ANTI-INFLAMMATORY AND ANTI-PYRETIC EFFECTS OF HEXANE FRACTION OF *ARDISIA CRISPA* THUNB. D.C

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Summary

Hexane fraction of *Ardisia crispa* root (ACHE) was used to investigate its anti-inflammatory and anti-pyretic activities in this study. For anti-inflammatory activity, 12-O-tetradecanoylphorbol-13-acetate (TPA) was applied to ear of mice to induce oedema and treated with 0.5,1 and 2mg/ear of ACHE topically. In cotton-pellet granuloma test, treated groups have received 3, 10, 30 and 100mg/kg of hexane extract administered orally for 7 days. For antipyretic activity, brewer's yeast was injected in mice to induce fever and later, ACHE at dose ranging from 10 to 300 mg/kg were administered to the rats orally. The results exhibited that 1 and 2mg/ear of ACHE produced significant suppression by 19.9% and 20.2% respectively. the lowest dose of ACHE showed no significant effect when compared with control. Results showed that ACHE showed significant anti-pyretic effect at all doses (10, 30, 100 and 300 mg/kg).   At 30, 100 and 300mg/kg, ACHE even exhibited higher efficacy when compared with 100 mg/kg acetaminophen. ACHE also elicited a significant ($P<0.05$) inhibition of granuloma tissue and exudate formation. Thus, it can be concluded that *Ardisia crispa* posseses anti-inflammatory and antipyretic effects

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Introduction

The root and leaves part of *Ardisia crispa* are commonly used in folklore medicine (1). The plant *Ardisia crispa* Thunb. D.C belongs to the family Myrsinaceae and it is widely distributed in Asia stretching from Japan and the Himalayas to Java and the Philipines . It can be found in the undergrowth and jungle fringes, dappled shades and shady edges in Malaysia (2).

Its root is reported to be used as one of the traditional ingredient in post-natal syndromes where the root is boiled and the boiled concoction is used to treat pain in the throat and chest as well as to treat rheumatism. The mixture of its leaves and root is used as skin liniment (3). The root juice is useful for treating earache, cough, fever, diarrhea and also for women after-birth. In Canton, it has been marketed as "sin-lo-san", a herbal decoction drunk for sprains and broken bones. In Thailand, the root will be mixed with other plants to wash "dirty blood' or in women with dysmenorhea (menstrual pain) (4).

It has been established by the villagers among the South East Asian countries and China that they consumed the root and leaves part of *Ardisia crispa* to reduce pain and swellings (5). Roslida and Kim (6) has reported on its anti-inflammatory and anti-hyperalgesic activity on the hexane fraction of the root extract of this plant. Therefore, by using different model, we have decided to study other potential anti-inflammatory activities including anti-pyretic effect of the root extract of *Ardisia crispa.*

Methods

Preparation of Plant Extract

The root of *Ardisia crispa* were collected from Machang, Kelantan, Malaysia was deposited as a voucher specimen (no: 20841) in the herbarium of Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia.

The roots were cut into small pieces and dried at 60^0C for 3 days. The dried root and leaves were then grounded using Wiley laboratory mill. Grounded dried plant materials were macerated in cold aqueous ethanol (70% ethanol) for 48 hours. The extract was concentrated under reduced pressure in a rotary evaporator. The crude aqueous ethanol extract was then fractionated successively with n-hexane, dichloromethane and methanol. The solvents were removed under reduced pressure in a rotary evaporator at 40^0C and the concentrates dried at room temperature to yield solid residues; hexane fraction (14.1% w/w), dichloromethane fraction (7.62% w/w) and methanol fraction (57.40% w/w). The hexane fraction of *Ardisia crispa* is labelled as ACHE.

Animals and Experimental Design

Healthy *Sprague dawley* rats of either sex weighing between 170-250 g and adult ICR strain 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 (UPM/FPSK/PADS/BR.UUH/00267). The animals were fed on standard laboratory diet and allowed free access to water.

Brewer's yeast - induced pyrexia

The anti-pyretic property of ACHE was evaluated by slightly modifying the method described by Fadeyi *et al* (7). Male Balb-c mice were fasted overnight with water ad libitum before the experiments. Pyrexia was induced by subcutaneously injecting 20% (w/v) brewer's yeast suspension (20 ml/kg) into the animals' dorsum region. Eighteen hours after the injection, the rectal temperature of each mouse was measured using a digital thermometer. Only mice that showed an increase in rectal temperature of at least 0.5°C were used for the experiments. Vehicle (saline), ACHE (10, 30,100 and 300 mg/kg) or acetaminophen (100 mg/kg) was administered orally and temperature was measured at 30, 60.90, 120,150 and 180 minutes after drug administration.

