International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 23-07-2021, Accepted: 07-08-2021, Published: 23-08-2021
Volume 6, Issue 4, 2021, Page No. 862-867

Effect of *Calotropis procera* leaf extracts and partitioned fractions on anti-inflammatory and analgesic activity

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Abstract

The present study was aimed to evaluate the anti-inflammatory and analgesic activities of *Calotropis procera* (named as CP) using various *in-vivo* and *in-vitro* models. Chloroform (CECP), ethanol (EECP) and methanolic (MECP) leaf extracts were screened for anti-inflammatory activity using carrageenan-induced rat paw swelling test and analgesic activity was performed for its central and peripheral pharmacological action by using acetic acid-induced writhing model. *In-vitro* anti-inflammatory activity of acetone (ACF) and ethyl acetate (EAF) fractions of methanolic extract of *C. procera* was assessed by using egg albumin denaturation and heat induced hemolysis models. Aspirin was used as a standard drug to evaluate anti-inflammatory and analgesic activities of *in-vivo* and *in-vitro* models. MECP significantly (P<0.01) decreased paw edema compared to control group animals. MECP (36.75% inhibition) revealed highly potent anti-inflammatory potential after 3 h, which was even greater than that of aspirin (32.45% inhibition). Similarly, it also exhibited highly significant and more analgesic activity (74.48% reduction) that of aspirin (61.12% reduction). In consistent with the *in- vivo* anti-inflammatory effects fractions, ACF and EAF also demonstrated its potential inflammatory activity in a concentration-dependant manner in *in -vitro* models. At 400 µg/mL concentration EAF showed greater % stabilization (46.05%) than aspirin (43.62%) in human red blood cell (HRBC) membrane stabilization assay. The findings of the present study revealed that the MECP possesses potent anti-inflammatory and analgesic activity against all the tested models. Whereas, EAF demonstrated significant *in-vitro* anti-inflammatory activity.

Keywords: *Calotropis procera,* anti-inflammatory, analgesic, carrageenan, acetic acid

Introduction

Inflammation is the reaction of vascularized living tissues to local injury and comprises of series of changes in vascular bed, in blood and connective tissues with eliminating irritants and to repair the damaged tissues [1]. Currently, various steroidal and non-steroidal anti-inflammatory (NSAIDs) drugs are used for treatment of inflammatory diseases. Generally, NSAIDs act by inhibiting the metabolism of arachidonic acid by both the cyclooxygenase and lipoxygenase enzyme pathway. Gastrointestinal bleeding and ulceration are the most common and severe side effects associated with NSAIDs [2]. Therefore search for drugs with low or no adverse effects are of prime importance to treat various inflammatory diseases. Ayurveda is an ancient Indian medicinal science which uses medicinal plants to cure various diseases. Certain herbal plants prevent structural damage of joints and are preferred for treatment of inflammatory diseases, and these are safe, relatively inexpensive and convenient for many patients [3].

Calotropis procera is a small shrub belonging to the family Asclepiadaceae, commonly known as milkweed or giant weed. It is commonly known as “Rui” in Marathi and “sodom of apple” in English. The *Calotropis* commonly have two main species i.e. *C. procera* (Ait.) R. Br. and *C. gigantea* (Linn) R. Br. *C. procera* occurs as single or many stemmed soft wooded shrub, and occasionally a tree reaching to 6 m height. All parts of the plant exude white milky latex when cut [4]. *C. procera* is most abundant and native to India, Pakistan, Nepal, Afghanistan, Algeria, Iran, Iraq, Israel, Kenya, Kuwait, Nigeria, Oman, Saudi Arabia, Yemen and Zimbabwe [5]. This plant can tolerate adverse climatic conditions and poor soil for its growth [6]. It has been widely used in traditional medicine due to its pharmacological active compounds found in all parts of plants: bark, roots, leaves and especially its latex which exudates from damaged or broken stem and leaves [7]. Latex has been used in leprosy, inflammation, eczema, cutaneous infection, syphilis, malaria, low hectic fever and as abortifacient [8]. Traditionally leaves are used in rheumatism, as an anti-inflammatory and antimicrobial, hepatoprotective, analgesic and in asthma [9, 10].

