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Anti-inflammatory and Anti-nociceptive activities of the ethanolic extract of *Annona muricata* leaf

A.H. Roslida*, C.E. Tay, A. Zuraini, Chan P.F.

Department of Biomedical Sciences, Faculty of Medicine & Health Sciences, University Putra Malaysia 43400 Serdang, Selangor, Malaysia

Abstract

Ethanolic extract of *Annona muricata* leaf (AML) was used to investigate its anti-inflammatory and antinociceptive activities by using carrageenan - induced oedema model and acetic acid induced writhing test. AML exhibited significant and dose-dependent anti-inflammatory activity at a dose of 10, 30, 100 and 300 mg/kg when administered orally. It is also demonstrated that the intraperitoneal administration of AML at a dose of 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 AML exhibits significant anti-inflammatory and antinociceptive effects.

Keywords: *Annona muricata*, acetic acid induced writhing, carrageenan-induced oedema.

1. Introduction

Annona muricata (Annonaceae) commonly known as soursop, is a typical tropical tree with heart shaped fruits and black seeds and widely distributed in most of tropical countries [1]. It has ethnobotanic uses as sedative, insecticidal, antiparasitic, antirheumatic, astringent and emetic [2]. The bark, leaves, and roots are considered sedative, antispasmodic, hypotensive, and nervine [3]. From *Annona muricata*, more than 80 acetogenins, mainly came from leaves, roots, and seeds, have been isolated. Acetogenins have been reported to possess anti-cancer activity and may also be responsible for other properties such as anti-parasitic and cytotoxic [4-5]. In the present study, we investigated the anti-inflammatory and analgesic effect of leaves extract of *Annona muricata* (AML) in carrageenan-induced oedema and acetic acid induced writhing tests.

2. Materials and methods

2.1. Plant Material

The leaves of *Annona muricata* were collected from Muar, Johor, Malaysia in November 2006

* Corresponding author
Email: roslida@medic.upm.edu.my

and identified by experts of the Herbarium of Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

2.2. Preparation of extracts and drugs

The leaves were cut into small pieces and dried at 60°C 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°C 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).

2.3. Experimental animals

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.

2.4. Carrageenan - induced paw oedema

The anti-inflammatory property of AML was evaluated using carrageenan-induced oedema on rat paw method, as described previously by Winter *et al* [6]. The animals were pretreated orally with AML (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

Table 1. Anti-inflammatory effects of ethanol extract of AML in carrageenan-induced paw oedema in rats.

Treatment with		Mean swelling in paw volume ± S.E.M. (%)							
(mg/kg)	0	30min	60min	90min	120min	150min	180min	210min	240min
5% EtOH	0	33.07 ± 2.45	37.33 ± 4.30	46.14 ± 5.80	39.32 ± 5.19	33.23 ± 4.49	41.61 ± 5.44	34.66 ± 5.16	32.22 ± 4.93
Indomethacin (10 mg/kg)	0	6.55 ± 1.81*	7.50 ± 1.37*	7.18 ± 1.36*	9.23 ±1.67*	6.30 ±1.15*	4.19 ±1.40*	3.35 ±1.63*	0.92 ±0.60*
AML Extract									
10 mg/kg	0	21.32 ±2.30*	24.73 ±3.75*	27.58 ±2.27*	20.43 ±2.78*	23.98 ±3.80	28.05 ±3.51	34.95 ±2.64	40.81 ±6.33
30 mg/kg	0	16.22 ±4.55*	15.41 ±4.23*	17.93 ±4.77*	26.78 ±5.76	32.47 ±5.67	30.29 ±5.52	28.41 ±6.36	20.90 ±5.46
100 mg/kg	0	7.37 ±1.47*	12.84 ±1.55*	17.42 ±2.10*	16.76 ±2.49*	17.93 ±4.30*	13.45 ±2.56*	10.66 ±1.48*	11.46 ±2.13*
300 mg/kg	0	5.12 ±1.31*	6.77 ±2.06*	9.55 ±2.06*	7.07 ±1.98*	7.86 ±1.79*	6.43 ±1.53*	6.07 ±1.77*	5.73 ±1.50*

Values are mean ± S.E.M. *$P < 0.05$ significantly different from mean value control (*t*-test)

Table 2. Percentage inhibition of carrageenan induced paw oedema at 90 minutes in rats on various doses of leaves extract of AML given orally and indomethacin administered intraperitoneally as compare to optimum oedema induced by carrageenan at 90 minutes.

