

Phytochemical and Pharmacological Evaluation of *Acalypha indica* Linn in Experimental Animal Models

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ABSTRACT

In the present study the plant was subjected to phytochemical evaluation and anti-inflammatory and analgesic activities by hiring carrageenan induced paw edema, human red blood cells membrane stabilization method (HRBC) as well as animal model of acute inflammation was adopted to probe the possible anti-inflammatory mechanism and eddy's hot plate and tail flick methods. Polyphenolic extract of *Acalypha indica* Linn (PPEA) produced significant anti-inflammatory (P < 0.001) and analgesic (P < 0.001) effects in dose dependent manner. The highest levels of phenolics and flavonoids (9.27 mg TA/g and 8.75 mg Ru/g, respectively). The total phenolic and flavonoid content indicates that these compounds are likely to be the main nociceptive and inflammatory contributing to the observed activities. PPEA exhibited significant anti-inflammatory mechanism of chemical constituents may be due, at least in part to the inhibition of PGE₂ levels. These finding suggest that of the medicinal herb studied in this paper are good source of inflammations. The results afford evidence to support the traditional linctus abuse of PPEA for the action of inflammation.

Keywords: *Acalypha indica* Linn, anti-inflammatory, analgesic, phenolic, flavonoid

INTRODUCTION

Inflammation is a dynamic and complex process that arises in response towards cellular injury. It has an important role in tissue repair, yet in some cases it can cause undesirable effects such as tissue damage and loss of function. Inflammation process is characterized by the production of a cascade of mediators that regulate important factors of the inflammatory response, as the increase in vascular permeability and recruitment of leukocytes in the blood¹. Once released, these inflammatory mediators may activate or sensitize nociceptors adjacent to the injured tissue resulting in pain sensation². Currently several analgesics and anti-inflammatory drugs are associated with important side effects, low efficacy and specificity. For this reason, studies are conducted to identify novel therapeutic options to develop and introduce new drugs with greater safety and efficacy. Medicinal plants were natural products known to be a significant source of new chemical substances with potential therapeutic effects³. Among natural products, use of essential oils is a promising option because of proven therapeutic action and for being also commonly added in food to obtain a specific taste⁴.

In this context, we can include the *Acalypha indica* Linn (Euphorbiaceae) (common name: Indian copperleaf) is an annual herb found throughout the Indian peninsula and in southern China, South Africa, Sri Lanka, Pakistan and Yemen. It is used by healers for the treatment of malarial fever, dysentery, diabetes and it possesses snake venom

neutralizing properties⁵⁻⁸. Cyanogenesis⁹ and antifertility activity¹⁰ in female albino rats of *Acalypha indica* Linn has long been known. Leaf extracts of this species have been studied as ovicides, larvicides and oviposition attractants for *Anopheles stephensi* Liston¹¹. There are numerous reports about the use of *Acalypha indica* Linn for the treatment of wound healing activity in rats¹². This is to the first report of PPEA from ethanolic extract of *Acalypha indica* Linn in showing activities. The main aim of the current study is to authenticate that traditional information of PPEA as both antinociceptive and anti-inflammatory. This plant will be useful for isolating newfangled inflammation.

MATERIALS AND METHODS

Plant Material: The whole plant of *Acalypha indica* Linn were collected from the local areas of Gulbarga, Karnataka and was authenticated from herbarium of the Laboratory of Botany, Gulbarga University, Gulbarga. A voucher specimen (HGUG No.279) was deposited in the Department of Botany, Gulbarga University, Gulbarga. Herbariums are made and their voucher specimen retained in the Department of botany for future reference.

Preparation of extraction: The collected plants were immediately dried in shade at 27° C for about 10 days and then powdered by a pulverizer. The powdered plant material (500 g) was Soxhlet extracted for a 48 hour with ethanol and the extract was filtered through a Whatmann

Table 1: Analgesic activity of the whole plant of *Acalypha indica* Linn extract on the hot-plate test in mice.

