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To cite this article: Shirin Marahel & Sharanaiah Umesha (2016) Anti-inflammatory and antinociceptive effect of *Pachygone ovata* leaves , *Pharmaceutical Biology*, 54:12, 3046-3054, DOI: [10.1080/13880209.2016.1203960](https://doi.org/10.1080/13880209.2016.1203960)

To link to this article: <https://doi.org/10.1080/13880209.2016.1203960>



Published online: 14 Jul 2016.



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RESEARCH ARTICLE

Anti-inflammatory and antinociceptive effect of *Pachygone ovata* leaves

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ABSTRACT

Context: *Pachygone ovata* (Poir.) Miers ex Hook. F. et Thoms (Menispermaceae) is a rich source of bioactive bisbenzylisoquinoline and aporphine alkaloids.

Objective: This study investigates the *in vitro* and *in vivo* anti-inflammatory and antinociceptive potential of *Pachygone ovata* leaves.

Materials and methods: Lipoxygenase (LOX) assay for anti-inflammatory activity was conducted using MeOH, EA, H and Aq extracts; followed by alkaloid isolation. The anti-inflammatory potential was determined using carrageenan-induced paw oedema and formalin tests for evaluation of *Pachygone ovata* analgesic effect. Different doses (100, 300 and 400 µg/kg) were administered orally to Wistar rats for a period of one week, once daily.

Results: MeOH and EA extract efficiently inhibited LOX (IC₅₀ 1.43 and 2.15 µg/mL, respectively). MeOH extract had better inhibiting capacity (57%) than indomethacin (51%) in carrageenan induced rats. MeOH extract (300 µg/kg) significantly reduced the increased levels of nitric oxide (8 ± 0.57 M), total leukocyte count (4.5 ± 0.05 cells 10³/cells) and C-reactive protein (55 ± 0.45 mg/mL). There was a decrease in various serum biochemical markers (ALT, AST). Histopathological studies revealed reduction in oedema and decreased cellular infiltration on supplementation with MeOH extract. Furthermore, MeOH extract (300 µg/kg) and alkaloid fraction (400 µg/kg) effected both phases (neurogenic and inflammatory) of formalin injected models.

Discussion and conclusion: Inflammatory mediators play a key role in inflammation; therefore, keeping it in control is of utmost importance. The usefulness of *Pachygone ovata* leaves on pain and inflammation has been described, probably due to its effect on inflammatory mediators and high alkaloid content.

ARTICLE HISTORY

Received 2 July 2015
Accepted 15 June 2016
Revised 18 May 2016
Published online 14 July 2016

KEYWORDS

Carrageenan;
histopathological studies;
toxicity; biochemical
markers

Introduction

Inflammation is the body's mechanism of defending itself against the cellular injury and challenges faced by the invader, which may result in release of inflammatory mediators to cause restoration of tissue function and structure. However, continuation and persistence of the inflammation can be harmful and contribute to genesis of various diseases; characterized by oedema formation, leukocyte infiltration and granuloma formation (Mitchell & Cotron 2000). Keeping in mind the adverse effects of oxidative stress and inflammatory processes in the development of many chronic diseases, it is worthwhile to look into the unexplored realm of medicinal plants with properties that can neutralize these repugnant effects.

According to Indian folk medicine, species of the Menispermaceae family have been used against diabetes, oedema, pain, rheumatoid arthritis, bone fracture, nephritis, pyrexia and hypertension (Kirtikar & Basu 1975; Pongboonrod 1979; Caius 1986; Chopra et al. 1996). Most species of the Menispermaceae family have been found to be rich in alkaloids, of which curare, an isoquinoline or indole alkaloid isolated from *Chondrodendron tomentosum* Ruiz and Rulz has been established as an arrow poison by indigenous people of Guyana. Further investigation advanced its use to an anaesthetic during surgeries (Krishnaswamy & Sundaresan 2013). *Chasmanthera dependens* Hoscht. has shown to have anti-inflammatory and analgesic effects on laboratory animals (Onabanjo et al. 1991; Morebise et al. 2001); its constituents

include berberine-type alkaloids (Iwu et al. 1999), quaternary alkaloids and non-phenolic alkaloids (Ohiri et al. 1982). *Cissamplos pareira* L. was used as a traditional folk medicine for its antiseptic properties against inflammation and for wound healing, which was reported to have high alkaloid content by Neuwinger (1994). *Tinospora crispa* (L.) Miers ex Hook. F. et Thoms. is claimed to be effective against rheumatism and arthritis (Quisumbing 1978). Benzylisoquinoline, protoberberines and aporphines constitute about 40% of the total alkaloids isolated from this family. Bisbenzylisoquinoline alkaloids have been reported to inhibit inflammatory mediators and play an important role in control of inflammation (Teh et al. 1990; Akiba et al. 1992).

Pachygone ovata (Poir.) Miers ex Hook. F. et Thoms. has been an unexplored and trivialized plant from the Menispermaceae family despite containing a number of interesting alkaloids such as coclaurine, reticuline, trilobine, pachyovotamine (benzylisoquinoline alkaloids) magnoflurine (aporphine alkaloid), norjuzipine (benzyltetrahydroisoquinoline alkaloid), liriodenine, pachygonine (erythrina alkaloid), etc. (Dasgupta et al. 1979; Bhat et al. 1980; El-Kawi et al. 1984; Uvais et al. 1985). Traditionally only its use as a fish poison and vermicide has been highlighted (Hooker 1961; Kirtikar & Basu 1975). Since there is no scientific validation for any pharmacological attributes to *Pachygone ovata*, this research investigates the crude extract and alkaloid fraction of *P. ovata* for anti-inflammatory potential both *in vitro* and *in vivo*. *In vitro* assay was based on the inhibition of the lipoxygenase enzyme (15-LOX) isolated from soybean and the *in vivo* analysis

was conducted using models of inflammatory pain (i.e., formalin-induced licking) and acute inflammation (i.e., carrageenan-induced oedema).