It has been found to possess analgesic [11], hepatoprotective [12, 13], antibacterial [14], antioxidant [15], antidiabetic [16], antitumor [17] and antiulcer properties [18]. Regardless of its traditional value and being most prevalent and commonly available plant, the analgesic and anti-inflammatory potential of the leaves of this plant has not been fully explored. Hence the present study was aimed to evaluate anti-inflammatory and analgesic activities of extracts and fractions of *C. procera* using various experimental models.

Materials and Methods

Collection and identification of plants

The leaves of *C. procera* was collected from the campus area of Swami Ramanand Teerth Marathwada University, Nanded, Maharashtra, India in the month of November 2014. It was identified and authenticated from Department of Botany, School of Life Science, Swami Ramanand Teerth Marathwada University, Nanded, Maharashtra, India (Voucher specimen no. 1/10/08/2015). The leaves were washed under tap water and shade dried. The dried leaves then powdered with electrical mixer into coarse powder. The dried powder was further stored in polythene bags for further use.

Preparation of extract and fractions

Five hundred gram powder of *Calotropis procera* leaves were used for extraction with 1000 mL of each chloroform, ethanol and methanol by Soxhlet extractor. The extracts were filtered and dried at 40 ^{0}C under vacuum using rotary evaporator and the yields were 3.71%, 8.07% and 15.35% respectively. Most active extract (methanolic) was further fractionated with ethyl acetate and acetone by using solid-liquid partition technique. Fractions were dried using rotary evaporator and were equivalent to 7.95% and 4.1% respectively of the dry methanolic extract. The extracts and fractions were preserved in vacuum desiccators at 4 ^{0}C until further use.

Animals

Albino rats of *Wistar* strain of either sex weighing between 150-200 g were used. They were housed in standard cages at room temperature (25 ± 2 ^{0}C) and provided with food and water at *ad libitum.* The study was conducted after obtaining Institutional Ethical Committee clearance (CPCSEA Registration No. 1613/PO/a/1, IAEC No. R-3-XIII dated 22/6/2014).

Anti-inflammatory activity of extracts of *C. procera*

Anti-inflammatory activity of various *C. procera* extracts was performed on albino Wistar rats. Acute inflammation was induced using the carrageenan-induced edema model. Five groups of six animals per group were used. Administration of aspirin (as a standard drug) and extracts was carried out 30 min. prior to injection of 0.1 mL of 1% carrageenan in the right hind paw sub-plantar of each rat [19]. Aspirin at a dose 150 mg/kg was given to positive control group; un-treated group (negative control) received distilled water, while, remaining groups were treated with the extracts of chloroform (CECP), ethanol (EECP) and methanol (MECP) at the oral dose of 200 mg/kg, 1 h before carrageenan injection. The paw volume was measured using a plethysmometer (ORCHID Scientific & Innovative India Pvt. Ltd.), before injection and at 1 h interval [20]. Anti-inflammatory effect of CECP, EECP and MECP was calculated by the following equation:

$$\text{Anti-inflammatory activity (\%)} = (1-V_t/V_c) \times 100$$

Where; V_t: Paw volume in drug treated animals and
V_c: Paw volume of control groups animals.

In vitro anti-inflammatory activity of the fractions of *C. procera*

HRBC membrane stabilization

The acetone (ACF) and ethyl acetate (ECF) fractions of methanol extract were further evaluated for in-vitro anti-inflammatory activity. *In vitro* anti-inflammatory activity of the fractions of *C. procera* was performed by using heat induced hemolysis model [21]. Fresh human whole blood was collected and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, 0.42% sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 min. and packed cells were washed three times with isosaline (0.85%, pH 7.2). The volume of blood was measured and reconstituted as 10% v/v suspension with saline. The assay mixture containing 1 mL of phosphate buffer (pH 7.4, 0.15 M), 2 mL of hyposaline (0.36%), 0.5 mL HRBC suspension (10% v/v) with 0.5 mL of plant fractions (ACF and EAF) and standard drug, aspirin at concentrations of 200, 400 and 800 µg/mL, and control (distilled water used instead of hyposaline) were incubated at 37^{0}C for 30 min. and centrifuged respectively. The hemoglobin content in the suspension was estimated by using spectrophotometer (GENEI Pvt. Ltd.) at 560 nm. The percentage of hemolysis of HRBC membrane was calculated by following equation.

