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in vivo Evaluation of Noxious Stimulus Induced Pain and Inflammation in Mice and Rats Using Methanolic Extract of *Galphimia glauca* Leaf

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This study assesses the analgesic and anti-inflammatory properties of methanolic extract of *Galphimia glauca* leaf using *in vivo* models. The dose range of 100, 200 and 400 mg/kg b.w was administered orally for assessing analgesic and anti-inflammatory activity respectively. Further studies were conducted for determining the involvement of central and peripheral receptor actions in the analgesic activity of the extract by pre-challenging it with naloxone and acetic acid respectively. The LD_{50} of the extract was found to be > 2000 mg/kg b.w. The leaf extract at 400 mg/kg dose exhibited significant ($P \leq 0.001$) and dose-dependent analgesic activity. It also exhibited central and peripheral analgesic actions when treated with naloxone and acetic acid respectively. However, the extract of leaf has shown anti-inflammatory activity, which is not comparable to that of standard drug. The results revealed that the methanolic extract of *Galphimia glauca* leaf has potential in terms of analgesic properties.

Keywords: Acetic acid, Carrageenan, Diclofenac sodium, Formalin, Granuloma, *Galphimia glauca* leaf.

INTRODUCTION

Galphimia glauca is a tropical habitat shrub, which can be found distributed across South India belonging to family of Malpighiaceae [1,2]. It is commonly known as "*Calderonaamarilla*" and "*Florestrella*" in Spanish [3]. The ethyl acetate extract of the shrub was found to have wide usage in the treatment of asthma and allergies [2]. Traditionally the shrub is used to relieve various forms of pain. A drink made from the yellow leaves of the *G. glauca* is used to relieve coronary pain as well as for soothing the nerves. The shrub is used to bring down the fever, to help women in labor and it is also used as emollient for injuries. There were no source reporting the use of *G. glauca* in treatment of pain and inflammation. Hence the present study is carried out to explore the beneficial effects of plant *G. glauca* in assessing the pain and inflammation using *in vivo* models.

EXPERIMENTAL

The shrub *G. glauca* was cultivated in the medicinal garden existing in the School of Pharmacy, Anurag Group of Institutions. *G. glauca* leaves were collected during November, 2013. The plant was identified and authenticated by taxonomist, Dr. E. Narsimha Murthy, Satavahana University, Karimnagar, Telangana state, India. A voucher copy is stored with the reference number No. 333, in the Department of Pharmacognosy and Phytochemistry, School of Pharmacy.

Extraction: *G. glauca* leaves were collected, dried in shade and powdered coarsely. 100 g of leaf powder was subjected to Soxhlet extraction using 500 mL of methanol. The extract was collected and then concentrated to dryness and stored. The yield obtained for *G. glauca* leaf methanol extract (GGLME), was 12 %.

Animals: Swiss albino mice of 6 to 8 weeks of age with 22.5 ± 2.5 g of either sex and Wistar albino rats of 12 to 14 weeks of age with 234 ± 24.8 g of either sex were used. Rodents were acclimatized for seven days to the laboratory conditions. The animals were retained in 12 h light/dark cycles at 22 ± 2 °C with 60 to 70 % relative humidity. Entire pharmacological studies were carried out randomly using six animals of either sex in each group. The experimental protocol was approved by the Institutional Animal Ethics Committee of the institute (IAEC), School of Pharmacy, Anurag Group of Institutions (the approval number: I/IAEC/LCP/032/2013/SM-35).

All the chemicals used were supplied from SD Fine Chemicals, India. Morphine was purchased from Troikaa Pharmaceuticals Inc. Gujarat, India. Carrageenan was procured from Sigma-Aldrich, USA. Diclofenac sodium and naloxone

drugs were procured from Novartis India Inc. and Samarth Pharma Inc. respectively as gift samples.

Acute toxicity studies: According to the Organization for Economic Co-operation and Development (OECD) guidelines, 423-2d, Acute oral toxicity studies were conducted [4].

Phytochemical screening: Phytochemical screening of the *G. glauca* leaf methanol extract (GGLME) was carried out using standard procedures [5,6].

Analgesic activity

Thermal stimulus model: The Swiss albino mice were placed on a hot plate which was set at a fixed temperature of 55 ± 1°C (V. J Instruments, India). Mice were distributed randomly into VII groups (n = 6) and the pre-treatment response time (withdrawing their paws) for each mice was recorded. After 1 h oral and 0.5 h intraperitoneal administration the post-treatment response was recorded with intervals of 0.5, 1.0 and 1.5 h, respectively [7].

