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Anti-nociceptive and anti-inflammatory activity of hydroalcoholic extract of leaves of *Amaranthus tricolor* L.

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ABSTRACT

Amaranthus tricolor Linn. (Family- Amaranthaceae) popularly known as Joseph's coat in English. The plant has been used as hepatoprotective and hypoglycemic in Indian system of traditional medicine. In present study the hydroalcoholic extract of leaves of *Amaranthus tricolor* L. (HAEAT) 100, 200 and 400 mg/kg body weight was studied for anti-nociceptive and anti-inflammatory activities in various animal models. Anti-nociceptive activity was carried out by using acetic acid-induced abdominal writhing test and hot plate test in mice. Anti-inflammatory activity was carried out by using carrageenan induced rat paw edema and cotton pellet induced granuloma tests in rats. The results suggested that HAEAT showed significant anti-nociceptive activity in acetic acid induced writhing model but not in hot plate model which reveals that anti-nociceptive activity of HAEAT is of the type produced by non-narcotic analgesics and anti-inflammatory activity was observed in both models. In carrageenan induced rat paw edema model the extract was found to exhibit a significant reduction in paw volume in late phase (3 to 5 h) and in cotton pellet induced granuloma model HAEAT decreased the increase in weight of cotton pellets. The observed pharmacological activities may be due to presence of phytochemicals like flavonoids, alkaloids, phenolic compounds, etc present in extract.

Keywords: Anti-inflammatory, Anti-nociceptive, *Amaranthus tricolor* L.

INTRODUCTION

Inflammation plays an important role in various diseases, such as rheumatoid arthritis, atherosclerosis and asthma, which all show a high prevalence globally. During an inflammatory response, mediators, such as pro-inflammatory cytokines, including interleukin (IL)-1, tumour necrosis factor (TNF), interferon (INF)-c, IL-6, IL-12, IL-18 and the granulocyte-macrophage colony-stimulating factor are released; this response is antagonized by anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, IFN-a and the transforming growth factor [1]. Early inflammatory changes in damaged tissues are known to involve the release of various biologically active materials from polymorph nuclear leukocytes, lysosomal enzymes and others. The vascular effects are primarily mediated by kinins, prostaglandins and vasoactive amines (histamine) released by mast cells [2]. Since prostaglandins are cytoprotective, long-term administration of nonsteroidal anti-inflammatory drugs (NSAIDs) may induce gastro-intestinal ulcers, bleeding, and renal disorders due to their non-selective inhibition of both isoforms of the COX enzyme, the constitutive (COX-1) and the inducible (COX-2) isoforms [3]. Despite the wide availability of clinically useful agents, and the great therapeutic efficacy of NSAIDs in controlling inflammatory symptoms, they usually have undesirable effects, which are closely connected with their mechanism of action [4].

On the other hand several medicinal plants have been proved to possess anti-inflammatory activity [5]. *Amaranthus tricolor* L. is a member of Amaranthaceae family. *Amaranthus tricolor* L. is a small annual or biennial herb grows up to 1 meter in height. Leaves are simple, alternate, ovate or rhomboid, acute or acuminate and dark brown colored. *Amaranthus tricolor* L. is a species which is very closely related to *Amaranthus spinosus*, *Amaranthus hybridus*, & *Amaranthus dubius* [6]. The methanol extract of the three plants (*Amaranthus viridis*, *Amaranthus caudatus* and *Amaranthus spinosus*) belonging to the family Amaranthaceae has been reported to be analgesic at the doses of 200 mg/kg and 400 mg/kg body weight [7]. *Amaranthus tricolor* L. contains a different betalains [8]. *Amaranthus spinosus* is also reported for its anti-inflammatory activity [9]. Three galactosyl diacylglycerols (1-3) with potent cyclooxygenase and human tumor cell growth inhibitory activities have also been isolated from the leaves of *Amaranthus tricolor* L. but no research was conducted to support in vivo anti-inflammatory activity of *Amaranthus tricolor* L. Linolenic, palmitic acid and spinasterol are also reported to be present in the leaves of the plant [10]. *Amaranthus tricolor* L. roots are considered as demulcent and in the form of decoction used for piles and diarrhea in children. The plant also possesses antioxidant and hepatoprotective activity. The presence of phytochemicals like carbohydrates, flavonoids (betacyanins A and B, amaranthin, isoamaranthin and quercetin), proteins and amino acids (proline, cysteine, tryptophan, leucine, glutamic acid, etc), steroids and fatty acids have been reported to be present in plant [11].

