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Antinociceptive activity of the aqueous leaves of *Pongamia pinnata* in rats

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Abstract: In Sri Lankan traditional medicine, the decoction of leaves of the plant *Pongamia pinnata* (L.) is claimed to possess antinociceptive activity. However, as yet, its antinociceptive potential has not been scientifically investigated. The aim of this study was to investigate the antinociceptive potential of an aqueous leaf extraction (ALE) of *P. pinnata* in rats, using three algesiometric methods (hot-plate, tail-flick and formalin tests) and different doses (500, 1000, 1500 and 3000 mg/kg, given orally). The result showed that the ALE possesses marked and significant ($p<0.05$) antinociceptive action when evaluated in hot-plate test and formalin tests (only the highest dose tested) but not in the tail-flick test. The antinociceptive action of ALE had a quick onset (within 1h) and a moderately long duration of action (up to 6h). The antinociceptive action of ALE was blocked by metoclopramide (D_2 type dopamine receptor antagonist) and atropine (a muscarinic receptor antagonist) but not by nalaxone (an opioid receptor antagonist). Moreover, the ALE has no sedative (as judged by hole board test) activity. ALE contained a wide range of chemical constituents of which alkaloids, flavonoids, steroids, and polyphenols which may have contributed to antinociceptive action of ALE. Collectively, these observations suggest that ALE-induced antinociception was mediated centrally, at supraspinal level, and peripherally. The antinociceptive action is likely to be mediated via dopaminergic and cholinoganic muscarinic mechanisms. The results also showed that ALE is effective against neurogenic and inflammatory pains. In conclusion, this study show, for the first time, that ALE of *P. pinnata* possesses moderately strong antinociceptive activity, justifying its therapetic claim in traditional medicine as a pain killer.

Key word: *Antinociceptive activity, dopaminergic mechanisms, muscarinic mechanisms, pain impairment, Pongamiapinnata, toxicology*

I. INTRODUCTION

Pongamia pinnata L. (Family: Leguminosae), *Indian beech* in English, *Pongam* in Tamil and *Magulkaranda* in Sinhala is a large tree with a soft, grey bark and slightly querulous buds. The leaves are compound, large, rachis about 12.5 cm long, glabrous, leaflets 5-9, each 7.5-12.5 cm long on thick stalks, oval, acute at base, acuminate, glabrous and shining on both sides, thin, bright green. The flowers are irregular, bisexual, greenish pink or white with calyx purplish brown, 1.5 cm long, pedicel rather long, slender, swollen at base, articulated often in pairs, racemes often two together, elongated, about equaling the leaves. This plant occurs in India, Sri Lanka, Malaya, Polynesia, Australia, Philippine Islands and Sri Lanka. It is common in the low-country in Sri Lanka on banks of streams and rivers, especially, near the coast area [1].

P. pinnata has been used as a medicinal plant, particularly in Ayurvedha and Siddha systems of Indian medicine. All parts of the plant have been used as a crude drug for the treatment of tumors, piles, skin diseases, itches, abscess, painful rheumatic joints, wounds, ulcers or diarrhoea. Besides, it is well known for its application as animal fodder, green manure and fish poison. It has also been recognized to possess applications in agriculture and environmental management, with insecticidal and nematicidal activity. More recently, the effectiveness of P. *pinnata* as a source of biomedicines has been reported, specifically, as antimicrobial and therapeutic agents [2,3]. The decoction of this plant is claimed to be effective as a diuretic in Sri Lankan traditional medicine[4]. Albeit, most recent animal study, using rats and aqueous leaf extract has failed to validate this claim [5].

Ayurvedic literature of India, different parts of this plant have been used for treating various inflammatory conditions. A hot infusion of leaves is used as a medicated bath for relieving rheumatic pains. Experimentally different extracts of roots (ethanol, petroleum ether and n-butanol) of P. *pinnata* have been shown to have analgesic activity [6]. Further, anti-inflammatory activity of root extracts (ethanol and acetate) and seed extracts (petroleum ether and aqueous) have been reported [6].

