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Antinociceptive Potential of the Sri Lankan Endemic Plant *Vernonia zeylanica*

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Abstract

In Sri Lankan traditional medicine, the endemic plant *Vernonia zeylanica* (L.) Less (Asteraceae) is recommended for the treatment of boils and bone fractures. A strong possibility thus exists that this plant could possess antinociceptive activity, but this has not been scientifically tested. The aim of this study was to investigate the antinociceptive potential of aqueous stem extract (ASE) of *V. zeylanica* in rats using two models of nociception (hot-plate and tail-flick tests) and different concentrations (750, 1500, and 2500 mg/kg, given orally). The results showed that ASE possessed marked and significant ($p < 0.05$) antinociceptive activity (in terms of prolongation of reaction time) when evaluated in the hot-plate test but not in the tail-flick test. The antinociceptive action had rapid onset (within 1 h) and moderately long duration of activity (up to 3 h), but was 2- to 5-fold weaker than morphine. Further, the ASE significantly suppressed the number of paw lickings and the time spent on paw licking in both phases of the formalin test. Naloxone, an opioid receptor antagonist, blocked the antinociceptive activity of ASE. Collectively, these observations suggest that ASE-induced antinociception was mediated centrally at the supraspinal level via opioid receptor mechanisms and is also effective against neurogenic and inflammatory pain. The ASE had mild antioxidant and moderate sedative actions, which are likely to contribute to its antinociceptive action. The ASE is well tolerated (in terms of overt signs, or renal, hepatic, or hemotoxicities) and nontoxic (by brine shrimp lethality assay). Phytochemical analysis revealed the presence of alkaloids, phenols, and steroids, which could contribute to the antinociceptive action. It is concluded that ASE of *V. zeylanica* has safe and moderate oral antinociceptive action.

Keywords: Antinociceptive activity, opioid mechanism, pain inhibition, *Vernonia zeylanica*.

Introduction

Vernonia zeylanica (L.) Less (Asteraceae), ''pupula'' in Sinhala and ''kuppilay'' in Tamil, is an endemic shrub in Sri Lanka. It is found in patanas and on dunes bordering forest scrub at sea level and up to 900 m. Shrubs are 1–2.5 m tall, usually erect or scrambling, occasionally prostrate. Young stems are densely pubescent or tomentose, later glabrescent. Leaves are pandurate or ovate, attenuate, subsessile and auriculate at the base, acute or obtuse at the apex, margins crenate-dentate, whitish pubescent on both surfaces, sparsely so on the upper surface and later glabrescent, dense or subtomentose on the undersurface. The tree flowers in the period June–April. The flowers are 6–8 per capitulum, corollas pale-mauve or violet, glandular, otherwise glabrous. Fruits are 2 to 2.5 mm long, brown, 4–5 ribs, glandular and puberulous (Dassanayake, 1980). Phytochemically, to our knowledge, no work has been reported.

In Sri Lankan traditional medicine, this plant, especially the stems, are recommended to be used in the treatment of boils and fractures of bones (Jayaweera, 1980). These uses suggest that the plant may have pain-relieving properties. As yet, however this has not been scientifically tested. In this study, we sought to explore the antinociceptive effect of the aqueous stem extract (ASE) of this plant in rats, using two models of nociception (hot-plate and tail-flick tests).

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Materials and Methods

Animals

Healthy adult, cross-breed male albino rats (weight 180–220 g) from our colony were used in the study. The animals were kept under standardized animal house conditions (temperature, 28–31°C; photoperiod, approximately 12-h natural light per day; relative humidity, 50–55%) with free access to pelleted food (Master Feed Ltd., Colombo, Sri Lanka) and tap water. Except at the time of the experimental procedures, the animals were handled only during cage cleaning. All efforts were made to minimize animal suffering and to reduce the number of animals used. The research was conducted in accordance with the internationally accepted principles for animal use and care.

Collection of plant

Aerial parts of *Vernonia zeylanica* were collected from Meerigama in the Gampaha district of Sri Lanka in August 2005 and identified and authenticated by Dr. (Ms) H. Kathriarachchi of the Department of Plant Science, University of Colombo, Sri Lanka. A voucher specimen (WDR/Papula 1) is deposited at the museum of the Department of Zoology, University of Colombo.

