sheet pre-coated with silica gel 60 F254, having thickness of 0.2 mm (E merck, Germany) using CAMAG LINOMAT SAMPLER-V. The TLC was developed using mobile phase of ethyl acetate: ethanol: water (6 : 2 : 1) up to the distance of 7 cm. The TLC were scanned using CAMAG TLC SCANNER-III and photo-documented at 254 nm and 366 nm using CAMAG REPROSTAR-III.
5. Quantification of Kaempferol in CT seed extract by HPTLC

1 g of CT seed and root extracts were separately dissolved in 50 mL of methanol and mixed with 20 mL of 2N H₂SO₄. The mixtures were heated on boiling water bath under reflux for 4 h. The aqueous phase is than extracted with chloroform (5 x 50 mL). The chloroform fractions were combined, filtered, and evaporated at room temperature to have pasty mass referred as acid hydrolyzed extracts of seed and root respectively. The acid hydrolyzed extracts were used for kaempferol quantification.

The acid hydrolyzed extracts of CT seed and root were dissolved in alcohol to have 1 mg/mL solutions. The standard solution of Kaempferol (1mg/mL) is applied as 3, 6, 9, 12, and 15 μL on the TLC aluminium sheet pre-coated with silica gel 60 F254, having thickness of 0.2 mm (E merck, Germany) using CAMAG LINOMAT SAMPLER-V. 10 μL each of acid hydrolyzed extracts of CT seed and root were also applied. The TLC was developed using mobile phase benzene: diethyl ether: hexane (8 : 6 : 2) up to the distance of 7 cm. The TLC were scanned using CAMAG TLC SCANNER-III and photo-documented at 254 nm and 366 nm using CAMAG REPROSTAR-III.

The standard curve was prepared by plotting area against μg of Kaempferol. The amount of Kaempferol in acid hydrolyzed CT extracts were calculated from the standard curve and expressed as % w/w of CT extracts.

E. Pharmacological studies
1. Animals

Albino rats (Wistar strain) were selected for the evaluation of anti-inflammatory, and hepatoprotective activities. Albino rats (SD strain) of either sex were used for the anti-hyperlipidemic and wound healing activities. Male Albino rats (Wistar strain) were used for immunomodulatory studies.

Animals weighing 150–200 g were obtained from the central facility and acclimatized for 6 days before each study. Throughout the studies, all rats were
housed individually under specific pathogen-free conditions in polypropylene cages at ambient temperature (25 ± 1°C), relative humidity (55 ± 5%), and under a 12/12 h light–dark cycle. Animals had free access to standard commercial pellet diet (Pranav Agro Industries Ltd., Sangli, India) and water ad libitum throughout the study period. This study was approved (Ref. No. LMCP/07/03) by the institutional animal ethics committee established in accordance with the guidelines of Committee for the Purpose of Supervision and Control of Experiments on Animals (CPCSEA).

2. Drugs and chemicals

The reference drugs - atorvastatin, dexamethasone, gemfibrozil, and silymarin, were received as gift samples from the Zydus Research Centre, Ahmedabad. All the chemicals and solvents used in the study were of analytical grade. Bovine serum albumin, carrageenan, p-hydroxyproline were purchased from the Sigma Aldrich Inc. (USA). Poloxamer-407 was obtained Cadila Healthcare Ltd., Ahmedabad. Gallic acid and catechin were provided by Department of Pharmacognocy, L. M. College of Pharmacy, Ahmedabad. All other chemicals and solvents were purchased from S.D. Finechemicals Ltd. Bombay.

3. LD$_{50}$ determination

Albino rats (Wistar strain) of either sex weighing between 150 – 200 g were divided into different groups, comprising of six animals each. Animals were treated with different doses 250, 500, 750 and 1000 mg/kg, p.o. of each extracts. After single dose administration, animals were observed for death or any other deformities up to 72 h.

4. ED$_{50}$ determination

The effective dose in 50 % of population (ED$_{50}$) was determined using carrageenan–induced rat hind paw edema models. Albino rats (Wistar strain) of either sex weighing between 150 – 200 g were divided into different groups,
comprising of six animals each. Animals were treated with different doses of CT seed and root extracts (100, 250, 300, and 500 mg/kg, p.o.) before the carrageenan (0.1 mL of 1% w/v solution) injection. The paw volume was measured by plethysmometry method using mercury displacement. The % inhibition of edema was plotted against log dose and the EC$_{50}$ values were calculated from the graph for each extracts.

