



Anti-inflammatory and anti-ulcer effects of *Calotropis gigantea* R.Br flowers in rodent

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Abstract

Objective: To evaluate the chloroform extract of *Calotropis gigantea* flowers (CCGF) and ethanol extract of *Calotropis gigantea* flowers (ECGF) for their possible anti-inflammatory (AI) and anti-ulcer (AU) activities. **Method:** The anti-inflammatory effect of CCGF and ECGF (200mg/kg; p.o.) was investigated in carrageenan induced rat paw edema and cotton pellet induced granuloma model. The AU activity studied in pylorus ligation and aspirin induced gastric ulcers model. The aspirin and ranitidine were used as standards for AI and AU studies respectively. **Results:** The CCGF and ECGF significantly reduced rat paw edema ($p > 0.05$ to > 0.01) and dry weight granuloma ($p > 0.01$). Similarly, both extracts treated group were significantly ($p > 0.01$) protected from pyloric ligation and aspirin induced gastric ulcers. These effects were statistically significant. **Conclusion:** The results suggest that the CCGF and ECGF possess significant AI and AU activities. The observed effect may be due to the presence of protease like bioactive constituent.

Keywords: *Calotropis gigantea* flower, anti-inflammatory, anti-ulcer, aspirin.

1. Introduction

Calotropis gigantea R. Br. (Asclepiadaceae) is a shrub or a small tree 8-10 feet height. *Calotropis* is a genus of about six species, among which *C. gigantea* and *C. procera* are the sister species which are commonly grown in waste land throughout India [1]. The hydroalcoholic extract of flowers are reported to possess hepatoprotective activity [2]. The aerial part of *C. gigantea* has been reported for its antidiarrhoeal property in castor oil induced

diarrhea [3]. Recently, alcoholic extract of the flowers has been reported to possess analgesic activity [4]. Traditionally, *C. gigantea* flowers used to cure inflammation, ulcer and asthma like diseases [5].

Inflammation continues to be an area of great interest for research probably due to non-availability of safer and more effective anti-inflammatory agents; Rheumatoid arthritis is a

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chronic autoimmune disease in which there is an inflammation of joints, synovial proliferation and destruction of articular cartilage [6]. Although a number of drugs like non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase (COX) inhibitors, immunosuppressants are being used clinically in treatment of arthritis, osteoporosis and joint pain like inflammatory complication; they are all not devoid of adverse effects. Gastric side effects like ulcer, gastric hemorrhages are very well known with use of aspirin, it is being used routinely. Hence, there is a need for a more effective drug with lower side effects. No scientific study has been reported for anti-inflammatory and anti-ulcer properties of *C. gigantea* flowers. The present investigation focuses on the anti-inflammatory and anti-ulcer potential of *C. gigantea* flower.

2. Materials and Method

2.1 Animals

Male albino Wistar rats weighing between 150 – 250 g were used for the present study. They were maintained under standard environmental conditions and were fed with standard pellet diet and water *ad libitum*. The experiments were performed followed by approval from Animal Ethical Committee of the establishment.

2.2 Preparation of the plant extracts

The flowers of *C. gigantea* were collected from Belgaum region in the month of December. The flowers were authenticated from Dept. Botany of RLS Institute, Belgaum (Karnataka). The flowers were dried at room temperature until they were free from moisture and was subjected to size reduction to get coarse powder of desired particle size. The powdered material was defatted with pet-ether and successively extracted in a Soxhlet apparatus using chloroform and 95% ethanol in increasing polarity. The prepared extract of CGCF and ECGF suspended in 5% Tween 80 at the time of use.

2.3 Acute Toxicity Studies

Acute toxicity studies were carried out following OECD guidelines [7] and were found to be safe up to 2000 mg/kg body weight in albino Wistar rats.

2.4 Carrageenan induced rat paw edema

Several groups of 6 rats each were selected. six animals each. They were starved overnight with water *ad libitum* prior to the day of experiment. The control group received 0.5 ml 5% Tween 80 suspension orally, while the other groups received different drug treatment as described below [Table-1]. Thirty minutes after drug administration, acute inflammation was induced by injecting 0.5 ml of 1% carrageenan (Sigma Co., St Louis) in normal saline into the sub plantar region of the left hind paw, as per the method of Winter *et al* [8]. A mark was applied on the leg at the malleolus to facilitate subsequent readings. The paw edema volume was measured with the help of plethysmometer (UGO, BASILE, Italy) at 0, 0.5, 1, 2, 3, 4 and 5 h after injecting carrageenan. The difference between 0 h and subsequent reading was considered as edema volume in various groups and calculated using formula:

$$\% \text{ edema inhibition} = 1 - V_t / V_c \times 100$$

V_t and V_c were edema volume in the drug-treated and control group respectively.