Preparation of the extract

The pieces of stem bark were washed under running water, air-dried, and cut into very small pieces. The pieces (800 g) were macerated with water and refluxed with distilled water (DW) for 2 days in a round-bottom flask fitted with a Leibig condenser. The dark-brownish colored solution was filtered and freeze-dried (29.9 g, yield 3.7%). The powder was stored air tight at room temperature (30–32°C). The freeze-dried powder was dissolved in DW to obtain the required dosages in 1 mL solution (750, 1500, or 2500 mg/kg). The mid-dose tested was 7.5 times higher than what is usually recommended by the traditional practitioners of Sri Lanka in prescribing herbal decoctions, which is within the accepted range for the rat model (Dhawan & Srimal, 2000).

Hot-Plate and tail-flick tests

Thirty-eight rats were selected and divided into five groups. Different concentrations of ASE or vehicle (control) were administered orally in the following manner: groups 1, 2, and 3 ($n = 8$ per group) with 750, 1500, and 2500 mg/kg ASE, respectively; group 4 ($n = 8$) with 1 mL DW; and group 5 ($n = 6$) with 15 mg/kg morphine sulfate (Pharmachemie B.V., Haarlem, The Netherlands) (an opioid receptor agonist) as a positive control.

Two hours before treatment (pretreatment) and then at hourly intervals for 6 h posttreatment, the rats were subjected to hot-plate and tail-flick tests (Langerman et al., 1995). A cut of time of 20 s was used to avoid tissue damage. In the hot-plate test, the time taken to lick either hind paw or to jump up (reaction time) when placed on an enclosed hot plate (Model MK 35 A; Muromachi Kikai Co., Ltd., Tokyo, Japan) maintained at 50°C was recorded. In the tail-flick test, time taken to flick the tail (the reaction time) when the tail was immersed (5–6 cm from the tip) in a water bath at 55°C was noted.

Investigation for opioid receptor mediation

Twelve rats were randomly divided into two groups. Those in group 1 ($n = 6$) were intraperitoneally injected with 5 mg/kg naloxone hydrochloride (Troikaa Pharmaceuticals Ltd, Gujarat, India) (an opioid receptor antagonist) and those in group 2 ($n = 6$) with isotonic saline. After 45 min, rats in both groups were orally administered 1500 mg/kg of extract. These rats were subjected to the hot-plate test before treatment (naloxone or saline) and 1 h after ASE treatment (Ratnasooriya & Dharmasiri, 1999).

Investigation for dopamine receptor mediation

Twelve male rats were randomly divided into two groups. Group 1 ($n = 6$) was orally treated with 1000 mg/kg metachlopramide (Glaxo SmithKline, Pakistan Ltd, Karachchi, Pakistan), dopamine antagonist in 1 mL 1% methylcellulose (Griffin and George Ltd., London, UK). Group 2 ($n = 6$) was orally treated with 1 mL 1% methylcellulose. One hour later, both groups of rats were orally treated with a 1500 mg/kg dose of ASE, and nociception was determined before treatment and 1 h posttreatment, using the hot-plate technique (Ratnasooriya & Dharmasiri, 1999).

Investigation for muscarinic receptor mediation

Twelve rats were divided into two equal groups. Those in group 1 ($n = 6$) were intraperitoneally injected with 2 mg/kg atropine sulfate (Laboratorre Renaudin, Paris, France) (a muscarinic receptor antagonist) and those in group 2 ($n = 6$) with isotonic saline. After 10 min, rats in both groups were orally administered 1500 mg/kg ASE. These rats were subjected to the hot-plate test before treatment (atropine or saline) and 1 h after extract treatment (Ratnasooriya & Dharmasiri, 1999).

Evaluation of membrane stabilization

To investigate the neuronal membrane stabilizing activity of ASE, the effect of ASE on heat-induced hemolysis in the rat erythrocyte model was tested (Ratnasooriya &

Dharmasiri, 1999). Briefly, 20 μL of uncoagulated, fresh rat blood was added into vials containing 1.00 mL of 0.15 M phosphate-buffered saline (PBS; pH 7.4) and to this either 5 μL of ASE in PBS (10, 15, 30, 60, or 125 μg/mL) (n = 6/concentration) or PBS (n = 8) was added. Thereafter, the vials were mixed thoroughly and incubated at 37°C for 15 min followed by 25 min at 54°C. The vials were centrifuged at 3200 × *g* for 5 min. The absorbance of the supernatant was measured at 540 nm using a spectrophotometer (Jasco V560; Jasco Corporation, Tokyo, Japan).

