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RESEARCH ARTICLE

Anti-inflammatory, Analgesic, Antinociceptive and Antipyretic Investigation of Bioactive Constituents from Partial Purified Dichloromethane Crude Extracts from Leaves of *Labisia Pumila*

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ABSTRACT

Labisia pumila, LP (Myrsinaceae), is a popular herb among the women in Malaysia known locally as ‘‘Kacip Fatimah. It has been claimed by the traditional medicinal practitioner as a pain reliever, treat inflammatory disorders, such as rheumatism and fever. This study was aimed to investigate the partially purified crude extracts of *Labisia pumila* (DELP) *for* its anti-inflammatory, antinociceptive and antipyretic effects. Acetic acid-induced abdominal writhing and carrageenan-induced paw oedema was used to evaluate the anti-inflammatory activity whereas hot plate test and formalin test was used for the antinocicpetive activity. Antipyretic activity was determined by yeast induced pyrexia method. Partial purification of DELP extract resulted in 5 different fractions (A-E). All the fractions of DELP showed significant anti-inflammatory, antinociceptive and antipyretic activity in the entire test used. Phytochemical screening revealed the presence of flavonoids, steroids, saponin, alkaloids and tannins. HPLC analysis revealed the presence of hesperidin. The present study confirmed that the extracts exhibited anti-inflammatory, antinociceptive and antipyretic activity, supporting its folk use and is in consonances with our earlier report.

KEYWORDS

Anti-Inflammation, Antinociception, Antipyretic, Analgesia, *Labisia Pumila*, Phytochemical

INTRODUCTION

For many generations, the indigenous people of the Malay Archipelago in Malaysia have used *Labisia pumila* as herbal medicine to induce and facilitate childbirth and eases delivery, as a post-partum medication to help contract the birth channel, to regain body strength, regulate menstrual cycle and avoid painful or difficult menstruation, inflammatory disorders such as rheumatism and as energy drink as well as other medical usage.[1,2] Since it is believed that current synthetic drugs including drugs such as opiates and NSAIDs are not useful in all cases, because of their side effects and potency, research into medicinal plants have been carried out to develop new drugs and it has continued to play a vital role in the progression of drug discovery in this modern world.[3] Previous study showed that the DELP *exhibited* anti-inflammatory, anti-nociceptive, antipyretic effects,[4] anti-histaminergic and anticholinergic effect.[5] In this study, the partial purification and anti-

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inflammatory, anti-nociceptive and anti-pyretic effects of DELP was investigated.

MATERIALS AND METHOD

Plant Material

The leaves of *Labisia pumila* (1kg) were freshly collected during the month of December 2012 from Sungai Perak Malaysia. The plant was identified by Dr. Shamsul Khamis, a plant taxonomist from the laboratory of Natural Products (NATPRO), Institute of Bioscience (IBS) University Putra Malaysia. The specimen voucher number IBS/UPM/LP 143 was assigned and deposited in the herbarium of laboratory of Natural Products (NATPRO), Institute of Bioscience (IBS) University Putra Malaysia.

Preparation of Extracts

The leaves were air-dried for almost 3 weeks and were then grounded into fine powder using a miller. Extraction of powdered leaf (1kg) with dichloromethane (5L) was carried out by successive maceration at room temperature for a week followed by filtration. The filtration process was repeated several times to make sure all the dirt and dust are completely removed. The filtrate obtained was then concentrated using rotary evaporator at temperatures of 35^{o}C until dryness this process was repeated a few times to obtain enough DCM crude extract (13.7g) of LP (DELP). Silica gel column chromatography was employed in this study to partially purify DELP into fraction A to fraction E.[5,6]

Animals

Healthy young adult Sprague-Dawley rats of both sexes weighing 200 g purchased from Institute of Medical Research Kuala Lumpur (IMR) were used in this study. The rats were screened and housed in standard polypropylene cages (three rats per cages), maintained under standard laboratory conditions (*i.e.* 12:12 hour light and dark cycle; at an ambient temperature of 25 ± 5°C; 50-70 % of relative humidity); the animals were fed with standard rat pellet diet and water was made available at all times. The ethical procedure of UCSI was approved and followed in this research.