Recently, 70% ethanolic extract of *P. pinnata* leaves have been evaluated in acute, sub-acute and chronic models of inflammation in rats [7]. However, as yet, its analgesic potential of aqueous extracts of leaves has not been scientifically evaluated. The present study is an attempt to address this issue. The objective of this study was to scientifically investigate the effectiveness of the decoction made from leaves of this plant as an oral antinociceptive agent. In Sri Lankan traditional medicine decoction made from leaves of *P. pinnata* is recommended as an antinociceptive.

II. MATERIAL AND METHODS

2.1 Plant collection and identification

Mature fresh leaves of *P. pinnata*were collected from the Faculty of Science, University of Colombo, Colombo 03 (N 6.90232°, E 79.85942°) in Sri Lanka. The leaves were identified and authenticated by Dr. (Ms) S. Ranawala, Department of Plant Science, University of Colombo. The voucher specimen (WDR/SAD/1008) was deposited at the Museum of the Department of Zoology, University of Colombo, Sri Lanka.

2.2 Preparation of the extract

The leaves were washed under running water, air dried for 3 days and ground into small pieces. The pieces were refluxed with water for 16 h in a round bottom flask fitted with a Liebig condenser (1 kg of plant material in 12 L of water). The brownish red solution was filtered using a sintered funnel and concentrated up to 1 L. The concentrated sample was freeze dried (yield: 18.90 w/w) and stored in airtight bottles at -20°C. The freeze-dried powder was dissolved in 1 ml distilled water to obtain the required dosage concentrations (500, 1000, 1500, and 3000 mg/kg) [8].

2.3 Experimental animals

Healthy adult crossbreed male rats (weight 200-250g) were used in study. The healthy animals were kept in plastic cages under standardized animal house conditions (temperature: 28-31^{o}C, photoperiod: approximately 12 hours natural light per day, and relative humidity: 50-55%) with free access to pelleted food (Ceylon Grain Elevators, Colombo, Sri Lanka) and clear drinking water. Except at the time of experimental procedure the animals were handled only during cage cleaning. The experiment was conducted in accordance with the internationally accepted laboratory animal use and care, and guidance and rules of the Faculty of Medicine, University of Colombo, Sri Lanka, of animal experimentation. Ethical clearance was obtained from Faculty of Medicine, University of Colombo, Sri Lanka. (EC/12/141)

2.4 Evaluation of antinociceptive activity

2.4.1 Hot Plate and Tail flick tests

Thirty six male albino rats were randomly selected and fasted for 24 h before the experiment with free access to water and separated into six groups (n=6 per group) and treated orally in the following manner: Group 1: with 1mL of distilled water, Groups 2, 3, 4, and 5: with 1mL of 500, 1000, 1500, 3000 mg / kg of freeze-dried aqueous extract, respectively, and Group 6: with 1mL of 15mg / kg of morphine sulphate (Pharmachemie B.V., Harlem, Netherlands), the reference drug of opioid receptor agonistas a positive control. One hour before treatment (pretreatment) and then at hourly intervals for 6h post treatment, these rats were subjected to hot plate and tail flick tests [9]. The aqueous leaf extract treated rats were observed for elicition of struab's tail reaction [10].

In the hot-plate test, each rat was placed on enclosed hot plate (Model MK 35A Muromachi Kikai Co., Ltd., Tokyo, Japan) which maintained at 50^{o}C and then the time taken to lick either hind paw or to jump up (reaction time) was recorded. Rats showing a pre-treatment reaction time greater than 15s in the hot-plate test were not selected for the experiment. A cut of time of 20 sec was used so as to avoid tissue damage[11].In the tail-flick test, time taken to flick the tail (the reaction time) when the tail was immersed (5-6cm from the tip) in a water bath at 55^{o}C was recorded using a stopwatch. Rats showing a pre-treatment reaction time greater than 5s in the tail flick test were not selected for the experiment. A cut off time of 5s was set to avoid tissue damage [11].

2.4.2 Formalin test

Twelve rats were randomly divided into two groups and treated orally in the following manner. Group 1: with 1mL of distilled water, Groups 2: with 1mL of 3000 mg / kg of freeze-dried aqueous leaves extract. Three hours after administration, each of these rats was subcutaneously injected with 0.05 ml 2.5% formalin solution (BDH Chemical, Poole, UK) into the sub plantar surface of the left hind paw. The rats were then observed for 30 min and the numbers of licking of hind paw, flinching, lifting and time spend on licking of the injected paw were recorded in two phases: first phase 0-5 min and second phase 20-30 min [8,12].

