

**INTERNATIONAL JOURNAL OF ADVANCES IN
PHARMACY, BIOLOGY AND CHEMISTRY****Research Article****Antinociceptive activity of aqueous fruit extract of
Dillenia retusa T. in rats****S.A.Deraniyagala¹, W.D.Ratnasooriya², P.S.Wijesekara¹.**¹Department of Chemistry, University of Colombo, Colombo 03, Sri Lanka.²Department of Zoology, University of Colombo, Colombo 3, Sri Lanka.**ABSTRACT**

Dillenia retusa Thunb (Family: Dilleniaceae) is an endemic plant in Sri Lanka and in Sri Lankan traditional medicine the mature fruit is used to treat and manage the pain of fractures and dislocations. The aim of this study was to investigate the antinociceptive potential of aqueous fruit extract (AFE) of *D. retusa* in rats. Different concentrations of the AFE (250, 500, 750, 1000 mg/kg) were orally administered to female rats and antinociceptive activity was determined using hot plate test, tail flick test and formalin test against water control. **The results showed that AFE has significant ($p \leq 0.05$) antinociceptive action when evaluated in hot plate and formalin test but not in tail flick test.** The antinociceptive activity had a quick onset (within 2 h) and fairly long duration of action (up to 5 h). The AFE induced antinociception was not blocked by metoclopramide (a dopamine receptor antagonist) or atropine (a muscarinic receptor antagonist) but was suppressed by naloxone (opioid receptor antagonist). Furthermore the AFE did not show sedative activity (in terms of rat hole board test) in rats. Stressful behaviors and any overt signs of toxicity were also not observed after acute administration of the AFE. All these results and observations suggest that AFE mediates its antinociceptive activity centrally at supraspinal level and peripherally and is effective against both neurogenic and inflammatory pain. The results also propose that the antinociceptive action was not due to dopaminergic, cholinergic and sedative mechanisms but can be due to opioid receptor mechanism. Phytochemical screening of the AFE showed the presence of flavonoids, leucoanthocyanins and 2-Deoxy sugars. Flavonoids may mostly contribute to the antinociceptive activity of *D. retusa*. This study shows for the first time, that AFE of *D. retusa* exhibits moderately strong oral analgesic activity supporting its use as a pain reliever in traditional medicine.

Key words: *Dillenia retusa*, antinociception, opioid mechanism, pain inhibition.**INTRODUCTION**

Dillenia retusa Thunb, (Family Dilleniaceae) 'Godapara' in Sinhala is a moderate size ever green tree and an endemic species in Sri Lanka.¹ It can be found in the moist low-country especially on creek banks.¹ The leaves are, large, simple, alternate, 15 - 24 cm long, 6.5 - 10 cm broad, obovate-oblong, rounded to retuse at apex, acute and decurrent at base and shining glabrous above with densely to sparsely strigose beneath.^{1, 2} The flowers are pubescent, sub terminal or leaf-opposed, regular, bisexual, often two flowers clustered, terminal 3.5 - 5.5 cm long peduncle, 2.5 - 4.5 cm long and cylindrical pedicels. There are five petals with 7.5 - 9.0 cm diameter and white with pink or purple stamens on long.¹ The bark

is brownish grey and wood is reddish brown, hard and durable.³ The ripe fruit is enclosed by much enlarge, thickened imbricate sepals forming a depressed-globose. It is a finely pubescent, 2.5 - 3.8 cm diameter orange fruit, with fleshy thin pericarp with many smooth seeds.¹

In Sri Lankan traditional medicine, the fruit of *D. retusa* is used as an ingredient for poultices applied to manage the pain on fractures and dislocations.^{1, 4} This traditional application indicates that the fruit of the plant may have antinociceptive potential and it has not been scientifically validated as yet. The attempt of this study was to investigate the antinociceptive effect of the plant and validate it scientifically.

MATERIALS AND METHODS

Plant Collection and Authentication :

Ripe fruits of the *D. retusa* were collected from Ambalangoda area in Galle district of Sri Lanka in February 2013. It was identified and authenticated by Dr. H. Kathirarachchi of the Department of Plant Science, University of Colombo and a voucher specimen (PW1) was deposited at the herbarium of the Department of Plant Science, University of Colombo.