Evaluation of antioxidant activity (TBARS method)

Into snap-capped vials, 10 μL of different concentrations of ASE (15, 30, 20, 125 μg/mL) (n = 6/concentration) and egg yolk 50 μL were added. DW (10 μL) was used as the control. Acetic acid (20% solution, 150 μL) and 0.8% thiobarbituric acid (TBA; 150 μL) were added to each snap-capped vial. Total volume was adjusted to 400 μL by adding DW. These mixtures were vortexed for 5 s and kept in a water bath (LCH-110 Lab Thermo Cool; Advantec, Tokyo, Japan) at 95°C for 60 min. Butanol (1 mL) was added to each tube and vortexed for 5 s. After centrifuging at 1500 × g for 5 min, the butanol layer was separated. Absorbance values were measured at 532 nm (Dorman et al., 1995). Ascorbic acid, butylated hydroxytoluene (BHT), and vitamin E (100 μg/mL) were used as positive controls. Antioxidant index was calculated as follows:

$$\text{Antioxidant index} = (1 - T/C) \times 100$$

where T = absorbance of test and C = absorbance of control.

Evaluation of sedative activity

Eighteen rats were randomly divided into two equal groups and rats in group 1 were orally administered 1 mL DW and those in group 2 were administered with 1500 mg/kg ASE. After 1 h, each of these rats was tested for sedative activity in the rat hole-board test (File & Wardill, 1975). Briefly, 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, the number of rears, number of head dips, cumulative time spent on head dips, and locomotor activity were monitored. The time spent per head dip was then computed.

Evaluation of effects on muscle coordination and strength

Eighteen rats were treated orally either with 1500 mg/kg ASE (n = 9) or vehicle (n = 9) and 1 h posttreatment, the rats were subjected to the bar-holding test (to evaluate muscle strength) (Plaznik et al., 1993) and to Bridge (Plaznik et al., 1993) and righting reflex tests (Mortin et al., 1993) (to evaluate muscle coordination), and their respective latencies (in seconds) were recorded.

Formalin test

Eighteen rats were divided into two groups and were orally administered ASE or vehicle as follows; group 1 (n = 8) with 1500 mg/kg of ASE; group 2 (n = 10) with 1 mL vehicle. One hour after administration, each rat was subcutaneously injected with 0.05 mL 2.5% formalin solution (BDH Chemicals, Poole, UK) into the subplantar surface of the left hind paw. Rats were then observed for 30 min, and the numbers of lickings, flinchings, liftings, and time spent on licking the injected paw were recorded in two phases: First phase, 0–5 min, and second phase, 20–30 min.

Evaluation of effect on hematologic parameters, serum glutamic-pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), urea, and creatinine levels

Twelve rats were randomly assigned into equal groups. The rats in group 1 (n = 6) were treated with 2500 mg/kg ASE and group 2 with DW once a day (09.00–10.00 h) for 21 consecutive days. On day 21 posttreatment, blood (1.5–2.0 mL) was collected under mild ether anesthesia using aseptic precaution. The white blood cell (WBC) count, red blood cell (RBC) count, and different cell (DC) count of the fresh blood were determined using standard techniques (Ghai, 1993). Another aliquot of blood was allowed to clot at room temperature (28–30°C) and centrifuged at 3200 × *g* for 5 min. The serum was collected and the SGOT (EC 2.6.1.1), SGPT (EC 2.6.1.2), urea, and creatinine levels were determined using Randox kits (Randox Labotories Ltd., Co., Antium, UK) and a spectrophotometer per the manufacturer's instructions.

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

Rats used in the above investigation were closely observed each day of treatment (6–8 h) and on day 1 posttreatment for presence of overt sings of toxicity (salivation, diarrhea, yellowing of hair, postural abnormalities, behavioral changes, marked impairments of food and water intake and body weight) stress (fur erection and exopthalmia), and aversive behaviors (biting and scratching behavior, licking at tail, paw, and penis, intense grooming behavior). The rectal temperature of these rats was also determined using a clinical

thermometer (TM-II, normal glass; Focal Corporation, Tokyo, Japan).