$$\text{\% Hemolysis} = (\text{Optical density of Test sample} / \text{Optical density of Control}) \times 100$$

The percentage of HRBC membrane stabilization can be calculated as follows:

$$\text{\% Stabilization} = 100 - [(\text{Optical density of Test sample} / \text{Optical density of Control}) \times 100]$$

Egg albumin protein denaturation

Egg albumin (0.2 mL), phosphate buffer saline of pH 6.4 (2.8 mL), and varying concentration (200, 400 and 800 µg/mL) of test fractions of ACF and EAF (2 mL) were used to prepare the reaction mixture (5mL). The mixtures were incubated at 37 ^{0}C for 15 min. and then heated at 70 ^{0}C for 5 min.

After cooling, the absorbance was measured at 660 nm by using vehicle as blank. Aspirin at concentration 200, 400 and 800 µg/mL was used as reference drug [22]. The percent inhibition of protein denaturation was calculated by following equation:

$$\text{\% inhibition} = 100 \times [A_t / A_c - 1]$$

Where, A_t = absorbance of test sample, A_c = absorbance of control.

Analgesic activity of extracts of *C. procera*

Albino Wistar rats were used for the evaluation of analgesic activity by using acetic acid-induced writhings model. Five groups of six animals each were used. Control group animals received distilled water. Standard group received aspirin at dose of 100 mg/kg intra-peritonealy before 30 min. of administration of 0.1 mL 0.6% of acetic acid. Treatment group animals received CECP, EECP and MECP at dose 200 mg/kg orally before 1 h of administration of acetic acid. Animals allowed elapsing for 5 min. and measuring the number of writhings for 20 minutes [23].

Statistical analysis

All values were expressed as mean ± standard error mean (SEM or SD). Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Dunnett test. *P* values < 0.05 were considered to be statistically significant when compared to control.

Results and Discussion

Anti-inflammatory activity

The most widely used screening method for acute anti-inflammatory activity is carrageenan-induced rat hind paw edema. Carrageenan, a mucopolysaccharide, is the most commonly used and well-studied phlogistics, producing edema in 3 hour [24]. The development of edema in the rats paw after injection of carrageenan is manifested in two phases [25, 26]. In the first phase, a rapid rise in edema occurs immediately after sub-plantar injection of carrageenan and observed during first hour is attributed to the release of histamine and serotonin. In the second phase at around 2 to 3 hour, a strong increase in edema occurs due to the release of prostaglandins like substances [27]. CECP, EECP and MECP extracts of *C. procera* were found to have noteworthy anti-inflammatory effects at the dose of 200 mg/kg. After carrageenan injection, aspirin showed prominent inhibition of paw edema at 1 hr, while, MECP demonstrated significant (P< 0.01) paw edema inhibition (36.75%) which was more than aspirin (32.45%) at 3 hr) (Figure 1).

Based on this, it could be argued that the reduction of the first phase may be due to inhibition of the release of the early phase mediators, histamine and serotonin and the activity in the second phase may be exhibited due to inhibition of cyclooxygenase [28]. A later experiment has shown that two or more mediators are released during carrageenan induced edema in two phases [29]. Carrageenan model is typically associated with activation of cyclooxygenase pathway and is sensitive to glucocorticoids and prostaglandin synthesis antagonists [30]. In the present work the MECP showed more activity than aspirin while, CECP (32.45%) exerted the same effect as that of aspirin at 3 h. This indicates that both the extracts exert prominent activity in the second phase of inflammation which suggests that the possible mechanism of the anti-inflammatory action of the extracts may be through prostaglandins synthesis inhibitions. Possibility inhibition of prostaglandins is further supported by potent analgesic activity of extracts assessed in acetic acid-induced writhing model (discussed below).

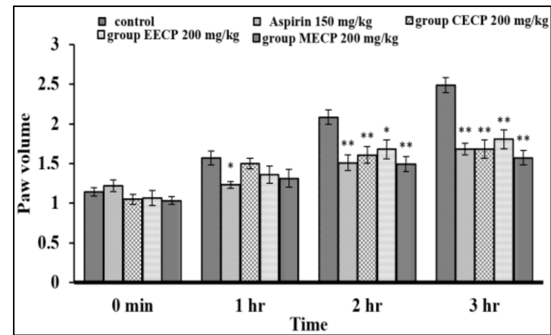

Fig 1: Effect of leaf extracts (CECP, EECP, and MECP) of *C. procera* on carrageenan-induced rat paw edema.