Materials and methods

Animals

The present study was conducted in accordance with internationally accepted principles for laboratory animal use and care. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) and received approval number UOM/IAEC/18/2013, dated 28/9/2013. The animals were procured from the Animal House Center, Department of Zoology, University of Mysore, India, and were maintained in colony cages at $25 \pm 3^\circ\text{C}$, with a relative humidity of 45–50%, under 12/12 h light and dark cycles. The experiments were conducted on male Wistar rats weighing 150–200 g. The animals were fed with laboratory chow and water *ad libitum*. The animals were allowed to acclimatize to the experimental room for 24 h before the experiments.

Reagents

All the chemicals and solvents were of analytical grade and procured from various sources. λ -Carrageenan from Sigma-Aldrich (Switzerland); indomethacin, vanadium chloride, Griess reagent, LOX enzyme, linoleic acid, ammonium hydroxide, disodium sulphide and other chemicals were purchased from Sigma-Aldrich (India), while Thiourea was from Sisco Research Laboratories (India). Kits were procured from Merck Millipore – USA and Cayman Bio Trend – Germany.

Plant collection and extraction

The aerial parts of *Pachygone ovata* were collected from Chammundi hills, Mysore, Karnataka, India in August 2012. Dr. G.R. Shivamurthi (former Professor, Department of Botany, University of Mysore, Mysore, India) botanically identified and authenticated the plant. An authenticated voucher specimen of the plant (UMDB/SU/Po-5) was deposited in the herbarium of Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore, India for future reference. The plant leaves were washed under running tap water and allowed to dry at room temperature. Further, 700 g of the dried leaves of *Pachygone ovata* was taken and crushed into a fine powder and extracted by exhaustive percolation following the method of Sultana et al. (2009) with slight modification using hexane (H), ethyl acetate (EA), methanol (MeOH) and water (Aq). The solvents were transferred to flash evaporator for complete evaporation resulting in dried hexane, ethyl acetate, methanol and aqueous extracts (Sultana et al. 2009).

In vitro anti-inflammatory potential

Lipoxygenase inhibition assay

The LOX assay was evaluated according to the method of Malterud and Rydland (2000), with slight modification. Serial dilutions of the extracts (MeOH, EA, H and Aq) were prepared in a 96-well microtitre plate over a range of 0.1–50 $\mu\text{g}/\text{mL}$. Briefly, 495 μL of phosphate borate (0.1 M, pH 9) was added to 5 μL of extracts and 500 μL of linoleic acid solution (0.15 mM in water).

The reaction was initiated by adding 495 μL of enzyme solution (0.28 U/mL of phosphate borate). Quercetin was used as a standard. Temporal pattern and enzyme activity was measured at 234 nm for 2 min with appropriate controls. Lipoxygenase inhibition was calculated from the following formula:

Inhibition (%) = $[(A-S)/A] \times 100$; where, A = absorbance of assay without extract and S = absorbance of assay with extract.

Partial alkaloid extraction

The methanol extract was further resuspended in hydrochloric acid (2 N) and washed with ethyl ether three times. The aqueous fraction was collected, alkalized using ammonium hydroxide and washed three times with ethyl ether. The ethereal fraction was allowed to dry, resulting in a purified alkaloid fraction (AF) (Silva et al. 2013).

The residue was dissolved in methanol, subjected to TLC using chloroform:methanol:acetic acid:water (6.25:2.77:0.830:0.138). The plates were observed under UV light (254 and 365 nm). Blue, blue-green and yellow fluorescent stains at 365 nm and pronounced quenching (blue-violet stain) at 254 nm are indicative of alkaloids (Wagner et al. 2006).

Estimation of alkaloids precipitable with Dragendorff's reagent

Spectrophotometric method was used to evaluate the amount of alkaloids precipitable with Dragendorff's reagent (Sreevidya & Mehrotra 2003). Based on this method the yellow bismuth complex that is formed in nitric acid medium with thiourea is in direct correlation with the amount of alkaloid present in the plant. Approximately 2 g crude extract was dissolved in ethanol and hydrochloric acid (1%) to give a final concentration of 60 mg/mL with a pH of 2–2.5. An aliquot of the extract solution (5 mL) was added to 2 mL of Dragendorff's reagent, the precipitate formed was centrifuged for 30 min at 2400 rpm. The residue was then washed with ethanol and treated with 2 mL of disodium sulphide solution. A brownish-black precipitate was formed which was again centrifuged and then dissolved in concentrated nitric acid (2 mL). This solution was made up to 10 mL using a standard flask with distilled water. This solution (1 mL) was taken and 5 mL thiourea solution (3%) was added and absorbance was measured at 435 nm against a blank containing nitric acid and thiourea. The amount of alkaloids present was calculated by the absorbance of the sample and the calibration curve. The quantification procedure indicated the percentage of alkaloids in the *Pachygone ovata* leaves to be 1.26%.

