Research Article

ANTI-INFLAMMATORY PROPERTIES OF METHANOLIC EXTRACT OF "*SIKARI LAHARO"* (*Periploca calophylla*)

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ABSTRACT

Inflammation, orchestrated in a series of cardinal signs is a pathophysiological condition that occurs during various clinical presentations. *Periploca calophylla* is a herbal plant which is used traditionally as an anti-inflammatory agent for a myriad of malaises in Nepalese countryside. The scientific research on the anti-inflammatory property of this plant is scanty, and if available elsewhere, its properties are not proven scientifically, except sporadic empirical evidence reported by the traditional faith healers. The main objective of this research was to establish a proof of concept on the anti-inflammatory property of *P. calophylla* based on the results obtained from a scientific experiment. Accordingly, Adult albino mice animal model was used for in vivo assessment of its property. Three different doses of 80% methanolic extract of the vine of *P. calophylla* (1.5 mg/kg, 2 mg/kg, and 2.5 mg/kg) were administered intra-peritoneally to the animals of the test groups. Indomethacin (25 mg/kg) and distilled water (3 mL/kg) were used as positive and negative controls, via the same route of administration. The anti-inflammatory property was evaluated by the Carrageenan-induced hind paw oedema model test, fresh egg albumin induced paw oedema test, formalin-induced paw oedema test, and haematology. Extract of *P. calophylla* (1.5 mg/kg) significantly ($p<0.01$) inhibited inflammatory responses in all the evaluated tests in the animal model. The data obtained from this study indicated that the phyto-extract of *P. calophylla* possessed a significant amount of anti-inflammatory property. This justifies the empirical and traditional use of this plant as an anti-inflammatory agent. Isolation of the particular compound related to this property needs further experimentation and scientific investigation.

Key words: *Periploca calophylla,* phyto-extract, inflammation, and animal model.

INTRODUCTION

Inflammation is one of the first protective physiological responses of the immune system to infection or cellular damages. This response can be against foreign pathogens, irritation, as well as autoimmune or neuro-degenerative diseases (Acharya et al., 2019). It is a complex biological response of living vascular tissue to any stimuli, such as pathogens, irritants, or damaged cells, thereby leads to an influx of neutrophils, resulting in activation of macrophages (Schett et al., 2013). This causes the release of various inflammatory mediators, such as nitric oxide (NO), pro-inflammatory cytokines, including TNF-α, interleukins (IL-6, IL-1β), and prostaglandins (PGs) (MacMicking et al., 1997; Tanaka et al., 2014). NO, produced by nitric oxide synthetase from L-arginine in response to inflammatory stimuli is a central inflammatory mediator. Increased NO level is one of the well-known causative agents of inflammatory disorders. NO and PGs largely act as secondary mediators of pro-inflammatory cytokines TNF-α and IL-6 (Skelly et al., 2013).

Rubor (redness), calor (increased heat), tumor (swelling), dolor (pain), and *functio laesa* (loss of function) are the cardinal signs of inflammation (Hakansson & Molin, 2011). The inflammatory response which plays an important role in host survival can also lead to the development of other diseases, such as mastitis, cancer, rheumatoid arthritis, and cardiovascular dysfunction (Grivennikov et al., 2010; Skeoch & Bruce, 2015; Libby, 2006). Currently, inflammation is the focus of global scientific research because of its association in all human and animal diseases (Adedapo et al., 2008). Most anti-inflammatory drugs, particularly NSAIDs inhibit the production of PGs and hence interfere with the inflammatory cascade (Ricciotti & FitzGerald, 2011). Prolonged use of such drugs can potentially cause adverse side effects such as inflammation of gastro-intestinal tract, renal failure and liver toxicity, just to name a few (Harirforoosh et al., 2013). This has led to the increased demand for safe, and efficacious new anti-inflammatory compounds with minimal toxicity (Tadiwos et al., 2017). On the other hand, the search for new natural compounds is escalating because of their efficacy, minimum side effects, and that they provide significant leadership in the development of more effective synthetic molecules and are relatively cheaper than chemical compounds (Koech et al., 2017).

Phyto-active natural alkaloids from plants have been used in complementary and alternative medicine (CAM) to treat inflammation and pain as well as other diseases since time immemorial. The knowledge of traditional, ethnic and herbal medicine, which is handed down from generation to generation, and has been used for over centuries, can be a better alternative medicine in the current era.

It is noteworthy that most of the synthetic anti-inflammatory and analgesic drugs, such as aspirin and morphine are derived from botanicals (Koech et al., 2017). Cragg et al., (1997) reported that approximately 70% of the anti-inflammatory and analgesic compounds are synthetic drugs, and most common ones such as aspirin were also prototyped from the phytoactive chemicals. This emphasizes that studies of plants believed to have anti-inflammatory phytoactive natural compounds, or used for such purposes are rewarding and logical (Nandani et al., 2018).