Group	% of oedema (mean ± S.E.M)	% inhibition of oedema (Obtained from average value)
Control (5% EtOH)	46.14 ± 5.80	0
AML 10 mg/kg	27.58 ± 2.27	40.22*
AML 30 mg/kg	17.93 ± 4.77	61.14*
AML 100 mg/kg	17.42 ± 2.10	62.25*
AML 300 mg/kg	9.55 ± 2.06	79.30*
Indomethacin 10 mg/kg (i.p)	7.18 ± 1.36	84.44*

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

Table 3. Effect of AML extract on acetic acid-induced writhing test in mice

Group	Mean of writhings (60 min)(mean ± S.E.M)	% inhibition
Control (5% EtOH)	128.5 ± 10.2	-
AML 10 mg/kg	125.7 ± 14.9	2.2
AML 30 mg/kg	82.5 ± 6.4	35.8*
AML 100 mg/kg	65.8 ± 2.7	48.8*
AML 300 mg/kg	38.3 ± 4.8	70.2*
Indomethacin 10 mg/kg	46.7 ± 4.6	63.7*

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

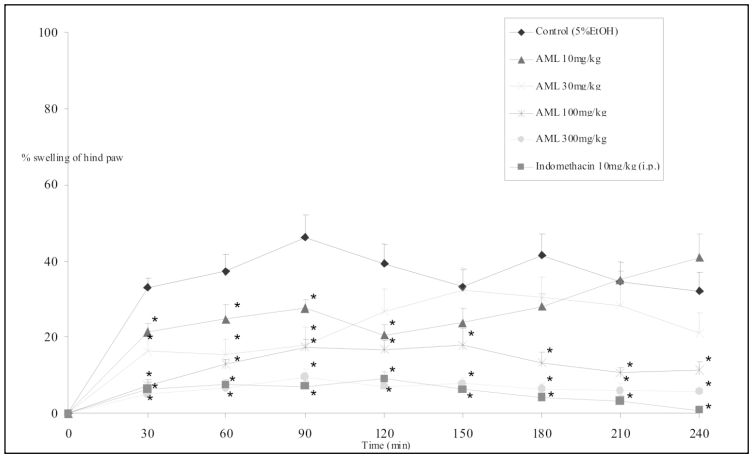

Figure 1. Carrageenan induced- paw oedema in rats and attenuating effects in different doses of AML and 5% EtOH orally and indomethacin 10 mg/kg administered intraperitoneally.
Data presented as mean ± S.E.M. (n = 6 animal). *P<0.05 compared with control group determined by t-test

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 according to the formula given as follows (7):

$$\% \text{ swelling} = \frac{Vr - Vr0}{Vr0} - \frac{Vl - Vl0}{Vl0} \times 100$$

Vr = Right Paw Volume

Vr0 = Right paw initial volume

Vl = Left paw volume

Vl0 = Left paw initial volume

2.5. Acetic acid induced writhing test

The method of Collier *et al* [8] 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; AML (10, 30, 100 and 300 mg/kg) via peritoneum administration while 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 previous reports [8-9], 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 60 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 [10].

2.6. Statistical analysis

The data for each experiment were expressed as the mean value ± S.E.M (standard error of mean) (n=6). Unless otherwise specified, differences between vehicle control and treatment groups were tested using one way Analysis of Variant (ANOVA) followed by Tukey's Test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Carrageenan-induced paw oedema

The anti-inflammatory effect of AML on carrageenan - induced paw oedema are summarized in Table 1. Optimum oedema volume in control group was achieved at 90 minutes. Thus, the optimum percentage of inhibition of each AML doses and 10 mg/kg indomethacin were calculated at 90 minutes as compared to the optimum oedema effect in control group as showed in Table 2. AML at 300 mg/kg showed 79.3% inhibition of oedema as compared to 84.4% of oedema inhibition by indomethacin (10 mg/kg).