Acute toxicity

Acute toxicity was conducted by estimating the LD₅₀ which is the lethal dose that can kill 50% of the animal population in 24 h post treatment with the plant extract. Different concentrations (500, 1000, 1500 and 2000 mg/kg) were administered orally. The animals were then observed for 24 h for any behavioural changes or even death (Upmanyu et al. 2011). At the end of the study the animals were counted for the calculation of LD₅₀ according to the method of Karber (1931). Using the equation: $LD_{50} = LD_{100} - \Sigma(a \times b)/n$

n = total number of animal in a group.

a = the difference between two successive doses of administered extract/substance.

b = the average number of dead animals in two successive doses.

LD_{100} = Lethal dose causing the 100% death of all test animals.

In vivo anti-inflammatory potential

Carrageenan-induced paw oedema

The anti-inflammatory effect was evaluated by the carrageenan-induced paw oedema method in rats (Winter et al. 1962) with modification (Ferreira et al. 2013). Extract samples were diluted using sterile distilled water. Methanol extract (MeOH) of *Pachygone ovata* (100 and 300 mg/kg), AF (400 mg/kg) and indomethacin (10 mg/kg) were given to the animals by oral route once daily for one week before inducing paw oedema. Animals were divided into six groups each containing six animals. Indomethacin was used as a positive control, the other three groups were used for treatment with the extracts, one group was only treated with carrageenan and the control was only fed with water. The group treated with carrageenan was considered as the maximum of inflammation and the other groups were compared to this group. The paw edema was induced by injecting carrageenan (0.1 mL, 0.1%, in 0.9% saline w/v) into the sub plantar tissue of the right hind paw, after half an hour of the treatment with the extract and standard drug. By measuring the water displacement by plethysmometer the paw thickness was measured before carrageenan injection and every hour after the oedema induction for 4 h (1, 2, 3 and 4 h). Paw swelling was expressed as thickness variation (Δ) (Hoffmeister et al. 2011).

Blood sample collection

Animals were anesthetized and further sacrificed following the ethical guidelines and blood samples were collected by heart puncture. For leukocyte count blood was collected in EDTA blood sample tubes and for analysis of C-reactive protein (CRP), NO, AST and ALT the serum was separated from the blood samples collected in different tubes.

Histological analysis of paw tissue

Entire paw sections (5 mm) were removed from all groups of animals and immersed in 10% formalin at room temperature. After decalcification, each sample was embedded in paraffin wax, thereafter, using microtome, sections of 5 μ m were cut and mounted on a glass slide and dried over night at 37°C. Sections were cleared, hydrated and stained with hematoxylin and eosin for histological examination and observed under Axio Imager 2 (Zeiss, Oberkochen, Germany) microscope with 10 \times objective followed by photographs taken of the structural abnormalities using AxioCam MRc 5. Analyses of the figures were carried out by Adobe® Photoshop® (Adobe Systems). To avoid bias judgment the slides were labelled with numbers.

Measurement of blood cell count and CRP

The total and differential cell counts were determined with a haemocytometer. Plasma CRP level was measured using C-reactive protein ELISA Kit, Rat (Merck Millipore, Temecula, CA).

Quantification of NO concentrations in serum

To determine nitric oxide (NO) Griess method (Grisham et al. 1996) adapted by Vysakh et al. (2014) was used. In the presence

of H₂O, NO gets converted rapidly to nitrite (NO₂⁻) and nitrate (NO₃⁻). Serum samples (0.1 mL) were transferred to cuvettes and vanadium chloride 0.05 M (0.15 mL) in HCl 1.0 M (0.05 mL) was added which cause reduction of nitrate to nitrite. Immediately, Griess reagent [1% sulphanilamide and 1% *N*-[naphthyl] ethylenediamine dihydrochloride: 1:1] was added and after 45 min incubation at 37°C absorbance was read at 550 nm in an ELISA plate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentrations were estimated from a sodium nitrite (NaNO₂) standard curve.

Biochemical markers of toxicity

The serum collected from the carrageenan inflamed rats was subjected to analysis for detecting the different levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST); using standard procedures that are provided with the commercially available kits (Alanine Transaminase and aspartate aminotransferase Colorimetric Activity Assay Kit-Cayman chemicals, Ann Arbor, MI). ALT and AST were spectrophotometrically measured.

Formalin test

The antinociceptive effect was evaluated by the method previously described by Meunier et al. (1998) with slight modifications, by administering 50 μ L of 5% formalin subcutaneously into the left hind paw of the rat. The number of licking (times) was recorded from 0 to 5 min (phase 1, neurogenic) and from 20 to 40 min (phase 2, inflammatory) after the formalin injection. The control group was treated with normal saline; other groups were treated with the extract (100 and 300 mg/kg) and AF (400 mg/kg) by oral route administration 30 min before formalin injection. Aspirin was used as a positive control. The antinociceptive effect was evaluated based on the reduction in licking/biting mean time in comparison to the control group.

Statistical analysis

In vitro results are obtained by three independent experiments performed in triplicate and presented as mean \pm SD. *In vivo* experiments are presented as mean \pm S.E.M. and each group contained six rats. Statistical analysis was conducted using SPSS statistics software. The statistical significance between groups was compared using one-way analysis of variance (ANOVA) followed by Tukey test when appropriate. p Values lower than 0.05 ($p < 0.05$) were considered to be indicative of significance.

Results

Extraction yield

The average yield obtained by MeOH was (55 to 60 g) followed by EA (27 to 33.5 g), Aq (18.5 to 20.5 g) and H (10.8 to 17.4 g).