5. Pharmacological screening

CT seed and root extracts were investigated for their anti-inflammatory, hepatoprotective, anti-hyperlipidemic, immunomodulatory and wound healing activities against various experimental models.

a) Anti-inflammatory Activity

Different extracts of the seeds viz. ethyl acetate, acetone, acid hydrolyzed, hydroalcoholic extracts and hydroalcoholic extract of the roots were used to evaluate anti-inflammatory activities in carrageenan–induced rat paw edema model. Based on these findings, only hydroalcoholic extracts of seeds and roots were used for further pharmacological screenings, and were referred as CT seed extract and CT root extract throughout the study.

i. Carrageenan–induced hind paw edema in rats

The method of Winter et al. (1962) was used for carrageenan–induced hind paw edema. Animals were divided into different groups each consisting of six animals. Rats in: Group I (control) received 0.1 mL vehicle (1% gum acacia solutions); Group II (inflammatory control) received sub-planter injection of 0.1 mL of 1% w/v carrageenan suspended in 1.0% w/v acacia solution; Group III (reference drug) received indomethacin in the dose of 10 mg/kg, p.o. by gavage; Group IV were treated with 300 mg CT seed extract/kg, p.o. by gavage; Group V were treated with 500 mg CT seed extract/kg, p.o. by gavage; Group VI were treated with 500 mg CT root extract/kg, p.o. by gavage; and Group VII were treated with 500 mg/kg, p.o. by gavage. All the treatments were given 1 h prior to
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sub-planter injection of 0.1 mL of 1% w/v carrageenan suspended in 1.0% w/v acacia solution. The paw volumes were measured by plethysmometry method using mercury displacement before the treatment and then at the interval of 1 h up to 3 h. The percentage inhibition of inflammation was calculated as shown below

\[
\% \text{Inhibition of Inflammation} = \left(1 - \frac{\text{Mean Paw Volume of test}}{\text{Mean Paw Volume of control}}\right) \times 100
\]

The percentage inhibition of inflammation produced by different extracts was compared with that of Indomethacine.

\section*{ii. Carrageenan-induced pleurisy in rats}

Pleurisy was induced in rats according to Vogel and Vogel (1997) by administration of 0.1 mL of 1.0% w/v solution of carrageenan (24 h previously soaked in distilled water) directly into the pleural cavity of right lung under light ether anesthesia. Animals were divided into different groups each consisting of six animals. Rats in: Group I (control) received 2 mL vehicle (1% gum acacia solutions), orally; Group II (inflammatory control) received intra-pleural injection of 0.1 mL of 1.0% w/v solution of carrageenan; Group III were treated with 500 mg CT seed extract/kg, p.o. by gavage; Group IV were treated with 500 mg CT root extract/kg, p.o. by gavage; and Group V (reference drug) were treated with indomethacin in the dose of 10 mg/kg, p.o. by gavage.

Animals in Group II to V were treated with intra-pleural injection of 0.1 mL of 1.0% w/v solution of carrageenan directly into the pleural cavity of right lung under light ether anesthesia at 0 h. the respective treatments were given at 0, 24, and 48 h. After 72 h, animals were administrated with 1 mL of heparinized saline into pleural cavity, gently massaged and the exudates were recovered with care and measured. The blood samples were collected by retro-orbital puncture technique and processed for serum, which was stored at deep freeze temperature for determination of turbidity. The leukocytes counts were also

estimated in the each exudate. The percentage of the leukocyte inhibition was calculated using following formula (Charles Dorni et al., 2006),

\[
\text{Leukocyte inhibition} = \left( 1 - \frac{T}{C} \right) \times 100
\]

Where, T represents the treated groups’ leukocyte count and ‘C’ represents the control group leukocyte count.