2.5 Evaluation of the mechanism of antinociceptive activity

2.5.1 Investigation for dopamine receptor mediation

Twelve male rats were randomly divided into two groups. Those in group 1 wereintraperitoneally injected with 1.5 mg/kg of metoclopramide (AivitaPharmaPvt Limited, Gujarat, India), a dopamine receptor antagonist, and those in group 2 with isotonic saline. 10 mins later, the rats in both groups were orally administered with 3000mg/kg of freeze-dried aqueous leaves extract. These rats were subjected to the hot plate test one hour before treatment, 1h and 3h after post treatment [13].

2.5.2 Investigation for muscarinic receptor mediation

Twelve male rats were randomly divided into two groups. Those in group 1 wereintraperitoneally injected with 5 mg/kg of atropine sulphate (Harson Laboratories, Borada, India), a muscarinic receptor antagonist, and those in group 2 with isotonic saline. 10 min later, the rats in both groups were orally administered with 3000mg/kg of freeze-dried aqueous leaves extract. These rats were subjected to the hot plate test before treatment, 1h and 3h after post treatment [13].

2.5.3 Investigation for opioid receptor mediation

Twelve male rats were randomly divided into two groups. Those in group 1 wereintraperitoneally injected with 1ml of 1.5 mg/kg of naloxone hydrochloride (Samarth Life Sciences Pvt. Ltd, Mumbai, India), an opioid receptor antagonist, and those in group 2 with 1ml of isotonic saline. After 45 min, the rats in both groups were orally administered with 3000mg/kg of freeze-dried aqueous leaves extract. These rats were subjected to the hot plate test one hour before treatment, 1h and 3h after post treatment [13].

2.6 Evaluation of effects on muscle coordination and strength

Twelve rats were randomly divided into two groups. Those in group1 were orally administered with 1ml of 3000 mg / kg of freeze-dried aqueous leaves extract and those in group 2 with1ml of distilled water. After 3h, these rats were subjected to the bar holding test (to evaluate muscle strength)[13] and Bridge test[14]and righting reflex test[15] (to evaluate muscle coordination) and their respective latencies (in second) were measured.

2.7 Evaluation of sedative activity

Twelve male rats were randomly divided into two groups. Those in group1 were orally administered with 1ml of 3000 mg / kg of freeze-dried aqueous leaves extract and those in group 2 with 1ml of distilled water. After 3h, each of these rats were tested for sedative activity in the rat hole-board test [16].Each of these rats were individually placed at the center of the standard rat hole board apparatus and observed for 7.5 min. During this period, number of rearing, number of crossing, number of head dipping, cumulative time spend on head dipping were recorded.

2.8 Evaluation of effect on hematologic parameters, serum urea, creatinine, ALT and AST levels

Fresh twelve rats were randomly divided into two equal groups (n=6). One group was orally daily administered with highest dose (3000 mg/Kg) and the other group with 1ml of distilled water consecutively for 21 days. On day 21 post treatment (22^{nd} day), these rats were anaesthetized with diether and blood (1.5-2.0ml)[8]was collected from tails of these rats with aseptic precautions (EC 12.141). Examination of serum urea and creatinine (to examine renal toxicity), ALT and AST (to examine liver toxicity) were made using respective kits (Randox Laboratories Ltd., Antrim, UK).

2.8.1 Observation of overt signs of toxicity, stress and aversive behavior

Rats used in the above investigation were closely observed each day of treatment and on day 1 post treatment for presence of overt signs of toxicity (salivation, diarrhea, yellowing of fur and loss of fur, ataxia, postural abnormalities, behavioral change, marked impairment of food and water intake and body weight), stress (erection of fur and exophthalmia) and aversive behaviors (biting and scratching, licking of tail, paw and penis, intense grooming or vocalization) [8].