Group I received distilled water [10 mL/kg, body weight (b.w.), per oral (p.o.)].

Group II was treated with morphine [10 mg/kg, b.w., intraperitoneally (i. p)].

Group III-V was treated with GGLME [100, 200 and 400 mg/kg, b.w., respectively, (p.o)].

Investigation of opioid receptors involvement in the analgesic activity of the extract: The GGLME with a dose of 400 mg/kg given orally was examined for this study. Specifically two groups of mice (n = 6) *i.e.*; Group VI & Group VII were pre-challenged intraperitoneally with naloxone (5 mg/kg) 15 min prior to the administration of morphine (10 mg/kg; i.p.) and oral administration of GGLME (400 mg/kg) respectively. The reaction time was recorded before and after the treatment as per the procedure mentioned in Thermal Stimulus Model [8,9].

Chemical stimulus model

Formalin test: Swiss albino mice (s) used for this study were abstained from food overnight and were used for assessing the formalin induced pain. Groups I to V was treated according to the procedure of thermal stimulus model. Group VI received diclofenac sodium (20 mg/kg; i.p.), which served as a standard drug. After 0.5 h of standard drug administration and after 1 h treatment with extract, formalin (20 µL of 2.5 % solution) was injected into the right hind paw of each animal subcutaneously. Individual animal was observed for pain responses in early (0-5 min) phase and in the late phase (15-30 min) respectively. The time (s) spent for biting or licking the hind paw was observed and recorded [10,11].

$$\text{Inhibition (\%)} = \frac{A - B}{A} \times 100$$

where, A = Reaction time [Control group], B = Reaction time [Treated group].

Writhing test: The Swiss albino mice (s) used for the study were grouped into VII groups (n = 6) and kept on a fast overnight. Group I received water (10 mL/kg), group II received diclofenac sodium (20 mg/kg; i.p.), whereas group III to V received the *G. glauca* extract treatment in accordance with the procedure of thermal stimulus model. After 0.5 h of drug/extract administration, all the animals were treated intraperitoneally with 0.7 % acetic acid (10 mL/kg) and the numbers of writhing's were recorded for a duration of 0.5 h [12,13].

$$\text{Inhibition (\%)} = \frac{A - B}{A} \times 100$$

where, A = Number of writhes [Control group], B = Number of writhes [Treated group].

Investigation of peripheral receptors involvement in the analgesic activity of the extract: Separately two groups of mice (n = 6), Group VI and Group VII were pre-challenged intraperitoneally with naloxone (5 mg/kg,) 15 min prior to the i.p. administration of diclofenac sodium (20 mg/kg) and oral administration of GGLME (400 mg/kg) respectively. After 0.5 h, the animals were subjected to writhing test and the results were recorded [8].

Mechanical stimulus model

Haffner's tail clip method: The Wistar albino rats used in this study were screened initially for inducing pain at root of the tail by applying a metal artery clip. Rats were grouped into V groups (n = 6) and pre-treatment response time for individual rat was recorded. Groups I to V was treated according to the procedure of thermal stimulus model.

After 1 h of oral and 0.5 h of i.p. administration of the leaf extract and standard drug, the same procedure was used for recording the post-treatment response time [14].

$$\text{Inhibition (\%)} = \frac{[\text{Post treatment latency}] - [\text{Pre treatment latency}]}{[\text{Cut of time} - \text{Pre treatment latency}]} \times 100$$

Anti-inflammatory activity

Carrageenan induced paw edema model: The carrageenan induced paw edema test was performed based on the method described by Laupattarakasem *et al*. [15] and Winter *et al.* [16]. The Wistar albino rats used in this study were grouped into V groups (n = 6). Group I received water (10 mL/kg), Group II was treated with saline for initial seven days and with diclofenac sodium (20 mg/kg; i.p.) on the day of treatment. Groups III to V were treated with appropriate doses of leaf methanol extract for seven consecutive days as mentioned in procedure of Thermal Stimulus Model. The rats were kept on fast overnight and on the 8th day *i.e.*, 1 h after the administration of diclofenac sodium and leaf methanol extract of varying doses, the rat paw edema was induced to all the groups by injecting carrageenan (0.1 mL, 1 % w/v in saline) into sub plantar region of right hind paw. The change in the paw volume was recorded immediately at different intervals of time (1st, 2nd, 3rd and 4th h) in both drug and extract treated groups before and after carrageenan challenge test using digital Plethysmometer (V.J. Instruments, India).

$$\text{Inhibition (\%)} = \frac{A - B}{A} \times 100$$

where, A = Mean edema in control group, B = Mean edema in drug treated group.