The animals were sacrificed and right lung were dissected. The lung tissues were immediately washed with chilled distilled water followed by 0.25 M sucrose solution and bloated. The middle lobe of each lung was used for histopathological investigation (Supratech Micropath Laboratory & Research Institute, Ahmedabad, Gujarat, India). The other part of lung was used for preparation of homogenate with 10% w/v tris–hydrochloride. Various bio–parameters like tissue protein (Lowery et al., 1951), malondialdehyde (MDA) (Okhawa et al., 1979), reduced glutathione (GSH) (Beutler et al., 1963), myeloperoxidase (MYP) (Zhang et al., 2001), catalase (Aebi, 1974), nitric oxide (NO) scavenging activity (Nakagawa and Yokozawa, 2002), and super oxide dismutase (SOD) (Misra and Fridovich, 1973) were estimated in lung homogenates.

iii. Cotton pellet granuloma in rats

Pellets of surgical cotton weighing 9+1 mg were sterilized in an air oven for 2 h and were implanted in both the axillae and groins under ether anesthesia according to the method of Meier et al. (1950). Animals were divided into different groups each consisting six animals. Rats in: Group I (control) received 2 mL vehicle (1% gum acacia solutions), by gavage, once daily for 6 days; Group II were treated with 500 mg CT seed extract/kg, p.o. by gavage, once daily for 6 days; Group III were treated with 500 mg CT root extract/kg, p.o. by gavage, once daily for 6 days; and Group IV (reference drug) were treated with indomethacin in the dose of 10 mg/kg, p.o. by gavage, once daily for 6 days. The pellets were dissected out on the 7th day under light ether anesthesia. They were
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kept separately in small glass vials, dried for 2 h at 150 °C and weighed after cooling. The percentage anti-inflammatory effect was calculated for each dose according to the following formula,

\[
\% \text{ Anti-inflammatory effect} = \left(1 - \frac{T}{C}\right) \times 100
\]

Where T and C are the mean weight of granulation tissue in drug treated and control groups respectively. The blood samples were also collected in heparinized saline, and total leukocytes as well as differential leukocytes count were estimated.

iv. **In vitro and in vivo studies**

a. **Inhibition of protein denaturation**

The reaction mixture (0.5 mL) consisted of 0.45 mL bovine serum albumin (5% aqueous solution) and 0.05 mL of test extract (250 µg/mL of final volume, pH was adjusted at 6.3 using a small amount of 1 N HCl. The samples were incubated at 37 °C for 20 min and then heated at 57 °C for 3 min. After cooling the samples, 2.5 mL phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm (Mizushima and Kobayashi, 1968). For control tests 0.05 mL distilled water was used instead of extracts where as product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows,

\[
\% \text{ Inhibition} = 100 - \frac{(O.D. \text{ of test} - O.D. \text{ of product control})}{O.D. \text{ of control}} \times 100
\]

Control sample reading represents 100% protein denaturation. The results were compared with acetyl salicylic acid (250 µg/mL of final volume) treated samples.

b. **Effect on membrane stabilization**

The reaction mixtures (4.5 mL) consisted of 2 mL hypotonic saline (0.25% NaCl), 1 mL of 0.15 M phosphate buffer (pH 7.4) and 1 mL test solution (250 µg/mL of final volume).
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μg/mL of final volume) in normal saline, 0.5 mL of 10% rabbit RBC in normal saline was added. For control tests, 1 mL of isotonic saline was used instead of tests solution and product control tests lacked red blood cells. The mixtures were incubated at 56 °C for 30 min. The tubes were cooled under running tap water for 20 min. The mixtures were centrifuged and the absorbance of the supernatants read at 560 nm (Sadique et al., 1989). Percent membrane stabilizing activity was calculated as follows,

\[
\% \text{ Stabilization} = 100 - \frac{(O.D. \text{ of Test} - O.D. \text{ of Control})}{O.D. \text{ of Control}} \times 100
\]

Control group represents 100% lysis. The results were compared with acetyl salicylic acid (250 μg/mL of final volume) treated samples.

c. Anti-histaminic activity

It is determined using the method of Spector (1959). Rats (Wistar strain) were divided into different groups consisting of six animals. The fur on the left lateral side of the back of rats (2.5 cm diameter) was shaved before 24 h of the study. Animals were divided into different groups each consisting of six animals. Rats in: Group I (control) received vehicle (1 % acacia solution) by gavage; Group II were treated with 500 mg CT seed extract/kg, p.o. by gavage; Group III were treated with 500 mg CT root extract/kg, p.o. by gavage; and Group IV were treated with pheniramine in the dose of 10 mg/kg, p.o. by gavage. After 1 h of test drug administration, rats were injected with 50 μL of 200 μg/mL histamine dihydrochloride subcutaneously into the skin where fur had been removed. After 2 min, the area of the wheel formation was measured. The results were compared with pheniramine treated group.

b) Hepatoprotective Activity

i. Paracetamol–induced hepatotoxicity

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Animals were divided in different groups each of six animals. Rats in: Group I (normal control) received 1 mL aqueous suspension of 1% carboxy methylcellulose (CMC) as vehicle orally at 0, 6, 12, and 18 h without any treatment; Group II (PCM control) were intoxicated with PCM (500 mg/kg body weight, p.o.) at 0, 6, 12, and 18 h; Group III were treated with silymarin (25 mg/kg body weight, suspended in 1% CMC solution, p.o.) 2 h before PCM intoxication; Group IV were treated with CT seed extract (500 mg/kg body weight, p.o.) 2 h before PCM intoxication; and Group V were treated with CT root extract (500 mg/kg body weight, p.o.) 2 h before PCM intoxication.