Acetic Acid-Induced Abdominal Writhing

Seven (7) groups of rats (n-5) were selected for the present study. Group one (1) received saline (1ml/kg) (control) and group two (2) received standard drug aspirin (100mg/kg). The remaining five groups (3-7) received (100mg/kg) of their respective plant extracts. Sixty (60) minutes later 0.6% acetic acid (10ml/kg body weight) was injected intraperitoneally. Immediately after the acetic acid injection, each rat was placed in a transparent observation cage and the number of writhes per rat was counted for 30 mins. The writhing activity consists of a contraction of the abdominal muscles together with a stretching of the hind limbs. The percentage inhibition was calculated using the following formula: (control mean – treated mean) × 100/control mean.[7,8]

Carrageenan-Induced Paw Oedema

Carrageenan-induced paw inflammation was induced according to the method described by [9]. One (1) hour after oral administration of the extracts (100mg/kg), reference drug (indomethacin, 10mg/kg) and saline (1ml/kg) as negative control, an injection of 0.1mL of carrageenan (1% carrageenan suspended in 0.9% NaCl) was made into the right hind limb of each rat under the sub-plantar aponeuvrosis. The paw volume was measured before and after carrageenan injection at 1 hr interval for 5 hr using plethysmometor. The inhibitory activity was calculated according to the following formula, Arul et al.[8]

$$\%\text{Inhibition} = 100 - \frac{\text{oedema volume in treated}}{\text{oedema volume in control}} \times 100$$

Hot Plate Test

In this method, a 24 cm diameter glass cylinder was placed on a hot plate with temperature set at 55 ± 0.5 °C. Latency of the rats was determined before and after the treatment. The latency was recorded at the time before and 15, 30, 45, and 60 minutes after intraperitoneal administration of the extracts or drugs. Each rat was placed on the hot plate in order to obtained the animals response to heat-induce antinociceptive pain stimulus. Response was defined as licking, or

biting of the paw, or jumping out of hot the plate. Time taken for each response was noted and recorded in seconds. A latency period of 30s was fixed as the cut off time to prevent tissue damage to the rats. Seven group of rats (n=5) received fraction A-E (100mg/kg), 0.9% saline (1ml/kg) and morphine (10mg/kg) each.

Formalin Test

The method used was similar to what has been described.[10] To induce nociception, rat were injected with 50 µl of 2.5% formalin in 0.9 % of saline solution into the sub plantar surface of the left hind paw, 1 hour after the administration of 0.9 % saline, 100mg/kg of Aspirin and 100mg/kg of each extracts. Rats were then observed for 30 minutes and the time spent licking the paw was recorded in two phases. The data were express as total licking time in the early phase (0-5 min) and the late phase (15-30min) after formalin injection.

Antipyretic Activity

Yeast induced pyrexia was used to evaluate the antipyretic activity of the extract. The rats were divided into seven (7) groups of five rats (n-5) each. Before the induction of fever a thermometer probe was inserted 3-4cm deep into the rectum, to record the basal rectal temperature. After the basal temperature has been taken, the animals were subcutaneously injected with 4ml/kg of 15% w/v brewer's yeast suspension to induce fever (pyrexia). 24 hours post yeast administration, rat's rectal temperature was recorded. Only rats with elevated rectal temperature of at least 0.5^{o}C were selected for further study. Immediately after rectal temperature measurement, rats were administered their respective designated treatments. Briefly, Group 1 received normal saline 4ml/kg (negative control), Group 2 received paracetamol 100mg/kg (positive control), Group 3-7 was treated with partially purified fractions A-E from the leaves of *Labisia pumila* respectively. After investigational drug administration, Physitemp's Thermalert TH-5 thermometer was used to measure rectal temperature at every 1 hour for 4 hours.