Cotton pellet induced granuloma experiment: The Wistar albino rats used in this study were grouped into V groups (n = 6) and were abstained from food overnight. Sterilized cotton pellets weighing about 20 mg each were used. The rats were anesthetized using urethane (1.5 g/kg; i.p.) and the skin

incision was made on the dorsal side of the rats and a cotton pellet was inserted subcutaneously, finally the incision was closed using surgical suture. Drug treatment started and continued for seven consecutive days. Group I received water (10 mL/kg), Group II was treated with diclofenac sodium (20 mg/kg; i.p.), whereas Groups III to V were treated with appropriate doses for seven days in accordance with the procedure of thermal stimulus model. Finally on the 8th day, the rats were anesthetized and cotton pellets were removed out and foreign tissues were taken off and dried for about 24 h at 60 °C and the dry weights were recorded. The transudative and granuloma weights were recorded and the percentage inhibition of granuloma tissue formation was determined applying the given formula [17].

$$\text{Inhibition (\%)} = \frac{A - B}{A} \times 100$$

where, A = Granuloma tissue weight [Control group], B = Granuloma tissue weight [Treated group].

Statistical analysis: The results were reported as mean ± SEM. Statistical analysis were performed with one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test to calculate the significance of results. All the statistical analysis was performed using Graph Pad Prism 5.0 software.

RESULTS AND DISCUSSION

Acute toxicity studies: The results showed no mortality/toxic symptoms in mice and rats treated with leaf extract (2000 mg/kg) until the 14th day of the treatment period according to the OECD 423-2d guidelines. Based on the results, we have selected 100, 200 and 400 mg/kg as low, moderate and high doses to assess the analgesic and anti-inflammatory studies.

Phytochemical screening: In the phytochemical screening leaf methanol extract showed positive results for carbohydrates, proteins, amino acids, flavonoids, tannins and phenolic compounds.

Analgesic activity

Thermal stimulus model: The GGLME increased the latency time in a dose dependent way. The onset of activity was observed at 0.5 h and it reached to a peak at 1.5 h. The activity was comparable ($P \leq 0.001$) with standard morphine (10 mg/kg). The results were tabulated in Table-1.

Opioid receptors involvement in the analgesic activity of plant extract: The central analgesic activity results of GGLME are depicted in Table-1. The naloxone treated group (5 mg/kg) had significantly reversed the pain relieving property of GGLME at a dose of 400 mg/kg thus confirming the central actions of the leaf extract.

Formalin test: The GGLME (400 mg/kg) had showed 51.96 %, 66.32 % inhibition of pain in the early and late phase respectively. The results are represented in Table-2.

Writhing test: The GGLME (400 mg/kg) exhibited significant (66.2 % inhibition) analgesic activity in a dose dependent manner when compared to that of diclofenac sodium (20 mg/kg; i.p.), which showed 74.97 % inhibition. The results are reported in Fig. 1.

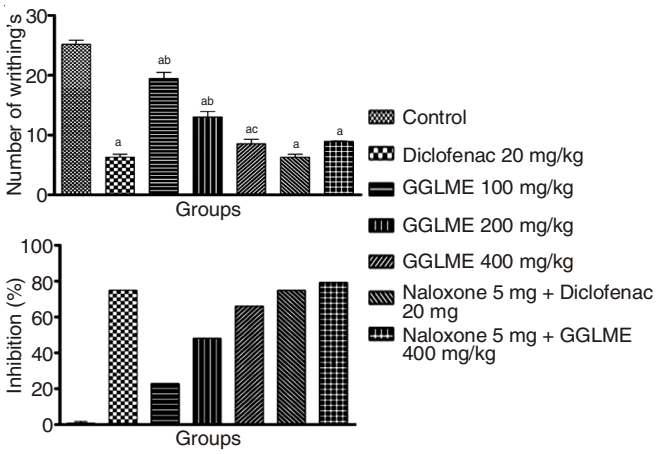

Fig. 1. Effects of *G. glauca* leaf methanol extract (GGLME) on acetic acid induced pain in mice (Writhing test) (a) Number of writhing's (b) Inhibition percentage (%); [a]$P \leq 0.001$ indicates comparison with group I. [b]$P \leq 0.01$ indicates comparison with group II. [c]$P \leq 0.001$ indicates the dose dependent activity on comparison of the high dose with respective low dose of the extract