Periploca calophylla (Wight), Falconer, of family *Asclepiadaceae,* is a trailing shrub, having lanceolate, long acuminate, leathery and shiny stalked leaves, 3.5-8.5 cm long, and 0.3-1.7 cm wide. Flowers are pinkish in lax cymes and fruit is cylindrical. It flowers during April-May and fruit in November-January. It propagates by seeds and is distributed throughout the shady places around the altitude of 1500-2000 meters above sea level (masl) in Nepal as well as in northern India, Bhutan, Tibet and Central West China. Along the altitude range of 500-900 masl in Nepal, the *P. calophylla* (locally named as "*Sikarilaharo*") is one of the commonly used plants for treating multiple ailments (Bhuju 2005). For example, the paste made from the whole-plant of *P. calophylla* is used to treat bone fractures and muscle pains (Aryal et al., 2016). Under this context, an experiment was done to establish a proof of concept on the anti-inflammatory property of *Periploca calophylla* based on the indications obtained from a scientific experiment by using an adult albino mice animal model.

MATERIAL AND METHODS

Collection and identification of the test plant

Vine along with bark of locally available *Sikarilahara* (literally translates as hunter vines in English) was collected from Ghalegaun of Lamjung district at 1800 meters above sea level in mid-western Nepal. A specimen was submitted to National Herbarium, Godawari, Lalitpur, Nepal for species identification. It was identified as *Periploca calophylla* by the taxonomists (Ref No 076/77, Dispatch No: 142, National Herbarium and Plant Laboratory, Godavari, Lalitpur, Nepal).

Methanol extraction

The collected plant parts were washed with clean water and air-dried in shade, powdered, and soaked in 80 % methanol for 48 hours in a capped conical flask inside a rotatory incubator. The plant-methanol mixture was sieved by using a double layer of fine muslin cloth followed by Whatman® filter paper number one. The filtrate was evaporated till dry at 55^0C in a rotary evaporator to get the methanolic extract. The extract was used for the assessment of anti-inflammatory properties.

Animal experiments

Healthy adult male Swiss albino mice, each weighing about 25-30 gm was used for this study. The animals were maintained as recommended by Acharya et al., (2019), but with some minor modifications to suit our laboratory conditions. They were housed at a temperature of 23-25^0 C with a relative humidity of 60-70% and given 12 hours of alternating light and dark cycle, in standard-sized plastic cages at the lab animal house, Faculty of Animal Science, Veterinary Science and Fisheries, Agriculture and Forestry University, Chitwan, Nepal. They were fed *ad libitum* on a commercial pellet diet and sterile filtered potable water. All the animal experiments were approved by the Animal Ethical Committee, Agriculture and Forestry University, Rampur, Chitwan, Nepal on 20th Jan. 2019.

Animal experiments

The experiments in the animal model were done using a completely randomized design (CRD). Experimental animals were randomly divided into three different treatment groups with five animals in each group. Each treatment group had five replications. The animal equivalent dose was calculated based on the total body surface area. Animal equivalent doses (mg/kg) for rat and mouse were calculated by multiplying human equivalent dose as recommended by the traditional healers (mg/kg) by factor 6.2 and 12.3, respectively (Nair and Jacob, 2016). The extract of *P. calophylla (Pc)* was tested at doses of 1.5 (T1), 2.0 (T2) and 2.5 (T3) mg/kg given intra-peritoneally (I.P), respectively. Indomethacin, at the rate of 25 mg/kg I.P was given as the positive control, while distilled water (3 ml/kg, I.P) was used as the negative control.

Evaluation of anti-inflammatory activity

Carrageenan-induced hind paw oedema model test

Carrageenan induced paw oedema test was performed according to the modified methods (Winter et al., 1962; Acharya et al., 2019). The model animals were divided into different groups (five animals per group) based on basal paw volume measured using plethysmometer at 0 hours. Inflammation was induced by the subcutaneous injection of λ-carrageenan (0.1 mL of 1 % solution in sterile normal saline) into the plantar surface of the left hind paw. The paw was marked with ink at the level of lateral malleolus and volume was measured up to the mark sixty minutes after the intraperitoneal administration of a test dose of the *Pc* extract. Simultaneously, 25µL sterile saline solution was injected similarly as above into the left hind paw. Paw oedema was then measured at an interval of every ninety minutes until six hours after induction of inflammation. The difference in footpad thickness was measured. Mean value of the treated group was compared with the value of the same footpad before injection of carrageenan (value at 0 hours) and those of a control group to analyze by using appropriate statistical methods. Indomethacin (25 mg/kg) was used as the reference anti-inflammatory drug (Acharya et al., 2019).