2.9 Phytochemical analysis

The aqueous leave extraction was subjected to phytochemical screening. Phytochemical screening procedures were carried out for the screening of four different chemical groups: alkaloids, polycyclic compounds, flavonoids, leucoanthocyanins and tannins [17].The freeze dried crude of aqueous leave extract was also subjected to column chromatography. The column was packed with normal phase silica gel using hexane. It was eluted with solvent of increasing polarity starting with hexane, mixture of hexane and ethyl acetate, ethyl acetate, mixture of ethyl acetate and methanol and finally with methanol [18]. The fractions with similar thin layer chromatography (TLC) spots under UV light (at 254nm and 365nm) were combined. The combined fractions were again subjected to TLC normal phase (Aldrich silica gel precoated on aluminium sheets). The mobile phases were 100% hexane, 30% ethyl acetate in hexane, 40% ethyl acetate in hexane, 100% ethyl

acetate, 20% methanol in ethyl acetate. These TLC plates were sprayed with colour reagent specific for various classes of compounds [19].

2.10 Statistical analysis of data

The data were expressed as the mean ± SEM. Statistical comparisons were made by one-way analysis of variance (ANOVA) using Minitab 13.0 version statistical package. Significant values were set as $P \leq 0.05$.

III. RESULTS

3.1 Hot Plate and Tail flick tests

The results obtained are summarized in Table 1. As shown, a 500 mg/kg dose of ALE caused a significant ($p \leq 0.05$) prolongation of the reaction time in the hot-plate test from the first hour to the sixth hour compared with the control (first hour by 70%, second hour by 78%, third hour by 66%, forth hour by 49%, fifth hour by 42% and sixth hour by 28%) and the first and second hour compare with the own pre-treatment value (first hour by 59%, second hour by 61%).

A 3000 mg/kg dose of ALE caused a significant ($p \leq 0.05$) prolongation of the reaction time in the hot-plate test from the first hour to the fifth hour compared with the control (first hour by 88%, second hour by 61%, third hour by 80%, forth hour by 55% and fifth hour by 37%) and from the first hour to the forth hour compare with the own pre-treatment value (first hour by 93%, second hour by 59%, third hour by 66%, forth hour by 34%).

A significant prolongation of the reaction time was also evident with 1000 mg/kg (with control: second hour by 62%, third hour by 68%, forth hour by 34% and sixth hour by 29%, with own pre-treatment: second hour by 50%, third hour by 45%) and 1500 mg/kg (with control: first hour by 42%, second hour by 49%, forth hour by 66%, fifth hour by 47% and sixth hour by 41%, with own pre-treatment: forth hour by 43% and sixth hour by 42%).

Morphine caused a huge and significant ($p<0.05$) increase in the reaction time up to fifth hour post-treatment compared with the control (first hour by 102%, second hour by 152%, third hour by 106%, forth hour by 64% and fifth hour by 19%) and to forth hour post treatment compared with its own pre-treatment (first hour by 85%, second hour by 121%, third hour by 69% and forth hour by 26%).

In contrast, in the tail flick test, there was no significant alteration ($p>0.05$) in the tail-flick reaction time inrats treated with any of the aqueous leaves extraction of *P. pinnata*compared with control rats (data not shown). Furthermore, none of the ALE treated rats exhibited characteristic straab's tail reaction.

3.2 Formalin test

The results of the formalin test shown in Table 2 indicate that oral administration of 3000mg/kg dose of ALE significantly ($p<0.05$) impaired the number of licking (first phase by 27% and second phase by 23%), licking time (first phase by 46% and second phase by 69%), cumulative time spent per licking (first phase by 25% and second phase by 60%) and total cumulative time spent on licking (by 54%). Howevernumber of flicking, number of lifting were not significantly ($p<0.05$) impaired by highest dose of ALE.

3.3 Investigation for dopamine receptor mediation

As shown in Table 3, with the hot plate technique, intraperitoneal administration of metochlopramide significantly ($p \leq 0.05$) impaired the prolongation of reaction time induced by 3000mg/kg of ALE in first and third hour compared with control.

3.4 Investigation for muscarinic receptor mediation

As shown in Table 4, with the hot plate technique, intraperitoneal administration of atropine did not significantly ($p>0.05$) impair the prolongation of reaction time induced by 3000mg/kg of ALE at first hour. However intraperitoneal administration of atropine significantly ($p<0.05$) impair the prolongation of reaction time induced by 3000mg/kg of ALE at third hour.