Peripheral receptors involvement in the analgesic activity of plant extract: The drug naloxone (5 mg/kg) has showed no effect on the abdominal constriction in mice, which were treated with GGLME (400 mg/kg) and diclofenac sodium (20 mg/kg). The results revealed that the activity was a result of the activation of peripheral receptors. GGLME (100, 200 mg/kg) results are not mentioned here. The above results are shown in Fig. 1.

Haffner's tail clip test: The GGLME showed maximum effect with 61.4 %, 39.3 % and 20.9 % inhibition of pain at high, medium and low doses respectively. The results are tabulated in Table-3.

TABLE-1
ANALGESIC EFFECT OF *G. glauca* LEAF METHANOL EXTRACT ON THERMAL STIMULUS INDUCED PAIN IN MICE

Group	Dose (mg/kg)	Reaction time after administering control/standard/extract (s)			
		0 h	0.5 h	1.0 h	1.5 h
I (Distilled water)	10 (mL/kg)	3.6 ± 0.08	3.3 ± 0.07	3.6 ± 0.50	3.6 ± 0.50
II (Morphine)	10	3.7 ± 0.10	7.9 ± 0.50 [a]	9.8 ± 0.50 [a]	12.1 ± 0.50 [a]
III (GGLME)	100	3.8 ± 0.20	4.0 ± 0.06 [ab]	4.7 ± 0.20 [ab]	5.4 ± 0.20 [ab]
IV (GGLME)	200	3.8 ± 0.10	4.2 ± 0.10 [ab]	6.0 ± 0.10 [abc]	5.9 ± 0.05 [ab]
V (GGLME)	400	3.7 ± 0.10	5.5 ± 0.02 [abc]	6.4 ± 00.5 [abc]	7.7± 0.50 [abc]
VI (Naloxone + morphine)	(5 + 10)	3.6 ± 0.08	4.0 ± 0.05 [a]	3.5 ± 0.30 [b]	3.6 ± 0.20 [b]
VII (Naloxone + GGLME)	(5 + 400)	3.6 ± 0.01	4.1 ± 0.15 [a]	3.7 ± 0.50 [b]	3.6 ± 0.50 [b]

Values are expressed as mean ± SEM; n = 6; the statistical significance done by ANOVA, followed by Tukey's multiple comparison tests and is represented by a symbol. [a]$P \leq 0.001$ indicates comparison with group I. [b]$P \leq 0.001$ indicates comparison with group II. [c]$P \leq 0.001$ indicates the dose dependent activity on comparison of the high dose with respective low dose of the extracts.

TABLE-2
ANALGESIC EFFECT OF *G. glauca* LEAF METHANOL EXTRACT ON FORMALIN INDUCED PAIN IN MICE

Group	Dose (mg/kg)	Paw licking time (s)			
		Early phase (0-5 min)	Inhibition (%)	Late phase (15- 30 min)	Inhibition (%)
I (Distilled water)	10 (mL/kg)	178.7 ± 5.5	-	118.8 ± 3.8	–
II (Morphine)	10	40.6 ± 1.3 [a]	77.28	4.6 ± 0.5[a]	96.12
III (GGLME)	100	141 ± 3.1[abc]	21.00	73 ± 0.9 [ab]	38.50
IV (GGLME)	200	111 ± 0.5[abc]	37.88	58 ± 0.6 [ab]	51.17
V (GGLME)	400	85 ± 0.5[abc]	51.96	40 ± 0.6 [b]	66.32
VI (Diclofenac sodium)	20	91 ± 1.7 [a]	49.07	14 ± 0.5[a]	88.21

Values are expressed as mean ± SEM.; n = 6; the statistical significance done by ANOVA, followed by Tukey's multiple comparison tests and is represented by a symbol. [a]P ≤ 0.001 indicates comparison with group I. [b]P ≤ 0.001 indicates comparison with group II. [c]P ≤ 0.001 indicates the dose dependent activity on comparison of the high dose with respective low dose of the extracts.