Fresh egg albumin induced paw oedema test

The fresh egg albumin induced hind paw oedema was used as a model for evaluation of acute inflammation. Hind paw oedema in test animals was induced by intraplantar (I.P.) injection of a phlogistic agent, such as fresh egg albumin at the rate of 0.5 mL/kg body weight (Ojewole, 2007) into the sub-plantar surface of the right hind paw. Pedal inflammation (oedema) is evident within 5–8 minute following the injection. The volume of the paw was measured using plathysmometer for three hours at an interval of every half an hour after the administration of the phlogistic agent. Increases in the diameter of the right hind paws were taken as indicators of paw oedema. Oedema was assessed in terms of the difference in the volume of the injected right hind paw before injection [‘time zero’ (C0)], and the volume at various time points of 1, 1.5, 2, 2.5 and 3 hours [‘time t’ (Ct)] following fresh egg albumin administration. The increase in the diameter of the right hind paw induced after injection of fresh egg albumin was compared to the diameter of the same paw before injection and that of the control group (Ojewole, 2007). Indomethacin (25 mg/kg) was used as the reference anti-inflammatory drug Acharya et al., 2019).

Formalin-induced paw oedema test

Acute hind paw oedema was produced by injecting 0.1 mL of 3 % formalin locally into the sub-plantar region of the right hind paw of the rat. The plant extract was administered intra-peritoneally (I.P) at different doses (at the rates of 100,200 mg/kg, I.P) to the test subjects along with the standard drug indomethacin (at the rate of 25 mg/kg, I.P) to the positive control group and normal saline (at the rate of 10 mL/kg I.P) to control groups one hour before the induction of paw oedema by formalin. Measurement of paw size was done by wrapping a piece of cotton thread around the paw, and the length of thread corresponding to paw circumference was determined using a meter rule. Measurement was done immediately before and one to three hours after the formalin injections. The inhibitory activity was calculated as suggested by Olajide et al.,(2000):

$$Percentage\ inhibition == \frac{[(Ct - Co)control - (Ct - Co)treated]}{(Ct - Co)control} * 100$$

Where Ct is the paw circumference at time t, Co is paw circumference before formalin injection, (Ct-Co) is oedema, and (Ct-Co) control is oedema or paw size after formalin injection to control rats at time t.

Haematology

Blood was collected from the tail vein of the rat after 72 hours of the treatment. Total leukocytes count (TLC), neutrophils and lymphocytes were counted by using a haemocytometer, total protein (TP) was determined by using a refractometer while fibrinogen was determined by heat precipitation method (Coles, 1986).

Data analysis

Data collection and management was done in Microsoft Office Excel 2010 and statistical analysis was done by using M-STAT version 3C using one-way ANOVA. Data obtained from experiments were expressed as mean along with the standard deviation (±SD). The results obtained for each treatment (different dosages of the extract) was compared with the control treatment (dosage of indomethacin). Differences between treatments were considered significant at $p<0.05$.

RESULTS

Carrageen an induced hind paw oedema in rats

The average paw volume increased significantly ($p<0.01$) in all the treatment groups. There was no statistically significant difference ($p>0.05$) in average paw volume at one, two, three, and four hours in the treatments group, except control (Table 1) The lowest volume of paw oedema was observed at the later stages of the experiments hours (Table 1).

Table 1. Effect of *P. calophylla* extract on carrageenan-induced oedema in rats

Time (hr)	Volume of paw (units)				
	Indomethacin (10mg/kg)	*Pc* (1.50 mg/kg)	*Pc* (2.00 mg/kg)	*Pc* (2.50 mg/kg)	Control
0	$7.70^{b} \pm 0.84$	$7.50^{b} \pm 0.71$	$7.40^{c} \pm 0.65$	$7.00^{c} \pm 0.35$	$6.60^{d} \pm 0.22$
1	$8.88^{a} \pm 0.91$	$8.90^{a} \pm 0.62$	$8.86^{ab} \pm 0.59$	$8.38^{ab} \pm 0.36$	$9.80^{c} \pm 0.91$
2	$9.06^{a} \pm 0.91$	$9.00^{a} \pm 0.80$	$9.06^{ab} \pm 0.67$	$8.44^{ab} \pm 0.34$	$10.74^{b} \pm 0.78$
3	$9.32^{a} \pm 0.85$	$9.46^{a} \pm 0.68$	$9.66^{a} \pm 0.49$	$8.74^{a} \pm 0.42$	$12.94^{a} \pm 0.36$
4	$8.70^{ab} \pm 0.90$	$8.60^{a} \pm 0.75$	$8.60^{b} \pm 0.64$	$8.06^{b} \pm 0.36$	$9.70^{c} \pm 0.91$
LSD	1.164	0.9422	0.8068	0.4847	0.9226
CV (%)	10.11	8.21	7.01	4.53	7.03
F- Value	2.472**	5.292**	9.281**	16.756**	53.331**

** $p<0.01$ compared to the same group at 0 hours. Treatment means followed by the same letter within the same column are not statistically significant.