Table 1 Effect of the oral administration of aqueous leaf extract (ALE) of *P. pinnata* (doses 500, 1000, 1500 & 3000 mg/kg) on the hot-plate reaction time of rats.

Dose mg/kg	Reaction time (sec) (Mean±SEM)						
	P-T	1h	2h	3h	4h	5h	6h
Control	6.73± 0.76	6.87±0.46	6.60±0.55	6.18±0.93	5.80±0.85	5.92±1.23	6.22±0.93
500	7.32± 1.39	11.65±1.63**	11.78±2.62**	10.23±3.01*	8.67±1.66*	8.42±1.57*	7.95±1.16*
1000	7.13± 1.10	7.28±2.04	10.70±1.97**	10.37±1.19**	7.77±1.33*	7.43±2.23	8.03±1.62*
1500	6.75± 1.12	9.73±3.11*	9.83±3.26*	9.67±3.95	9.62±2.75**	8.73±1.95*	8.83±1.65**
3000	6.70± 1.29	12.92±1.67**	10.62±1.91**	11.10±2.05**	8.97±0.94**	8.13±1.84*	7.80±1.49
Morphine	7.52± 1.27	13.90±0.95**	16.62±1.21**	12.72±1.27**	9.50±1.15**	7.05±0.66	6.77±0.95

Values are significant at p<0.05 (*-compared with control and *-compared with pretreatment) PT:Pre-treatment.

Table 2 Effect of oral administration of 3000mg/kg dose of aqueous leaf extract (ALE) of *P. pinnata* on formalin test

Treatment	First Phase			Second Phase			Total cumulative time spent per licking	Number of flicking	Number of lifting
	Number of licking	Licking time (s)	Cumulative time spent per licking	Number of licking	Licking time (s)	Cumulative time spent per licking			
Control	14.00 ±1.79	109.6 ±14.65	7.84 ±0.44	49.00 ±9.01	532.00 ±88.60	10.9 ± 0.81	10.21 ±0.68	6.83 ±2.79	5.33 ±2.73
3000mg/kg	10.17 ±1.84*	59.00 ±9.38*	5.87 ±0.71*	37.50 ±7.42*	163.67 ±28.81*	4.41 ±0.42*	4.69 ±0.29*	8.00 ±2.97	7.83 ±3.66

Values are significant at *p<0.05 compared with respective controls.

Table 3 Effect of intraperitoneal injection of metochlopramide on the hot-plate reaction time of aqueous leaves extract (ALE) (3000mg/kg) of *P. pinnata*.

Treatment	Hot-plate reaction	time (s)	(Mean±SEM)
	Pretreatment	First hour	Third hour
Saline + extract (n=6)	6.97± 1.15	13.90±1.88	11.82±3.30
Metochlopramide	7.03±1.65	7.73±1.43*	8.15±1.17*

Values are significant at *p<0.05.

Table 4 Effect of intraperitoneal injection atropine on the hot-pate reaction time of aqueous leaves extract (ALE) (3000mg/kg) of *P. pinnata*.

Treatment	Hot-plate reaction	time (s)	(Mean±SEM)
	Pretreatment	First hour	Third hour
Saline + extract (n=6)	6.97± 1.15	13.90±1.88	11.82±3.30
Atropine	6.57±1.34	12.97±3.63	6.73±1.10*

Values are significant at *p<0.05.

3.5 Investigation of opioid receptor mediation

Intraperitoneal administration of naloxone did not significantly (p>0.05) impair the prolongation of reaction time induced by 3000mg/kg of ALE (naloxone + ALE vs. saline + ALE: at first hour 14.27±3.71 sec vs. 14.40±4.14sec, at second hour 16.20±1.27sec vs. 13.77±2.97sec).

3.6 Muscle strength and coordination

None of the latencies of these tests were significantly ($p>0.05$) altered by a 3000mg/kg dose of ALE (control vs. treatment: bar-hold test, 6.94±5.47 sec vs. 4.88±2.88 sec; Bridge test, 5.52±3.32 sec vs. 7.80±2.86 sec; righting reflex test, 1.15±0.17 sec vs. 1.11±0.14 sec).