AML leaves extract produced a dose-dependent anti-inflammatory effects showed in Table 1. Further statistical analysis (ANOVA followed by Tukey Comparison Test) showed that AML at 300 mg/kg and 10 mg/kg indomethacin are the homogenous subset where they produce the nearly same effect. However, AML at 10 mg/kg and 30 mg/kg did not show significant difference in anti-inflammatory response. The anti-inflammatory response has significant decrease when dose was increased to 100 mg/kg.

3.2. Abdominal writhing test

The antinociceptive effect of AML extract (i.p) on the abdominal writhes of mice induced by 0.6% acetic acid is summarized in Table 3. The i.p administration of AML extract at doses of 30, 100 and 300 mg/kg produced significant and dose dependent reduction in the number of abdominal writhes with 35.8%, 48.85 and 70.2% of inhibition respectively, as compared in relation to the respective control value. Indomethacin exerted a significant inhibitory effect, inducing an inhibition of 63.7% at a dose of 10 mg/kg. Therefore, the effect of AML at 300 mg/kg is comparable to indomethacin at 10 mg/kg.

4. 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 [11]. 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 [12]. In addition, it is a method that has been frequently used to assess the anti-oedematous effect of natural products [13-14]. Furthermore Mossa *et al* [15] 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 AML showed that the ethanol extract has significantly reduced the paw volume (edema) in every dose tested (10, 30, 100 and 300 mg/kg). This indicated possible anti-inflammatory activity of leaves part of the plant. It is ubiquitously known that carrageenan-induced paw oedema involves many mediators which induce inflammatory reaction in two different phases [16]. 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 [17]. 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 [18]. Histamine was one of the important inflammation mediators and it was a potent vasodilator substance and increases the vascular permeability [19]. The edema 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 [20]. The second phase is correlated with the oxygen-derived free radicals and production of inducible cyclooxygenase besides elevated production of prostaglandin [21]. It has been reported that the second phase of edema is sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agents [22-23]. 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 *Annona muricata L* was able to effectively inhibit oedema during the early phase of the inflammation at low and higher doses (10, 30, 100, and 300 mg/kg). However, the inhibitory effect also continue occurred later phase only at higher doses (100 and 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 edema.

Oral administration of the ethanol extract of *Annona muricata L* at 100 and 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 all doses tested (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 *Annona muricata L* leaves at higher doses (100 and 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 [24]. However, studies have also indicated that such pseudo inhibition can only be caused by the local application of a counter irritant [24]. 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 [25]. Acetic acid causes analgesia by liberating endogenous substances and many other that excite pain at nerve ending [26-27].

According to Deraedt *et al* [28], 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 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 68.75% of inhibition and was comparable with the reference drugs used, indomethacin at 10 mg/kg (67.71%) (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 [29-31]. Thus, the results obtained for the writhing test using acetic acid are similar to those obtained for the oedematogenic test using carrageenan, since AML (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 [32], 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 AML 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 [33]. This model involves different nociceptive mechanisms, such as sympathetic system (biogenic amines release), cyclooxygenase and their metabolites [29] and opoid mechanisms [8]. 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 [34].

In conclusion, AML ethanolic extract possesses significant anti-inflammatory effect in carrageenan-induced paw oedema test. The effect of the extract is dose-dependence. It was demonstrated in the present study that lower doses of extract cause inhibition of early phase of oedema induced by carrageenan. Higher dose of extract show inhibition in first and second phase of oedema. AML extract at higher dose may also be a potential COX inhibitor as it possessed the effect similar to indomethacin. Indeed, AML ethanol extract presents a peripheral antinociceptive effect in acetic acid-induced writhing test in dose dependent manner. As it was demonstrated in the present study that AML 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 AML. 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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