TPA – induced ear oedema

Anti-inflammatory activity of ACHE was evaluated using a modification of the methods of Gschwendt *et* al (8) and Hirota *et al* (9). A solution of 2.5 µg 12-O-tetradecanoylphorbol-13-acetate (TPA) in 25 µl of acetone was topically applied to groups of BALB-c male mice (25-30g) on both the inner and outer surface of the right and the left ear. Topical administration of ACHE (0.5, 1 and 2mg/ right ear) and indomethacin (0.5 mg/right ear) dissolved in acetone was performed 30 min after TPA treatment. The other ear which acted as a control was applied with acetone, the sample vehicle. In all groups, the oedema was allowed to develop for 6 h, afterwards the animals were sacrificed and plugs (diameter of 7 mm) of the central portion were taken from both ears and weighed. The swellings induced by TPA was assessed in terms of the increase in the weight of the right ear punch biopsy over that of left ear. The inhibitory effects (IE%) of ACHE and indomethacin were then calculated as the ratio of the weight increase of the ear sections, according to the following formula:

% Inhibitory effect (IE) = $\dfrac{L - R}{L - C} \times 100$

$$= \frac{L - R}{L - (L/2.41^*)} \times 100$$

where L = weight of left ear which is treated with TPA only
R = weight of right ear which is treated with TPA plus tested extract
C = calculated weight of untreated ear
(* treating with 0.5 µg TPA resulted in a 2.41 times increase in weight of the ear)

Cotton pellet-induced granuloma

In this study, the effects of ACHE and piroxicam on the proliferation phase of inflammation were investigated by cotton pellet model (9). Cotton pellets weighing 3.0 ± 1.0 mg were sterilized. Under anaesthesia, the pellets were introduced subcutaneously through a skin incision at the sternum level of rats. The administration (p.o) of vehicle (5% Tween 80), 200 mg/kg of piroxicam (as a positive control) and 3, 10, 30 and 100 mg/kg of ACHE was followed 30 min after cotton pellet implantation. ACHE is administered once daily for next 7 days. On day 8, animals were killed by overdose of ether. The pellets were dissected out, free of tissue attachments and dry in the oven overnight at 60^0C. The dry pellets were weighed and mean weight of granuloma tissue formed around each pellet was determined. The level of inhibition of granuloma tissue development is calculated

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.

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Results

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Brewer's yeast induced pyrexia

The anti-pyretic effects of ACHE on brewer's yeast induced pyrexia are summarized in Table 1. ACHE significantly suppressed the pyrexia induced by yeast in mice at all doses (10, 30, 100 and 300 mg/kg) at 30, 60, 90, 120 and 180 min after oral administration. At 30, 100 and 300mg/kg, ACHE even exhibited higher efficacy when compared with 100 mg/kg acetaminophen. These three dosages of ACHE reduced temperature effectively ($p < 0.001$) after 30 minutes of treatment. Besides that, rectal temperature was maintained below 36 ºC. The lowest dose of ACHE ie 10 mg/kg exhibited antipyretic activity comparable to acetaminophen..

TPA- induced ear oedema

The anti-inflammatory effects of ACHE on TPA-induced ear oedema are summarized in Table 2. ACHE significantly suppressed oedema ($p < 0.001$) at 1 and 2 .0 mg/ear with reduction swelling of 19.9% and 20.2% respectively. However at lowest concentration ie 0.5 mg, ACHE did not significantly suppress the oedema when compared with other treated groups. At 1 mg/ear, percentage of oedema inhibition is comparable to that of indomethacin.

Cotton pellet-induced granuloma

ACHE also elicited a significant ($P < 0.05$) inhibition of granuloma tissue and exudate formation in dose dependent manner as tabulated in Table 3. At doses of 3, 30 and 100 mg/kg, ACHE significantly reduced the pellets from 46.53 ± 2.66 mg to 36.97 ± 2.42 , 35.75 ± 2.92 and 34.38 ± 4.46 mg respectively. In terms of oedema reduction, the granuloma tissue formation was reduced to 19.7%, 23.2% and 26.1% respectively. At 100 mg/kg, ACHE exhibited the effect which was near to that of 200 mg/kg piroxicam.

