ANTI-INFLAMMATORY AND ANTINOCICEPTIVE ACTIVITIES OF THE ETHANOLIC EXTRACT OF *PLUCHEA INDICA* (L) LESS LEAF

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Summary

Ethanolic extract of *Pluchea indica* leaf (PIL) was used to investigate its anti-inflammatory and antinociceptive activities by using carrageenan – induced oedema model and acetic acid induced writhing test. PIL exhibited significant and dose-dependent anti-inflammatory activity at a dose of 300 mg/kg when administered orally. It is also demonstrated that the i.p administration of PIL at a dose of 10, 30, 100 and 300 mg/kg produced significant inhibition of abdominal constriction induced with 0.6% (v/v) acetic acid in dose dependent manner. These results indicate that PIL exhibits significant anti-inflammatory and antinociceptive effects.

Key words: *Pluchea indica* , acetic acid induced writhing, carrageenan-induced oedema,

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Introduction

Pluchea indica (L.) Less is a wild plant that grows naturally in littoral areas of many countries. In Malaysia, herbal medicine practitioners claim that the leaves of this plant are capable of treating dysentery, rheumatism, leucorrhea, bad breath and body odour, and also boils and ulcers. The roots on the other hand are used to treat fever, lumbago, indigestion and headache (1).

The plant has been reported to possess hypoglycaemic as well as diuretic effects (2-4). The leaves are given internally to treat lumbago (5). The root of this plant has multifarious activities. It is proven that the roots can act as anti-depression and able to induce sleep. It also possesses hepatoprotective, anti-inflammatory and anti-ulcer activity (6-8). The roots are also effective as antidote for venom poisoning.

Recently, the root extract is reported to possess antioxidant effect and also antimicrobial property (9-11). In addition, the phytochemical examinations of this plant have indicated the presence of sterols, terpenes and lignan glycosides (11 – 15).However, no study has been done yet on efficacy of ethanolic leaf extract of *Pluchea indica* (L.) Less in inhibiting inflammation and pain stimulus. Therefore, aqueous ethanolic crude extract of *Pluchea indica (L.)* Less leaves were assessed for their anti-inflammatory and antinociceptive effects on mice and rats.

Methods

Preparation of Plant Extract

The leaves of *Pluchea indica* were collected from Kuantan, Pahang, Malaysia and identified by experts of the Herbarium of Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia and was retained as voucher specimen no KEP46462.

The leaves were cut into small pieces and dried at 60^0C for 3 days. The dried leaves (500 g) were then grounded using Wiley laboratory mill and macerated in cold aqueous ethanol (70% ethanol) for 48 hours. The extract was concentrated under reduced pressure in a rotary evaporator at 40^0C and the concentrates dried at room temperature to yield solid AML residues of approximately 5.1% w/w on dry weight basis.. The extract were dissolved in 5% aqueous ethanol solution at desired concentration (10, 30, 100 and 300 mg/kg) just before use and administered orally 30 minutes prior to the administration of inducers. Acetic acid, carrageenan and indomethacin (IND) were purchased from Sigma Chemical Co. (St Louis, Mo).

Animals and Experimental Design

Healthy *Sprague dawley* rats of either sex weighing between 170-250 g and adult Balb/c mice of either sex (20-30 g) were obtained from Animal Unit of Faculty of Medicine & Health Sciences, Universiti Putra Malaysia with ethics approval from the Animal Ethics

Committee of Universiti Putra Malaysia (00219). The animals were fed on standard laboratory diet and allowed free access to water.

Carrageenan – induced paw oedema

The anti-inflammatory property of PIL was evaluated using carrageenan-induced oedema on rat paw method, as described previously by Winter *et al* (16). The animals were pretreated orally with PIL (10, 30, 100 and 300 mg/kg). Negative control animals received a similar volume of 5% aqueous ethanol solution (oral) and positive control animals received indomethacin (IND; 10 mg/kg) intraperitoneally. After 30 minutes, 0.1 ml of 1% w/v suspension of carrageenan was injected subcutaneously onto the plantar surface of right hind paw to all the groups. Equal volume of saline was injected onto the plantar surface of the left hind paw. The volumes of both hind paws of each rat were measured using a Plethysmometer (Model 7140,Ugo Basile) at every half-hourly interval until the period of four hours after the injection of the carrageenan. For a consistent measurement, a line was marked just above the ankle joint of both rat's hind limbs. Hind paw swelling was measured when the paw was immersed at the line marked and was calculated as oedema percentage (17) according to the formula:

$$\% \text{ swelling} = \left[\frac{V_r - V_{r0}}{V_{r0}} - \frac{V_l - V_{l0}}{V_{l0}} \right] \times 100$$