The blood samples were collected by the retro-orbital puncture method under light ether anesthesia at 24 h. At the end of the study, animals were anaesthetized and liver was dissected quickly, collected, washed thoroughly in normal saline, bloated and preserved at -40°C for further analysis. The liver tissues were subjected to biochemical and histopathological investigations.

ii. Carbon tetrachloride–induced hepatotoxicity

The method of Agarwal et al. (2006) was used to produce carbon tetrachloride (CCl4)-induced hepatotoxicity. Albino rats (Wistar strain) of either sex weighing 150–200 g were selected for the study. Animals were divided in different groups each of six animals. Rats in: Group I (control) received only aqueous suspension of 1% CMC as vehicle orally without any treatment for 7 days; Group II (CCl4 control) were intoxicated with 1:1 (v/v) mixture of CCl4 in Arachis oil (1.5 mL/kg body weight, twice a week on day 3 and day 6); Group III were intoxicated with CCl4 and received silymarin (25 mg/kg body weight, suspended in 1% CMC solution, p.o.) once daily for 7 days; Group IV were intoxicated with CCl4 and received CT seed extract (500 mg/kg body weight, p.o.) once daily for 7 days; and Group V were intoxicated with CCl4 and received CT root (500 mg/kg body weight, p.o.) once daily for 7 days.

At the end of the study, the blood was collected by retro-orbital puncture method under light ether anesthesia and serum was separated by centrifuging at
2000 rpm for 15 – 20 min. At the end of the study, animals were anaesthetized and liver was dissected quickly, collected, washed thoroughly in normal saline, bloated and preserved at – 40°C for further analysis.

iii. **Assessment of liver function**

At the end of the study, the blood was collected by retro-orbital puncture under light ether anesthesia and serum was separated by centrifuging at 2000 rpm for 15 – 20 min. The serum levels of serum glutamic oxaloacetic transaminase (SGOT) (Tietz, 1970), serum glutamic pyruvic transaminase (SGPT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Kind and King, 1954), creatinine (Bonsnes and Taussky, 1945), and total bilirubin (Malloy and Evelyn, 1937) were estimated colorimetrically using UV-Visible spectrophotometer (Shimadzu–UV–1601, Japan). All the tests were carried out with commercial diagnostic kits supplied by Span Diagnostic Ltd., India.

iv. **Lipid peroxidation and anti-oxidant parameters in liver**

The liver homogenates were prepared in 10 mL of tris-hydrochloride buffer (0.1M, pH 7). They were subjected to estimation of protein (Lowry et al., 1951), malondialdehyde (MDA) (Okhawa et al., 1979), superoxide dismutase (SOD) (Misra and Fridovich, 1973), catalase (Aebi, 1974), and reduced glutathione (GSH) (Beutler et al., 1963).

v. **Phenobarbitone–induced sleeping time**

To investigate the hepatic microsomal enzymes inhibition, potentiation of the phenobarbitone–induced sleeping time was measured in rats (Walker and Parry, 1949). Animals were divided into different groups, each consisting of six. The control group received single dose of phenobarbitone (80 mg/kg, i.p.). The treatment groups received 500 mg/kg body weight, p.o. of each extracts one hour before phenobarbitone injection. The animals were observed for righting reflex. If the animals failed to maintain normal posture when placed on one side within 30 sec; it was considered as loss of righting reflex.
vi. *Determination of hepatic hydroxyproline content*