A significant difference in paw volume of different treatment groups was observed at each hour. There was no significant difference between the increment in paw volume of the groups treated with different doses of *Pc* and indomethacin at one, two and four hours, but the increment in paw volume of the group treated with *Pc*2.5 mg/kg was significantly different with the increment in paw volume of the groups treated with indomethacin and *Pc* 1.5 mg/kg.at 3 hours and, minimum increment being at the group treated with *Pc* 1.5 mg/kg in all the hours (Table 2). In biological terms, the output at the final period is more biologically significant than the en-route outputs so it can be safely inferred that these outputs indeed have a biological significance.

Table 2. Effect of *P. calophylla* extract on the increment of oedema in carrageenan-induced hind paw of rats

Treatments	Increment of oedema			
	1 (hr)	2 (hr)	3 (hr)	4 (hr)
Indomethacin (10mg/kg)	$1.38^{b} \pm 0.08$	$1.44^{b} \pm 0.25$	$1.740^{c} \pm 0.27$	$1.06^{b} \pm 0.09$
Pc (1.5 mg/kg)	$1.18^{b} \pm 0.13$	$1.36^{b} \pm 0.11$	$1.620^{c} \pm 0.19$	$1.00^{b} \pm 0.10$
Pc (2.0 mg/kg)	$1.40^{b} \pm 0.10$	$1.50^{b} \pm 0.19$	$1.960^{bc} \pm 0.30$	$1.10^{b} \pm 0.12$
Pc (2.5 mg/kg)	$1.46^{b} \pm 0.11$	$1.66^{b} \pm 0.23$	$2.260^{b} \pm 0.23$	$1.20^{b} \pm 0.07$
Control	$3.20^{a} \pm 1.04$	$4.140^{a} \pm 0.88$	$6.340^{a} \pm 0.54$	$3.10^{a} \pm 0.96$
LSD	0.6244	0.5705	0.4356	0.5796
CV (%)	27.48	21.43	11.85	29.41
F- Value	15.416**	37.796**	184.324**	21.114**

** $p<0.01$ compared with control at the same hour. Treatment means followed by the same letter within the same column are not statistically significant.

A significant difference was observed when the inhibition percentage of inflammation of different treatment groups was compared with the control group at each hour. There was no significant difference in inhibition percentage of inflammation among the groups treated with indomethacin, *Pc* 2 mg/kg and 2.5 mg/kg at 1 hour. The group treated with *Pc* 1.5 mg/kg had significantly higher inhibition percentage of inflammation than the other dosage groups. There was no significant difference in inhibition percentage of inflammation between the groups treated with different doses of *Pc* with indomethacin at two hours. At three hours, inhibition percentage of inflammation of group treated with *Pc* 2.5 mg/kg was significantly lower than the groups treated with other doses of *Pc* and indomethacin. There was no significant difference in inhibition percentage of inflammation between the groups

treated with indomethacin and other doses of *Pc* except 2.5 mg/kg at four hours. Maximum inhibition percentage of inflammation was observed in the group treated with *Pc* 1.5 mg/kg for all the hours (Table 3).

Table 3. Effect of *P. calophylla* extract on inhibition percentage of oedema in carrageenan-induced hind paw oedema of rats

	Inhibition percentage of oedema			
	1 (hr)	2 (hr)	3 (hr)	4 (hr)
Treatments				
Indomethacin (10 mg/kg)	56.88^{b}± 2.62	65.22^{ab} ± 6.06	72.56^{ab} ± 4.26	65.81^{a} ± 2.88
Pc (1.5 mg/kg)	63.13^{a} ± 4.07	67.15^{a} ± 2.75	74.45^{a} ± 3.03	67.74^{a} ± 3.23
Pc (2.0 mg/kg)	56.25^{b} ± 3.13	63.77^{ab} ± 4.52	69.08^{b} ± 4.68	64.52^{ab} ± 3.95
Pc (2.5 mg/kg)	54.38^{b} ± 3.56	59.90^{b} ± 5.56	64.35^{c} ± 3.63	61.29^{b} ± 2.28
Control	0.00^{c} ± 0.00	0.00^{c} ± 0.00	0.00^{d} ± 0.00	0.00^{c} ± 0.00
LSD	3.997	5.771	4.662	3.708
CV (%)	6.57	8.54	6.30	5.42
F- Value	368.087**	215.956**	399.570**	534.960**

** p<0.01 compared with control at the same hour. Treatment means followed by the same letter within the same column are not statistically significant.

Fresh egg albumin induced paw oedema in rat

There was a significant difference in average paw volumes at one and two hours of *P. calophylla* and indomethacin treated group compared to the 0 hours of the same group. Average paw volumes of the group treated with *Pc* at 1.5 mg/kg and 2 mg/kg at 2.5 and 3 hours were not significantly different from that of 0 hours of the same group. Similarly, results shown by *Pc* at 2 mg/kg and 2.5 mg/kg were not significantly different from the paw volumes at 0 hours of the same group. There was a significant difference in average paw volumes at all hours of the control group when compared to the 0 hours. In all cases, maximum volume was observed at 1.5 hours (Table 4).