TABLE-3
ANALGESIC EFFECT OF *G. glauca* LEAF METHANOL EXTRACT ON
TAIL CLIP INDUCED PAIN IN RATS (HAFFNER'S TAIL CLIP TEST)

Group	Dose (mg/kg)	Pre-treatment reaction latency (s)	Post-treatment reaction latency (s)	Inhibition (%)
I (Distilled water)	10 (mL/kg)	1.50 ± 0.02	1.54 ± 0.02	–
II (Morphine)	10	1.68 ± 0.20	11.6 ± 0.30 [a]	85.51
III (GGLME)	100	1.74 ± 0.20	2.2 ± 0.10 [b]	20.90
IV (GGLME)	200	1.76 ± 0.02	2.9 ± 0.10 [ab]	39.30
V (GGLME)	400	1.62 ± 0.20	4.2 ± 0.10 [abc]	61.40

Values are expressed as mean ± SEM.; n = 6; the statistical significance done by ANOVA, followed by Tukey's multiple comparison tests and is represented by a symbol. [a]P ≤ 0.001 indicates comparison with group I. [b]P ≤ 0.001 indicates comparison with group II. [c]P ≤ 0.001 indicates the dose dependent activity on comparison of the high dose with respective low dose of the extracts.

Anti-inflammatory activity

Carrageenan induced paw edema model: The obtained experimental results are reported in Table-4. *G. glauca* leaf methanol extract at 400 mg/kg dose showed 29.62 and 37.82 % inhibition of paw edema at 3rd and 4th h respectively when compared with standard diclofenac sodium at respective time points.

Cotton pellet induced granuloma test: The experimental results are reported in Table-5. The GGLME (400 mg/kg) showed 96 mg reduction in transudative weight of cotton pellets and 33 % inhibition of granuloma formation when compared to diclofenac sodium, which showed 98 mg reduction in transudative weight and 76.19 % inhibition of granuloma formation.

Methanol extract of *Galphimia glauca* leaf was explored for assessing *in vivo* analgesic and anti-inflammatory properties.

TABLE-4
ANTI-INFLAMMATORY EFFECT OF *G. glauca* LEAF METHANOL
EXTRACT ON CARRAGEENAN INDUCED PAW EDEMA TEST IN RAT

Group	Dose (mg/kg)	Changes in paw edema volume after administration of control/standard/extracts (h)							
		1 h	IPE (%)	2 h	IPE (%)	3 h	IPE (%)	4 h	IPE (%)
I (Distilled water)	10 (mL/kg)	1.19±0.002	–	1.32±0.002	–	1.62±0.09	–	1.93±0.01	–
II (Diclofenac sodium)	20	0.72±0.002[a]	39.49	0.58±0.01[a]	56.06	0.49±0.02[a]	69.75	0.32±0.01[a]	83.41
III (GGLME)	100	1.11±0.10	6.72	1.22±0.01[ab]	9.00	1.39±0.01[ab]	14.00	1.58±0.01[ab]	18.10
IV (GGLME)	200	1.00±0.08	15.96	1.09±0.01[abc]	17.42	1.26±0.05[abc]	22.22	1.44±0.2[abc]	25.38
V (GGLME)	400	0.93±0.16	21.84	1.03±0.2[abc]	21.96	1.14±0.01[abc]	29.62	1.20±0.01[abc]	37.82

Values are expressed as mean ± SEM; n = 6; the statistical significance done by ANOVA, followed by Tukey's multiple comparison tests and is represented by a symbol. [a]P ≤ 0.001, indicates comparison with group I. [b]P ≤ 0.001 indicates comparison with group II. [c]P ≤ 0.001 indicates the dose dependent activity on comparison of the high dose with respective low dose of the extracts. IPE (%) indicates percentage inhibition of paw edema.

TABLE-5
ANTI-INFLAMMATORY EFFECT OF *G. glauca* LEAF METHANOL EXTRACT
ON COTTON PELLET INDUCED GRANULOMA TEST IN RAT

Group	Dose (mg/kg)	Granuloma wet weight (mg)	Granuloma dry weight (mg)	Transudative weight (mg)	Granuloma weight (mg) (mg/mg cotton)	Inhibition of granuloma (%)
I (Distilled water)	10 (mL/kg)	189 ± 2.54	62 ± 2.5	127 ± 1.5	2.1 ± 0.1	–
II (Diclofenac sodium)	20	128 ± 2.90 [a]	30 ± 1.5[a]	98 ± 2.0 [a]	0.5 ± 0.1 [a]	76.19
III (GGLME)	100	177 ± 2.1[b]	59 ± 1.2 [b]	118 ± 2.8 [b]	1.95 ± 0.9 [b]	7.10
IV (GGLME)	200	169 ± 1.16 [a]	56 ± 1.5 [b]	113 ± 1.7 [b]	1.8 ± 0.09 [b]	14.20
V (GGLME)	400	144 ± 1.3 [ac]	48 ± 2.0 [a]	96 ± 3.2 [a]	1.4 ± 0.06 [a]	33.00