Group	Dose mg/kg	Pain dormancy after administration (s)						
		0 h	1 h	2 h	3 h	4 h	5 h	6 h
Control	-----	13.6±1.96	13.2±1.39	14.6±1.16	15.4±0.67	14.6±1.15	13.4±1.62	14.8±0.80
DS	0.13	12.4±1.29	12.8±0.86	12.2±0.85	11.8±0.58***	12±0.70	12.4±0.93	12.6±0.97
PPEA	200	13.4±0.50	12.4±0.92	12.6±0.91	10.8±1.5*	10.6±0.51*	12±1.86	11.8±0.66
PPEA	400	10.8±1.15	11.6±2.06*	11.4±1.07	10.0±0.71***	11.8±1.46	12.6±1.32**	13±1.22

Values are expressed as mean±Standard error (n=5) experiments, *P<0.05, ** P<0.01 and ***P<0.001 as compared with control (ANOVA followed by Student's t-test).

Table 2: Analgesic activity of the whole plant of *Acalypha indica* Linn extract by the tail-flick test in rats.

Group	Dose mg/kg	Pain threshold after administration (s)						
		0 h	1 h	2 h	3 h	4 h	5 h	6 h
Control	-----	7.8±0.85	8.4±0.92	07±1.15	8.8±0.85	07.6±1.20	08.4±1.20	8.8±1.62
DS	0.9	07±0.89	6.4±1.20	8.2±1.06	6.2±0.58*	6.4±0.80	07.2±0.85	07.2±1.15
PPEA	200	06±0.89	6.2±1.65	5.2±1.59	5.8±0.85*	6.8±1.27	6.6±1.07	5.6±1.56
PPEA	400	7.8±0.84	6.2±0.85	6.6±1.35	4.2±0.58**	5.2±1.87	05±1.41*	5.2±1.46

Values are expressed as mean±Standard error (n=5) experiments, *P<0.05 and **P<0.0001, compared with control (ANOVA followed by Student's t-test).

filter paper and the solvent was removed on vacuum rotary evaporator under reduced pressure to get the dried extract (yield: 30.8%). The dried extract was stored at refrigerator (4 °C) till its further use.

Preparations of polyphenolic extract (PPEA): The crude EEAI was dissolved in water and the aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The lower layer was then treated with ethyl acetate containing glacial acetic acid (10 mL/l). Extraction of polyphenols was carried out for 4 h at room temperature and the combined ethyl acetate layer was concentrated. The residue was desiccated and stored at 4 °C. The total polyphenolic content and flavonoid of the extract were assayed using the standard methods. The test extracts were subjected to various phytochemical tests, the methods of analysis employed were those described by Trease and Harbone^{13, 14}.

Determination of Total Phenol Content: Total phenolic content in the PPEA was determined by established method with suitable modification¹⁵ with Folin-Ciocalteu's reagent. Ethanolic extract was diluted to the concentration of 1mg/mL were taken in separate test tubes. Then 0.5 mL of the soluted extract was mixed with 2.5 mL of Folin-Ciocalteu's reagent (Previously diluted 10 fold with distilled water) and 2 mL of NaHCO₃ (7.5 %) were added sequentially in each tube. Soon after mixing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was measured at 765 nm versus blank sample on spectrophotometer (Paekin Elmer, Shelton, CT, U.S.A). Tannic acid was used as the standard, and the results are expressed as milligrams per liter of tannic acid equivalents (mg TAE/g extract). All measurements were performed in triplicate.

Determination of Flavonoid Content: Total flavonoid content in the PPEA was determined by established method with suitable modification¹⁶. An aliquot of 0.5 mL extract was mixed with 2 mL of distilled water and subsequently with 0.15 mL of 5% NaNO₂ solution. After

6 min, 0.15 mL of 10% AlCl₃ solution was added and allowed to stand for 6 min, and then 2 mL of 4% NaOH solution was added to the mixture. Immediately distilled water was added to bring the final volume to 5 mL. Then the mixture was thoroughly mixed and allowed to stand for another 15 min. The absorbance was measured at 510 nm versus blank sample on spectrophotometer (Paekin Elmer, Shelton, CT, U.S.A). The flavonoid content was expressed in terms of standard rutin equivalent (milligrams per gram of extracted compounds). The assay was carried out in triplicate.

Experimental Animals: Swiss albino mice (20-25 g) and Wistar albino rats (180-220 g) of either sex were procured from the Central animal house M. R. Medical College (MRMC) Gulbarga and were distributed into different experimental groups. The mice and rats were kept in the departmental house at an ambient temperature of 20 ± 2 °C with a 12 h light/ dark cycle. The animals had free access to standard pellet chow with tap water *ad libitum*. Experiments were conducted between 9.00 and 16.00 h. the behavioural testing was done during the light phase. Animals were acclimatized for at least one week before using them for experiments and exposed only once to every experiment. The experimental protocol was approved by the Institutional Animal Ethical Committee (I.A.E.C) (HKES COP/IAEC/2012/54) for conducting the experiments.