Statistical Analysis

The data obtained were expressed as the mean ± standard deviation. Experiment groups were compared using one way and two way analysis of variance (ANOVA test) followed by the student t-test.

Phytochemical Analysis

Simple colorimetric biochemical test were performed to identify the possible bioactive fractions present in the fraction of the *LP*. The bioactive fractions that were tested for are flavonoids, alkaloids, tannins, steroids and saponins.[11,12]

RESULTS AND DISCUSSION

Pain, swelling (oedema) and fever (pyrexia) are the signs and symptoms of both acute and chronic inflammation and stress. Acetylsalicylic acid (ASA) and other non-steroidal anti-inflammatory drugs (NSAID) are prominent agents used in treating these symptoms. The result presented in these studies showed that the partially purified fraction A, B, C, D, E at 100 mg/kg from the leaves of *L. pumila* possesses anti-inflammatory, analgesic, antinociceptive and antipyretic effect.

The results of the current study showed that oral administration of Fraction A, B, C, D, E at 100 mg/kg showed a highly significant (p<0.05) reduction in the number of abdominal constriction when compared to the negative control (Table 1). The acetic acid-induced abdominal constriction method is widely used for investigating compounds with anti-inflammatory activity and its use has been reported many times.[9,13,14] Administration of acetic acid (AA) is commonly employed to evaluate visceral pain since AA directly activates visceral and somatic nociceptors innervating the peritoneum and induces inflammation not only in sub diaphragmatic visceral organs, but also in subcutaneous muscle walls. AA causes damage and releases pain-producing substances including prostaglandins that activate nociceptors on the terminals of the sensory nerve fibers. Such painful stimuli caused by AA reach higher centers by a number

of spinal nerve pathways.[15] The writhing in the rats abdomen are due to an increase in the peritoneal fluid levels of prostaglandin (PGE_2 and PGF_2), serotonin and histamine which lead to a dilation of the capillary vessels and the increase in vascular permeability.[16,17,18] Based on the results, the inhibiting effect of the extract on acetic acid-induced writhing in the rat models, maybe due to the inhibition of the inflammatory mediators such as PGs (PGE_2 and PGI_2), serotonin and histamine and this results is in line with the earlier report.[4]

The oral administration fraction A, B, C, D, E at 100 mg/kg significantly (p<0.001) reduce the carrageenan-induced paw oedema observed at the fifth hour. From the results obtained, fraction A-E showed a highly significant anti-inflammatory activity when compared to the negative control, saline (100 l/kg) (Table 2). The presence of oedema is one prime sign of inflammation. Carrageenan-induced paw oedema is a test, which has significant predictive value for anti-inflammatory agents, acting by inhibiting the mediators of acute inflammation.[19,20,21] Oedema formation as a result of carrageenan induction is the biphasic event. It is comprised of early, exudative, and late stage of inflammation. Immediately after carrageenan injection, there is a sudden increase of paw volume that is attributed to the release of histamine and serotonin.

The second phase of oedema is due to the release and over secretion of bradykinin, prostaglandins and kinins in paw tissue, which accompanies leukocyte migration.[20,21] The result of this study revealed that all the fractions of *Labisia pumila* significantly (P<0.001) reduced the first and second phase of carrageenan – induced paw oedema in the experimental rat model in concentration dependent manner. The significant activity observed as reduction of carrageenan-induced paw oedema during the first and second phase maybe attributed to inhibition of release of early mediators such as histamine, serotonin and kinins. Additionally, the effects of the second phase may also be due to inhibition of cyclooxygenase, prostaglandin derivative and this effect is in line with the previous report.[4]

The administration of Fraction A, B, C, D, E intraperitoneally at 100 mg/kg exhibited significant increase in the hot plate reaction time.