Preparation of the aqueous fruit extract of *D. retusa* :

Air shade dried fruits were grinded and 1000 g of these were refluxed with distilled water (5 - 6 L) for 16 hours in a round bottom flask fixed with a Leibig condenser. The brownish AFE was filtered and its volume was reduced by further boiling to obtain a concentrated extract. This was freeze-dried (yield: 67%) and stored air tightly below at 4°C. The freeze-dried powder was dissolved in distilled water to obtain the required concentrations of AFE (250, 500, 750, 1000 mg/kg) to treat rats.

Experimental animals :

Healthy, adult albino female rats (weight: 180 - 225 g) were used in this study. The animals were kept in plastic cages with wire mesh roof (6 per cage) under standardized rat housing conditions (temperature: 28 - 31°C, 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. All the experiments were conducted in accordance with the guidelines and the rules of Ethics Review Committee of Institute of Biology for animal experiments in Sri Lanka (Reg. No. ERC IOBSL 105/06/13).

Evaluation of analgesic activity :

Hot plate and tail flick test :

Thirty-five rats were randomly divided in to six groups. Food was withheld from these rats for 16 h and different concentrations of AFE or vehicle (control) was orally administered in the following manner: Group 1 (n = 6) with 1 ml of distilled water; Group 2 (n = 6) with 1 ml of 250 mg/kg of AFE; Group 3 (n = 6) with 1 ml of 500 mg/kg of AFE; Group 4 (n = 6) with 1 ml of 750 mg/kg of AFE; Group 5 (n = 6) with 1 ml of 1000 mg/kg of AFE; and Group 6 (n = 5) with 1 ml of 15 mg/kg of morphine sulphate (Teva pharmaceutical works Pvt.Ltd, Hungary) (an opioid receptor agonist) as the positive control.⁵ Then the rats were subjected to hot plate and tail flick test.⁶ Hot plate and tail flick

reaction time of each rat were recorded before treatment (pre-treatment). Post treatment reaction time was taken hourly intervals for 6 h. In the hot plate test, the rats were placed in a hot plate (Model MK 35 A, Muromachi Klkai Co.Ltd., Tokyo, Japan) maintained at 50 °C and the time taken to lick either of the hind paws or jump up was recorded. The rats showing a pre treatment reaction time greater than 15 S for the hot plate test were not used for the test. A cut off time of 20 S was set to prevent possible tissue damages. In the tail flick test, the tail of the rat up to 5 cm - 6 cm from the tip was immersed in a water bath maintained at 55 °C and the time taken to flick the tail was recorded. The rats showing a pre treatment reaction time greater than 5 S for the tail flick test were not used for the experiment. A cut off time of 5 S was set to avoid the possible tissue damages.⁷

Formalin test :

Twelve rats were randomly divided in to two equal groups (n = 6/group) and were orally administered with AFE or distilled water (control) as follows; Group 1 with 1 ml of distilled water; Group 2 with 1 ml of 500 mg/kg of AFE. 2 h after administration each rat was subcutaneously injected with 0.05 ml of 2.5% formalin solution in to the sub plantar surface of the left hind paw. Then the rats were observed for 1 h and number of licking, flinching, lifting, and time spent on lifting and licking the injected paw were recorded in two phases; early phase; 0 - 5 min and late phase 20 - 60 min.⁷

Evaluation of mechanisms of antinociceptive activity :

Investigation for muscarinic receptor mediation :

Twelve rats were randomly divided into two equal groups (n = 6/group). Those in group 1 were intraperitoneally injected with 1 ml of isotonic saline (0.9% w/v sodium chloride solution) and rats in group 2 were intraperitoneally injected with 1 ml of 2 mg/kg atropine sulphate (Harson Laboratories, India) a muscarinic receptor antagonist.⁵ Ten minutes later the rats in both groups were orally administered with 1 ml of 500 mg/kg AFE. These rats were subjected to hot plate test, 1 h before treatment with muscarinic receptor antagonist and 2 h after AFE treatment.⁸

Investigation for dopamine receptor mediation :