Table 4. Effect of *P. calophylla* extract on egg albumin induced oedema of rats

	Volume of paw (Unit)				
Time (hr)	Indomethacin (10mg/kg))	*Pc* (1.50 mg/kg	*Pc* (2.00 mg/kg)	*Pc* (2.50 mg/kg)	Control
0	7.20^{c} ±0.57	7.500^{d} ± 0.50	6.40^{d} ± 0.65	6.60^{c} ± 0.65	6.40^{d} ± 0.65
1	8.44^{a} ± 0.67	8.840^{b} ± 0.55	7.88^{ab} ± 0.56	8.14^{ab} ± 0.65	10.30^{c} ± 0.84
1.5	8.64^{a} ± 0.59	10.20^{a} ± 0.55	8.20^{a} ± 0.50	8.62^{a} ± 0.75	13.24^{a} ± 0.55
2	8.14^{ab} ± 0.66	8.640^{bc} ± 0.56	7.66^{abc} ± 0.54	8.04^{ab} ± 0.74	12.18^{b} ± 0.60
2.5	7.58^{bc} ± 0.54	8.060^{cd} ± 0.49	7.24^{bc} ± 0.72	8.20^{ab} ± 1.05	11.64^{b} ± 0.51
3	7.32^{c} ± 0.54	7.820^{d} ± 0.38	6.92^{cd} ± 0.67	7.22^{bc} ± 0.77	9.96^{c} ± 0.85
LSD	0.7810	0.6630	0.7993	1.017	0.8853
CV (%)	7.59	5.97	8.29	9.98	6.39
F- Value	5.096**	18.107**	5.846**	4.586**	62.336**

** p<0.01 compared to the same group at 0 hours. Treatment means followed by the same letter within the same column are not statistically significant.

The increment in paw oedema of all the groups at all hours was significantly different when was compared to the control group at the same hour. When compared with the gold standard of indomethacin, the plant extract at 1.5 mg/kg had a statistically significant contribution to inhibit the oedema thereby exhibiting the maximum potential as an anti-inflammatory agent (Table 5).

Table 5. Effect of *P. calophylla* extract on increment of oedema in egg albumin-induced hind paw of rats

Treatments	Increment of oedema				
	1(hr)	1.5 (hr)	2 (hr)	2.5 (hr)	3 (hr)
Indomethacin (10mg/kg)	$1.34^{b} \pm 0.17$	$1.68^{bc} \pm 0.13$	$1.14^{bc} \pm 0.11$	$0.56^{cd} \pm 0.11$	$0.32^{b} \pm 0.13$
Pc(1.5 mg/kg)	$1.24^{b} \pm 0.11$	$1.44^{c} \pm 0.21$	$0.94^{c} \pm 0.24$	$0.38^{d} \pm 0.11$	$0.12^{b} \pm 0.08$
Pc(2.0 mg/kg)	$1.48^{b} \pm 0.13$	$1.80^{bc} \pm 0.16$	$1.26^{bc} \pm 0.17$	$0.84^{bc} \pm 0.11$	$0.52^{b} \pm 0.13$
Pc(2.5 mg/kg)	$1.54^{b} \pm 0.11$	$2.02^{b} \pm 0.19$	$1.44^{b} \pm 0.18$	$1.02^{b} \pm 0.15$	$0.62^{b} \pm 0.13$
Control	$3.90^{a} \pm 0.74$	$6.84^{a} \pm 0.62$	$5.78^{a} \pm 0.66$	$5.24^{a} \pm 0.48$	$3.56^{a} \pm 0.90$
LSD	0.4646	0.4213	0.4454	0.3177	0.5471
CV (%)	18.55	11.59	15.97	14.93	40.37
F- Value	50.878**	257.651**	186.183**	363.135**	59.243**

** $p<0.01$ compared with control at the same hour. Treatment means followed by the same letter within the same column are not statistically significant.

Inhibition percentage of oedema of treatment groups as compared to the control group of the same hour was statistically significant. At one and two hours, inhibition percentage of oedema of the group treated with *Pc* 1.5 mg/kg was non-significant as compared to the group treated with indomethacin, but statistically significant with other doses of *Pc* at 1.5, 2.5. At 3 hours, inhibition percentage of oedema of the group treated with *Pc* 1.5 mg/kg was statistically significant as compared to indomethacin and other doses of *Pc* as well. *Pc* at the rate of 1.5 mg/kg at all the hours showed maximum inhibition percentage of inflammation (Table 6).