As no scientific data on the anti-nociceptive and anti-inflammatory activity of hydroalcoholic extract of leaves of *Amaranthus tricolor* L. (HAEAT) is reported, hence the present study was done to evaluate scientifically the usefulness of this plant.

MATERIALS AND METHODS

2.1. Experimental animals and approval.

Female wistar rats weighing (180-220 g) and Female swiss albino mice (25-30 g) were purchased from National Toxicology Centre, Pune, India. They were maintained at a temperature of 25 ± 1 °C and relative humidity of 45 to 55% under 12-h light : 12-h dark cycle. The animals had access to food pellets (Manufactured by Pranav Agro Industries Ltd., Sangli India) and water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA), India (CPCSEA/22/12).

2.2 Preparation of *Amaranthus tricolor* L.

Amaranthus tricolor L. leaves (1 kg) were air dried at room temperature, powdered and macerated in water: methanol (50:50) for 7 days with intermittent shaking. The cold macerated extract was then filtered and the filtrate was dried on tray dryer at 40 °C (yield – 11.4 % w/w). The dry extract was stored in a refrigerator at 4-5 °C and used for pharmacological studies.

2.3 Collection and authentication of plant

Amaranthus tricolor L. was collected during August 2011 from the local market of Pune, Maharashtra, India. The plant was identified and authenticated at the Agharkar Research Institute, Pune, India and voucher specimen (WP-089) was deposited at that institute for future reference.

2.4 Preliminary phytochemical screening

Qualitative phytochemical analysis of the extract was carried out using standard procedures and tests for the presence of phytochemicals such as glycosides, alkaloids, saponins, flavonoids, phenols and steroidal compounds [12].

2.5 Acute toxicity study

Acute toxicity study was performed as per OECD guidelines 425. Five female Swiss albino mice were used for study. The animals were kept fasting for overnight providing only water. The test drug was administered orally at one dose level of 2000 mg/kg body weight. In further, animals were observed continuously for the first 4 h and then periodically up to 24 h for toxic symptoms and mortality.

2.6 Anti-nociceptive activity

2.6.1. Acetic acid-induced abdominal writhing

Swiss albino mice were randomly divided into five groups (n=6);

Group 1-Vehicle control,

Group 2-Acetyl salicylic acid (100 mg/kg, p.o.),

Group 3-HAEAT (100 mg/kg, p.o.),

Group 4- HAEAT (200 mg/kg, p.o.),

Group 5-HAEAT (400 mg/kg, p.o.).

The mice were pretreated orally with HAEAT and acetylsalicylic acid, 60 min before the administration of acetic acid solution at a dose of 10 ml/kg (0.6%, i.p). The numbers of abdominal constrictions (full extension of both hind paws) were cumulatively counted over a period of 15 min [13]. The anti-nociceptive activities were expressed as mean number of writhes (Table 1) and percentage inhibition was calculated by the following formula:

$$\% \text{ Inhibition} = (1 - \text{WT} / \text{WC} * 100)$$

Where, WC and WT were mean number of writhes observed in vehicle control group and treatment group respectively.

2.6.2. Hot plate test

Swiss albino mice were randomly divided into five groups (n=6);

Group 1-Vehicle control,

Group 2-Pentazocine (30 mg/kg, p.o.),

Group 3-HAEAT (100 mg/kg, p.o.),

Group 4- HAEAT (200 mg/kg, p.o.),

Group 5-HAEAT (400 mg/kg, p.o.).

Mice were screened by placing them on Eddy's hot plate maintained at 55 ± 1 °C and the reaction time was recorded in seconds. The pain threshold was considered to be reached when animals showed the signs of paw licking or jumping. Only mice which reacted within 15s and which did not show large variations when tested on four separate occasions, each 15 min apart, were taken for the test. The time for paw licking or jumping on the Eddy's hot plate was considered as a reaction time.