Hydroxyproline (HYP) was determined colorimetrically in duplicates from 0.2 g of liver tissues using a modified method of Jamall *et al.* (1981). Briefly, the frozen tissue was homogenized in 4 mL of 6N HCl and hydrolyzed at 110°C for 16 h. the hydrolysate was filtered, and then 30 μL aliquot of these samples was evaporated under vacuum, the sediment was dissolved in 1.2 mL of isopropanol and incubated with 0.2 mL of 0.84% chloramines-T in acetate-citrate buffer (pH 6.0) for 10 min at room temperature. Then, 1.0 mL of Ehrlich’s reagent was added and the mixture was incubated at 60°C for 25 min. The absorbance of the sample solution was measured at 560 nm (Simadzu–UV–1601, Japan). Next, the hydroxyproline content in 100 mg of liver was calculated from the standard curve of 4-hydroxyl-L-proline (Sigma, USA) and expressed as μg/100 mg liver weight.

vii. *Number of mast cells in liver tissues*

It was carried out at Pathology laboratory, Ahmadabad, India. Toluidine blue staining for mast cells was performed by immersion of liver sections in 0.01% toluidine blue (Sigma, USA) for 1 min at room temperature. The number of mast cells was quantified in 25 randomly selected, non-overlapping fields, and expressed as the number of mast cells/mm².

viii. *Histopathological studies*

The rats were sacrificed under deep anesthesia and the livers were excised quickly and fixed in 10% buffered neutral formalin. Paraffin sections (5–10 μ) were prepared, stained with Haematoxylin–Eosin, and finally mounted in neutral medium. Histopathological studies were carried out in the pathology laboratory.

c) Antihyperlipidemic Activity

i. *Poloxamer-407- induced hyperlipidemia in rats*

The acute hyperlipidemia was induced in rats using Poloxamer-407 (P-407) (Johnston and Palmer, 1993). Albino rats (SD strain) were divided into
different groups each consisting of six animals. Rats in: Group I (normal control) received vehicles (1 mL each of 1% acacia and 1% CMC) by gavage; Group II (hyperlipidemic control) were treated with P-407 (1 mL of 30% w/v solution, i.p.); Group III (standard–1) were treated with P-407 (1 mL of 30% w/v solution, i.p.) and atorvastatin (50 mg/kg., p.o.); Group IV (standard – 2) were treated with P-407 (1 mL of 30% w/v solution, i.p.) and gemfibrozil (50 mg/kg., p.o.); Group V were treated with P-407 (1 mL of 30% w/v solution, i.p.) and CT seed extract (500 mg/kg, p.o.); and Group VI were treated with P-407 (1 mL of 30% w/v solution, i.p.) and CT root extract (500 mg/kg, p.o.).

All the extracts and drugs were administered orally about 1 h before the i.p. injection of 1 mL of 30% w/v solution of P–407. Animals were fed with normal chew diet throughout the study. The blood samples were collected at 15 and 24 h after P–407 injection and investigated for lipid profiles.

ii. Diet–induced hyperlipidemia in rats

The method of Blank et al. (1963) with modification was used to produce diet–induced hyperlipidemia. Albino rats (SD strain) were divided into different groups each consisting of six animals. Rats in: Group I (normal control) received vehicles (1 mL each of 1% acacia and 1% CMC) by gavage for 7 days; Group II (hyperlipidemic control) have free access to high cholesterol diet for 7 days without any other treatment; Group III (standard–1) were treated with atorvastatin (50 mg/kg., p.o.); Group IV (standard–2) were treated with gemfibrozil (50 mg/kg, p.o.); Group V were treated with CT seed extract (500 mg/kg, p.o.); and Group VI were treated with CT root extract (500 mg/kg, p.o.). The normal control group received standard chew diet, and all other groups received high cholesterol diet consisting of normal chew diet 92% w/w, cholesterol 2.0% w/w, cholic acid 1% w/w, and coconut oil 5% w/w for 7 days.

The reference drugs and extracts were administered once daily between 8:00 to 9:00 a.m. for seven days. The daily food intake was determined before treatments. On the last day, animals were deprived of foods but not water. Blood
samples were collected by retro-orbital puncture technique under light anesthesia. The animals were sacrificed and liver tissues were collected and preserved at −40°C for further analysis. The fecal matters of last 24 h before fasting were collected, immediately dried in oven at 80°C for 1 h and stored at −40°C for further analysis.