Brine shrimp lethality assay

Brine shrimp (*Artemia salina* Leach) eggs (Ocean Star International, Snowaville, Utah, USA) were placed in a Petri dish containing seawater for 48 h. Freeze-dried powder (50 mg) was dissolved in 100 mL seawater to prepare stock solution of 500 μg/mL. From the stock solution, 10, 2, and 0.4 mL were transferred to a 50-mL volumetric flasks and made up with seawater so that the final concentrations of the solutions were 100, 20, and 4 μg/mL, respectively. The controls contained 50 mL seawater. Each concentration (n = 6/group) and controls (n = 6) contained 10 shrimp nauplii per Petri dish (60 shrimps per concentration). The dishes were allowed to remain at room temperature and the survivors were counted after 24 h (Mackeen et al., 2000) (any shrimp that was moving but not making forward progress was counted as dead). Percentage deaths at each dose were calculated, and the LC_{50} was determined by linear regression analysis.

Phytochemical analysis

Phytochemical screening of the ASE was carried according to Farnsworth (1978). Further, the ASE was also subjected to column chromatography on reverse-phase C-18 silica gel (Fluka Chemie G, Buchs, Switzerland) packed with water. The column was eluted with water, mixtures of methanol and water, methanol, mixtures of methanol and ethyl acetate, ethyl acetate, mixtures of ethyl acetate and methylene chloride, methylene chloride, mixtures of methylene chloride and hexane, and finally with hexane. The fractions showing similar spots under UV light in the thin-layer chromatograms were combined. The combined fractions were again subjected to thin-layer chromatography (TLC) (Aldrich silica gel precoated on plastic plates and Fluka Chemie G reverse-phase C-18 precoated glass plates). The mobile phases were 50% ethyl acetate in methanol for reverse-phase TLC and 15% methylene chloride in hexane for normal-phase TLC. The plates were sprayed with color reagents specific for various classes of compounds (Stahl, 1965) ($AlCl_3$ test for flavonoids; *para*-toluene sulfonic acid for steroids and flavonoids; vanillin–phosphoric acid for steroids; diazotized *para*-nitroaniline spray for phenols; Libermann Burchardt spray for triterpenoid glycosides; and Dragendorff's reagent and iodoplatinate reagent for alkaloids).

Analysis of data

Data are given as mean ± standard error of mean (SEM). Data were analyzed with Mann-Whitney *U*-test. Significance was set at $p \leq 0.05$.

Results

Hot-plate and tail-flick tests

The results obtained are summarized in Table 1. As shown, a 1500 mg/kg dose of ASE caused a significant ($p \leq 0.05$) prolongation of the reaction time in the hot-plate test from the first h to the third h compared with the control (first h by 33%, second h by 22%, and third h by 36%). A significant prolongation of the reaction time was also evident with 750 mg/kg (by 38%) and 2500 mg/kg (by 51%) ASE but only at the first h posttreatment. The antinociceptive effect of ASE at the first h ($r^2 = 0.87$, $p \leq 0.05$) was dose-dependent. Morphine provoked a huge and significant ($p < 0.05$) increase in the reaction time up to the third h post-treatment (first h by 89%, second h by 108%, and third h by 71%).

In contrast, in the tail-flick test there was no significant alteration ($p > 0.05$) in the tail-flick reaction time with any of the ASE-treated rats compared with control rats (data not shown).

Table 1. Effect of oral administration of different doses of aqueous stem extract (ASE) of *Vernonia zeylanica* on the hot-plate reaction time of rats.

	Hot plate reaction time (sec) (mean ± SEM)						
		Treatment					
Treatment (mg/kg)	Pretreatment	1 h	2 h	3 h	4 h	5 h	6 h
Control (n = 8)	8.2 ± 0.5	7.2 ± 0.4	7.7 ± 0.8	7.6 ± 0.6	8.8 ± 1.1	7.6 ± 0.9	8.0 ± 1.1
750 (n = 8)	7.9 ± 0.4	9.9 ± 0.4*	8.8 ± 0.9	7.8 ± 1.0	7.6 ± 0.9	7.6 ± 0.9	8.2 ± 0.6
1500 (n = 8)	7.5 ± 0.4	9.6 ± 1.1*	9.4 ± 0.6*	10.3 ± 1.2*	8.8 ± 1.0	8.6 ± 1.5	7.5 ± 0.6
2500 (n = 8)	8.4 ± 0.6	10.9 ± 0.8*	9.7 ± 1.3	9.8 ± 1.3	8.6 ± 0.7	7.7 ± 0.6	9.0 ± 0.8
Morphine (n = 5)	8.2 ± 0.8	13.6 ± 0.7*	16.0 ± 1.0*	13.0 ± 1.2*	11.5 ± 1.5	9.0 ± 1.5	9.4 ± 1.3

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

Table 2. The effect of naloxone intraperitoneal injection on the hot-plate reaction time of aqueous stem extract (ASE) (1500 mg/kg) of *Vernonia zeylanica*.