Acute Toxicity Studies: The median lethal dose (LD₅₀) of PPEA from *Acalypha indica* Linn dried whole plant of ethanol extract was determined in the mice according to a modified method by Lorke¹⁷. Mice fasted for 24 h were randomly divided into groups of five mice per group either of sex. Graded doses of PPEA were separately administered orally to the mice in each of the test groups. The mice in test groups were then allowed free access to food and water and observed over a period of 7 days for signs of acute toxicity. The number of deaths within this period of time was recorded. Log-dose response plots are constructed for the plant extract, from which the median

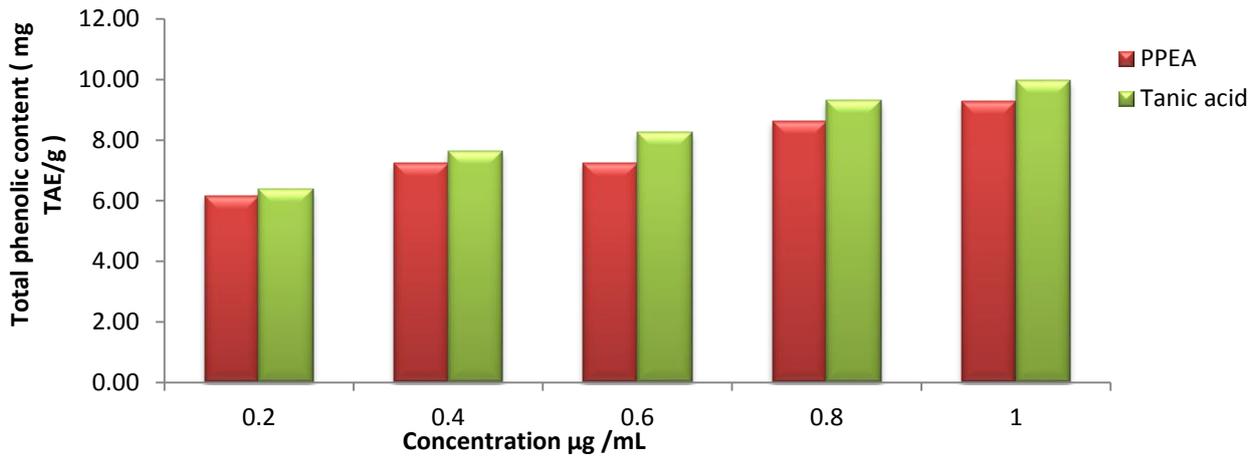


Fig. 1: Total phenolic content in the plant extracts expressed in terms of Tannic acid equivalent (mg TAE/g extract). Values are expressed as mean±Standard deviation.

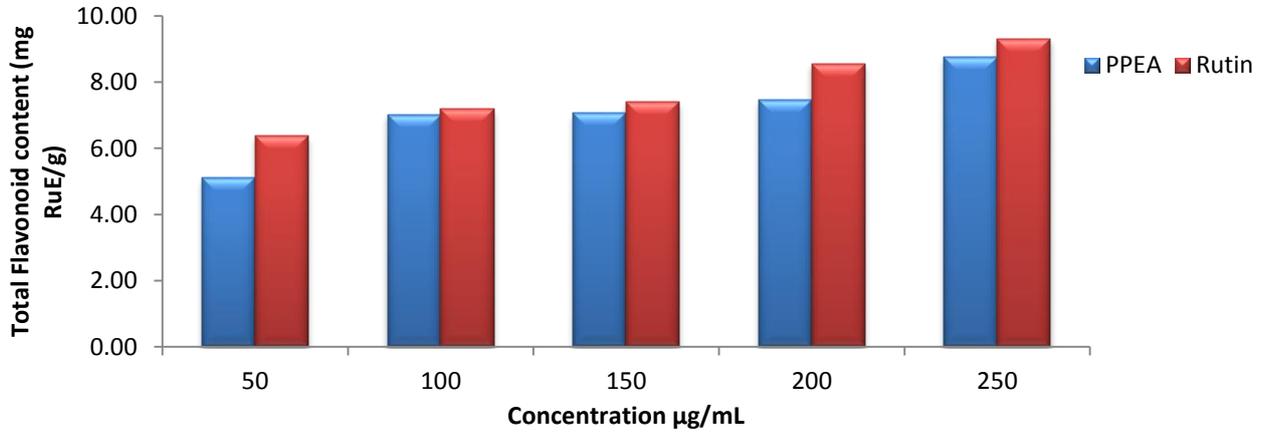


Fig. 2: Total flavonoid content in the plant extracts expressed in terms of rutin equivalent (mg RuE/g extract). Values are expressed as mean±Standard deviation.