Table 1: Effect of ACHE on yeast induced pyrexia in mice

Group	Dose(mg/kg)	Rectal temperature (ºC)±S.E.M						
		After 18 hrs	30min	60min	90min	120min	150min	180min
Control	-	38.79±0.03	37.74±0.21	37.89±0.23	38.60±0.21	38.39±0.46	38.36±0.56	38.34±0.53
acetaminophen	100	38.61±0.13**	37.17±0.13**	37.06±0.18**	37.34±0.26**	38.00±0.35*	38.27±0.31	38.04±0.31*
ACHE	10	38.44±0.12**	38.01±0.13**	37.31±0.34**	37.54±0.42**	37.7±0.37**	38.04±0.37*	38.01±0.32*
ACHE	30	38.36±0.16**	37.49±0.27**	35.50±0.58**	35.10±0.76**	35.16±0.77**	35.50±0.78**	35.43±0.68**
ACHE	100	38.76±0.16	37.56±0.33*	34.31±0.40**	32.93±0.37**	32.50±0.42**	32.54±0.48**	32.29±0.29**
ACHE	300	38.69±0.14**	36.66±0.25**	35.56±0.39**	34.89±0.25**	34.16±0.14**	33.91±0.11**	32.00±0**

*$p<0.05$, **$p<0.001$ compared with control (ANOVA followed by Tukey test). Each value represents the mean ± S.E.M. n=6 animals

Table 2: Effects of the topical application of indomethacin and ACHE on TPA-induced ear oedema model in mice

Group	Dose(mg/ear))	Ear swelling (mg)	Swelling reduction (%)
Control	-	-1.74 ± 0.000785	
Indomethacin	0.5	2.54 ± 0.000876	13.0*
ACHE	0.5	2.83 ± 0.000876	12.7
ACHE	1	4.61 ± 0.00111	19.9**
ACHE	2	4.50 ± 0.00164	20.2**

Results are means ± S.E.M (n=6 animals).
*$p<0.05$., **$p<0.001$ compared with vehicle control

Table 3: Effect of the hexane extracts of *Ardisia. crispa* on Cotton-pellet granuloma test in rat

Group	Dose(mg/kg)	Granuloma tissue(mg) mean $\pm$ S.E.M	Inhibition(%)
Control	-	46.53 $\pm$ 2.66	
Piroxicam	200	28.88 $\pm$ 2.49**	37.9
ACHE	3	36.97 $\pm$ 2.42*	19.7
ACHE	10	36.83 $\pm$ 2.11	20.9
ACHE	30	35.75 $\pm$ 2.92*	23.2
ACHE	100	34.38 $\pm$ 4.46*	26.1

*$p<0.05$, **$p<0.001$ compared with control (ANOVA followed by Tukey test). Each value represents the mean $\pm$ s.e.m.

Discussion

The results demonstrate that hexane fraction (ACHE) obtained from the crude ethanolic extract of *Ardisia crispa* root exhibited anti-pyretic and anti-inflammatory activities in experimental animal models. Priorly, Roslida and Kim (6) has reported on its acute anti-inflammatory activity on carrageenan-induced oedema model and anti-hyperalgesic activity on carrageenan-induced – hyperalgesia by using Plantar test method. Nevertheless, none has reported on its anti-pyretic as well as chronic anti-inflammatory activity yet.

ACHE significantly reduced the pyrexia induced by brewer's yeast in mice. The reference drug, acetaminophen also suppressed the pyrexia. It is currently accepted that prostaglandin E2 (PGE2) is the final fever mediator in the brain, specifically in the preoptic area of the anterior hypothalamus (10), thus it maybe plausible to conclude that ACHE inhibits the synthesis of prostaglandins. However it must be noted that several biochemical events occur leading ultimately to the synthesis of PGE2.

Fever is believed to result from a finely tuned, complex event that involves both the peripheral immune system and the brain, through which a series of inflammatory and metabolic processes are regulated (11-12). It is established that there are two pathways leading to the transcription and induction of cyclooxygenase (COX)-2, the rate limiting enzyme for PGE2 synthesis (11). Both pathways are activated by by cytokines eg IL-1α, IL-6 and tumor necrosis factor (TNF) which trigger central mechanisms that act via transcription factors such NFκ B and signal transducer and activator of transcription (11). It may therefore be worthwhile to investigate the exact point in the biochemical events where the extract exerts its anti-pyretic effect.

In addition to its anti-pyretic properties, ACHE also showed anti-inflammatory effect on an acute inflammatory process like in TPA-induced ear oedema in mice. The TPA induced oedema test is a screening method to evaluate the ability of test extracts or compounds to prevent an inflammatory reaction in response to the edemogen. It is known that phorbol esters, such as TPA induce skin inflammation and a hyperproliferative response with an infiltration of neutrophils (13).