Values expressed as mean± SD (n=6). ANOVA followed by Dunnett test. $^{*}p < 0.05$, when compared with control group. $^{**}p < 0.01$, when compared with control group. The paw volume of various extracts shows time dependent anti-inflammatory potential as revealed by aspirin. The MECP demonstrated more significant reduction in paw volume than aspirin after 3 h.

HRBC membrane stabilization effect

HRBC membrane stabilization is one of the important *in-vitro* methods for the evaluation of anti-inflammatory effects. The HRBC membrane is analogous to the lysosomal membrane and its stabilization indicates the stabilization of lysosomal membranes [31]. Stabilization of the membranes of these cells inhibits lysis and subsequent release of the cytoplasmic contents, which, in turn, limits the tissue damage and exacerbation of the inflammation response [32]. The effect of fractions and aspirin on the heat-induced hemolysis is tested in present study (Table 1). All the fractions exhibited significant anti-inflammatory effects when compared with control. EAF of methanolic extract showed more potent anti-inflammatory activity (46.05 % stabilization) than aspirin (43.62 % stabilization) at the same concentration i. e. 400 μg/mL. This anti-inflammatory activity of all fractions was found to be in a concentration-dependent manner. Exposure of red blood cells to hypotonic medium or heat results in the lysis of membrane accompanied by hemolysis and oxidation of hemoglobin [33]. Therefore this technique can be used to evaluate anti-inflammatory activity. Although both the fractions demonstrated significant membrane stabilization, especially, EAF revealed more comparable percent stabilization as that of aspirin. These fractions appear to inhibit the lysis and subsequent release of cytoplasmic contents, which may possibly responsible for limiting the tissue damage and the exacerbation of inflammatory reaction.

Table 1: HRBC membrane stabilization effect of different fractions of methanolic extract of *Calotropis procera* leaves

Design of treatment	Concentration (μg/mL)	Mean ± SEM	% Hemolysis	% Stabilization
Group-I (Control)	_	0.7933 ± 0.0328	100.00	-
Group-II (Aspirin)	200	0.5120 ± 0.0049**	64.54	35.46
	400	0.4473 ± 0.0158**	56.38	43.62
	800	0.2857 ± 0.0069**	36.01	63.99
Group-III (ACF)	200	0.6743 ± 0.0143**	84.99	15.01
	400	0.5947 ± 0.0035**	74.96	25.04
	800	0.4787 ± 0.0235**	60.34	39.66
Group-IV (EAF)	200	0.5227 ± 0.0122**	65.89	34.11
	400	0.4280 ± 0.0285**	53.95	46.05
	800	0.3630 ± 0.0267**	45.76	54.24

Values expressed as mean ± SEM, (n=3). ANOVA followed by Dunnett test. $^{*}p < 0.05$, when compared with control group. $^{**}p < 0.01$, when compared with control group.

Protein denaturation effect

The maintenance of structural hierarchy of proteins by cell system is necessary for proper functioning of metabolic activities. Denaturation of tissue proteins is one of the well documented causes of inflammatory and arthritic diseases [34]. Table 2 Presents the protein denaturation activity of fractions of *C. procera*. Ethyl acetate fraction showed more effective inhibition of protein denaturation at 800 µg/mL (57.46%), which is comparable to that of aspirin, which showed 69.70% inhibition of protein denaturation at the same concentration. Production of auto-antigens in certain arthritic diseases may be due to denaturation of tissue proteins [35]. Results of the present work indicate that both EAF and ACF exert the effects through inhibition of tissue proteins.

Table 2: Effect of fractions of methanolic extract of *Calotropis procera* leaves on egg albumin denaturation

Design of treatment	Concentration (µg/mL)	Mean ±SEM	% Inhibition
Group-I (Control)	-	1.6400 ± 0.0225	-
Group-II (Aspirin)	200	0.6840 ± 0.0160**	58.29
	400	0.6277 ± 0.0307**	61.73
	800	0.4970 ± 0.0026**	69.70
Group-III (ACF)	200	1.1340 ± 0.0727**	30.85
	400	1.0330 ± 0.0404**	37.01
	800	0.9790 ± 0.0136**	40.31
Group-IV (EAF)	200	0.9770 ± 0.0274**	40.43
	400	0.9150 ± 0.0252**	44.21
	800	0.6977 ± 0.0274**	57.46

Values expressed as mean± SEM. (n=3). ANOVA followed by Dunnett test. $^{*}p < 0.05$, when compared with control group. $^{**}p < 0.01$, when compared with control group.