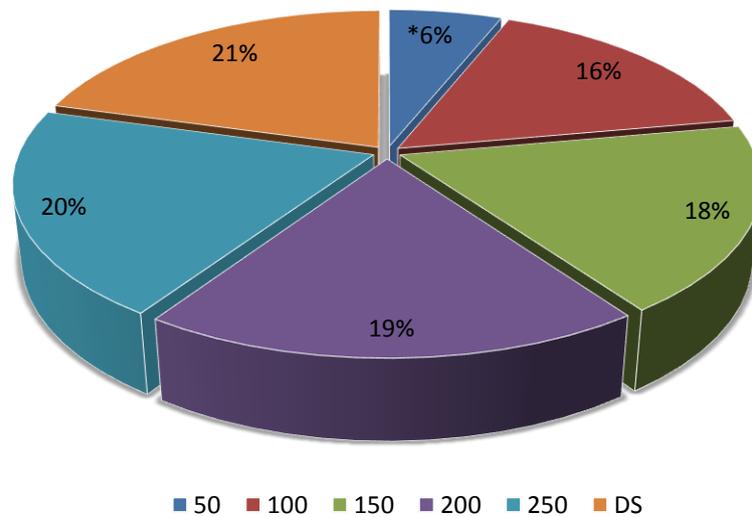


Fig. 3: Percentage protection of PPEA at different concentrations (50 to 250 µg/mL) displayed significant stabilization towards HRBC membranes, indicates higher concentration 250 µg/mL is more when compared to the other concentrations. Values are expressed as mean triplicate analyses.

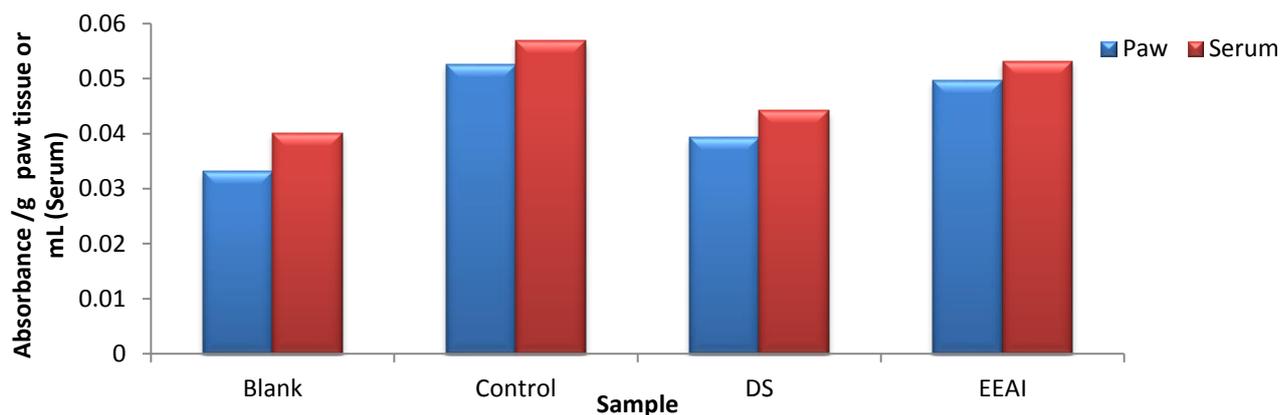


Fig. 4: Effects of PPEA and DS on the PGE₂ levels of paw tissue and serum at 6 h after injection of carrageenan in right hind paw, compared to rats were treated with blank and control.

lethal dose (LD₅₀) of the polyphenolic extract was determined.