Various responses such as paw licking or jumping were recorded before and after 30, 60, 90, 120, 150 and 180 min after the oral administration of HAEAT and Pentazocine [14]. A cut-off time of 15s was used to avoid injury to the animals (Figure 1).

2.7 Anti-inflammatory activity

2.7.1 Carrageenan - induced hind paw edema in rats (acute study)

The wistar rats were divided into five groups (n=6);

Group 1-Carrageenan control,

Group 2-Diclofenac (10 mg/kg, p.o.),

Group 3-HAEAT (100 mg/kg, p.o.),

Group 4- HAEAT (200 mg/kg, p.o.),

Group 5-HAEAT (400 mg/kg, p.o.).

Acute inflammation was produced by injecting 0.1ml of 1% lambda carrageenan (Sigma Chemical Co., USA) in sterile normal saline into the sub plantar region of the left hind paw of the rat [15].

Rats were pretreated orally with HAEAT and diclofenac 1h before the carrageenan injection. The paw volume was measured from 0-6 h, at an hourly interval using plethysmometer (Ugo Basile, Italy). The mean changes in injected paw volume with respect to initial paw volume were calculated. Percentage inhibition of paw volume between treated and control group was calculated by the following formula (Table 3).

$$\% \text{ Inhibition} = (1 - VT / VC * 100)$$

Where, VT and VC are the mean increase in paw volume in treated and control groups, respectively.

2.7.2 Cotton pellet induced granuloma in rats (chronic study)

The wistar rats were divided into five groups (n=6);

Group 1-Vehicle control,

Group 2-Diclofenac (10 mg/kg, p.o.),

Group 3-HAEAT (100 mg/kg, p.o.),

Group 4- HAEAT (200 mg/kg, p.o.),

Group 5-HAEAT (400 mg/kg, p.o.).

Chronic inflammation was produced by implanting the pre-weighed sterile cotton pellets (50mg) in the axilla region of the each rat through a small incision [16, 17]. Before implanting the cotton pellets, rats were anaesthetized with anesthetic ether.

HAEAT and diclofenac were administered orally for seven consecutive days after the cotton pellet implantation. On the eight day animals were sacrificed by cervical dislocation and the cotton pellets were removed from animal's body, freed from the extraneous tissues, dried at 60 °C for 24 h and weighed.

Statistical Analysis

Data was expressed as mean \pm SEM and statistical analysis was carried out by using GraphPad 5.0 software (GraphPad, San Diego, USA) by applying one-way ANOVA with Dunnett's test or two-way ANOVA with Bonferroni test $p < 0.05$ was considered to be significant.

RESULTS

3.1 Preliminary phytochemical screening

Preliminary phytochemical analysis of HAEAT showed the presence of glycosides, alkaloids, saponins, flavonoids, phenols and steroidal compounds in the extract.

3.2 Acute oral toxicity

Administration of HAEAT (2000 mg/kg, p.o) did not produce any toxic symptoms and mortality. Hence, the extract was found to be safe at the dose of 2000 mg/kg b.w. Therefore three doses (100, 200 and 400 mg/kg b.w) were selected for pharmacological studies.

3.3 Anti-nociceptive activity

3.3.1 Acetic acid induced writhing

The number of writhes in HAEAT (100, 200 and 400 mg/kg) pretreated animals was 63 ± 1.6 , 60 ± 1.2 and 56 ± 2.6 respectively. The percentage reduction was for 100 mg/kg (9.08%), 200 mg/kg (14.67%) and 400 mg/kg (20.27). The number of writhes in the acetic acid vehicle control group was found to be 70 ± 1.8 . The result thus indicated that HAEAT (200 mg/kg) significantly ($p < 0.01$) and HAEAT (400 mg/kg) significantly ($p < 0.001$) decreased the number of acetic acid induced writhes. Acetyl salicylic acid (100 mg/kg) appears to be more effective than the highest dose of HAEAT in reducing the number of acetic acid induced writhes (Table 1).