3.7 Sedative effect

In the hole-board test, none of the parameters were significantly ($p>0.05$) altered by a 3000mg/kg dose of ALE (control vs. treatment: number of rears, 4.83±2.64 vs. 6.17±2.64; number of crossing, 9.67±5.24 vs. 9.50±2.74; number of head dipping, 5.33±3.67 vs. 2.67±1.21; dipping time, 6.42±3.92 sec vs. 4.41±2.80 sec; time per dip, 1.29±0.25 sec vs. 1.67±0.55 sec).

3.8 Evaluation of effect on hematologic parameters, serum urea, creatinine, ALT and AST levels

None of the enzyme levels (control vs. treatment: ALT, 27.733±5.708 vs. 24.008±6.921 U/L; AST, 22.553±3.440 vs. 22.931±2.080 U/L), serum urea (control vs. treatment: 45.27±3.10 vs. 49.63±16.22 mg/dl), creatinine (control vs. treatment: 2.31±0.07 vs. 2.30±0.09 mg/dl) were significantly ($p>0.05$) altered by a 3000mg/kg dose of ALE.

3.9 Observation of overt signs of toxicity, stress and aversive behavior

No overt signs of toxicity, stress, or aversive behaviors were observed in rats during this study. Further, none of the treated or control rats became morbid or died up to day 1 post treatment.

3.10 Phytochemical analysis

Phytochemical screening of the aqueous extract showedthe presence of flavonoids, alkaloids, unsaturated steroids, terpenes, tannins, polyphenols and leucoanthocyanins. The normal phase TLC's obtained from the fractions collected from the normal phase column chromatogram (eluting solvent: hexane, mixture of hexane and ethyl acetate, ethyl acetate, mixture of ethyl acetate and methanol and finally with methanol) on spraying with characteristic reagents showed the presence of alkaloids (mobile phase - 70% hexane: 30% ethyl acetate R_f 0.86; 100% ethyl acetate R_f0.88, 0.70, 0.06; 80% ethyl acetate: 20% methanol R_f 0.80, 0.12, 0.04), flavonoids(mobile phase - 100% hexane R_f 0.89; 60% hexane: 40% ethyl acetate R_f0.52; 100% ethyl acetate R_f 0.92), steroids (mobile phase - 100% hexane R_f 0.90,0.64; 70% hexane: 30% ethyl acetate R_f 0.18,0.04; 60% hexane: 40% ethyl acetate R_f0.00), triterpenoids glycosides (mobile phase - 100% hexane R_f 0.84,0.76,0.12; 70% hexane: 30% ethyl acetate R_f 0.74,0.56; 100% ethyl acetate R_f0.04,0.00), saponins (mobile phase - 100% hexane R_f 0.92; 70% hexane: 30% ethyl acetate R_f 0.78; 60% hexane: 40% ethyl acetate R_f0.44,0.28,0.08; 100% ethyl acetate R_f0.88,0.78,0.67; 80% ethyl acetate: 20% methanol R_f0.61,0.08), phenols (mobile phase - 60% hexane: 40% ethyl acetate R_f0.56; 100% ethyl acetate R_f0.12), terpenoids (mobile phase - 100% hexane R_f 0.84,0.68; 70% hexane: 30% ethyl acetate R_f 0.68, 0.38, 0.19; 60% hexane: 40% ethyl acetate R_f0.19,0.11; 100% ethyl acetate R_f0.92,0.16; 80% ethyl acetate: 20% methanol R_f0.18,0.12).

IV. DISCUSSION

The results convincingly show, for the first time that, an aqueous extract of fresh mature leaves of *P. pinnata* possesses antinociceptive activity in rats, when given orally (in doses acceptable in rat models), and evaluated in the hot plate (in terms of prolongation of reaction time) and the formalin (in terms of shortening of measured parameters) algesiometric tests. However, antinociceptive action was not evident when assessed on the tail flick test: these tests are scientifically validated widely used standardized methods employed in the evaluation of potential antinociceptive agents. Compared to morphine, ALE was less efficacious in eliciting the antinociceptive action. Further, ALE of *P. pinnata*neither induced motor deficits (as reflected from bar test and unimpaired locomotory activity in the rat hole-board test) nor nervous incoordination (as judged by bridge and righting reflex tests). Thus, the results obtained are reliable, valid and meaningfully interpreted.