iii. Estimation of biochemical parameters

a. Lipid profile

The serum lipid profile was determined at 15 and 24 h after P–407 injection, and on day 8 in case of diet–induced hyperlipidemia. The total cholesterol (TC), triglycerides (TG), and high density lipoprotein–cholesterol (HDL–C) levels were estimated using commercially available kits (ERBA, Transasia Bio–medicals Ltd., Daman, India). Very low density lipoprotein–cholesterol (VLDL–C) was calculated as TG/5. Low density lipoprotein–cholesterol (LDL–C) levels were calculated using Friedewald's formula (Friedwald et al., 1972). The Atherogenic index (AI) was calculated using following formula:

\[
Atherogenic Index (AI) = \frac{(VLCL - C + LDL - C)}{HDL - C}
\]

b. HMG–CoA reductase activity

The activity of the enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) reductase was determined by indirect method (Rao and Ramakrishnan, 1975). The method estimates the HMG–CoA to mevalonate ratio as an index of the activity of HMG–CoA reductase. The liver tissue was removed as quickly as possible and a 10% homogenate was prepared in saline arsenate solution. The homogenate was deproteinized using an equal volume of dilute perchloric acid and allowed to stand for 5 min, followed by centrifugation. To 1 mL of the filtrate, 0.5 mL of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG–CoA) was added. It was mixed and 1.5 mL of ferric chloride reagent was added after five min. The absorbance was read after 10 min.
at 540 nm against a similarly treated saline arsenate blank. The ratio of HMG–CoA to mevalonate was calculated.

c. *Fecal cholesterol and bile acid excretion*

Fecal matter was collected during the last 24 h before fasting in diet-induced hyperlipidemia model. The dried and powdered fecal matter was extracted with alkaline methanol. The resultant extract was then analyzed for cholesterol content in a manner similar to that of the serum. The cholesterol excreted in the fecal matter was calculated and expressed as mg per g of fecal matter. The method of Evrard and Janssen (1968) modified by Manes and Schneider (1971), was used for fecal bile acid extraction and bile acid levels were estimated by colorimetric method (Snell and Snell, 1954) and expressed as cholic acid equivalent per g of fecal matter.

d. *Serum ascorbic acid levels*

Serum total ascorbic acid (TAA), L-ascorbic acid (LAA) and dehydroascorbic acid (DAA) levels were estimated according to method of Schaffert and Kingsley (1955).

d) *Immunomodulatory Activity*

i. *Treatment regimens*

CT seed and root extracts were suspended in distilled water using 1% w/v gum acacia. Dexamethasone (DXM) was suspended at a concentration of 0.8 μg/mL in distilled water using 1% w/v CMC. In the studies herein unless elsewise indicated, treatment rats received CT seed and root extracts at 500 mg/kg body weight (BW) in 1 mL doses daily by gavage. The control group rats received vehicle, i.e., a single 2 mL bolus bearing 1 mL each of the 1% w/v gum acacia and 1% w/v CMC solutions, in parallel daily. The rats in the reference drug group received DXM at the dose of 0.25 mg/kg BW in 1 mL volume, daily by gavage.

ii. *Antigen preparation*
Fresh blood was collected from sheep sacrificed in the local slaughter house, and placed in Alsever's solution. During the experiment, adequate amount of stock solution of sheep red blood cells (SRBC) stored in Alsever's solution, was taken and allowed to stand at room temperature. It was washed three times with normal saline. The settled SRBC were then suspended in normal saline. The SRBC of this suspension were adjusted to a concentration of 5x10^9 SRBC/mL for immunization and challenge (Bafna and Mishra, 2005).

iii. **SRBC–induced humoral antibody (HA) titer**

The method described by Atal et al. (1986) was utilized to examine the rats provided CT seed and root extracts once daily by gavage, starting 7 days prior to sensitization and continuing up to the second time of challenge (i.e., Day -7 up to and through Day +14; for a total of 21 days). Control and DXM–treated rats received vehicle or the drug, respectively, in parallel each day.

To specifically assess effects on antibody formation, groups of six rats per treatment were immunized with 20 μL of SRBC suspension (5x10^9 SRBC/mL) injected subcutaneously into right hind foot pad. The day of immunization was referred to as Day 0. Seven days later (Day +7), the rats were challenged by injecting 20 μL of SRBC suspension (5x10^9 SRBC/mL) intradermally into the left hind foot pad. Blood samples were collected from all the animals separately by retro–orbital puncture under light ether anesthesia on Day +7 (after challenge) for assessment of primary antibody titer and on Day +14 (after challenge) for measures of secondary antibody titer. Antibody levels were determined by the method described by Shinde et al. (1999). After allowing the collected blood to clot, serum was isolated and 25 μL was placed into one well of a 96–well microtiter plate. Serial two–fold dilutions of the serum were made using 25 μL of normal saline each time of transfer across the plate. To the 25 μL of diluted serum in each well was then added 25 μL of 1% v/v SRBC suspension in normal saline. The microtiter plate was maintained at room temperature for 1 h and then well contents examined for haemagglutination i.e., until control wells showed
unequivocally negative patterns. The value of the highest serum dilution showing haemagglutination was defined as the antibody titer for the given rat.