The results showed that the fraction A-E showed a highly significant (p<0.001) antinociceptive activity (MPE) when compared with the negative control (saline, 100ml/kg) (Table 5). The MPE was measured at 45 minutes. The standard hot-plate test, a central analgesic activity testing model, measures two behavioral components including paw licking and jumping which are both considered to be supraspinally integrated responses.[6]

This model usually employed morphine as a reference drug, it demonstrated potent analgesic effects in this model indicating the sensitivity of this test. Rats show clear and quantifiable behavior when tested on hot plate, the strength of a drug against nociceptive pain were indicated by increase of reaction time or response latency of the rat.[6] The effect observed by the extracts may be as a result of its ability to deactivate the C-fibers in the central located dorsal horn or inhibition of the release of prostaglandins and glutamate and neuropeptides (especially substance P) from the primary afferent neuron or both.

Fraction A, B, C, D, E given orally at 100 mg/kg inhibited both the early and late phases in the formalin test. The results showed that the partially purified fraction A-E has a potent antinociceptive (p<0.001) effect against the chemical stimuli provoked by the formalin sub plantar injection when compared with the negative control (Table 4). Formalin injection into the rat hind paws results in biphasic pain-related behaviors (such as licking, flinching and biting of the injured paw) that seem to involve two distinct mechanisms. The first phase (acute pain) appears immediately following formalin injection lasting only few minutes and is believed to be driven by primary afferent nociceptor activity.

Table 1: Effects of fraction A-E of *L. pumila* leaf extract and aspirin on acetic acid induced writhing

Group	Dose (mg/kg)	Number of Writhings	Inhibition (%)
Control	-	117 ± 2.1	-
Aspirin	100	17.8 ± 1.5*	84.9
Fraction A	100	42.6 ± 1.8*	63.8
Fraction B	100	36.4 ± 1.7*	69.0
Fraction C	100	32.0 ± 1.6*	72.8
Fraction D	100	32.8 ± 4.4*	73.4
Fraction E	100	26.0 ± 2.9*	77.9

Table 2: Effects of fraction A-E of L. pumila leaf extract and indomethacin on carrageenan induced-paw oedema

Group	Dose (mg/kg)	% Inhibition				
		1h	2h	3h	4h	5h
Control	-	-	-	-	-	-
Indomethacin	10	44.1	41.3	49.4	49.7	51.4
Fraction A	100	37.2	30.0	35.1	44.4	51.4
Fraction B	100	42.1	40.7	40.5	45.1	37.9
Fraction C	100	21.4	20.7	24.4	21.6	17.6
Fraction D	100	36.6	37.3	39.9	39.9	43.6
Fraction E	100	29.0	31.3	30.4	41.2	50.7

Table 3: Effect of *L. pumila* extracts on the latency time of rat submitted to the hot plate test and Maximum Possible Effects (MPE) % versus treatment

Treatment	Dose	Time (min) (MPE %0				
		0	15	30	45	60
(Saline)		3.45 ± 0.07	3.63 ± 0.21	3.62 ± 0.25	3.59 ± 0.23	3.57 ± 0.23
(Morphine)	10	5.37 ± 0.49**(7.23)	6.58 ± 0.31***(11.19)	7.62 ± 0.38***(15.16)	9.06 ± 0.47***(20.71)	5.59 ± 0.32**(7.64)
Fraction A	100	3.97 ± 0.24**(1.96)	4.37 ± 0.14**(2.81)	4.55 ± 0.17**(3.53)	4.86 ± 0.07***(4.81)	3.81 ± 0.16(0.91)
Fraction B	100	4.05 ± 0.19***(2.26)	6.86 ± 1.41***(12.25)	8.58 ± 0.75***(18.80)	7.60 ± 1.08***(15.18)	4.02 ± 0.22* (1.70)
Fraction C	100	4.68 ± 0.40**(4.63)	6.73 ± 0.52**(11.76)	8.47 ± 0.28***(18.39)	9.80 ± 0.28***(23.51)	4.89 ± 0.63**(4.99)
Fraction D	100	4.52 ± 0.63*(4.03)	5.97 ± 0.34***(8.87)	7.71 ± 0.24***(15.50)	6.02 ± 0.14***(9.20)	3.63 ± 0.11 (0.23)
Fraction E	100	4.31 ± 0.20***(3.24)	6.17 ± 0.11***(9.63)	7.49 ± 0.08***(14.67)	6.16 ± 0.09***(9.73)	4.78 ± 0.15***(4.58)