In vitro anti-inflammatory activity

LOX assay

The anti-inflammatory potential was evaluated using the inhibition percentage of lipoxigenase enzyme in correlation to their IC₅₀ values. The MeOH and EA fraction efficiently inhibited the lipoxigenase by 70.33 \pm 0.88% and 66 \pm 1.15%, respectively; while

H extract showed least inhibiting capacity ($17.9 \pm 1.1\%$ inhibition). Aq extract did not exhibit any inhibition and therefore is not included in the table. The IC_{50} values are listed Table 1 with MeOH extract having least value with an IC_{50} of 1.43 ± 1.19 ($\mu\text{g}/\text{mL}$) followed by EA ($2.15 \pm 0.9 \mu\text{g}/\text{mL}$) and H ($14.4 \pm 1.0 \mu\text{g}/\text{mL}$). Quercetin exhibited an IC_{50} of ($1.02 \pm 0.9 \mu\text{g}/\text{mL}$).

Partial alkaloid extraction

The different types of alkaloids present in the extract have been listed in Table 2. At a wavelength of 254 nm four and at 365 nm seven different types of alkaloids were separated based on their R_f values.

Acute toxicity

There were no behavioural changes or toxic symptoms observed 24 h post administration, implying their safety margin up to 1500 mg/kg. At 2000 mg/kg there was 100% death observed, therefore the LD_{50} was recorded to be 1500 mg/kg body weight.

In vivo anti-inflammatory potential

Carrageenan-induced paw oedema

Pretreatment with different doses of extract and AF considerably reduced the paw volume (μL) in the 3 and 4 h and almost neutralized the swelling as compared to the control group by the 4 h.

Table 1. Results of lipoxygenase inhibitory effect of crude *Pachygone ovata* extracts.

Samples	% of Inhibition ($\mu\text{g}/\text{ml}$)*	IC_{50} ($\mu\text{g}/\text{ml}$)
MeOH	70.33 ± 0.88^b	1.43 ± 1.19
EA	66 ± 1.15^a	2.15 ± 0.9
H	17.9 ± 1.1^b	14.4 ± 1.0
Quercetin	92 ± 1.07	1.02 ± 0.9

*Each value is represented as mean \pm SEM ($n=3$). He: Hexane extract, EA: Ethyl acetate extract, MeOH: Methanol extract. Values differ significantly at $^a p < 0.01$, $^b p < 0.05$ when compared with quercetin (control).

Table 2. Substances characterized by TLC.

Mobile phase	Wavelength	R_f
Chl: MeOH: AcOH: H ₂ O 6.25: 2.77: 0.830: 0.138	254 nm: blue-violet stain	0.47
		0.40
		0.20
		0.11
365 nm: yellow fluorescence	0.56	
	0.44	
	0.26	
Blue fluorescence	0.37	
	0.19	
	0.16	
Blue-Green fluorescence	0.12	

Chl: Chloroform; MeOH: Methanol; AcOH: Acetic acid and H₂O: Water.

Percentage of paw swelling from 1 to 4 h has been tabulated (Table 3). Figure 1 shows the maximum effect at the 4th h by MeOH extract (300 mg/kg) on the paw oedema reduction.

Histopathological analysis of paw tissue

The histopathological results from oedema paws are shown in Figure 2. The stratum corneum has remained intact and no cellular infiltration or oedema was observed in control groups (Figure 2(a)). In contrast, the carrageenan injected models showed absence of stratum corneum, excess polymorphonuclear (PMN) infiltration, septal panniculitis and oedema. The collagen fibres were degenerated and foreign-body granuloma (macrophages, lymphocytes, and neutrophils) accumulation was observed (Figure 2(b)). Administration with MeOH extract (300 mg/kg) and AF (400 mg/kg) significantly reduced the PMN infiltration and oedema. The stratum corneum was intact in models treated with MeOH extract (300 mg/kg) (Figure 2(e,d)). However, only slight improvements were observed in PMN infiltration and oedema using MeOH extract at 100 mg/kg body weights (Figure 2(c)). The reference drug indomethacin exhibited similar effects as that of MeOH extract at 300 mg/kg body weights (Figure 2(f)).

Measurement of CRP and leukocyte count

The concentration of CRP (Table 4) was increased significantly (70 ± 0.42 versus $48 \pm 0.56 \text{ mg}/\text{mL}$) in carrageenan-induced rats when compared to the control group (normal rats). However, treatment with MeOH extract (300 mg/kg) significantly reduced CRP concentration (55 ± 0.45 versus $70 \pm 0.42 \text{ mg}/\text{mL}$, $p < 0.05$) (Table 4).

Similarly, there was a notable increase in the leukocyte count (7.0 ± 0.07 versus $4.0 \pm 0.05 \text{ cells} \times 10^3$) in carrageenan induced rats compared to normal rats. However, treatment with MeOH extract (300 mg/kg) significantly reduced the leukocyte count (4.5 ± 0.05 versus $7.0 \pm 0.07 \text{ cells} \times 10^3$, $p < 0.05$) (Table 4).

Determination of NO concentrations in serum

It is clearly evident from the present study that an increase in *P. ovata* concentration caused a decrease in NO concentration. MeOH extract at 300 mg/kg reduced the NO concentration back to normal. Similarly AF reduced the NO levels to more than 50% of the nitrite accumulation (Table 4). Increasing the concentration did not have any significant effect on the NO concentration further.

Biochemical markers of toxicity

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are indicators of liver, muscle and kidney injury. In carrageenan-induced rats the ALT and AST increased to a small

Table 3. Percentage paw swelling in carrageenan inflamed rats at different time intervals.