iv. **SRBC-induced delayed–type hypersensitivity (DTH) Response**

   The method of Lagrange *et al.* (1974) was used to analyze effects on DTH responses in the treated rats. Daily treatment with CT seed and root extracts (500 mg/kg, by gavage) began 14 days prior to the challenge i.e., starting on the same day as immunization with SRBC. Control and DXM–treated rats received vehicle or the drug, respectively, in parallel each day.

   On Day 0, all rats were immunized with 20 μL SRBC solution (5 x 10⁹ SRBC/mL) injected subcutaneously into their right hind footpad. After 14 days of gavage treatment, the thickness of each rat's left footpad was measured just before the challenge; using a Schnelltaster caliper (H.C. Kroplin Hessen, Schluchtern, Germany) that could measure to a minimum unit of 0.01 mm. The rats were then challenged by injecting 20 μL SRBC solution (5 x 10⁹ SRBC/mL) intradermally into their left hind footpad (deemed time 0). Foot thickness was the re–measured after 24 h. The difference between the thicknesses of left foot just before and 24 h after challenge (in mm) was taken as a measure of DTH (Doherty, 1981).

v. **Neutrophil adhesion test**

   The method described by Wilkenson (1978), was used for evaluating the effect of CT seed and root extracts on neutrophil adhesion. After 14 days of gavage treatment, blood samples were collected from rats in each group by retro–orbital puncture under light ether anesthesia in heparinized vials and subjected to total as well as differential leukocyte count. After performance of the initial counts, the each blood sample was incubated with 80 mg/mL of nylon fibers at 37°C for 15 min. The incubated samples were again analyzed for total and differential leukocyte count. The product of total leukocyte count and the percentage (%) neutrophil, known as neutrophil index, was determined for each
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rat of the respective groups (Fulzele et al., 2002). The % neutrophil adhesion for each of the test rat was then calculated as,

\[
\% \text{ Neutrophil Adhesion} = 100 \times \frac{(Nl_0 - Nl_t)}{Nl_0}
\]

Where ‘Nl_0’ is the neutrophil index of the blood samples before nylon fiber treatment and ‘Nl_t’ is the neutrophil index after nylon fiber treatment.

vi. Carbon clearance test

The method of Biozzi et al. (1953) was used to analyze phagocytic activity among the white blood cells in the rats. For each treatment regimen, a total of 6 rats were utilized. Daily treatment with CT seed extract (500 mg/kg, by gavage) occurred for 5 day prior to the assessment of in situ phagocytic activity. Control and dexamethasone – treated rats received vehicle or the drug, respectively, in parallel each day. A colloidal carbon ink suspension was injected via the tail vein into each rat 48 h after the final treatment. From each rat, blood samples (25 μL) were then withdrawn from the retro–orbital plexus under mild ether anesthesia, immediately after the injection and then 5, 10, and 15 min thereafter. Each blood sample was lysed with 2 mL of 0.1% acetic acid and the absorbance of the resulting solution evaluated at 675 nm (Damre et al., 2003). A graph of absorbance vs. time post–injection was prepared for each animal and the in situ phagocytic index was calculated using following formula,

\[
\text{Phagocytic Index} = \frac{K_{\text{sample}}}{K_{\text{standard}}}
\]

Wherein \(K_{\text{sample}}\) represents the slope of the absorbance vs. time curve of blood samples from rats in the extract – treated or dexamethasone – treated group and \(K_{\text{standard}}\) represents the slope of the absorbance vs. time curve of blood samples for the rats in the control group.

vii. Hematological profile
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After 8 days of the repeated gavage treatment, blood was collected from each rat via their retro-orbital plexus under light ether anesthesia. Various parameters such as total white blood cell (WBC), differential WBC, red blood cell (RBC), platelet counts, as well as hemoglobin (Hb) levels were then evaluated using a Sysmax XS800i automated hematology analyzer (TOA Medical Electronic Co., Tokyo, Japan).