The pronounced inflammation induced by TPA when applied topically to the mouse ear is thought to be mediated by protein kinase C and the stimulation of phospholipase A2 which results in the release of arachidonic acid and prostaglandins (14). Protein kinase C is family of specific protein kinases which play a role in a range of signal transduction processes. Although the mechanism by which TPA causes inflammation is not completely clear, it seems to be related in part to the release of eicosanoid mediators. Inhibitors of prostaglandin and leukotriene biosynthesis have been shown to be active in the TPA model and it has therefore been used extensively as a compound screen for this class of compounds (15). However, according to Puignero and Queralt (16), TPA-induced local inflammation is more effectively controlled with COX-inhibitors than LOX inhibitors. This actually proved the effectiveness of indomethacin, a COX inhibitor in reducing the mice's ear oedema.

In the present experiment, it was shown that topical application of ACHE at doses of 1 and 2 mg/ear were active in reducing the oedema, 1.5 times higher than indomethacin, the well known cyclooxygenase inhibitor used in this assay as a reference. This is a valid model to screen either effective extracts or compounds for potential topical anti-inflammatory therapy. A single application of TPA induces oxidative stress, cutaneous inflammation and epidermal hyperplasia due to enhance keratinocyte proliferation. TPA induces TNF-a production and the formation of LTB4 with a resultant increase in vascular permeability and neutrophil influx (17).

Another experiment has been done in investigating the effects of ACHE and piroxicam on chronic phase of inflammation by using cotton pellet granuloma model. ACHE also showed significant anti-inflammatory effect in cotton pellet – induced granuloma test. At 100 mg/kg, ACHE decreased the weight of cotton pellets inserted under the skin 26.1% compared with control group (Table 3). It is known that the inflammatory granuloma is a typical response of a chronic inflammatory process, and it has been established that the dry weight of the pellets is well correlated with granulomatous tissue (18). Weight of cotton pellets were also reduced in piroxicam group (37.9%) compared with the control group. The decreasing weight of cotton pellets at the end of the experiments indicated that both ACHE and piroxicam exhibited its antiproliferative effect.

Chronic inflammation is the reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes a proliferation of

fibroblasts and infiltration of neutrophils with exudation of fluid. It occurs by means of development of proliferative cells which can either spread or form granuloma. Efficacy of anti-inflammatory agents in chronic inflammatory states is indicated by their ability to inhibit the increase in the number of fibroblasts during granular tissue formation (19)

Monocyte infiltration and fibroblast proliferation rather than neutrophil infiltration and exudation take place in chronic inflammation (20-21). This proliferation becomes widespread by proliferation of small vessels or granuloma (21). During the repair process of inflammation, there is proliferation of macrophages, neutrophils, fibroblasts and multiplication of small blood vessels, which are the basic sources of forming a highly vascularized reddish mass, termed granulomatous tissue (22).

Non-steroidal anti-inflammatory drugs (NSAIDs) decrease the size of granuloma (cotton pellet), which results from cellular reaction by inhibiting granulocyte inflammation preventing generation of collagen fibers and suppression mucopolysaccarides (23-24). Thus, it is possible that ACHE also inhibits monocyte infiltration and fibroblast proliferation as it has showed significant anti-inflammatory effect in this particular model.. This fact is related with the capability of the extract to act in the proliferative events of granulation tissue formation (25). It also reflected its efficacy to a high extent to reduce an increase in the number of fibroblasts and synthesis of collagen and mucopolysaccarides which are natural proliferative events of granulation tissue formation (26)

In conclusion, ACHE possesses significant anti-pyretic effect throughout the duration of period of three hours. In addition, it can also act as topical inflammatory agent as it significantly reduce the ear oedema induced with TPA in mice. Possibly, triterpenoid as reported in previous study (6) is the bioactive compounds in ACHE responsible in inhibiting this activity. Several reports of the literature has shown the effectiveness of triterpenoid in TPA-induced ear oedema model (27-28). This result may also justify use of *Ardisia crispa* in folk medicine as topical anti-inflammatory agent, although further pharmacological research is necessary to fully understand its mechanism of action. On the other hand, it also presents anti-proliferative effect in chronic inflammation model of cotton pellet induced granuloma in dose dependent manner. Therefore, further study viz biochemical analysis should also be conducted to elucidate and confirm the exact mechanism underlying the effects of ACHE. .

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