	Hot-plate reaction time (s) (mean ± SEM)	
Treatment	Pretreatment	First hour
Saline + extract (n = 6)	7.4 ± 0.5	8.8 ± 0.5*
Naloxone + extract (n = 6)	8.2 ± 0.5	6.9 ± 1.0

*Values are significant at $p \leq 0.05$.

Opioid receptor mediation

As shown in Table 2, with the hot-plate technique, intraperitoneal administration of naloxone significantly ($p \leq 0.05$) curtailed the prolongation of reaction time induced by 1500 mg/kg ASE.

Dopamine receptor mediation

Metochlopramide did not significantly ($p > 0.05$) impair the prolongation of reaction time induced by 1500 mg/kg ASE (metochlopramide + ASE vs. 1% methylcellulose + ASE: 8.0 ± 0.4 vs. 8.8 ± 0.4 sec).

Muscarinic receptor mediation

Intraperitoneal administration of atropine did not significantly ($p > 0.05$) impair the prolongation of reaction time induced by 1500 mg/kg ASE (atropine + ASE vs. saline + ASE: 10.0 ± 1.1 vs. 8.8 ± 0.5 sec).

Plasma membrane stabilizing activity

All the concentrations of ASE tested failed to significantly ($p > 0.05$) change the absorbance values in the heat-induced hemolysis test of rat blood cells (data not shown).

Antioxidant activity

ASE showed mild antioxidant activity compared with BHT, vitamin E, and ascorbic acid as indicated by the antioxidant index (Table 3).

Sedative effect

The treatment with 1500 mg/kg ASE significantly ($p < 0.05$) impaired three of the parameters monitored, namely number of rearings (by 46%), locomotor activity (by 38%), and number of head dips (by 45%) in the rat hole-board test compared with control (Table 4).

Table 3. In vitro antioxidant activity of the aqueous stem extract (ASE) of *Vernonia zeylanica*.

Sample	Concentration (μg/mL)	Antioxidant index
BHT (n = 9)	100	69.37 ± 2.21
Vitamin E (n = 9)	100	57.95 ± 1.05
Ascorbic acid (n = 9)	100	73.34 ± 2.66
ASE (n = 6)	15	39.85 ± 4.52
	30	30.54 ± 2.67
	60	36.95 ± 7.59
	125	38.40 ± 3.41

Antioxidant index = $(1 - T/C) \times 100$, where T is absorbance of extract and C is absorbance of control.

Muscle strength and coordination

None of the latencies of these three tests were significantly ($p > 0.05$) altered by a 1500 mg/kg dose of ASE: bar-holding test, control vs. treatment, 54.0 ± 4.2 vs. 58.1 ± 1.3 sec; Bridge test; control vs. treatment, 59.6 ± 0.3 vs. 58.9 ± 0.8 sec; and righting reflex test, control vs. treatments, 0.44 ± 0.04 vs. 0.39 ± 0.04 sec.

Formalin test

The ASE significantly ($p < 0.05$) impaired two parameters investigated both in the early and late phases (number of lickings in early phase; control vs. treatment, 14.4 ± 1.4 vs. 7.0 ± 1.0; number of lickings in late phase, 13.5 ± 2.5 vs. 7.5 ± 1.4; time spent on licking early phase, 80.7 ± 7.6 vs. 45.9 ± 2.3 sec; time spent on licking late phase, 56.8 ± 6.9 vs. 38.6 ± 3.6 sec).