Table 6. Effect of *P. calophylla* extract on inhibition percentage of oedema in egg albumin-induced hind paw oedema of rats

Treatments	Inhibition percentage of oedema				
	1 (hr)	1.5 (hr)	2 (hr)	2.5 (hr)	3 (hr)
Indomethacin (10mg/kg)	$65.64^{ab} \pm 4.29$	$75.44^{b} \pm 1.91$	$80.28^{ab} \pm 1.97$	$89.31^{b} \pm 2.18$	$91.01^{b} \pm 3.66$
Pc(1.5 mg/kg)	$68.21^{a} \pm 2.92$	$78.95^{a} \pm 3.03$	$83.74^{a} \pm 4.17$	$92.75^{a} \pm 2.09$	$96.63^{a} \pm 2.35$
Pc(2.0 mg/kg)	$62.05^{bc} \pm 3.34$	$73.68^{b} \pm 2.31$	$78.20^{bc} \pm 2.90$	$83.97^{c} \pm 2.18$	$85.39^{c} \pm 3.66$
Pc(2.5 mg/kg)	$60.51^{c} \pm 2.92$	$70.47^{c} \pm 2.81$	$75.09^{c} \pm 3.14$	$80.53^{d} \pm 2.83$	$82.58^{c} \pm 3.66$
Control	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$
LSD	0.4646	3.013	3.709	2.758	3.991
CV (%)	5.96	3.82	4.43	3.02	4.25
F- Value	445.000**	1077.066**	802.561**	1743.050**	879.449**

** $p<0.01$ compared with control at the same hour. Treatment means followed by the same letter within the same column are not statistically significant.

Formalin induced hind paw oedema model

When compared to the 0 hours of the same group, there was no significant difference in average paw volumes with different doses of *P. callophyla* and indomethacin but there was a significant difference in average paw volumes at all hours of the control group. The maximum volume was recorded at 3 hours in all groups (Table 7).

Table 7. Effect of *P. calophylla* extract on formalin-induced oedema of rat

Time (hr)	Volume of paw (unit)				
	Pc (1.50 mg/kg)	*Pc* (2.00 mg/kg)	*Pc* (2.50 mg/kg)	Indomethacin (10mg/kg)	Control
0	$6.24^{a} \pm 0.64$	$6.40^{a} \pm 1.19$	$6.30^{a} \pm 0.84$	$5.70^{a} \pm 0.67$	$5.60^{b} \pm 0.65$
1	$6.92^{a} \pm 0.53$	$7.34^{a} \pm 1.14$	$7.28^{a} \pm 0.83$	$6.46^{a} \pm 0.76$	$7.98^{a} \pm 0.74$
2	$6.84^{a} \pm 0.61$	$7.12^{a} \pm 1.21$	$7.10^{a} \pm 0.89$	$6.56^{a} \pm 0.95$	$8.04^{a} \pm 0.51$
3	$6.96^{a} \pm 0.57$	$7.28^{a} \pm 1.05$	$7.40^{a} \pm 0.74$	$6.92^{a} \pm 1.21$	$9.22^{a} \pm 0.56$
LSD	0.7875	1.540	1.109	1.235	1.540
CV (%)	8.72	16.33	11.78	14.37	8.07
F- Value	1.645**	0.712**	1.795**	1.551**	29.795**

** $p<0.01$ compared to the same group at 0 hours. Treatment means followed by the same letter within the same column are not statistically significant.

Overall, there were significant differences with increment in paw volume of different treatment groups compared with the control group at the same hour (Table 8). At 1 hour, there was no significant difference between the increment in paw volume of different groups treated with different doses of *Pc* and indomethacin but there was a significant difference in the paw volume of groups treated with 1.5 mg/kg, 2 mg/kg and 2.5 mg/kg of *Pc*. There was no significant difference among any treatments at 2 and 3 hours. The minimum increment was observed in the group treated with 1.5 mg/kg of *Pc* at all the hours (Table 8).

Table 8. Effect of *P. calophylla* extract on the increment of oedema in formalin-induced oedema of rats

Treatments	Increment of oedema (unit)		
	1 (hr)	2 (hr)	3 (hr)
Indomethacin (10 mg/kg)	$0.76^{bc} \pm 0.11$	$0.70^{b} \pm 0.07$	$0.92^{b} \pm 0.08$
Pc (1.50 mg/kg)	$0.68^{c} \pm 0.18$	$0.60^{b} \pm 0.16$	$0.72^{b} \pm 0.26$
Pc (2.00 mg/kg)	$0.94^{b} \pm 0.09$	$0.72^{b} \pm 0.08$	$0.88^{b} \pm 0.16$
Pc (2.50 mg/kg)	$0.98^{b} \pm 0.08$	$0.80^{b} \pm 0.12$	$1.10^{b} \pm 0.16$
Control	$2.38^{a} \pm 0.30$	$2.44^{a} \pm 0.38$	$3.62^{a} \pm 0.80$
LSD	0.2285	0.2605	0.5194
CV (%)	15.19	18.77	27.15
F- Value	80.546**	77.836**	48.270**

** $p<0.01$ compared with control at the same hour. Treatment means followed by the same letter within the same column are not statistically significant.