V_r = Right Paw Volume
V_{r0}= Right paw initial volume
V_l= Left paw volume
V_{l0}= Left paw initial volume

Acetic acid induced writhing test

The method of Collier *et al* (18) was adopted with slight modification. Adult Balb/c albino mice weighing 20-30 g were used in this study. Animals were first pretreated with either control (5% ethanol) or the extracts; PIL (10, 30, 100 and 300 mg/kg) via peritoneum administration whilst for the standard drug, indomethacin was given intraperitoneally at 10 mg/kg. Extract were administered 30 minutes before the intraperitoneal injection of 0.15 ml/10 g body weight of 0.6% acetic acid to induce the typical stretching response. Control animals received similar volume of the vehicle. As described by Collier *et al.* (18) and Santos *et al*. (19), abdominal constriction known as writhing reflex was induced by 0.6% acetic acid was observed on the abdominal muscle together with a stretching of hind limb.

After induction, pairs of mice were placed in separate boxes and the writhings or stretchings per animal were counted for a period of 5 minutes under a double blind observation for the duration of 15 minutes. The antinociceptive effect was measured by calculating the mean reduction in the number of abdominal constriction for each extract as compared with the control group. The evaluation of antinociceptive activity was expressed as inhibition or reduction percentage of the number of total abdominal writhes (20).

Statistical analysis

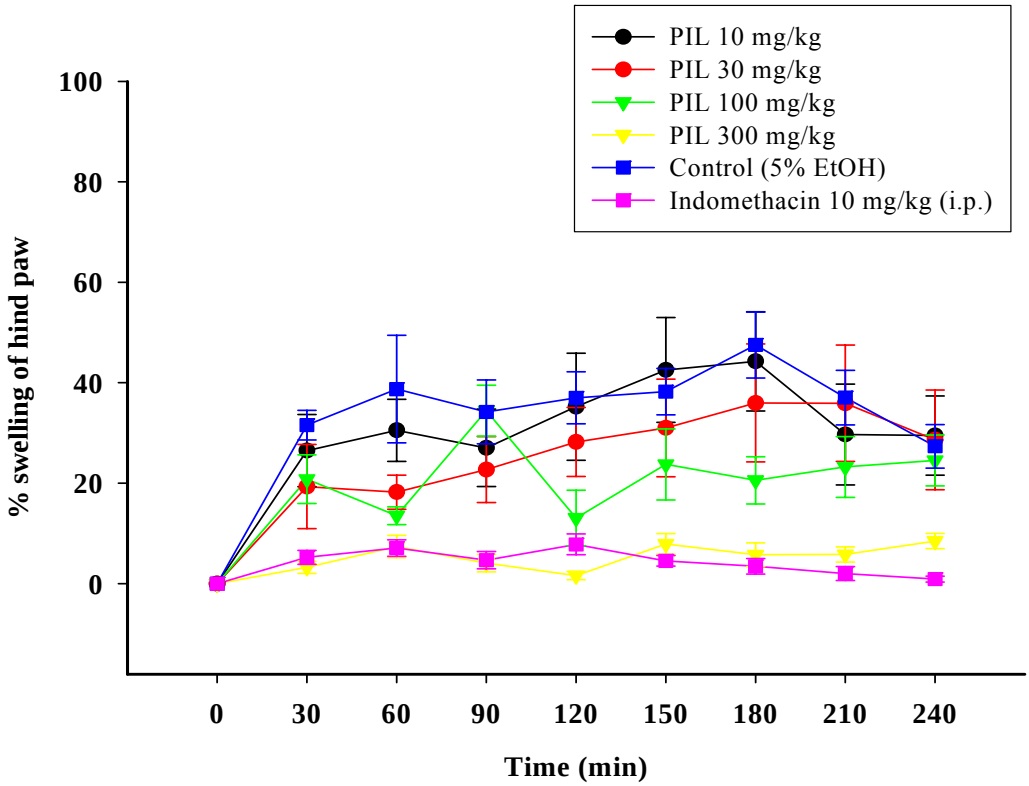
Data was expressed as mean ± S.E.M. The results of of the experiments were expressed as changes of percentage from control values. Data was analyzed by two-way analysis of variance (ANOVA), followed by Duncan's multiple comparison test for post-hoc comparison of group means. Student's *t*-test was used to compare between two groups For all tests, effects with probability of $p<0.05$ were considered significant.