Table 1:- Effect of hydroalcoholic extract of leaves of *Amaranthus tricolor* L. (HAEAT) in acetic acid induced writhing in mice.

| Treatment | Dose (mg/kg, p.o.) | Number of writhing | Percentage reduction |
|-----------------------|--------------------|--------------------|----------------------|
| Vehicle control | - | 70 ± 1.8 | - |
| Acetyl salicylic acid | 100 | $26 \pm 1.5^{***}$ | 63.26 |
| HAEAT | 100 | 63 ± 1.6 | 9.08 |
| HAEAT | 200 | $60 \pm 1.2^{**}$ | 14.67 |
| HAEAT | 400 | $56 \pm 2.6^{***}$ | 20.27 |

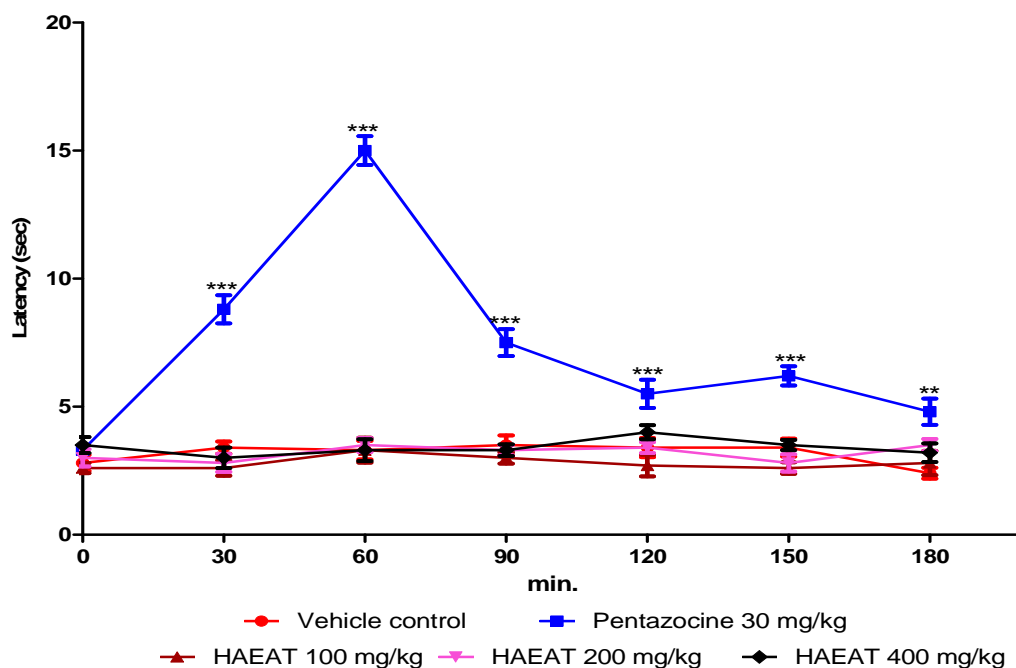
Values are expressed as mean \pm SEM for six animals and analysed by One way ANOVA followed by Dunnett's test, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared to vehicle control.

3.3.2 Hot plate

Pentazocin (30 mg/kg, p.o.) significantly increased the pain latency whereas HAEAT pretreatment failed to increase the pain latency at all the doses (Figure 1).

Figure

Figure 1:- Effect of hydroalcoholic extract of leaves of *Amaranthus tricolor* L. (HAEAT) on response latency (sec) in Eddy's hot plate model.



Values are expressed as mean \pm SEM for six animals and analysed by Two way ANOVA followed by Bonferroni post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to healthy control.

Table 2:- Effect of hydroalcoholic extract of leaves of *Amaranthus tricolor* L. (HAEAT) on carrageenan-induced rat paw edema

| Treatment | Dose (mg/kg, p.o.) | Change in paw volume (ml) | | |
|---------------------|--------------------|-------------------------------|-------------------------------|-------------------------------|
| | | 1 h | 3 h | 5 h |
| Carrageenan Control | - | 0.83 \pm 0.04 | 1.90 \pm 0.05 | 2.70 \pm 0.09 |
| Diclofenac | 10 | 0.43 \pm 0.07*** (48.19) | 0.57 \pm 0.05*** (70.00) | 0.37 \pm 0.08*** (86.30) |
| HAEAT | 100 | 0.77 \pm 0.03 (7.23) | 1.80 \pm 0.05 (5.26) | 2.10 \pm 0.09*** (22.22) |
| HAEAT | 200 | 0.68 \pm 0.07 (18.07) | 1.30 \pm 0.08*** (31.58) | 0.99 \pm 0.07*** (63.33) |
| HAEAT | 400 | 0.65 \pm 0.05 (21.69) | 1.10 \pm 0.12*** (42.11) | 0.62 \pm 0.06*** (77.04) |

Values are expressed as mean \pm SEM for six animals and analysed by Two way ANOVA followed by Bonferroni post-hoc test, *** $p < 0.001$ when compared to carrageenan control. The figures in parenthesis indicate the percent inhibition.