Samples	1 h (%)	2 h (%)	3 h (%)	4 h (%)
Carrageenan	73.1 ± 133.3	78 ± 72.6	72.6 ± 57.7	69 ± 28.8
Indomethacin	51.4 ± 104^a	43 ± 66.6^b	27 ± 76.3^a	10.8 ± 5.7^a
MeOH extract (100 mg/kg)	56.4 ± 93.3^b	49 ± 88.1^b	36 ± 66.6^c	20.5 ± 20.8^a
MeOH extract (300 mg/kg)	52.7 ± 75^b	37.5 ± 50^b	14.3 ± 90^c	#
AF (400 mg/kg)	54.7 ± 120.1^b	46 ± 92.7^b	32 ± 83.3^c	12.5 ± 16^b

#Same size as control group. All samples are treated with carrageenan except positive control. Values differ significantly at $^a p < 0.01$, $^b p < 0.03$, $^c p < 0.05$ in each sample compared to carrageenan control at each specified hour.

extent of 117 ± 8.5 and 312 ± 15.6 U/L, respectively and were reverted back to normal using 300 mg/kg MeOH extract and AF. MeOH extract (100 mg/kg) did not have a significant effect on reduction of the ALT (112.6 ± 13) and AST levels (301 ± 12) (Table 4).

Formalin test

Two spontaneous behaviours (flinching/shaking and licking/biting) indicative of pain were recorded immediately after the subcutaneous injection of formalin in the right hind paw. In the control rats (formalin-treated rats), a subcutaneous injection of formalin resulted in biphasic flinching and licking of the injected paw. Oral administration of MeOH extract (100 and 300 mg/kg) 30 min prior to formalin injection significantly decreased the sum of flinching in MeOH extract (300 mg/kg), not in MeOH extract (100 mg/kg) in phase 1 as compared with the control group. In phase 2 both 100 and 300 mg/kg inhibited the licking/biting up to 20.7% and 42.3%, respectively, at 30 min. Similarly, AF showed potential reduction in the phase 1 flinching, however, in phase 2 its potency to reduce the licking/biting was almost similar to MeOH extract (100 mg/kg). Administration of aspirin did not have an effect on the flinching (phase 1), while it significantly reduced the licking/biting in the second phase. The inhibitory effect of MeOH extract at 300 mg/kg (87.7%) was slightly better compared with aspirin (83.3%) (Figure 3).

Discussion

The rapidly burgeoning research to find an anti-inflammatory drug with minimal side effects, better potential and devoid of dependence liability has inspired this research work.

Acute and chronic inflammations are complex processes that are inducible by a variety of means and involve many activated immune cells. In the present study LOX assay is used as an *in vitro* indicator of anti-inflammatory potential of *P. ovata* and carrageenan induced oedema is used to evaluate its effects *in vivo*. Controlling the LOX enzyme is of significance as it initiates the formation of pro-inflammatory leukotrienes (LTs) from

arachidonic acid. LTs have different biological effects amongst which leukocyte aggregation, smooth muscle contraction and vascular permeability are some of the few to name; that these biological effects have been seen in inflammatory disorders such as asthma, rheumatoid arthritis, inflammatory bowel disease and psoriasis (Vickers 1995). Additionally, 15-lipoxygenase is also of interest due to its possible role in the development of atherosclerosis (Henriques et al. 1987), therefore, keeping it in control is of utmost importance especially in the inflammation process. In the present work the inhibitory capacity amongst different extracts varied from 17.9 to 70.33, methanol extract having the highest inhibitory effect on LOX enzyme as expected and hexane extract having the lowest inhibitory effect. Since the *in vitro* studies has provided evidence of the methanol extract showing better activity, a semi fractionation was carried out to separate the alkaloids to give us a better insight of the effect of alkaloid components on the inflammation induced by carrageenan in rats. Quercetin is mainly used as a standard as it is a known flavonoid that is distributed widely in nature and its anti-inflammatory effects have been well established in various reports (Guardia et al. 2001; Hamalainen et al. 2007).

Carrageenan-induced inflammation is a well-established method for anti-inflammatory evaluation of drugs and is also known to cause biphasic acute inflammation (Henriques et al. 1987). The initial phase (0–1 h) has been attributed to the release of histamine, 5-hydroxytryptamine (serotonin), bradykinin, cytokines, as well as TNF- α and ILs (Di Rosa & Willoughby 1971; Dé ciga-Campos et al. 2007). While, the second phase (1–6 h) is accelerated by over production of prostaglandins and cyclo-oxygenase (COX-2) with local neutrophil infiltration and activation contributing alongside (Di Rosa & Willoughby 1971; Vinegar et al. 1971; Seibert et al. 1994; Dé ciga-Campos et al. 2007).