There was a significant difference at each hour when the inhibition percentage of inflammation of different treatment groups was compared with the control group at the same hour (Table 9). There was no significant difference ($p>0.05$), however, between inhibition percentage of inflammation in the groups treated with indomethacin and *Pc* 1.5 mg/kg at all hours, but it was significantly different with *Pc* 2mg/kg and 2.5 mg/kg at 1 hour. The inhibition percentage of inflammation in the group treated *Pc* 1.5mg/kg was significantly different with the groups treated with *Pc* 2 mg/kg and 2.5 mg/kg at 1 hour, *Pc* 2.5 mg/kg at 2 and 3 hours. The maximum percentage of inhibition was observed at the group treated with 1.5 mg/kg *Pc* for all the hours (Table 9).

Table 9. Effect of *P. calophylla* extract on inhibition percentage of oedema in formalin-induced hind paw oedema of rats

Treatments	Inhibition percentage of oedema		
	1 (hr)	2 (hr)	3 (hr)
Indomethacin (10 mg/kg)	$68.07^{a} \pm 0.11$	$71.31^{ab} \pm 0.07$	$74.59^{ab} \pm 0.08$
Pc (1.50 mg/kg)	$71.43^{a} \pm 0.18$	$75.41^{a} \pm 0.16$	$80.11^{a} \pm 0.26$
Pc (2.00 mg/kg)	$60.50^{b} \pm 0.09$	$70.49^{ab} \pm 0.08$	$75.69^{a} \pm 0.16$
Pc (2.50 mg/kg)	$58.82^{b} \pm 0.08$	$67.21^{b} \pm 0.12$	$69.61^{b} \pm 0.16$
Control	$0.00^{c} \pm 0.30$	$0.00^{c} \pm 0.38$	$0.00^{c} \pm 0.80$
LSD	6.072	5.515	5.785
CV (%)	15.19	18.77	27.15
F- Value	80.546**	77.836**	48.270**

** p<0.01 compared with control at the same hour. Treatment means followed by the same letter within the same column are not statistically significant.

Effect on haematological parameter

The results of the blood analysis indicated a significant decrease in the fibrinogen levels for all the three treatment doses (1.5, 2.0 and 2.5 mg/kg body weight) and indomethacin compared to control group. There were no significant variations in the TLC, neutrophils, lymphocyte and TP in the rats of the treatment groups as compared to control group (Table 10).

Table 10. Effect of *P. calophylla* extract on the haematological parameter in mice

Group	Haematological parameters				
	Total WBC count	Neutrophil (%)	Lymphocyte (%)	Total protein	Fibrinogen
Control	3366.67 ± 642.91	14.67 ± 3.51	84.67 ± 5.86	10.83 ± 0.59	1.67 ± 0.15
Pc (1.5 mg/kg)	4433.33* ± 642.91	18.00 ± 4.36	72.67 ± 6.66	11.7 ± 1.47	0.70** ± 0.10
Pc (2.0 mg/kg)	4900.00* ± 435.89	18.00 ± 9.17	79.00 ± 11.14	11.8 ± 1.74	0.73** ± 0.12
Pc (2.5 mg/kg)	5000.00* ± 100.00	10.33 ± 1.53	85.67 ± 2.08	12.3 ± 0.26*	0.63** ± 0.06
Indomethacin	3300.00 ± 556.78	13.67 ± 4.51	80.67 ± 5.51	10.93 ± 1.79	0.93** ± 0.23

*p<0.05 as compared with the control group, ** p<0.01 compared with control at the same hour.

The results obtained from all the tested models to evaluate these properties suggest that the plant possesses statistically significant anti-inflammatory property. In all the tests, Pc 1.5 mg/kg showed better results followed by 2 and 2.5 mg/kg.

DISCUSSION

The carrageenan-induced inflammatory response was described in 1962 in the rat paw, and in 1969 in mice. Since then, this is widely used as a simple and reliable model to assess anti-inflammatory activity and has been increasingly used to test new anti-inflammatory drugs (Ashok et al., 2010; Acharya et al., 2019). The carrageenan test is highly sensitive to non-steroidal anti-inflammation drugs, it has long accepted as a useful phlogistic tool for investigating new systemic anti-inflammatory drugs (Just et al., 1998). In general, the inflammatory response to a local injection of carrageenan into a rat paw is a complex process involving mast cell mediators, prostaglandins, the kinin system (Damas and Remacle-Volon, 1986; Kulkarni et al., 1986) and several cytokines (Loram et al., 2007). Nitric oxide (NO) is equally crucial mediator contributing to the generation of the inflammatory response in carrageenan-induced inflammation (Sakaguchi et al., 2006). It is a biphasic event, during the early phase of inflammation (0–2hrs) mediators like histamine, 5-hydroxytryptamine and bradykinin play an important role, while during the late accelerating phase (post 2 hrs) there is elevated production of PGs, and production of COX-2 (Di Rosa et al., 1971; Sakat et al., 2014, Acharya et al., 2019). Therefore the receptors of these mediators and also enzymes responsible for their production could be a target for the anti-inflammatory activity of drugs (Dharmasiri et al., 2003).