3.4 Anti-inflammatory activity

3.4.1 Carrageenan-induced rat hind paw edema (Acute study)

Maximum phlogistic response of carrageenan was observed at 3–5 h after the injection in the control animals. HAEAT (200 mg/kg and 400 mg/kg) produced a significant ($p < 0.001$) reduction in paw volume at 3rd and 5th h when compared to carrageenan control animals. HAEAT (100 mg/kg) showed significant ($p < 0.001$) inhibition in paw volume at 5th h when compared to carrageenan control animals. HAEAT at 100, 200, and 400 mg/kg showed 5.26%, 31.58%, and 42.11% of inhibition at 3rd h and 22.22%, 63.33%, and 77.04% of inhibition at 5th h respectively. Diclofenac (10 mg/kg) produced a significant ($p < 0.001$) inhibition in paw volume at 1st, 3rd, and 5th h

when compared to carrageenan control animals. Diclofenac significantly inhibited the edema formation by 70.00% and 86.30% at 3rd and 5th h respectively when compared to carrageenan control animals (Table 2).

3.4.2 Cotton pellet-induced granuloma in rats (chronic study)

In cotton pellet induced granuloma, the HAEAT (200 and 400 mg/kg) significantly ($p < 0.001$) inhibited the granuloma formation when compared to vehicle control group. The degree of inhibition was dose dependent. The increase in weight of cotton pellet in HAEAT (100, 200 and 400 mg/kg) pretreated animals was 80 ± 1.3 , 60 ± 1.5 and 41 ± 0.97 respectively. The percentage inhibition was for 100 mg/kg (6.98), 200 mg/kg (30.23) and 400 mg (52.33). Diclofenac (10 mg/kg) significantly ($p < 0.001$) inhibited the granuloma formation by 67.44% (Table 3).

Table 3:- Effect of hydroalcoholic extract of leaves of *Amaranthus tricolor* L. (HAEAT) on cotton pellet-induced granuloma in rats

| Treatment | Dose (mg/kg, p.o.) | Increase in weight of cotton pellet (mg) | Percentage inhibition |
|-----------------|--------------------|--|-----------------------|
| Vehicle control | - | 86 ± 1.4 | - |
| Diclofenac | 10 | $28 \pm 1.4^{***}$ | 67.44 |
| HAEAT | 100 | $80 \pm 1.3^*$ | 6.98 |
| HAEAT | 200 | $60 \pm 1.5^{***}$ | 30.23 |
| HAEAT | 400 | $41 \pm 0.97^{***}$ | 52.33 |

Values are expressed as mean \pm SEM for six animals and analysed by One way ANOVA followed by Dunnett's test, * $p < 0.05$, *** $p < 0.001$ when compared to vehicle control.

DISCUSSION

Over the centuries, a number of medicinal plants have been used for the treatment of the disorders associated with the inflammatory conditions. These medicinal plants owe their activities due to the phytoconstituents and may exert anti-inflammatory effect by interfering generally with the inflammatory pathways or specifically with certain components of the pathway, such as release of pro-inflammatory mediators, migration of leukocytes under inflammatory stimulus with consequent release of the cytoplasmic contents at inflammatory sites [18]. Therefore, the present study was aimed at evaluating the scientific basis for the traditional use of *Amaranthus tricolor* L. using *in vivo* nociceptive and inflammatory models.

The present study demonstrates that systemic administration of the HAEAT, at doses that did not produce any motor performance alteration, produced consistent anti-nociceptive and anti-inflammatory effects in different models of pain and inflammation. The anti-nociceptive effect of HAEAT was observed in acetic acid-induced writhing model, but not in the hot plate model. Moreover, HAEAT produced anti-inflammatory effect on carrageenan-induced rat paw edema and cotton pellet induced granuloma in rats. The preliminary phytochemical study performed showed the presence of glycosides, alkaloids, saponins, flavonoids, phenols and steroidal compounds in the extract. Acute toxicity studies revealed the non-toxic nature of extract at the dose of 2000 mg/kg. There was no toxic reactions or mortality found at selected doses until the end of study period.