Effect on hematologic parameters, SGPT, SGOT, urea, and creatinine levels

None of the enzyme levels (SGOT, control vs. treatment; 30.1 ± 1.2 vs. 30.7 ± 1.1 U/L; SGPT, control vs. treatment, 13.8 ± 0.3 vs. 14.0 ± 0.4 U/L), creatinine (0.6 ± 0.02 vs. 0.5 ± 0.02 mg/dL), urea (16.3 ± 0.3 vs. 16.9 ± 0.3 mg/dL) or hematologic parameters investigated (RBC count, control vs. treatment; $5.5 \pm 0.2 \times 10^6$ vs. $5.5 \pm 0.5 \times 10^6$ cells/mm^3; WBC count, $9.0 \times 10^3 \pm 1.7 \times 10^2$ vs. $9.0 \times 10^3 \pm 1.4 \times 10^2$ cells/mm^3; DC count: lymphocytes, 65.0 ± 1.8% vs. 64.8 ± 2.3%; neutrophils, 30.8 ± 0.9% vs. 29.7 ± 1.5%; monocytes, 1.2 ± 0.4% vs. 1.5 ± 0.6%; eosinophils, 0.7 ± 0.3% vs. 0.8 ± 0.3%; basophils, 0.0 ± 0.0% vs. 0.0 ± 0.0%) were altered significantly ($p > 0.05$) by the ASE.

Toxicity, stress, and aversive behavior

Subchronic treatment with ASE did not elicit overt signs of toxicity, stress, or aversive behavior. Further, none of

Table 4. Effect of orally administered aqueous stem extract (ASE) (1500 mg/kg) of *Vernonia zeylanica* on sedative parameters of the rat hole-board test (mean ± SEM) at 1 h posttreatment.

Treatment	Number of rears	Locomotor activity	Number of head dips	Total time spent on head dips (sec)	Time/head dip (sec)
Control (n = 9)	18.9 ± 2.3	19.6 ± 2.8	8.4 ± 1.3	14.06 ± 1.53	1.83 ± 0.20
1500 mg/kg (n = 9)	10.3 ± 3.2*	12.1 ± 1.4*	4.6 ± 0.9*	9.61 ± 1.99	1.99 ± 0.23

*Values are significant at $p \leq 0.05$.

the treated rats died during the study period. The rectal temperature was also not significantly ($p > 0.05$) altered (control vs. treatment, 101.0 ± 0.4°F vs. 101.2 ± 0.3°F).

Brine shrimp lethality assay

The percentage deaths of the concentrations 4, 20, 100 μg/mL and the control were 1, 2, 4, and 0, respectively. The effect was dose-dependent ($r^2 = 0.9$, $p < 0.05$) and the LC_{50} value was 1429.6 μg/mL.

Phytochemical analysis

Phytochemical screening of the extract showed the presence of alkaloids, flavonoids, steroids, triterpenoids, polyphenols, and saponins. The TLC of the water, methanol/water, methanol, methanol/ethyl acetate, ethyl acetate, ethyl acetate/methylene chloride, methylene chloride, methylene chloride/hexane fractions showed the presence of alkaloids (R_f 0.40, 0.71 for reverse-phase TLC; R_f 0.65, 0.73 for normal-phase TLC), flavonoids (R_f 0.66, 0.71, 0.76 for reverse-phase TLC) (R_f 0.40, 0.73, 0.09 for normal-phase TLC), steroids (R_f 0.62, 0.71 for reverse-phase TLC; R_f 0.64, 0.34, 0.13 for normal-phase TLC), phenols (R_f 0.98, 0.56, 0.23 for normal-phase TLC), and triterpenoid glycosides (R_f 0.61, 0.74, 0.71 for reverse-phase TLC; R_f 0.66 for normal-phase TLC) on spraying with characteristic reagents.

Discussion

The results show, for the first time, that the ASE of *Vernonia zeylanica* when given orally possesses antinociceptive activity as evaluated from the hot-plate test (in terms of prolonged reaction time) but not in the tail-flick-test. The positive results in the hot-plate test suggest that the ASE has antinociceptive activity against phasic transient pain and it is mediated centrally at the supraspinal level (Wong et al., 1994). Lack of a significant effect in the tail-flick test suggests that spinal mechanisms are not likely to be operative in the induction of antinociception by ASE (Wong et al., 1994). In the hot-plate test, the antinociceptive activity of the ASE had a rapid onset of action (within 1 h) and moderately long duration of action (up to 3 h), but its effectiveness was 2- to 5-fold lower than morphine, one of the most potent analgesics (Rang et al., 2003). Lack of motor deficiencies (in terms of Bridge, righting, and bar-holding tests) and hypothermia suggests that the ASE-induced antinociception was genuine. The mid-dose (1500 mg/kg) had the highest antinociceptive activity, whereas the low dose (750 mg/kg) and high dose (2500 mg/kg) had lower antinociceptive activities (in terms of prolongation of hot-plate reaction time). Such response may result from desensitization (Scuka & Mazrzymas, 1991) or down-regulation of receptors (Stewart & Badiani, 1993). Alternatively, lower activity of the antinociceptive effect with the high dose may have resulted from the coexistence of component(s) in the $extract_{max}$, which blocks pain inhibition pathways of the brain. Such a mode of action is proposed for opioid analgesics such as morphine (Roumy & Jean-Marie, 1998). Further, the ASE suppressed both phases of the formalin test. This indicates that the ASE has peripherally mediated antinociceptive action against both neurogenic and inflammatory pain (Farsam et al., 2000).