Ten rats were randomly divided into two equal groups (n = 5/group). The rats in group 1 were orally treated with 1 ml of 1% methylcellulose. Rats in group 2 were orally treated with 1 ml of 1.5 mg/kg metoclopramide (Ipca laboratories Pvt.Ltd, India) a dopamine receptor antagonist in 1% methylcellulose.⁵

One hour later, both groups were orally treated with 1 ml of 500 mg/kg AFE and the nociception was determined before treatment with dopamine receptor antagonist and 2 h post treatment, using the hot plate technique.⁸

Investigation for opioid receptor mediation :

Ten rats were randomly divided into two equal groups (n = 5/group). Those in group 1 were intraperitoneally injected with 1 ml of isotonic saline (0.9% w/v sodium chloride solution) and those in group 2 were intraperitoneally injected with 1 ml of 1.5 mg/kg naloxone hydrochloride (Troikaa Pharmaceuticals Ltd, India), an opioid receptor antagonist.⁵ After 45 min, the rats in both groups were orally administered with 1 ml of 500 mg/kg AFE. These rats were subjected to hot plate test, 1 h before treatment with opioid receptor antagonist and 2 h after the AFE treatment.⁹

Evaluation of muscle coordination and strength :

Twelve rats were orally treated either with 1 ml of distilled water (n = 6) or 1 ml of 500 mg/kg AFE (n = 6). Two hour after the treatment these rats were subjected to the bar holding test bridge test and righting reflex test and respective latencies (in seconds) were recorded.^{10, 11}

Evaluation of sedative activity :

Twelve rats were randomly divided into two equal groups (n = 6/group). The rats in group 1 were orally administered with 1 ml of distilled water and those in group 2 with 1 ml of 500 mg/kg AFE. After 2 h each of these rats was individually placed in the center of the rat hole board apparatus and was observed for 7.5 min. During this period, number of head dipping, cumulative head dipping time, number of rearing, number of crossings, and number of fecal boluses produced were recorded.¹²

Phytochemical analysis :

AFE was subjected to phytochemical screening procedures for the screening of four different chemical groups; alkaloids, polycyclic compounds, flavonoids and leucoanthocyanins and tannins and poly phenols.¹³

Statistical analysis :

The data were expressed as the mean \pm standard error of mean (SEM). Statistical analysis was performed using Mann-Whitney U-test *via* Minitab 16.0 statistical package. Significance was set at $P \leq 0.05$.

RESULTS

Hot plate and tail flick test :

As shown in Table 1, 500 mg/kg dose of the AFE caused a significant ($P < 0.05$) prolongation of the hot plate reaction time from the 2nd h to 5th h (2nd h by 45.6%, 3rd h by 50.0%, 4th h by 45.2%, 5th h by 24.6%) compared to water control. The 750 mg/kg and 1000 mg/kg of the AFE significantly increased the hotplate reaction time at 2nd h by 30.9% and at 3rd h by 32.3% respectively compared to water control. In contrast the 250 mg/kg of the AFE failed to significantly alter the hot plate reaction time at any hour. Morphine induced a remarkable prolongation of reaction time from 1st h to 6th h compared to water control (1st h by 57.3%, 2nd h by 133.8%, 3rd h by 132.2%, 4th h by 98.4%, 5th h by 67.7% and 6th h by 34.4%) and effective hours of the 500 mg/kg (2nd h by 60.6%, 3rd h by 54.8%, 4th h by 36.7% and 5th h by 34.6%).

In contrast, in the tail flick test there was no significant ($P > 0.05$) prolongation of the reaction time provoked by any dose of AFE compared to water control (data was not shown).

Formalin test :

As shown in the table 2, 500 mg/kg dose of AFE significantly ($P < 0.05$) impaired two of the tested parameters in the early phase (mean number of licking by 46% and cumulative licking duration by 41%) and all the parameters in the late phase (mean number of licking by 47%, cumulative licking duration by 61%, mean number of lifting by 49%, cumulative lifting duration by 34% and mean number of flinching by 59%) compared to water control.

Muscarinic receptor mediation :

Intraperitoneally administration of atropine did not significantly ($P > 0.05$) alter the hot plate reaction time induced by 500 mg/kg of AFE (control vs. treatment: 8.6 ± 0.2 vs. 8.4 ± 0.3 Sec).