Amongst other important mediators produced is NO which is a potent vasodilator and increases vascular permeability which causes oedema through changes in the blood flow (Moncada et al. 1991). Reports suggest NO exerts multiple cytotoxic effects during inflammation including increase in prostaglandin (PG) production and formation of peroxynitrite (a potent oxidizing molecule) which is capable of evoking lipid peroxidation and thereafter

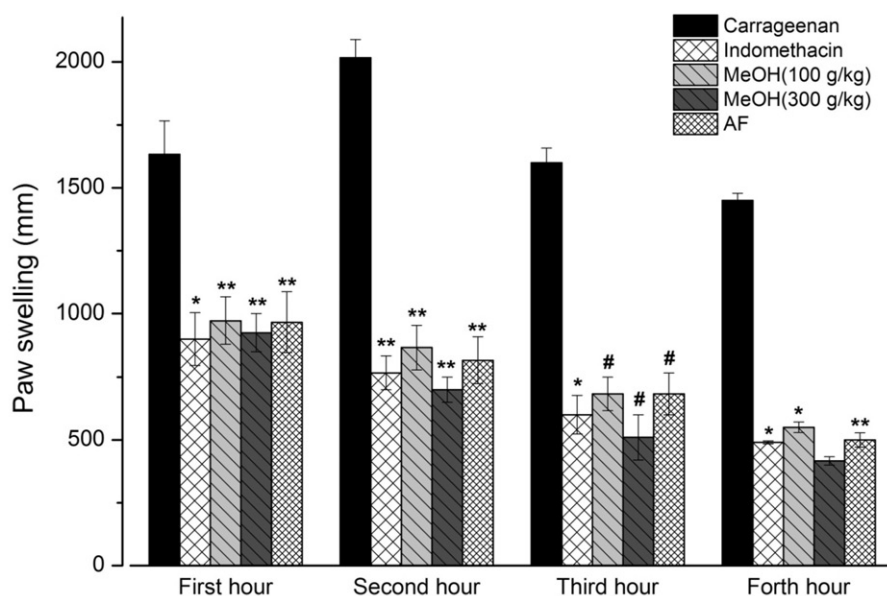


Figure 1. Carrageenan-induced rat paw oedema. Effect of MeOH extract (100 and 300 mg/kg), AF (400 mg/kg) and indomethacin on rat paw oedema induced by carrageenan. Results represent the mean \pm S.E.M. of percentage of variation ($n = 6$). Values differ significantly at * $p < 0.01$, ** $p < 0.03$, # $p < 0.05$ in each sample compared to carrageenan control at each specified hour. MeOH: methanol extract and AF: alkaloid fraction.