The acetic acid induced writhing test has long been used as a screening tool for the assessment of anti-nociceptive properties of new substances [3]. Acetic acid causes an increase in peritoneal fluids of prostaglandin E2 (PGE2) and prostaglandin F2a (PGF2a) and it is a very sensitive model for screening anti-nociceptive effect of extracts. Experiments on mice showed that acetic acid injection induces an inflammatory process by causing tissue injury [19]. The HAEAT exhibited anti-nociceptive activity in acetic acid induced writhing model. It significantly inhibited the abdominal constrictions in mice. The observed effects of the HAEAT suggested that prostaglandins may be involved in the action of the extract. HAEAT produced significant results at the dose of 200 mg/kg ($p < 0.01$) and 400mg/kg ($p < 0.001$) compared to vehicle control group in the acetic acid induced writhing study which indicates high level of analgesic activity at that doses. HAEAT failed to exhibit any significant anti-nociceptive activity in the hot plate model which reveals that the anti-nociceptive activity of HAEAT is of the type produced by non-narcotic analgesics [14].

It is well known that the inflammatory process is caused by the release of mediators from tissues and migrating cells, and most strongly implicated are the prostaglandins (PGs), leucotrienes (LTs), histamine, bradykinin, platelet-activating factor (PAF) and interleukin-1 [20]. The carrageenan induced rat paw edema is a suitable test for evaluating anti-inflammatory activity of extracts [15]. Carrageenan is a family of linear sulphated polysaccharides extracted from the red seaweed marine algae *Chondrus crispus*. Lambda carrageenan is used in animal models of

inflammation to test activity of new compounds, because dilute carrageenan solution (1–2%) injection causes swelling and pain [21]. The oedema development in rat after the injection of carrageenan has been described as a biphasic event [1]. The first phase of which results from the concomitant release of histamine, serotonin and kinins and a second phase correlates with the elevated production of prostaglandins, oxygen-derived free radicals, production of inducible cyclooxygenase and local neutrophil infiltration and activation [22]. The edema induced by carrageenan is highly sensitive to NSAIDs and has been accepted as a useful indicator for identifying the new anti-inflammatory molecules. The results of the carrageenan induced paw inflammation revealed that HAEAT (100, 200 & 400 mg/ kg) produced significant inhibition of paw edema as compared to carrageenan control group. The effect of HAEAT was observed between 1 to 6 h. The result indicated that the HAEAT was effective in preventing a more sustained late phase (3 to 5 h) regulated by neutrophilic infiltration and sustained production of arachidonic metabolites prostanoids (primarily by cyclooxygenase) or nitric oxide from inducible nitric oxide synthase.

Chronic anti-inflammatory activity was investigated against a foreign body granuloma produced by implanting a pellet of cotton in a model of sub chronic proliferative inflammation [16]. The cotton pellet granuloma method has been widely employed to assess the transudative, exudative and proliferative components of chronic inflammation [23]. The repair phase of inflammation starts as proliferation of fibroblasts, as well as multiplication of small blood vessels. Such proliferating cells penetrate the exudates producing a highly vascularised reddened mass known as granulation tissue. The fluid absorbed by the pellet greatly influences the wet weight of the granuloma and the dry weight correlates well with the amount of granulomatous tissue formed [24]. In this study, HAEAT decreased the increase in weight of cotton pellets as compared to vehicle control group. This may be due to its ability in reducing the number of fibroblasts and synthesis of collagen and mucopolysaccharide, which are natural proliferative agents of granulation tissue formation.

CONCLUSION

In conclusion, this study clearly showed that HAEAT showed anti-nociceptive activity against acetic acid induced writhing model and anti-inflammatory activity against carrageenan induced rat paw edema and cotton pellet induced granuloma in rats. This activity can be attributed to its phytochemical constituents like glycosides, alkaloids, saponins, flavonoids, phenols and steroidal compounds present in the extract.

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