Dopamine receptor mediation :

Oral administration of metoclopramide did not significantly ($P > 0.05$) alter the hot plate reaction time induced by 500 mg/kg of AFE (control vs. treatment: 9.9 ± 0.3 vs. 9.0 ± 0.3 Sec).

Opioid receptor mediation :

Intraperitoneally administration of naloxone significantly ($P < 0.05$) impaired the prolongation of the hot plate reaction time induced by 500 mg/kg of AFE (results are shown in table 3).

Effects on muscle coordination and strength :

None of the latencies in the bridge test, bar holding test and righting reflex tests were significantly ($P > 0.05$) altered the 500 mg/kg of AFE (control vs.

treatment: Bridge test: 16.58 ± 2.79 vs. 17.07 ± 1.27 Sec; Bar holding test: 14.10 ± 1.36 vs. 14.93 ± 1.54 Sec; righting reflex: 1.00 ± 0.00 vs. 1.00 ± 0.00 Sec).

Sedative activity :

The 500 mg/kg dose of AFE failed to significantly ($P > 0.05$) alter any of the parameters in rat hole board test (control vs. treatment: number of crossings: 17.7 ± 1.2 vs. 17.3 ± 1.9 ; number of rears: 20.5 ± 2.1 vs. 19.8 ± 3.4 ; number of dips: 7.8 ± 1.1 vs. 6.8 ± 1.1 ; cumulative dipping duration: 12.92 ± 1.27 vs. 13.82 ± 2.40 ; number of faecal boluses: 0.8 ± 0.4 vs. 0.2 ± 0.2).

Phytochemical analysis :

Phytochemical analysis of the AFE showed the presence of flavonoids, leucoanthocyanins and 2-Deoxy sugars.

DISCUSSION

Thermal and chemical models of nociception were used in this study to evaluate the antinociception potential of *D. retusa*. These models are accepted as accurate and reliable when evaluating antinociception.¹⁴ The results showed, for the first time, that AFE consists of acute, moderately strong antinociceptive activity with fairly long duration of action and considerable quick onset of action. Nociceptive pathways and inhibitory controls can be activated *via*, supra-spinal level, spinal cord level or/and peripheral mechanisms.⁵ In order to find out the relevant pathways involved for the antinociceptive effect of *D. retusa* plant, hot plate method, tail flick method and formalin test were followed. Hot plate method is responsible for antinociception mediated through supra-spinal level and tail flick test measures antinociception mediated through spinal reflexes.¹⁵ Positive hot plate results and negative tail flick results suggest that antinociceptive activity is mediated centrally at supra spinal level and it is not mediated *via* spinal mechanisms. In the hot plate test among all tested doses, neither 250 mg/kg nor 750 mg/kg showed the maximum effect. However it was the 500 mg/kg which showed the maximum mean reaction time suggesting that the dose-response curve for the antinociceptive effect of the AFE may be bell shaped. Such an action may be shown due to the receptor desensitization or receptor down regulation.^{16,17} However antinociception action of AFE was nearly two fold less effective than morphine, one of the most potent analgesic used to treat severe visceral pain.

Results obtained for the formalin induced antinociception were significant compared to the

water control. In the case of formalin test early phase is caused due to direct action of nociceptors (neurogenic pain) and late phase is a measure of peripheral mechanisms (inflammatory pain).^{9,18} According to the results, in the early phase (0 - 5 min) of formalin test, out of the five parameters, two parameters significantly impaired the corresponding values compared to control suggesting that there is a direct effect on nociceptors and AFE is active against neurogenic pain. This also agrees with the positive results of the hot plate test which responds to centrally acting antinociception. In the late phase (15 - 60 min) of formalin test, all the five parameters significantly impaired the corresponding values, compared to the control showing that involvement of the peripheral antinociceptive mechanisms. Centrally acting analgesic drugs like narcotics generally inhibit both phases of the formalin test in an equal manner whereas non steroidal anti inflammatory drugs only inhibit the late phase of formalin test.¹⁷ AFE inhibits both early phase and late phase of formalin test suggesting that it acts both centrally and peripherally. However the late phase of formalin test depends on the inflammatory reaction in the peripheral level (inhibition of synthesis of peripheral mediators such as prostaglandin) and AFE suppressed the parameters in the late phase more conspicuously than the early phase. This suggests that AFE might be more effective against inflammatory pain than the neurogenic pain.