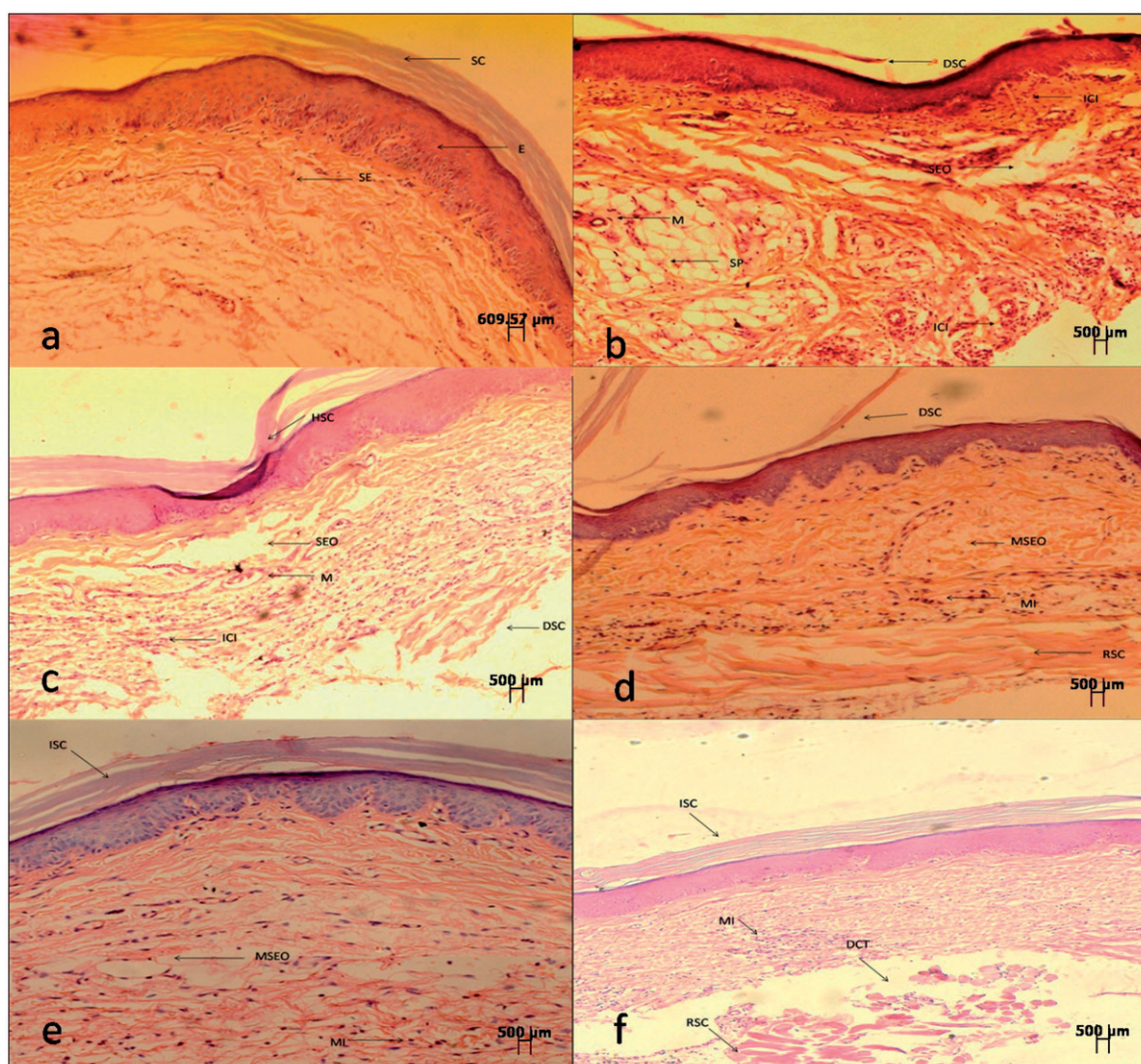


Figure 2. Histopathologic analysis of the effects of MeOH extract (100 and 300 mg/kg) and alkaloid fraction (AF) on cell infiltration in carrageenan injected paws. (a) Control group (b) Carrageenan injected group (c) MeOH extract (100 mg/kg) treated group (d) AF (400 mg/kg) treated group (e) MeOH extract (300 mg/kg) treated group (f) Indomethacin treated group. Cross sections of paws shows intact stratum corneum (ISC) in control, indomethacin and MeOH extract (300 mg/kg) treated groups, damaged stratum corneum (DSC) in carrageenan injected and AF-treated groups, hyper stratum corneum in groups treated with MeOH extract (100 mg/kg). In control group epidermal (E) and sub epidermal (SE) layer remain unchanged. In carrageenan injected group there is an influx of inflammatory cells infiltration (ICI), sub epidermal oedema (SEO), macrophages and septal paniculitis (SP). In MeOH extract (100 mg/kg) treated rats hyper stratum corneum (HSC) and damage of subcutaneous (DSC) layer was observed. In AF and MeOH extract (300 mg/kg) treated groups mild infiltration (MI), mild sub-epidermal oedema (MSEO) and restored subcutaneous (RSC) layer was observed. Damaged connective tissue (DCT) was seen in some places in indomethacin-treated paw tissues.

Table 4. Inhibitory effect of *Pachygone ovata* on the concentration of plasma CRP, leukocyte count, NO, ALT and AST activity.

Groups	CRP (mg/ml)	leukocytes (cells 10^3 /cells)	NO (M)	ALT (U/L)	AST (U/L)
Control	48 ± 0.56	4.0 ± 0.05	2.2 ± 0.14	93.4 ± 18	275 ± 8.2
Carrageenan	70 ± 0.42	7.0 ± 0.07	26.6 ± 0.8	117 ± 8.5	312 ± 15.6
MeOH (100 mg/kg)	63 ± 0.78 ^{a,b}	5.9 ± 0.02 ^{a,b}	19.6 ± 0.3 ^{a,b}	112.6 ± 13 ^{a,b}	301 ± 12 ^{a,b}
MeOH (300 mg/kg)	55 ± 0.45 ^{a,b}	4.5 ± 0.05 ^{a,b}	8 ± 0.57 ^{a,b}	98 ± 20 ^{a,b}	290 ± 17.2 ^{a,b}
AF (400 mg/kg)	58 ± 0.72 ^{a,b}	4.8 ± 0.05 ^{a,b}	14.3 ± 1.2 ^{a,b}	98.7 ± 10 ^{a,b}	293 ± 10.3 ^{a,b}
Indomethacin	53 ± 0.34 ^{a,b}	4.1 ± 0.02 ^{a,b}	11.6 ± 0.8 ^{a,b}	95 ± 4.3 ^{a,b}	285 ± 7.3 ^{a,b}

Values are expressed as average of 6 samples ± S.E.M in each group. MeOH: Methanol extract and AF: Alkaloid fraction.

^aValues differ statistically with control at $p < 0.05$.

^bStatistical difference with carrageenan induced rats at $p < 0.05$.

cellular damage, stimulating the recruitment of leukocytes, amplifying the inflammatory response (Beckman et al. 1990; Bhatia 2010). Another important inflammatory marker are the CRP, these were described as ‘acute-phase reactants’ by Avery and McCarty due to their increase in serum in patients with inflammatory stimuli. These CRP molecules bind to the pathogens and

initiate their destruction via the complement system (McCarty 1947; Chandrashekar 2014).

Our results suggest that MeOH extract and alkaloid fraction may owe its oedema inhibitory effect, at least in part, to the inhibition of some of the several mediators released during inflammation. Administration of carrageenan induced rats with