Food restriction induces antinociception in rats (McGivorn et al., 1979), but such a mode of action is unlikely, as food was available throughout the study period. Stress can give rise to analgesia (Rang et al., 2003); however, the ASE was not stressogenic (in terms of fur erection and exopthalmia). Therefore, in this study, antinociception was unlikely to be due to stress. Antinociceptive activity could be mediated from cholinergic mechanisms (Anonymous, 2000); however, ASE-evoked antinociception was not impaired by atropine, a muscarinic receptor anatagonist, indicating such a mode of action is unlikely to be operative here. Dopamine agonists are shown to act as analgesics (Rang et al., 2003); however, metachlopramide, a dopamine receptor antagonist, failed to inhibit ASE-induced antinociception. This suggests that the antinociception is not mediated via dopaminergic mechanisms. Antinociception can result from a membrane stabilizing effect and/or rising of the nociception threshold, as reported with some herbal drugs (Dharmasiri et al., 2003), but the ASE failed to inhibit heat-induced hemolysis of rat erythrocytes *in vitro*. Thus, ASE-induced antinociception was not mediated via membrane stabilization.

On the other hand, the antinociceptive activity of ASE was blocked by naloxone, an opioid receptor antagonist, indicating that the antinociception was mediated via opioid mechanisms (Wong et al., 1994). Opioids are known to inhibit both phases of the formalin test (Tjolsen et al., 1992) and a similar effect was seen with ASE, which provides additional support for the above inference. Several natural plant alkaloids induce antinociception through opioid mechanisms (Elisabetsky et al., 1995; Menzies et al., 1998). It is possible that ASE induced antinociception mainly via its alkaloid constituents that were present in the ASE. However, in the formalin test, the inhibition of pain at both initial and late phases could also arise from phenolic (de Campos et al., 1997) and steroidal (Miguel et al., 1996) constituents that were present in the extract.

Some sedatives have been shown to induce analgesia (Rang et al., 2003). Three of the parameters monitored in the hole-board test were significantly suppressed by ASE. Herbal drugs commonly used as sedatives in traditional medicine of several countries contain flavonoids to which their sedative actions are ascribed (Vincent et al., 2001; Akah et al., 2002). It is therefore likely that the flavonoids that were present in the ASE also may be involved in inducing sedation. Further, oxygen free radicals are implicated in pain (Halliwell, 1994). A mild antioxidant activity was present in the ASE when tested by thiobarbituric acid reactive substances assay. Thus, the mild antioxidant activity of ASE could also play a role in inducing antinociception. Antioxidative activity of the ASE may be attributed to flavonoids and phenols (Kim et al., 1996; Carlo et al., 1999) present in the ASE.

Subchronic treatment with ASE was well tolerated and did not produce overt signs of clinical toxicity (in terms of salivation, diarrhea, rectal temperature, yellowing of hair, postural abnormalities, behavioral changes, impairment of food and water intake and body weight) hepatotoxicity (in terms of SGOT and SGPT levels), neprotoxicity (creatinine and urea levels), hematotoxicity. Further, the ASE had an LC_{50} value of 1429.6 μg/mL in the brine shrimp lethality assay, indicating that it is nontoxic: in this study, LC_{50} value less than 1000 μg/mL is considered toxic (Meyer et al., 1982). This inference is further substantiated by lack of mortality after subchronic administration of the high dose of ASE.

In conclusion, the study shows for the first time that the ASE of *V. zeylanica* possesses moderate oral antinociceptive activity and that it may be useful as an herbal pain-alleviating agent.

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