Results

Carrageenan-induced paw oedema

The anti-inflammatory effect of PIL on carrageenan – induced paw oedema are summarized in Table 1. Optimum oedema volume in control group was achieved at 180 minutes as showed in Figure 1.Thus, the optimum percentage of inhibition of each PIL doses and 10 mg/kg indomethacin were calculated at 180 minutes as compared to the optimum oedema effect in control group as showed in Table 2. PIL at 300 mg/kg showed 82.9% inhibition of oedema as compared to 92.7% of oedema inhibition by indomethacin (10 mg/kg). However, PIL at 10, 30 and 100 mg/kg did not show any significant difference in anti-inflammatory response.

Figure 1: Carrageenan-induced rats' paw oedema and attenuating effect of different dose of PIL given orally and indomethacin (10 mg/kg) administered intraperitoneally. Data presented as mean ± S.E.M. (n=6 animals).



* $P<0.05$ indicated significant difference compared to the control group determined by *t*-test

Table 1: Anti-inflammatory effects of aqueous crude extract of *Pluchea indica (L.) Less* leaves (PIL) in carrageenan-induced rat paw oedema

Group (mg/kg)	% of oedema (Mean ± S.E.M.)								
	0 min	30 min	60 min	90 min	120 min	150 min	180 min	210 min	240 min
Control (5% ethanol)	0.00±0.00	31.57±2.95[a]	38.73±10.70[a]	34.19±6.35[a]	36.98±5.17[a]	38.21±4.62[a]	47.51±6.56[a]	37.04±5.43[a]	27.35±4.34[a]
PIL 10	0.00±0.00	26.47±7.18[a]	30.53±6.18[a]	27.04±7.70[a]	35.23±10.65[a]	42.55±10.42[a]	44.25±9.88[a]	29.70±10.05[a]	29.50±7.89[a]
PIL 30	0.00±0.00	19.32±8.39[b]	18.21±3.39*[a]	22.72±6.57[a]	28.20±6.88[a]	30.99±9.71[a]	35.98±11.76[a]	35.90±11.58[a]	28.63±9.92[a]
PIL 100	0.00±0.00	20.80±4.84[a]	13.53±1.76*[a]	34.44±5.05[a]	13.07±5.51*[b]	23.74±7.08[a]	20.57±4.70*[a]	23.25±6.05[a]	24.53±5.07[a]
PIL 300	0.00±0.00	3.27±1.21*[b]	7.41±2.22*[b]	4.09±1.73*[b]	1.61±0.82*[a]	7.91±2.07*[b]	5.75±2.37*[b]	5.83±1.52*[b]	8.48±1.52*[a]
Indomethacin (10)	0.00±0.00	5.26±1.38*	7.09±1.63*	4.67±1.71*	7.82±2.07*	4.54±1.06*	3.46±1.56*	2.00±1.38*	0.92±0.60*

N = 6, data = mean ± S.E.M.
* Significant (P< 0.05) when compared to the control (5% ethanol)
[a] Significant (P< 0.05) when compared to indomethacin
[b] Not significant (P< 0.05) when compared to indomethacin

Table 2: Percentage inhibition of carrageenan induced paw oedema at 180 minutes in rats on various doses of leaves extract of PIL given orally and indomethacin administered intraperitoneally as compare to optimum oedema induced by carrageenan at 180 minutes..