Pharmacological Studies: Analgesic effect on the hot plate test: The hot-plate analgesic test method employed in this study was modified from those described in detail by Eddy and Leimback¹⁸. For this purpose, Swiss albino mice (20-25 g) randomly assigned to four groups of five mice in each. The group I and II were given 2% acacia suspension (control) and standard group received the diclofenac sodium (DS) (0.13 mg/25 g/mice, i.p) and group III and IV were orally administered PPEA at doses of 200 and 400 mg/kg. Analgesic activity was measured 1 h after administration of extracts and standard drugs. Mice were placed on the hot plate at 55±0.5 °C (Analgesiometer Eddy's hot plate, Dolphine Mumbai, India) and the analgesic activity were estimated by measuring the dormancy era preceding the animal reaction of paw licking and jumping response was observed. Animals showing a reaction time greater than 20 sec were removed to evade gashes to the animals paw.

Analgesic effect by the tail flick method: The analgesic activity of the extracts was done by employing tail-flick method with suitable modification¹⁹. The pre-screened rats (reaction time: 3-5 sec) were divided into control, standard and test group with five rats in each group. DS was used as the standard drug. The drugs were administered orally with saline, PPEA (200 and 400 mg/kg), DS (0.9 mg/200 g/rat body weight i.p). The dose of DS administered was extension of human dose to rat based on body surface area. The distal part of the tail of the animals was assessed for analgesic activity by using (Analgesiometer Dolphine Mumbai, India), maintained at 55±0.5 °C. The time taken to withdraw the tail was noted as reaction time. A cut off time of 12 second was maintained at 55 °C to prevent tissue damage.

Anti-inflammatory activity (in vitro): Human red blood cell (HRBC) membrane Stabilization method with suitable modification²⁰ was used for the estimation of anti-inflammatory activity *in vitro*. Blood was collected from healthy volunteer and it was mixed with equal volume of sterilized Alsever's solution. This blood solution was centrifuged at 3000 rpm and the packed cells were

separated. The packed cells were washed with isosaline solution and a 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory property. Different concentrations of PPEA, reference sample and control were separately mixed with 1ml of phosphate buffer, 2ml of hypo saline and 0.5ml of HRBC suspension. All the assay mixtures were incubated at 37 °C for 30 minute and centrifuged at 3000 rpm. The supernatant liquid was decanted and the hemoglobin content was estimated at 560nm, using a spectrophotometer (Paekin Elmer, Shelton, CT, U.S.A). The percentage haemolysis was estimated by assuming the haemolysis produced in the control as 100%. Percentage protection = $[1 - OD \text{ sample} / OD \text{ control}] \times 100$. **Animal models of acute inflammation:** The anti-inflammatory activity was tested according to the method described by Winter et al²¹. For this purpose, Wistar albino rats of either sex weighing 180-220 g body weight were used. Rats were divided into four groups of five rats each. The group I and II were given 2% acacia suspension (control) and standard group received the DS (0.9 mg/200 g/rat, i.p), and group III and IV were orally administered PPEA at doses of 200 and 400 mg/kg, 1 h before the injection of carrageenan. Thirty minute before the injection carrageenan (0.1 ml of 2% w/v) subcutaneously into the subplantar region of the right hind paw for the induction of edema. In the left hind paw used as a control 0.1 ml of the sterile saline was injected. The paw edema volume of the rats was measured plethysmographically immediately after the injection of carrageenan and then 0- 6th h. The difference between the initial and subsequent values gave the actual edema volume which was compared with standard and the percent inhibition of edema was determined by using the formula. Percent inhibition = $[1 - V_t / V_c] \times 100$

Where 'V_c' represents edema volume in control and 'V_t' edema volume in group treated with test extract. After determining the extent of the paw edema (6 h), rats were sacrificed. Rats' right hind paw tissue and serum were kept for assay on PGE₂ concentration according to the method described by Zhang et al.²². The PGE₂ levels of serum and

paw tissue were expressed as the absorbance of PPEA, DS, Control and Blank, respectively.

Statistical analysis: The data were expressed as mean \pm SEM and the statistical significance was determined using analysis of variance (ANOVA) followed by Student's t-test. Values were considered to be significant ranged from $P < 0.05$ and $P < 0.001$.

RESULTS

Phytochemical Screening: Preliminary phytochemical study indicated that the presence of glycosides, saponins, terpenoides, tannins, flavonoids and phenolic compounds respectively, which may be responsible, at least in part, for the analgesic and anti-inflammatory effects of the polyphenolic extract of *Acalypha indica* Linn.