All Values given were expressed I mean = SD, N = 5rats. Asterisks indicated significant difference from control. *P<0.05, **P<0.01, ***P<0.001 (ANOVA followed by paired t-test).

Table 4: Effect of formalin test on rats and its percentage of inhibition

Group	Dose (mg/kg)	Number of licking			
		Early Phase (0-5min)	% inhibition	Late phase	% inhibition (15-30)
Saline	-	44 ± 3.16	0.00	58 ± 5.29	0.00
Aspirin	100	23 ± 2.41***	47.73	21 ± 1.00***	63.79
Fraction A	100	21 ± 3.58***	52.27	20 ± 2.70***	65.52
Fraction B	100	31 ± 4.09**	29.55	20 ± 3.96***	65.52
Fraction C	100	40 ± 3.21*	9.09	60 ± 4.82	0.00
Fraction D	100	31 ± 5.26**	29.55	69 ± 6.80	0.00
Fraction E	100	37 ± 2.74*	15.91	66 ± 2.88	0.00

All Values given were expressed I mean = SD, N = 5rats. Asterisks indicated significant difference from control. *P<0.05, **P<0.01, ***P<0.001 (ANOVA followed by paired t-test)

Table 5: Effects of DCM extract and the eluted fractions (A, B, C, D and E) of L. pumila leaves on yeast-induced pyrexia

Group	Dose	BT	YIP	1	2	3	4
Saline	-	36.16 ±0.34	37.54 ± 0.21	36.94 ± 0.18	37.78 ± 0.28	38.98 ± 0.38	38.98 ± 0.26
Paracetamol	100	35.46 ± 0.83	37.10 ± 0.33	36.58 ± 0.36 (1.4%)	36.20 ± 0.55(2.43 %)	35.80 ± 0.59(3.50%)	35.36 ± 0.61 (4.69%)
Fraction A	100	36.54 ± 0.49	35.54 ± 0.49	37.36 ± 0.39 (4.1%)	35.84 ± 0.91(4.55 %)	35.66 ± 0.87(5.19%)	34.68 ± 1.04 (7.17%)
Fraction B	100	36.32 ± 0.36	36.82 ± 0.36	37.66 ± 0.30 (-2.3%)	37.70 ± 0.31(-2.39%)	38.08 ± 0.42 (-3.42%)	38.22 ± 0.34(-3.80%)
Fraction C	100	36.68 ± 0.31	37.46 ± 0.06	37.58 ± 0.08 (-0.33%)	37.72 ± 0.08(-0.69%)	37.80 ± 0.12(-0.91%)	37.90 ± 0.12(-1.17%)
Fraction D	100	37.06 ± 0.39	37.78 ± 0.40	37.92 ± 0.31 (-0.37%)	37.94 ± 0.31(-0.42%)	38.12 ± 0.30(-0.89%)	38.30 ± 0.31(-1.38%)
Fraction E	100	36.96 ± 0.18	37.86 ± 0.23	37.82 ± 0.21 (0.11%)	37.70 ± 0.14(0.42 %)	37.62 ± 0.19(0.63%)	37.40 ± 0.07(1.22 %)

All Values given were expressed I mean = SD, N = 5rats. Asterisks indicated significant difference from control. *P<0.05, **P<0.01, ***P<0.001 (ANOVA followed by paired t-test).