2.5 Cotton pellet- induced granuloma

Sub acute inflammation was induced by a slightly modified method of D' Arcy *et al* [8]. Four groups of six rats each were used. Under light ether anesthesia, hair in the axilla and the groin were clipped, two sterile cotton pellets weighing 10mg each and two sterile grass pith (25 x 2mm) were implanted subcutaneously, through a small incision, either in the axilla or the groin, at random. The wounds were then sutured and the animals were caged individually

after recovery from anesthesia. Aseptic precautions were taken throughout the experiment. The rats then received treatments as shown below. The treatment was started on the day after the implantation and was repeated every 24 h, regularly for 10 days [Table 2]. On the 11th day the rats were sacrificed with an overdose of ether anesthesia, and the cotton pellets, grass pith and stomach were removed. The pellets, freed from extraneous tissue, were dried overnight at 60°C and their dry weight measured. Net granuloma formation was calculated by subtracting the initial weight of cotton from the final weights. Mean granuloma dry weight for the various groups was calculated and expressed as mg/100gm body weight. The grass pith was preserved in 10% formalin for histopathological study after H and E staining.

2.6 Ulcer index

The stomach was cut open along the greater curvature and gently washed with normal saline. Gastric mucosa was examined for the presence of erosion, hemorrhagic spot, ulcer and perforation, if any, with the help of magnifying lens. The severity of the ulcer was determined by arbitrary scoring system [9]. The ulcer index was calculated as a mean score of ulcer severity in all the treated groups and was compared with that of control.

2.7 Pylorus ligation method

Twenty-four rats of either sex were randomly divided into four groups and fasted for 48h with free access to water. Pylorus ligation was performed under light ether anesthesia to each animal [10]. Animals were given 5% Tween 80, CCGF, ECGF (200mg/kg) or ranitidine (50 mg/kg) orally immediately after ligation and recovery from anesthesia. Animals were sacrificed 4 h later, the stomach was carefully removed and the gastric content was collected. The gastric juice was centrifuged at 3000 rpm for 30 min

and the volume of gastric juice was measured. Free and total acidities in the supernatant were determined by titration with 0.1 N NaOH and expressed as mEq/L/100g [11]. The stomach was cut open along the greater curvature and pinned on a soft board for evaluating gastric ulcers and ulcer index [12].

The percentage inhibition of ulcer was calculated as:

$$\% \text{ Inhibition of ulcer} = \frac{\text{Mean ulcer index of control} - \text{mean ulcer index of test}}{\text{Mean ulcer index of control}} \times 100$$

Scoring for ulcer index: 0 – Normal colored stomach, 0.5 – Red colorations, 1 – spot ulcers, 1.5 – Hemorrhagic streaks, 2 – Ulcers ≥ 3 but ≤ 5 , 3 – Ulcers > 5 .

2.8 Aspirin induced gastric ulcers

Twenty-four rats of either sex were randomly divided into four groups and fasted for 24 h with free access to water. Animals were given 5% Tween 80, CCGF, ECGF (200mg/kg) and ranitidine at 50 mg/kg orally. After 1 h 200 mg/kg of aspirin was given orally to each rat [13]. Animals were sacrificed 4 h later, stomachs were isolated, ulcer index and percentage inhibition of ulcer was determined as explained in pylorus ligation model.

2.9 Statistical analysis

The results are expressed as mean \pm SEM. The data was analyzed by One-way analysis of variance (ANOVA) followed by Dunnett's test, p-value < 0.05 was taken as significant.

3. Results

3.1 Carrageenan induced rat paw edema

The CCGF, ECGF and aspirin treated groups have significantly ($p > 0.05$ – $p > 0.01$) inhibited carrageenan induced edema at 200mg/kg, when compared to vehicle treated control group

[Table 1]. The mean paw volume at 3h in CCGF and ECGF was comparable to the corresponding value of aspirin group, indicating significant ($p > 0.01$) inhibition of edema when compared to control group. The CCGF and ECGF were highly significant at 3h and 2 h respectively.