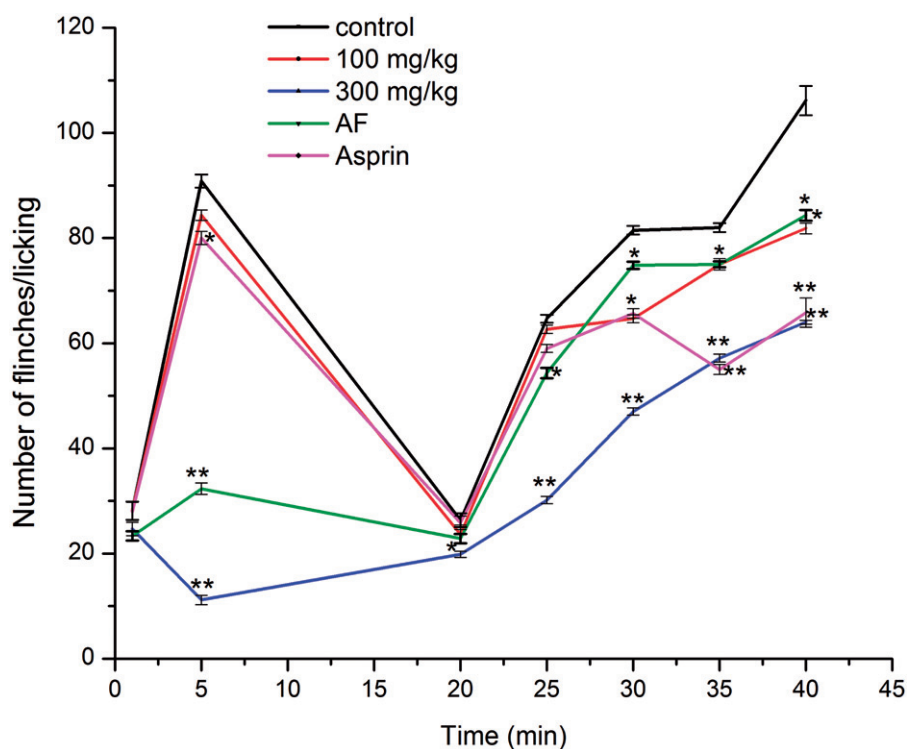


Figure 3. Effect of oral administration of MeOH extract (100 and 300 mg/kg), AF (400 mg/kg) and aspirin on the time course of the flinches/licking observed after the formalin injection. The number of flinches and licking/min is plotted versus time after formalin injection. Each line represents the group mean \pm S.E.M. ($n = 6$). Values differ statistically with control at * $p < 0.05$, ** $p < 0.1$ in each sample.

different concentrations of extract promoted a significant reduction in the oedema from the 1st h dose dependently. This suggests that the anti-edematogenic effect is related to the modulation of various enzymes and has various anti-inflammatory mechanisms of action mainly focusing on inhibiting the production of PGs and NO. To complement this, NO levels that had increased significantly at the 4h after carrageenan induction was notably reduced with using higher concentration of the extract. This confirms the effect of methanol extract on inhibiting the inflammation with respect to monocyte and neutrophil migration at the inflammatory site. Additionally, CRP and leukocyte count were increased with carrageenan administration pointing out the inflammation since they act as acute phase reactants, but there was a notable decrease observed upon administration with methanol extract and alkaloid fraction. This was comparable to that of indomethacin treated rats, which supports the inhibition of paw swelling in *P. ovata* treated rats.

Furthermore, chronic inflammation causes the disruption of the lysosomal membrane leading to leakage of lysosomal enzymes (ALT and AST) into the blood stream (Tanaka & Lizuka 1968). The slight increase in these biochemical markers were decreased by the methanol extract and alkaloid fraction, although, both were not as effective as the nonsteroidal anti-inflammatory drug used (indomethacin). But overall, the results from carrageenan-induced oedema demonstrated that methanol extract and alkaloid fraction were able to reduce the oedema suggesting their significance in acute inflammation.

In the model of formalin-induced pain, a biphasic behavioural change was observed in the rats such as flinching/shaking and licking/biting, the first being of neurogenic origin (Hunskar & Hole 1987) and the second being of inflammatory origin due to release of inflammatory mediators (PGs, NO, histamine) (Sulaiman et al. 2010). Our results showed that methanol extract at 100 mg/kg had no effect on the first phase while its nociceptive

effect was apparent in the second phase, but at 300 mg/kg there was an apparent inhibition of both phases (neurogenic and inflammatory). The alkaloid fraction followed a similar pattern; it notably inhibited the phase 1 and had a significant inhibitory effect on phase 2 as well. Although in the case of aspirin (positive control) only in phase 2 the licking/biting was notably reduced, this is in corroboration with previous studies that non-steroidal anti-inflammatory drugs (NSAID) are effective in the second phase of formalin test due to the inhibitory activity on PG synthesis in the modulation of pain (Hunskar & Hole 1987; Tsolen et al. 1992). It is well known that drugs such as morphine act primarily on the central nervous system and can therefore inhibit both phases equally (Shibata et al. 1989), from this we can understand that methanol extract of *P. ovata* at a concentration of 300 mg/kg body weights might have central antinociceptive effect and can exert its inhibitory effect peripherally as well as on the central nervous system. Since the alkaloid fraction followed a similar pattern, it can be hypothesized that this property of the methanol extract may be partially related to the alkaloid content present in it.

Therefore, from our studies it can be concluded that *P. ovata* can be used as an alternative NSAIDs with further investigation on individual effect of the components. This is the first report of *P. ovata* being studied for its pharmacological activity. It is evident from our study that pretreatment with methanol extract and alkaloid fraction can reduce swelling during inflammation owing this property to its inhibitory effect on NO and in turn on PGs, therefore, reducing cell infiltration and causing a decrease in oedema. The nociceptive effect of *P. ovata* also has been justified in the formalin induced pain model. The mechanism of anti-inflammatory and antinociceptive activities from *P. ovata* may be explained by the presence of alkaloids such as coclaurine, reticuline, norjuzophine, trilobine (Dasgupta et al. 1979; Bhat et al. 1980) and different components of the plant acting

simultaneously and amplifying the individual effect in a complementary manner. In our study the alkaloid fraction alone was not as significant in terms of anti-inflammatory as compared with the methanol extract; this could be due to the complex mixture of phytochemicals from the plant acting through a combination of additive and/or synergic interactions also indicating that the pharmacological effect of *P. ovata* is dependent on other phytochemicals apart from the alkaloids alone in it. Further line of studies is being undertaken to determine the various components and to analyze their effect individually on various *in vitro* and *in vivo* studies. To summarize, this work demonstrates that fractions from the leaves of *P. ovata* exhibit anti-inflammatory activity when given by oral route and even at higher concentrations did not possess any toxic effect on the rodents.


Acknowledgements

We express our gratitude to Institute of Excellence (IOE) for allowing us to use their facilities and to Dr. Ravi Shankar for his support and assistance throughout this work.

Disclosure statement

The authors report no declaration of interest.

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