The second phase (tonic pain) is observed 15 min after formalin injection, lasts at least for 60 min, and is thought to arise from nociceptive spinal neuron hyperactivity. In this second phase various mediators operate in a sequence to produce an inflammatory response and have been correlated with the elevated production of prostaglandin (PG), induction of cyclooxygenase (COX) and release of nitric oxide (NO).[22,23]

The results of the antipyretic activity of the compounds are presented in (Table 5). Administration of the yeast to the rats produced significant increase in rectal temperature 1 h after yeast injection. Yeast induced pyrexia is preferred animal model because it is simple, inexpensive and easy to standardize.[14,24] Yeast cause infection and damage to tissue and cells by release of cytokines such as interleukin-1 and 6 (IL-1 and IL-6), and tumor necrosis factor (TNF). Interleukin-1 migrates to the hypothalamus to activate the arachidonic pathway. Cyclooxygenase enzymes (COX-1 and COX-2) metabolize the free arachidonic acid to initiate the synthesis of prostaglandins PGE_2, the concentration of which is elevated in experimental rats with pyrexia.[25,26,27] Current studies shows that only fraction A and fraction E displays antipyretic activity in the yeast induce pyrexia model of rats over the period of 1-5 hours after the yeast injection. The possible effect of the extracts might be as a result of the interference with interleukin-1 which is responsible for pyrexia by activating signal transduction that resets thermoregulatory centre in the hypothalamus.[27] Also, the extracts may be competitive antagonists of COX enzyme, which is responsible for the generation of prostaglandins and thromboxanes.[28,29]

Preliminary phytochemicals screening of the *Labisia pumila* extracts indicated the presence of flavonoids, tannins, saponin, alkaloids and steroids (Table 6). The biological effect of the extract may be due to the presence of phytochemicals such as flavonoids, saponins, steroids, alkaloids and tannins. Flavonoids have been reported to have anti-inflammatory activity.[30,31] Saponins are well known for anti-inflammatory and cell stabilizing activity. It inhibits the formation of cyclooxygenase metabolites; prostaglandins and thromboxanes and the putative antipholgistic activity of saponins was ascribed to the inhibition of arachidonic acid metabolism.[32]

Table 6: The analysis of phytochemical in the sub fractions of *L. pumila*

Phytochemicals	A	B	C	D	E
Flavonoids	-	-	+	++	-
Tannins	-	+	++	++	-
Alkaloids	++	+	+	+	+
Saponins	++	++	++	++	++
Steroids	++	+	-	-	-

+ = weakly present; ++ = strongly present; - = absent

Another mechanism of action behind the anti-inflammatory effects of saponins is due to inhibition of histamine, bradykinin and serotonin along with its antioxidant effects, which in turn inhibits the formation of reactive oxygen species having important role in inflammation.[33] Tannins have been reported to inhibit COX enzyme, decrease vascular permeability and antioxidant activity. Steroids possess a very strong anti-inflammatory activity by inhibiting cytokine release IL-1, 2 and 6; migration of leucocytes, and induction of lipocortin.[34,35,36] Besides tannins, purified alkaloids from plants provide many pharmacologically active agents, including leading chemotherapy drugs and some of the well-known drugs in the market today such as atropine and codeine[37] Although all the fractions of LP showed effect on the anti-inflammatory, antinociceptive and anti-pyretic activity, fraction A showed the highest effect.

CONCLUSION

The administration of partially purified fraction A-E extract produced a significant anti-inflammatory, analgesic, antinociceptive and antipyretic effect, fraction A showed the highest effect except for hotplate test where fraction C

was the highest. These effects maybe as a result of the phytochemicals present in the extract which have been previously reported to have similar effect. Further study is required to identify the actual bioactive component responsible for the activities and to elucidate the mechanism action of the extracts.

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