Values are expressed as mean ± SEM.; n = 6; the statistical significance done by ANOVA, followed by Tukey's multiple comparison tests and is represented by a symbol. [a] P ≤ 0.001 indicates comparison with group I. [b]P ≤ 0.01 indicates comparison with group II. [c]P ≤ 0.001 indicates the dose dependent activity on comparison of the high dose with respective low dose of the extracts.

The hot plate and tail clip test methods were used for confirming the central actions, while the formalin and writhing's tests were conducted for the peripheral effects. The GGLME was given orally with 400 mg/kg dose showed significant and dose dependent analgesic activity in both models of pain (central and peripheral). Further the central opioid receptors involvement is confirmed by the reversed pain relieving property through the administration of naloxone. The above study results suggest the central analgesic actions of the leaf extract was perhaps mediated by the inhibition of opioid receptors. Similar kind of results was earlier reported by Zakaria *et al*. [18].

Morphine inhibits both the phases of formalin induced pain whereas diclofenac inhibits only late inflammatory phase. In formalin test the GGLME showed significant dose dependent action in both phases of pain. The acetic acid induced caused writhings, which is used for screening peripheral analgesic activity. The drugs which were used act through inhibition of COX enzyme in the peripheral tissues by blocking the synthesis and or release of inflammatory mediators like Prostaglandins [19]. *G. glauca* leaf methanol extract lessened the number of writhings in mice in a dose dependent way to that of diclofenac sodium. The results revealed significant central and peripheral actions of GGLME acting at both the phases in the formalin test and in acetic acid induced pain.

Carrageenan induced paw edema model is applied to study acute inflammation processes. The paw edema that was induced is biphasic; the initial phase involves the release of histamine, kinins and serotonin while the late phase is mediated by prostaglandins. However, the extract of leaf has shown anti-inflammatory activity, which is not comparable to that of standard drug diclofenac sodium, suggesting its action to be insignificant [20].

Usually cytokinins are of two types, proinflammatory and anti-inflammatory. Among the proinflammatory cytokinins TNF (tumor necrosis factor), interleukin-1 (IL-1) and interleukin-6 (IL-6) have been most widely studied with respect to metabolic regulation after inflammation. Tumor necrosis factor stimulates the release of IL-1 and both the TNF and IL-1 stimulate the release of IL-6. Other cytokinins involved in inflammation includes interleukin-4 (IL-4), interleukin-7 (IL-7), interleukin-8 (IL-8). In chronic inflammation, there is a macrophage stimulation by IL-1α, IL-1β, IL-2 and TNF-α and proliferation of macrophages by M-CSF (Macrophage colony stimulating factor), proliferation of fibroblasts and multiplication of small blood vessel. The anti-inflammatory drugs act by altering the endogenous factors involved in the migration of substances to the site of inflammation or by inhibiting the release of inflammatory mediators TNF-α, IFN-γ, IL-1, IL-2 [21]. In cotton pellet induced granuloma test, the GGLME treated rats exhibited insignificant inhibitory effects in transudative and proliferative phases of inflammation. The activity found to be insignificant; however the effect showed by the leaf extract may be due to the above said mechanisms acting on inflammatory mediators.

The phytochemical results of *G. glauca* leaf methanol extract showed the presence of secondary metabolites like flavonoids, tannins and phenolic compounds. One or a combination of the above plant constituents may be responsible for the analgesic and anti-inflammatory properties of GGLME in the present study.

Conclusion

The results showed that the GGLME has potential antinociceptive activity involving both central and peripheral mechanisms. However, the extract of leaf has shown anti-inflammatory activity, which is not comparable to that of diclofenac sodium. Further studies are planned to explore the phytochemical profile using HPLC/HPTLC. The bioactivity studies on different fractions of the GGLME have to be performed to identify the probable active compound(s).

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