Total Phenolic and Flavonoid Content: In this experiment, the yields of EEAI (30.8 %) by a Soxhlet extraction method and the estimation of phenolic and flavonoid contents from PPEA. The total phenolic and flavonoid contents were (6.16 to 9.27 mg TAE/g and 5.11 to 8.75 mg RuE/g) found to be higher in PPEA (9.27%) and (8.75%), respectively. The polyphenolic extract had highest total phenolic and flavonoid contents in *Acalypha indica* Linn. The results the present study strongly suggest that phenolics and flavonoids were important component of the plant as shown (Figure 1 and 2) and some of their pharmacological effects could be attributed to the presence of these constituents.

Acute Toxicity: PPEA did not exhibit acute toxicity up to the maximum dose of 5 g/kg and the weight of the mice had a normal variation after 7 days of observation. Common side effects such as, mild diarrhea, lose of weight and depression were not recorded. Orally administration of grade doses of *Acalypha indica* Linn dried whole plant polyphenolic extract in mice give a LD₅₀ value of 5 g/kg. This finding probably suggests that the plant extract is relatively safe in mice.

Analgesic activity: Oral administration of the PPEA (200 and 400 mg/kg) significantly increased latency time on the hot-plate in the evaluated time (3 h), compared to control. Likely, treatment with standard diclofenac sodium (0.13 mg/ 25 g, i.p.) induced a significant increase in latency time in the hot plate test, throughout the experimental period (Table 1). Moreover the extract by radiant heat on tail flick model has shown decreased pain threshold significantly ($P < 0.05$) during the period of observation as shown in (Table 2). The DS (0.9 mg/200 g, i.p) was used as a reference drug. This indicates significant difference from the respective group.

Anti-inflammatory activity (*in vitro*): *In vitro* anti-inflammatory effects of PPEA at different concentrations (50-250 μ g/ml) showed that the extract exhibited statistically significant stabilization towards HRBC membranes. The percentage protection at a higher concentration 250 μ g/ml was more when compared to the other concentrations. However, on higher concentration the percentage protection was found to be increased (Figure 3).

Anti-inflammatory activity (*in vivo*): Subplantar injection of carrageenan promoted an increase in paw volume dependent on the time, this increase was maintained until the six hour, getting the maximum value at the third hour (Table 3). Paw edema was significantly decreased by diclofenac sodium ($P < 0.05$) throughout the experimental period, with a maximal inhibition of 61.5%, at the third hour. The PPEA were evaluated at the doses of 200 and 400 mg/kg, where was observed significant inhibition of edema at a dose of 400 mg/kg at all times evaluated, granting the maximum of inhibition at the third hour, 92.3 % ($P < 0.001$) respectively, when compared to the control group. Because the dose of 400 mg/kg afforded most of the protection against the inflammatory effects caused by carrageenan induced paw edema. In order to investigate the possible anti-inflammatory mechanism of PPEA, we assayed PGE₂ levels of serum and paw tissue. The results clearly demonstrated that treatment with PPEA significantly reduced PGE₂ levels in both serum and paw tissue compared to the control group, as evident from (Figure 4). The reference drug, diclofenac sodium (0.9 mg/kg, i.p.), inhibited paw edema induced by PGE₂ inflammatory mediator significantly.

DISCUSSION

In the present study, we evaluated the potential anti-inflammatory and anti-nociceptive effects of PPEA, through the use of some pharmacological tools. Our results showed that the PPEA present significant anti-inflammatory and anti-nociceptive effects, ascribable to high level of total phenolic and flavonoid contents in the extract. Chemical evaluation of the PPEA reveals the presence of saponins, phenolic compounds, flavonoids, tannins were detected. The acute oral toxicity study, we did not observe any mortality case up to the doses of 5000 mg/kg of the PPEA. Therefore, we may suggest that the extract has no lethal toxicity in mice. The analgesic effect of PPEA demonstrated using a novel and objective behavioural test in which quantitation is independent of the observer and was sensitive to all classes of analgesics²³. Interestingly the extracts showed very good peripheral and central analgesic activities as evident from the hot plate and tail flick methods. The hot plate test is a specific central nociceptive test in which agents exert their analgesic effects via supraspinal and spinal receptors²⁴. The results obtained showed that PPE presented significant anti-nociceptive effect in the hot-plate test. These data suggest the participation of central mechanisms in the anti-nociceptive effects of the PPEA since both behavioral components that were evaluated in this experimental model, namely paw licking and jumping, are considered to be supraspinally integrated responses²⁵. The achieved results in current study evidenced that the PPEA strongly confirms its ability to act at central nociceptive level, by inhibiting the neurogenic phase, corroborating with the results found in the hot plate test, besides acting also at the peripheral level, by inhibiting the inflammatory phase.