Group	% of oedema (mean ± S.E.M)	% inhibition of oedema (Obtained from average value)
Control (5% EtOH)	47.51 ± 6.56	0
PIL 10 mg/kg	44.25 ± 9.88	6.9
PIL 30 mg/kg	35.98 ± 11.76	24.3
PIL 100 mg/kg	20.57 ± 4.70	56.7
PIL 300 mg/kg	5.75 ± 2.37	87.9*
Indomethacin 10 mg/kg (i.p)	3.46 ± 1.56	92.7*

*$P<0.001$ indicate significant difference compare with control using ANOVA followed by Duncan Multiple Comparison Test

Abdominal writhing test

The antinociceptive effect of PIL extract (i.p) on the abdominal writhes of mice induced by 0.6% acetic acid is summarized in Table 3. Intraperitoneal administration of PIL extract had significantly inhibited the writhing effects in mice. Number of stretching response observed in 15 minutes for control group (5% ethanol) was 51.7 ± 4.6 counts. PIL at 10, 30, 100 and 300 mg/kg significantly reduced the writhings to 33.0 ± 3.9, 27.2 ± 7.7, 21.8 ± 3.7 and 20.3 ± 2.5 respectively.. Indomethacin (10 mg/kg), also given intraperitoneally had also significantly reduced the writhings to 19.0 ± 2.4 counts, which is almost comparable to PIL at 300 mg/kg (Table 3).

Table 3: Effect of PIL extract on acetic acid-induced writhing test in mice

Group	Mean of writhings (15 min) (mean ± S.E.M)	% inhibition
Control (5% EtOH)	51.7 ± 4.6	-
PIL 10 mg/kg	33.0 ± 3.9	36.2
PIL 30 mg/kg	27.2 ± 7.7	47.4*
PIL 100 mg/kg	21.8 ± 3.7	57.8*
PIL 300 mg/kg	20.3 ± 4.8	60.7*
Indomethacin 10 mg/kg	19.0 ± 2.4	63.2*

Values are mean ± S.E.M. *$P < 0.05$ significantly different from mean value control (ANOVA followed by Tukey's test)

Discussion

It has been documented that carrageenan-induced rat paw edema is a suitable *in vivo* model to predict the value of anti-inflammatory agents, which act by inhibiting the mediator of acute inflammation (21).This method was chosen for this study since oedema induced by carrageenan is the most prominent acute experimental model in search for new anti-inflammatory drugs (22). In addition, it is a method that has been frequently used to assess the anti-oedematous effect of natural products (23,24). Furthermore Mossa *et al* (25) found carrageenan-induced inflammatory model to be very useful in the search for oral anti-inflammatory drugs acting peripherally via inhibiting the mediator of acute inflammation.

The result of the anti-inflammatory test carried out on the crude extract of PIL showed that the ethanolic extract has significantly reduced the paw volume (edema) only at 300 mg/kg. This indicated possible anti-inflammatory activity of leaves part of the plant at higher dose. It is ubiquitously known that carrageenan-induced paw oedema involves many mediators which induce inflammatory reaction in two different phases (26). These two different phases have caused two peaks which can be clearly observed in the effects of the control group (Figure 1). The initial phase, which occurs between 0 and 2.5 hours after the injection of the phlogistic agent, has been attributed to the action of mediators such as histamine, serotonin and bradykinin on vascular permeability (27)(Maity *et al*., 1998). It has been reported that histamine and serotonin are mainly released during first 1.5 hours while bradykinin is released until 2.5 hours after carrageenan injection (28).

Histamine was one of the important inflammation mediators and it was a potent vasodilator substance and increases the vascular permeability (29). The oedema volume reaches its maximum approximately 3 hours post-treatment and then begins to decline. The late phase, which is also a complement-dependent reaction has been shown to be a result of overproduction of prostaglandins in tissues and may continue until 5 hours post-carrageenan injection (30). The second phase is correlated with the oxygen-derived free radicals and production of inducible cyclooxygenase besides elevated production of prostaglandin (31). It has been reported that the second phase of edema is sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agents (32,33). This can be observed in the positive control, indomethacin (10 mg/kg) where it has significantly reduced in the second phase of oedema (Figure 1).

According to the result of this study, the ethanol extract of *Pluchea indica L* was able to effectively inhibit oedema during the early and later phase of the inflammation at higher dose (300 mg/kg (Figure 1). Based on this observation and the biphasic nature of carrageenan-induced paw edema, it is possible to propose that the significant activity observed in the suppression of the first phase of inflammation may be due to the ability of the extract to inhibit the release and/or activity of the early mediators involved in carrageenan-induced paw oedema.