Analgesic activity

All the extracts of leaves of *C. procera* i.e. CECP, EECP and MECP demonstrated noteworthy analgesic activity (Table 3).

The methanolic extract at a dose of 200 mg/kg revealed more potent activity (74.8% protection) than the aspirin (61.12 % protection) at dose of 100 mg/kg (Fig 2). Acetic acid is a sensitive agent for production of constriction of abdominal responses. It causes an increase in peritoneal fluid level of prostaglandins PGE2 and PGF2-α during the first 30 min after acetic acid injection, involving in inflammatory pain by inducing capillary permeability. Intra-peritoneal administration of acetic acid induces the release of prostaglandins and other sympathetic nervous system mediators [36-38].

Therefore, extracts CECP, EECP and MECP may exert analgesic activity by inhibition of prostaglandin release or synthesis; as acetic acid-induced pain mainly involves cyclooxygenase pathway and prostaglandin biosynthesis.

Table 3: Acetic acid induced writhing effect of different extracts of *Calotropis procera*

Group	Treatment and dose	No. of writhings	% Inhibition
Group I (Control)	distilled water, 10 mL/kg, p.o	56.17± 7.19	-
Group-II (Test group I)	CECP, 200 mg/kg, p.o	34.67± 4.41**	38.279
Group-III (Test group II)	EECP, 200 mg/k, /p.o	35.67± 6.80**	36.498
Group-IV (Test group III)	MECP, 200 mg/kg, p.o	14.33± 4.93**	74.481
Group-V (Positive control)	Aspirin, 100 mg/kg, p.o	21.83± 3.55**	61.128

Values expressed as mean± SD (n=6). ANOVA followed by Dunnett test. $^{*}p < 0.05$, when compared with control group. $^{**}p < 0.01$, when compared with control group.

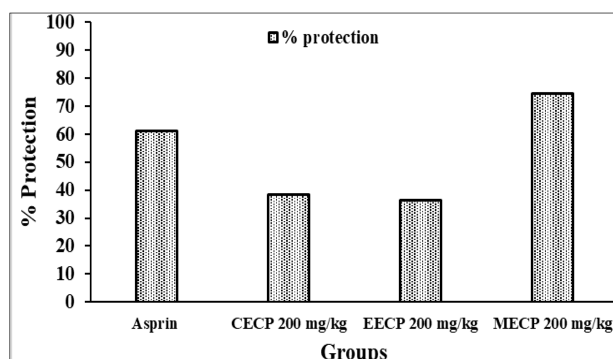

Fig 2: Acetic acid-induced writhings effect of extracts (CECP, EECP and MECP) of *C. procera*.

Percentage of protection towards the acetic acid induced Writhings shown greater for MECP than the standard analgesic drug aspirin. ANOVA followed by Dunnett test, $*P < 0.05$, $**P < 0.01$ vs. control group.

Conclusion

Our study provides insight into the anti-inflammatory and analgesic activity of *Calotropis procera* leaves. The results of the present study suggest that *C. procera* extracts (CECP, EECP and MECP) have significant analgesic and anti-inflammatory potential. *In-vitro* anti-inflammatory studies of fractions (ACF and EAF) further augment this claim. Especially MECP demonstrated more significant analgesic and anti-inflammatory activity than aspirin; ethyl acetate fraction also showed significant anti-inflammatory potential. Its anti-inflammatory activity can be supported by inhibition of cyclooxygenase and prostaglandin biosynthesis.

Acknowledgement

The authors are thankful to the Director of School of Pharmacy and School of Life Sciences, Swami Ramanand Teerth Marathwada University, Nanded, MS, India. Authors also grateful to the Principal, Nanded Pharmacy College,

Nanded, Maharashtra, India for providing experimental facilities to carry out the work.

Conflict of interest

We declare that we have no any conflict of interest.

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