3.2 Cotton – pellet induced granuloma

Mean granuloma dry weight in aspirin, CCGF and ECGF were significantly lower than the control group ($p > 0.01$) AI potential of treatment [Table 2]. However in gastric mucosal study the mean ulcer index of the aspirin (200 mg/kg) group was significantly higher than the control group. The UI in other groups was comparable to that of control [Table-2]. The histopathological study of the granuloma revealed a decrease in the thickness of granulation tissue and the fibroblast number in all treated groups as compared to control [Fig.1].

3.3 Effect on pylorus ligation induced gastric ulcers

The result of CCGF, ECGF and ranitidine are illustrated in [Table 3]. The severity of gastric ulceration was accessed on the basis of UI. The UI in CCGF (2.46), ECGF (2.26) and ranitidine (25.43), which is significantly lower as compared to control (4.51). The all treated groups have significantly ($p > 0.01$) protected stomach. In control animals, without any drug, the mean pH is 2.5, the CCGF and ECGF treated groups mean pH is 3.68 and 3.75, which indicate significant ($p > 0.01$) pH rise. Ranitidine as standard drug increases the pH to 3.85 which is statistically significant. The gastric free acidity (27.75mEq/ litre) and total acidity (46.6 mEq/ liter) is considerably high in control group after pylorus ligation, whereas the CCGF, ECGF and ranitidine groups showed significant ($p > 0.01$) reduction in the same when compared with control [Table3].

Table 1. Effect of various treatments in rats on carrageenan induced rats paw edema

| Treatment mg/ kg | Rat paw volume in ml | | | | |
|------------------|----------------------|-------------------|-------------------|-------------------|-----------------|
| | 1h | 2h | 3h | 4h | 5h |
| Tween 80 | 5.91 \pm 0.05 | 6.60 \pm 0.08 | 6.89 \pm 0.04 | 6.90 \pm 0.03 | 5.97 \pm 0.04 |
| Aspirin | 4.85** \pm 0.07 | 5.45** \pm 0.16 | 4.17** \pm 0.04 | 5.17** \pm 0.13 | 5.07 \pm 0.06 |
| CCGF | 5.69* \pm 0.05 | 5.23** \pm 0.21 | 4.94** \pm 0.07 | 6.35** \pm 0.05 | 5.74 \pm 0.06 |
| ECGF | 5.66* \pm 0.06 | 4.09** \pm 0.14 | 4.60** \pm 0.12 | 5.02 \pm 0.11 | 5.33 \pm 0.03 |

Values are mean \pm S.E.M; n = 6 in each group; All drug given 30 min prior; p * > 0.05, p** > 0.01

Table 2. Effect of CCGF and ECGF on cotton pellet- induced granuloma.

| Treatment | Weight of Cotton- pellet (mg) | % inhibition | Ulcer index |
|-------------|----------------------------------|--------------|-------------------|
| 5% Tween 80 | 14.01 \pm 0.07 | - | 11.66 \pm 7.51 |
| Aspirin | 6.21 \pm 0.05** | 55.68 | 40.00 \pm 0.00* |
| CCGF | 10.45 \pm 0.048 ** | 25.42 | 10.00 \pm 6.35 |
| ECGF | 8.63 \pm 0.24 ** | 38.41 | 11.66 \pm 7.51 |

Values are mean \pm S.E.M; n = 6 in each group; All treatment given for period of 10 days orally once daily p* > 0.01, p** > 0.001 when compared to control.

Table 3. Effect of different extracts of *C. gigantea* flowers for anti-ulcer activity in pyloric-ligation method.