e) Wound healing activity

i. Preparation of ointment

CT seed and root extracts were suspended in distilled water using 1% w/v gum acacia in order to achieve a 25% w/v aqueous phase. The simple ointment base BP (as described in British Pharmacopoeia, 1993) was melted by heating at 60°C over a water bath and the above aqueous phase was incorporated with constant stirring to yield an ointment containing 10% w/w extract. They were referred to as CT seed ointment and root ointment respectively.

ii. Excision wound model

The rats in these studies were inflicted with an excision wound as described by Morton and Malone (1972), under light ether anesthesia. Specifically, a single circular wound of \( \approx 300 \text{ mm}^2 \) was made on a depilated ethanol-sterilized dorsal thoracic region of the rats. The animals were then randomized into five groups (\( n = 6 \)/group); each treatment outlined here was utilized each day (for 10 consecutive days) after the wound infliction. Rats in: Group I (control) received the earlier-described 2 mL vehicle (i.e., 1 mL of 1% gum acacia and 1 mL of 1% CMC solutions) only by gavage; Group II (ointment control) received a topical application of 50 mg of the simple ointment BP; Group III (reference drug standard) were treated with a topical application of 50 mg of 1% (w/w) cotrimoxazole cream; Group IV were treated with 500 mg CT seed extract / kg BW (by gavage); Group V were treated with 500 mg CT root extract / kg BW (by gavage); Group VI were treated topically with 50 mg CT seed ointment; and, Group VII were treated topically with 50 mg CT root ointment.

the determination of wound closure, the wound area was traced onto mm\(^2\) graph paper on Days 0 and 10, and the percentage of wound closure was then calculated.

iii. Incision wound model

In this model, 6 cm long paravertebral incisions were made through the full thickness of the skin on either side of the vertebral column of rats as described by Ehrlich and Hunt (1969). The wounds were then closed with interrupted sutures 1 cm apart. The animals were then randomized into five groups (n = 6/group); each treatment outlined here was utilized each day (for 8 consecutive days) after the wound infliction. Rats in: Group I (control) received the earlier-described 2 mL vehicle (i.e., 1 mL of 1% gum acacia and 1 mL of 1% CMC solutions) only by gavage; Group II (ointment control) received a topical application of 50 mg of the BP ointment base; Group III (reference drug standard) were treated with a topical application of 50 mg of 1% (w/w) cotrimoxazole cream; Group IV were treated with 500 mg CT seed extract / kg BW (by gavage); Group V were treated with 500 mg CT root extract / kg BW (by gavage); Group VI were treated topically with 50 mg CT seed ointment; and, Group VII were treated topically with 50 mg CT root ointment. The skin breaking strength of the wound was measured 2 day later, as described by Lee (1968).

iv. Dead-space wound model

Dead-space wounds were created (under light ether anesthesia) by subcutaneous implantation of sterilized cylindrical grass piths (2.5 cm x 0.3 cm), one on either side of the dorsal paravertebral surface of the rat (Turner, 1965). Animals were then randomized into five groups (n = 6/group) each treatment outlined here was utilized each day (for 10 consecutive days) after the pith implantations. Rats in: Group I (control) received the earlier-described 2 mL vehicle (i.e., 1 mL of 1% gum acacia and 1 mL of 1% CMC solutions) only by gavage; Group II (ointment control) received a topical application of 50 mg of the simple ointment BP; Group III (reference drug standard) were treated with a
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topical application of 50 mg of 1\% (w/w) cotrimoxazole ointment; Group IV were treated with 500 mg CT seed extract / kg BW (by gavage); Group V were treated with 500 mg CT root extract / kg BW (by gavage); Group VI were treated topically with 50 mg CT seed ointment; and, Group VII were treated topically with 50 mg CT root ointment. To assess the extent of wound healing in the rats, the granulation tissues that had formed on the grass piths in each rat were excised on Day 10 post-wounding and weighed.

In each of the three wound healing activity studies performed here, rats were individually observed for 1.5 h after each topical application to make sure there was no medication spoilage. In addition, each animal group contained three extra rats (each treated with the given regimen, in parallel) in case there was any need to compensate for unexpected deaths or infections during the protocol periods. Any rat showing signs of infection during the study period was excluded from the study, and replaced by one of the alternates in its regimen.

F. Statistical analysis

Statistical analysis was carried out using One Way ANOVA followed by Tukey’s test, using the SigmaState™ 2.03 software, and computer with Intel Pentium® dual core™ processor. A value of $p < 0.05$ was considered statistically significant difference between analyzed groups.