The positive results in the hot plate test suggest that aqueous leaf extract of *P. pinnata* is effective against transient phasic pain which is centrally mediated at the supra spinal level: hot plate technique predominately measures supra spinal reflexes [20].On the other hand, impairment of different parameters, namely, number of licking, licking duration, cumulative time spent on licking (on both phases of the test) suggest that aqueous leaf extract of *P. pinnata* is effective against periphcial pain of both neurogenic and inflammatory origins [21]. This may result from ALE included impairment of inflammatory mediators such as cytokines, prostaglandins, bradykinin, serotonin or histamines[22], possibly via phenolic and steroidal phytoconstituents present in the extract. Continuous inflammatory pain is one of the most common types of pathological pain in clinical practical and persistent pain is known to have a major impact on the quality of life [23].Conversely, a lack of an effect of ALE of *P. pinnata*in tail flick test suggests that spinal mechanisms are not involved in its antinociceptive action [20].

The antinociceptive activity of ALE of *P. pinnata* had a quick onset (within 1 hour) and moderately long duration of action (up to six hour). This is presumably due to fast absorption of the active phytoconstituent/s and

its/their quick transport to the final site/s of action. Having a rapid onset of action of antinociceptive action is a much soughted featureof a pain killer.

Food restriction imparts antinociception in rats [24],but such a mode of action is unlikely to be operative here, as food was available through the study period and there was no apparent hypophagia. Stress is known to provoke antinociception [14,25].But, this mechanism of antinociceptive can be ruled out, in this study, as there were no signs of expothalamia, fur erection, diarrhea or aversive behaviors. Sedation is implicated with antinociception [26], and several sedatives have shown to possess marked antinociceptive activity [27].Albeit, this mechanism too is unlikely to be operative in this study as none of the parameters (number of crossings, number of level dippings, number of rears, dipping time and time per dip) was impaired.

Naloxone, the universal opioid receptor antagonist, failed to block ALE of *P. pinnata* induced antinociception. This indicates that opioid mechanisms are unlikely to be operative in this study. This notion is further reinforced by the fact that ALE of *P. pinnata* failed to elicit characteristic Straub's tail reaction which is characteristic of opioid receptor mediated drugs [10]. On this context, it is worth noting that ALE contained alkaloids and several plant alkaloids which are known to induce antinoceciptive*via* opioid mechanisms [28,29]. Although it was not the case in this study. This discrepancy may be attributed structural differences between alkaloids.

Dopamine is now recognized to play an important role in pain modulation and dopamine receptor blockers and known to suppress pain [25]. In this study, ALE induced antinociception was inhibited (both at 1st and 3rd hours) by metaclopramide, a dopamine recapture (D_2 type) antagonist. This is indicative of dopamine D_2 receptor mediation in ALE induced antinociception.

Cholinergic mechanisms are also now linked with pain [30]. In this study, ALE of *P. pinnata* induced antinociception was blocked by atropine, a well-known muscarinic cholinergic receptor antagonist at 3rd hour but not at 1st hours. This suggests the involvement of muscarinic cholinergic mechanisms, at least, at the 3rd hour (mid period) of ALE induced antinociception. However, an absence of a synergetic antinociceptive action at 3rd hour, compared to the 1st hour, argues against this mode of action.

Interestingly, even with daily sub chronic administration of a high dose of ALE, there was no morbidity, motility or overt signs of clinical toxicity (in term of salivation, diarrhea, excessive urination, yellowing of fur, loss of fur, postural abnormalities, behavioral change, impairments of food and water intake), renotoxicity (in terms of serum creatinine and urea level) or hepatotoxicity (in term of serum ALT and AST levels) indicating its safety with oral administration.

V. CONCLUSION

This study, shows for the first time, that aqueous leaf extract of mature leaves of *P. pinnata* can act as a natural safe, orally active, moderately strong antinociceptive. The results also justify the therapeutic claim in Sri Lanka traditional medicine that *P. pinnata* leaves has painkilling activity.

VI. ACKNOWLEDGMENT

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