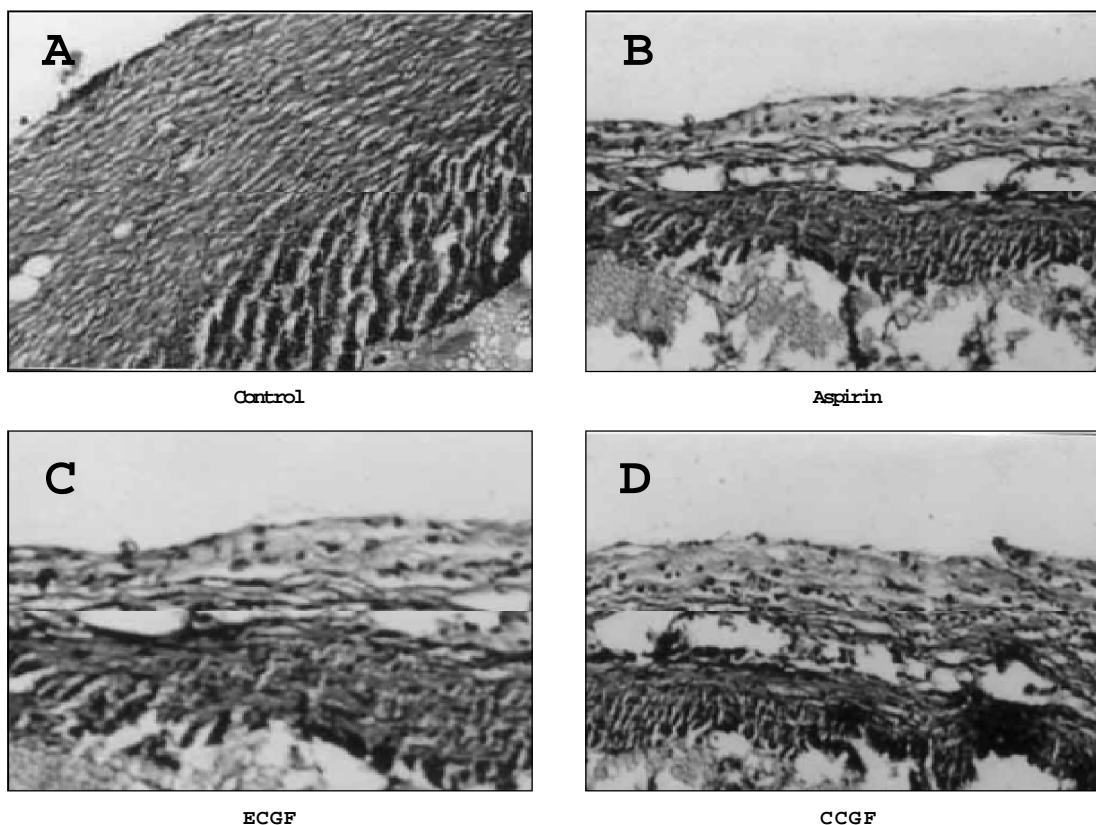
| Treatment | pH | Free acidity | Total acidity | Ulcer index |
|--------------|-------------------|-------------------|-------------------|--------------------|
| 5 % Tween 80 | 2.5 \pm 0.035 | 27.75 \pm 0.028 | 46.6 \pm 0.43 | 4.51 \pm 0.14 |
| CCGF | 3.68* \pm 0.008 | 18.08* \pm 0.15 | 36.91* \pm 0.83 | 2.46* \pm 0.03 |
| ECGF | 3.75* \pm 0.013 | 16.13* \pm 0.19 | 34.08* \pm 0.15 | 2.26* \pm 0.05 |
| Ranitidine | 3.85* \pm 0.016 | 12.50* \pm 0.25 | 30.4* \pm 0.48 | 25.43* \pm 0.007 |

Values are in Mean \pm S.E.M; p* >0.01 vs control

Table 4. Effect of different extract from *C.gigantea* flowers on aspirin induced gastric ulcers in rats.

| Treatment | Ulcer index | % Inhibition |
|--------------|------------------|--------------|
| 5 % Tween 80 | 0.66 \pm 0.01 | — |
| CCGF | 0.29 \pm 0.01* | 56.1 |
| ECGF | 0.20 \pm 0.02* | 69.1 |
| Ranitidine | 0.23 \pm 0.01* | 65.2 |

Values are in Mean \pm S.E.M; p* >0.01 vs control

**Fig. 1.** Microphotograph of granulation tissue stained with (H & E X 100).

A- Control group, B- Aspirin treated group, C- ECGF treated group, D- CCGF treated group

3.4 Aspirin induced gastric ulcers

The treatments of CCGF, ECGF and ranitidine have protected significantly ($p > 0.01$) the rat's stomach from aspirin induced gastric ulcers [Table 4].