Sedation is known to cause analgesia and sedative drugs such as benzodiazepines (use to treat insomnia and anxiety) are already used as co-analgesics.¹⁹ Since any of the parameters checked in rat hole board test were not significantly altered compared to water control, it can be suggested that AFE has no sedative effect and also does not create any anxiolytic action on rats. Rat hole-board is a standard and widely used model to evaluate the sedative effect.²⁰ Results of bar holding test, bridge test and righting reflex suggest that the pain impairment of the AFE is not a false positive result and results are genuine since the AFE does not induce motor deficiencies.⁹ Unsuppressed locomotory activity (number of crossings) of rat hole board test also proves this.

Stress induces analgesia *via* opioid dependent or opioid independent pathways.²¹ Since stressful behaviors of rats like fur erection, exophthalmia, aversive behavioral patterns (biting and scratching behaviors, licking the tail and paw) could not be observed after administration of the AFE, the antinociceptive action of AFE is unlikely to be due to stress. Food deprivation can induce antinociception.²¹

Table 1: The effect of oral administration of different dosages of aqueous fruit extract on the hot plate reaction time (mean \pm SEM).

| Treatment (mg/kg) | Hot plate reaction time (S) mean \pm S.E.M | | | | | | |
|-------------------|--|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| | Pre-treatment | First hour | Second hour | Third hour | Fourth hour | Fifth hour | Sixth hour |
| 250 (n=6) | 8.5 \pm 0.2 | 8.0 \pm 0.2 | 6.6 \pm 0.2 | 6.8 \pm 0.4 | 6.7 \pm 0.2 | 6.6 \pm 0.3 | 6.3 \pm 0.4 |
| 500 (n=6) | 8.8 \pm 0.3 | 8.8 \pm 0.2 | 9.9 \pm 0.6* | 9.3 \pm 0.6* | 9.0 \pm 0.6* | 8.1 \pm 0.3* | 7.1 \pm 0.3 |
| 750 (n=6) | 8.3 \pm 0.2 | 8.4 \pm 0.3 | 8.9 \pm 0.6* | 7.0 \pm 0.5 | 6.5 \pm 0.5 | 6.0 \pm 0.3 | 6.0 \pm 0.2 |
| 1000(n=6) | 7.9 \pm 0.2 | 7.7 \pm 0.2 | 6.0 \pm 0.2 | 8.2 \pm 0.4* | 7.3 \pm 0.4 | 7.2 \pm 0.5 | 5.6 \pm 0.3 |
| Control (n=6) | 8.6 \pm 0.2 | 8.2 \pm 0.3 | 6.8 \pm 0.3 | 6.2 \pm 0.1 | 6.2 \pm 0.2 | 6.5 \pm 0.2 | 6.4 \pm 0.3 |
| Morphine (n=5)** | 8.7 \pm 0.2 | 12.9 \pm 0.4* | 15.9 \pm 0.3* | 14.4 \pm 0.7* | 12.3 \pm 0.6* | 10.9 \pm 0.4* | 8.6 \pm 0.2* |

*values are significant at $p \leq 0.05$ vs. control

** Reference drug 15 mg/kg

Table 2: The effect of oral administration of water (control) and 500 mg/kg of aqueous fruit extract on formalin test (mean \pm SEM).