In this experiment, the suppression of inflammation at the early phase of inflammation can be contributed by PG synthesis inhibition and antihistamine activities shown by *Pc*. The anti-inflammatory activity at the second phase also indicates the inhibition of synthesis of inflammatory mediators such as bradykinin, leukotrienes, polymorphonuclear cells. Nitric oxide (NO) is one of the well-known pro-inflammatory mediators in the pathogenesis of inflammation. For the expression of inducible nitric oxide synthase (iNOS), the mammalian cells should be triggered by specific stimulants such as pro-inflammatory cytokines and bacterial lipopolysaccharide (Jeon et al., 2008). Since iNOS-derived NO is involved in inflammation (Singh et al., 2002), suppression of iNOS might be closely linked with the anti-inflammatory action of *Pc*. Opioid receptor-mediated activities can be the cause of suppression of the inflammation in the carrageenan-induced paw oedema (Planas et al., 2000). A similar mechanism might have caused regarding inhibition of inflammation in *Pc* treated groups. The reduction of anti-inflammatory inhibition percentage after three hours indicates a short duration of action of *Pc.*

Fresh egg albumin induced paw oedema test is the model of inflammation used to screen agents for anti-inflammatory effect (Adzu et al., 2014; Dzoyem et al., 2017). The characteristic swelling of the paw is due to oedema formation. Inhibition of increased vascular permeability, and hence attendant oedema, modulates the extent and magnitude of the inflammatory reaction. Many chemical mediators such as histamine, serotonin (5-HT), kinins, and prostanoids mediate an acute inflammatory response induced by phlogistic agents including egg albumin (Marsha-Lyn et al., 2002). Inflammation occurs through three distinct phases: an early phase mediated by histamine and serotonin (up to two hours), an intermediate phase involving the activity of bradykinin. In this experiment, suppression in the first phase of inflammation can be contributed by PG synthesis inhibition and antihistaminic activity of *Pc.*

The formalin-induced paw oedema test is a popular chemical assay of injury-produced inflammatory pain. It is regarded as a robust model of clinical pain and is a useful model for the screening of novel compounds, as it encompasses inflammatory, neurogenic, and central mechanisms of nociception (Hunskaar & Hole, 1987; Tjølsen et al., 1992; Tjølsen & Hole, 1997; Sofidiya et al., 2014). The advantage of the formalin assay over other models of inflammatory pain is that the injection of a dilute solution of formalin into the surface of a mouse or rat's hind paw allows modelling of both acute and tonic pain using a single chemical in a relatively limited time.

In all the anti-inflammatory test models, the lower dose (1.5 mg/kg) was found to be more effective than higher doses. Lack of increase in potency at higher doses of the extracts suggests that at the dose beyond 1.5 mg/kg is not necessarily e□ective for required anti-inflammatory property. This phenomenon is been explained by the hypothesis that some of the active constituent(s) of the plant extract at high concentrations may exhibit pro-inflammatory (Rezazadeh et al., 2005). Similarly, Tadiwos et al., 2017, explains that the plant extract has multiple compounds acting di□erently via anti- or pro-inflammatory responses being activated by various pharmacological pathways and pharmacodynamics. Pharmacodynamics of the active phyto-compounds are governed by multiple variables, for example, ligand-receptor affinity, and regulatory genomic networks acting on the signal transduction pathways forming a complex intracellularly interacting components. These mechanisms are time-dependent owing to the enzymatic activities and the half-life of such compounds. Therefore, evaluations at every interval of time do not carry much biologically significant interpretation as compared to the endpoint biological efficacy which was the focus of this study.

CONCLUSION

The extract of *P. calophylla (Pc)* was tested at doses of 1.5 (T1), 2.0 (T2) and 2.5 (T3) mg/kg given intra-peritoneally (I.P), in albino mouse model experiments. Maximum inhibition percentage of inflammation was observed in the group treated with *Pc* 1.5 mg/kg followed by 2 and 2.5 mg/kg. Similarly, a minimum increment of oedema was observed with *Pc* 1.5 mg/kg. The results obtained from all the tested models to evaluate these properties suggest that the plant possesses anti-inflammatory property. In all the tests, Pc 1.5 mg/kg showed better results followed by 2 and 2.5 mg/kg. Based on the results obtained from these tested models performed to evaluate these properties, it is affirmed that the plant *P. calophylla* possesses a good anti-inflammatory property. This study validates the traditional use of plant parts as an anti-inflammatory agent while providing scientific evidence for this effectiveness. However, the active phytochemicals present in the extract needs to be purified and further tested for their exact physiological mechanism of action at the cellular level along with its pharmacokinetics studies including ED_{50}, dosing interval and the toxicological studies including LD_{50} of the plant.

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Conflict of Interests

All the authors declare that there are no conflicts of interests that might affect the findings of this study.

Authors Contribution

JA prepared the experimental design, executed the experiments and collected the data, ST, SS and MKS provided the mentorship on animal experiments, JA and NP analyzed the data, and drafted the manuscript and finalized the draft. All authors have read and agreed upon the submission of this manuscript.

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