Table 3: *In vivo* anti-inflammatory activity of the whole plant of *Acalypha indica* Linn extract by carrageenan-induced paw edema in rats.

Group	Dose mg/kg	Carrageenan-induced paw edema in rats (Volume in ml)						
		0 h	1 h	2 h	3 h	4 h	5 h	6 h
Control	-----	0.1±0.027	0.11±0.024	0.13±0.048	0.13±0.02	0.10±0.034	0.09±0.048	0.08±0.02
DS	0.9	0.08±0.048	0.07±0.025	0.04±0.018*	0.05±0.022*	0.06±0.017	0.09±0.02	0.06±0.024
PPEA	200	0.06±0.031	0.05±0.029	0.03±0.019*	0.02±0.015*	0.04±0.024	0.08±0.041	0.07±0.037
PPEA	400	0.04±0.022	0.06±0.035	0.02±0.038	0.01±0.011**	0.03±0.019	0.06±0.031	0.09±0.048

Values are expressed as mean±SEM. (n=5), *P<0.05, **P<0.0001 as compared with control.

Inflammation is common phenomenon and it is a reaction of living tissues towards injury. Steroidal anti-inflammatory agents will lyse and possibly induce the redistribution of lymphocytes causing rapid and transient decrease in peripheral blood lymphocyte counts to effects longer term response. Here anti-inflammatory activity was performed based on the folk lore information using two methods. HRBC method was selected for the *in vitro* evaluation of anti-inflammatory property because the erythrocyte membrane is analogous to the lysosomal membrane²⁶ and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release. The result indicated that the PPEA at various concentrations had significant anti-inflammatory property. Carrageenan-induced paw edema is a well-established animal model to assess the anti-inflammatory effect of natural products as well as synthetic chemical compounds²¹. Carrageenan-induced paw edema is widely used for determining the biphasic event of inflammation that involves the participation of diverse set of inflammatory mediators and intense neutrophil infiltration²⁷. The first phase of this experimental model has been attributed to the action of mediators such histamine, serotonin and bradykinin on vascular permeability²⁸. The second phase is characterized by an excess production of prostaglandins and an intense neutrophil infiltrate^{29,30}. In the present work, we evaluated the inhibitory potential of the PPEA on the carrageenan edema, where we observed that the PPEA caused significant reduction of edema at all times evaluated, suggesting that its anti-edematogenic effect is probably due to the inhibition of different aspects and chemical mediators of inflammation.

In order to investigate the possible anti-inflammatory mechanism of PPEA, we assayed PGE₂ levels of serum and paw tissue. The results clearly demonstrated that treatment with PPEA significantly reduced PGE₂ levels in both serum and paw tissue compared to the control group. These indicated that PPEA might exhibit its anti-inflammatory effect by means of the inhibition of COX-2 expression, which ultimately led to the inhibition of the synthesis, release or action of inflammatory mediators PGE₂ involved in inflammation. Mediators produced at the sites of inflammation have been known to produce pain

through the activation or sensitization of nociceptors adjacent to the injured tissue³¹. Experimental models of inflammatory pain in rodents have been successfully employed to reproduce this kind of pain and are used to search new anti-inflammatory and analgesic drugs². These activities affirm the presence of biologically active compounds from the whole plant of *Acalypha indica* Linn. Currently, isolation of bioactive compounds is underway in our laboratory to evaluate the pharmacological activities of the target compounds of the lead plant.

CONCLUSION

This study suggest that the whole plant of *Acalypha indica* Linn possesses very good anti-inflammatory and anti-nociceptive activities, ascribable to high level of phytoconstituents for instance phenolic and flavonoid contents in the extract. The PPEA was proved a natural safe remedy for the treatment of algnesia and inflammation. Our current findings demonstrated scientific rationale for the traditional use of the plant as anti-inflammatory and anti-nociceptive activities. Further, the isolation of pure secondary metabolites from the plant will help us in understanding the mechanism of action and identification of lead compounds for clinical utility.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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