| Parameter | Early Phase (0-5 min) | | Late phase (20-60 min) | |
|---------------------------------|-----------------------|--------------------|------------------------|---------------------|
| | Control(n=6) | 500 mg/kg (n=6) | Control(n=6) | 500 mg/kg (n=6) |
| Mean number of licking | 15.5 \pm 1.3 | 8.3 \pm 1.0* | 85.7 \pm 10.1 | 45.5 \pm 5.9* |
| Cumulative licking duration (S) | 142.20 \pm 7.92 | 83.60 \pm 11.20* | 548.30 \pm 35.10 | 211.80 \pm 40.80* |
| Mean number of lifting | 9.5 \pm 1.3 | 6.3 \pm 0.8 | 41.3 \pm 3.3 | 21.2 \pm 3.2* |
| Cumulative lifting duration (S) | 45.94 \pm 6.80 | 37.02 \pm 4.34 | 188.10 \pm 14.90 | 124.50 \pm 10.10* |
| Mean number of flinching | 4.3 \pm 1.1 | 3.5 \pm 0.9 | 52.2 \pm 3.7 | 21.2 \pm 4.5* |

*values are significant at $p \leq 0.05$ vs. control**Table 3: The effect on hot plate reaction time of rats tested for the opioid receptor mediation mechanism (mean \pm SEM).**

| Treatment | Pre-treatment(S) | Post treatment(S) (after two hours of treatment) |
|------------------------------------|------------------|--|
| Naloxone hydrochloride + AFE (n=5) | 7.8 \pm 0.3 | 5.0 \pm 0.2* |
| Saline + AFE (n=5) | 7.8 \pm 0.3 | 8.4 \pm 0.2 |

*values are significant at $p \leq 0.05$ vs. control

However such mode of action is impossible since the experimental conditions were same for the control rats and treatment rats and statistical analysis was done comparing with the control rats.

Nociception is known to precipitate *via* cholinergic mechanisms.²² Mediation of cholinergic mechanisms

are unlikely in this study as atropine, an anti muscarinic drug failed to inhibit AFE induced antinociception. Antinociception can also be evoked by dopaminergic mechanisms.²³ However such mechanism is unlikely to be active since metoclopramide, a dopamine receptor antagonist

failed to suppress AFE induced antinociception. In contrast the rats treated with naloxone hydrochloride which is an opioid receptor blocker, significantly curtailed the hot plate reaction time compared to its control. Therefore this result indicates that opioid mechanism can involve with the antinociceptive action of the AFE. Naloxone with the affinity to all three types of opioid receptors inhibits the action of endogenous opioid peptides and action of morphine like drugs. Therefore antinociceptive action can be due to opioid receptor agonist substances (opioidmimetics) present in the AFE or/and AFE may cause to release endogenous opioids like endorphines, enkephalines. Opioid peptides mediate analgesia both centrally and peripherally and since opioids suppress both phase of formalin test, results of the formalin test is also an evidence to support the idea that antinociceptive action of AFE mediates *via* opioid mechanism.

Flavonoids and leucoanthocyanins are the characteristic constituents present in the AFE. Flavonoids are well known for suppressing the parameters of the formalin test and responsible for the anti-inflammatory action.²⁴ Flavonoids have ability to decrease the formation of free radicals and scavenge free radicals.²⁵ Generally oxygen derived free radicals cause tissue damage and as a result inflammatory responses are elicited in the body. Although the antioxidant activity of the AFE was not tested in this study, antioxidant activity due to flavonoids may play an auxiliary role against the inflammatory pain of the AFE. Several flavonol type flavonoids like quercetin, (+)-dihydroquercetin, kaempferol, (+)-dihydrokaempferol have been identified from the bark, wood, and the fruit of *D. retusa* plant.²⁶ Furthermore these flavonoids have shown the antinociceptive activity in other plants.^{27, 28, 29, 30} Therefore antinociceptive activity of AFE may be due to these types of flavonoids. Moreover the betulinic acid which has shown the anti-inflammatory activity and antinociceptive activity in other plants is also present in *D. retusa*.^{31, 32} This suggests that antinociceptive activity and anti-inflammatory activity of AFE may be also due to the betulinic acid.

CONCLUSION

The findings of the study scientifically show for the first time that fruit of *D. retusa* consists of acute, moderately strong antinociceptive activity with fairly long duration of action. The antinociception was mediated supra-spinally *via* opioid mechanisms. Although the fruit is applied externally to manage the pain in traditional medicine, this study suggests that AFE is also orally active against the pain. Therefore isolation and characterization of the active compound

causing analgesia may be helpful for the development of a new analgesic drug with highest efficacy, quality and safety.

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