Oral administration of the ethanol extract of *Pluchea indica L* at 300 mg/kg suppressed the oedematous response 30 minutes after carrageenan injection and the effect continued up to 4 hours (Figure 1). The observed effect was similar to 10 mg/kg indomethacin, a well known NSAID, which is also a COX-1 inhibitor. In fact, the ethanol extract caused a statistically significant reduction at optimum oedema (90 minutes) at 300 mg/kg (Table 2,). The inhibitory effect was comparable in magnitude with the inhibition action of indomethacin. Based on the result obtained, it is likely that the mechanisms of action of the *Pluchea indica L* leaves at higher dose (300 mg/kg) are similar to that of non steroidal anti-inflammatory drugs, namely inhibition of prostaglandins biosynthesis. However, this can only be clarified by doing further study on determining its prostaglandin or COX contents in rat paw in the end of the experiment.

It is also well known that irritating compounds, may cause pseudo inhibition of oedema induced by carrageenan (34). However, studies have also indicated that such pseudo inhibition can only be caused by the local application of a counter irritant (34). In the present study, since extract was given orally, their activity could not be due to their counter irritant property.

In this study, we also utilized another model for pain on abdominal constriction response induced by acetic acid. Regarding this model, it is known that the intraperitoneal administration of agents that irritate serous membrane, such as acetic acid, provokes a stereotypical behavior in mice characterized by abdominal contractions, movements of the body as a whole, twisting of dorsoabdominal muscles, and a reduction in motor activity and coordination (35). Acetic acid causes analgesia by liberating endogenous substances and many other that excite pain at nerve ending (36,37).

According to Deraedt *et al* (38), the quantification of prostaglandins by radioimmunoassay in the peritoneal exudates of rats, obtained after intraperitoneal injection of acetic acid demonstrated high levels of prostaglandins $PGE_2\alpha$ and $PGF_2\alpha$ during 30 minutes after stimulus. The extract at 10, 30, 100 and 300 mg/kg administered intraperitoneally, significantly inhibited the acetic acid-induced writhing in mice (Table 3). These may be related to the level of prostaglandin. The results strongly suggested that the mechanism of action of the extract may be linked partly to lipoxygenases and/or cycloxygenases which are enzymes for prostaglandin synthesis. The effect of the extract is in dose-dependent manner. At 300 mg/kg, the extract produced 60.7% of inhibition and was comparable with the reference drugs used, indomethacin at 10 mg/kg (63.2%) (Table 3).

Nevertheless, it was found that the intraperitoneal administration of acetic acid induces the liberation not only of prostaglandin, but also the sympathetic nervous system mediators (39-41). Thus, the results obtained for the writhing test using acetic acid are similar to those obtained for the oedematogenic test using carrageenan, since PIL (10,30, 100 and 300 mg/kg) was effective in inhibiting the acetic acid induced writhing in mice. Therefore, an anti-inflammatory substance may also be involved in the peripheral antinociceptive activity.

According to Hosseinzadeh & Younesi (42), the antinociceptive activity of most plant extracts tested in the writhing test was not inhibited by naloxone. Therefore, these finding indicated that the extracts may not act via opiod reaction and may exert their activity via peripheral mechanism. Thus, the preliminary results of PIL extract also suggested that its antinociceptive activity might be via a peripheral mechanism.

Although, the abdominal constriction response induced by acetic acid is a very sensitive procedure that enable the detection of peripheral antinociceptive activity of compounds using animal protocols, but it is not a specific model. (43). This model involves different nociceptive mechanisms, such as sympathetic system (biogenic amines release), cyclooxygenase and their metabolites (39) and opoid mechanisms (18). Therefore, the writhing test may not be conclusive enough to determine the mechanism of action of antinociceptive effects of the extracts. On the other hand, it was reported that it was impossible to evaluate the duration of an analgesic as the frequency of cramps decrease spontaneously with time and the number cramps was subject to a great deal of variability (44).

In conclusion, PIL ethanolic extract possesses significant anti-inflammatory effect in carrageenan-induced paw oedema test at higher dose. On the other hand, it presents a peripheral antinociceptive effect in acetic acid-induced writhing test in dose dependent manner. As it was demonstrated in the present study that PIL ethanol extract possess both anti-inflammatory and antinociceptive responses, therefore, further studies should be done to elucidate the exact mechanism action underlying the effects of PIL. In this experiment, the exact nature of the active compound has yet to be determined. It is highly recommended that further studies should be carried out, especially, in identifying the

composition of the active compound itself, in order to help build a profile of its bioactive constituents.

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