4. Discussion

The carrageenan induced rat paw edema model in rats is known to be sensitive to cyclooxygenase (COX) inhibitors and has been used to evaluate the effect of non-steroidal anti-inflammatory agents, which primarily inhibit the COX involved in prostaglandin synthesis [14,15]. The time course of edema development in this model is generally represented by a biphasic curve [16]. The first phase, which occurs between 0 to 2.5 h after injection of the phlogestic agent, has been attributed to the release of histamine or serotonin [17]. The edema volume reaches its maximum approximately 3 h post treatment and then begins to decline. The second phase of inflammatory reaction that is measured at 3 h is caused by the release of bradykinin, protease, prostaglandin and lysosomes [16,17]. Therefore it can be inferred that the inhibitory effect of CCGF and ECGF could be due to inhibition of the enzyme COX leading to inhibition of prostaglandin synthesis.

Chronic inflammation is mediated by both immunological and nonimmunological mechanisms and is frequently observed with reparative response, namely, granulation tissue and fibrosis. The fibroblast proliferation and activation result in an increased and extra cellular matrix, which contains fibronectin like component which can be chemoattractant [18]. In cotton pellet induced granuloma, the CCGF and ECGF showed significant AI activity, thus found effective in chronic inflammatory condition. The histopathological study revealed the significant decrease in vascularity, granuloma,

fibroblast number and collagen content as compared to control group. Several cystein proteases are isolated and characterized from the latex of *C. gigantea*, but their pharmacological action are not known yet [19, 20]. The crude extract of *C. gigantea* latex hydrolyses strongly all the subunit of fibrinogen and crude fibrin clot even at lower concentration and at higher concentration it induces hemorrhage at the site of injection [21]. The clot inducing and clot dissolving enzymes might act sequentially and facilitates the wound healing process, which manifest as decrease in the granuloma.

Inhibition of cyclooxygenase (COX) is one mechanism by which NSAIDs including aspirin exert analgesic and anti-inflammatory effect. NSAIDs block COX-2, induces formation of prostaglandin's, thereby mitigating pain and inflammation [22]. However, they also affect COX-1 and lead to decreased homeostatic functions, resulting in gastric and renal adverse effect. In ulcer index study aspirin has significantly induced ulcers along with hemorrhages, whereas CCGF and ECGF are devoid of such effect indicating involvement of COX-2 like mechanism for AI along with gastro protective properties. Ulcers are local defects on the surface of an organ produced by inflammation [23]. It has been proposed that in pylorus ligation, the digestive effect of accumulated gastric juice and interferences of gastric blood circulation are responsible for induction of ulceration [24]. The anti-ulcer activity of CCGF and ECGF is evident from its significant reduction in gastric volume, free acidity, total acidity and ulcer index. Because the all treated animals significantly inhibited formation of pylorus ligation ulcer in the stomach and also decrease both acid concentration and gastric volume. It is suggested that the CCGF and ECGF can suppress gastric damage induced by aggressive factors. NSAIDs like aspirin, indomethacin cause gastric damage by inhibition

of prostaglandin synthesis [25]. The extracts were significantly protected by gastric mucus against aspirin induced ulcers. The *C. gigantea* latex extract is reported to possess the procoagulant activity, which is due to cysteine protease like constituent [21]. The protease family has drawn special attention for drug target for cure of several diseases such as osteoporosis, arthritis and cancer [26]. The latex is present abundantly throughout the plant especially in flower. Therefore, the anti-inflammatory and anti-ulcer activities of CCGF and ECGF may be due to the inhibition of COX-2 pathway of prostaglandin synthesis and procoagulant properties. However, the exact mechanism involved and the bioactive constituent responsible for said potential is remained to be explored.

Thus, in the present investigation, the data strongly suggests that the AI activities of CCGF and ECGF in acute and chronic model is by involvement of more than one mechanism. The activity could be attributed to cysteine protease like constituent of plant. The anti-ulcer potential of the above extracts studied using pylorus ligation and aspirin induced ulcer in which the extracts protect significantly, the aggressive factor and prostaglandin pathway induced ulcer, the gastro protective activity could be attributed to the procoagulant activity of cysteine proteases like bioactive compound. Hence, a detail study is needed to pinpoint exact mechanism indicating antiphlogestic, anti-inflammatory activity devoid of gastric side effect.

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