International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 2(3): May-June 2012

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Pharmaceutical Sciences **Research Article……!!!**

Received: 05-04-2012; Revised; Accepted: 22-06-2012

ANTINOCICEPTIVE EFFECT OF INNER STEM BARK OF SRI LANKAN ENDEMIC PLANT, *KOKOONA ZEYLANICA*, IN RATS

U.E. Illangakoon[1], M.D.J. Wijayabandara[2], S.A. Deraniyagala[1], W.D. Ratnasooriya[3]*

1. Department of Chemistry, University of Colombo, Colombo 03, Sri Lanka
2. B.Pharm Program, Faculty of Medical Sciences , University of Sri Jayawardenapura, Sri Lanka
3. Department of Zoology, University of Colombo, Colombo 03, Sri Lanka

Keywords:

Kokoona zeylanica;

Antinociception;

Dopaminergic activity

For Correspondence:

Prof. W.D. Ratnasooriya

Department of Zoology,

University of Colombo,

Colombo 03, Sri Lanka

E-mail:

sd@chem.cmb.ac.lk

ABSTRACT

Kokoona zeylanica Thw is an endemic plant in Sri Lanka and has been used for many centuries past in the traditional system of medicine for alleviation of many ailments including headaches. The aim of this study was to scientifically investigate the antinociceptive potential of bark of Sri Lankan endemic plant, *Kokoona zeylanica* Thw. Different concentrations (750, 1500, 2500 mg kg^{-1}) of aqueous inner stem bark extract (AISBE) was prepared. AISBE was orally administered to rats and antinociceptive activity was determined using three models of nociception (tail flick, hot plate and formaline tests). The results showed that AISBE has significant ($P < 0.05$) and dose -dependent antinociceptive activity (when evaluated in hot plate and formaline tests but not in the tail flick test). The antinociceptive activity had a rapid onset (1 h) and fairly long duration of action (5 h).The AISBE induced antinociception was not blocked by naloxone or atropine but was inhibited by metochlopramide indicating dopaminergic mode of action. Further, AISBE had mild sedative and moderate antioxidant activities which may play an auxiliary role in inducing antinociceptive activity. AISBE was not toxic (in terms of mortality in brine shrimp assay) and was well tolerated (in terms of overt signs, renal, hepatic and haematological toxicities). It is concluded that AISBE of *K. zeylanica* has safe and moderate oral antinociceptive activity supporting its use as painkiller in traditional medicine.

INTRODUCTION

Sri Lanka is an island rich in floral biodiversity. Over 3500 flowering plants are found in this island, of which more than 1400 species have been used in the traditional health care systems. It has also been estimated that about 25 % of the islands endemic plants could be characterized as medicinal plants.[1] *Kokoona* species occur in the Asian subcontinent. *Kokoona zeylanica* Thw. (Sinhala- kokun, pottueta, wanapotu) is an endemic plant belonging to the family Celastraceae.[2] The powdered bark is used as a snuff to alleviate headaches. The powdered bark is mixed with water to a paste and is dried to form pieces of soap which are used by villagers in Sri Lanka.[3] It is reported that the inner stem bark is also used for the treatment of pimples and for removing marks from the face. The inner stem bark has also been used for treatment of snake bites , swollen joints and eye disease. The oil from the seeds has been used as an insect repellent.[2] Many of the plant based decoctions used in the treatment of diabetes mellitus contains the inner stem bark of *K. Zeylanica*.[4]

Phytochemical studies have led to the isolation of many triterpenoids from the inner stem bark and zeylasterone, demethylzeylasterone, zeylasteral, demethylzeylasteral (6-oxo phenolic triterpenoids), pristimerin, celastranhydirde and minor terpenoids from the outer stem bark of *K. zeylaninca.*[4,5] During the latter years this plant has been studied scientifically in order validate the claims made in use of it in folk medicine to alleviate many ailments.

Demthylzeylasteral has showed inhibitory action on hepatitis *C virus,*[6] antifertility,[7] antitumoural,[8] immunosuppressive and anti inflammatory[9,10]activity. Zeylasteral, demethyl zeylasteral have also shown antimicrobial activity.[11] Demethylzeylasterone has been shown to be an inhibitor of topoisomerase II alpha (IC(50) = 17.6 uM). Demethylzeylasterone selectively inhibits the growth of non small cell lung cancer (NCI-H460) and CNS glioma (SF-268) cell lines.[12] The aqueous bark extract of *k. zeylanica* has been also show to contain safe, moderate and acute hypoglyceamic activity.[4]

However, to our knowledge, there are no scientific studies reported on the antinociceptive activity of the inner stem bark of *K.zeylanica* to substantiate its ethno medicinal use. Hence, in the present study, the aqueous extract of inner stem bark (AISBE) was examined for its antinociceptive activity.

MATERIALS AND METHODS

Plant Material

The stem bark of *K.zeylanica* was collected from a mature tree in peak wilderness sanctuary in the Ratnapura district of Sri Lanka in June 2011 and was identified and authenticated by Dr. H. Kathriarachchi Department of Plant Science, University of Colombo. A voucher specimen (WDR/Kokun 1005) was deposited at Colombo University herbarium.

Preparation of the Extract

The outer grey bark of the *K.zeylanica* was removed by using a sharp knife. The pieces of yellow coloured inner bark were dried under the shade for two days and powdered by using a mechanical grinder. The powdered inner bark (750 g) was macerated with water and was then refluxed with 4 L distilled water (DW) for two days in a round bottom flask fitted to a Leibig condenser. The yellowish coloured solution was filtered and freeze-dried (10 g, yield 1.3 %) and stored air tight at room temperature (28 - 30 ^{0}C). The freeze-dried powder was dissolved in DW to obtain the required dosages in 1 ml solution (750,1500 or 2500 mg kg^{-1}). The mid dose tested was 7.5 times higher than that is usually recommended by the traditional practitioners of Sri Lanka in prescribing herbal decoctions.[13] These doses are within the accepted range for testing drugs in rat models.[14]

Experimental Animals

Healthy adult cross-bred albino male rats (weight: 175-225 g) were used in the study. The animals were kept in plastic cages under standardized animal house conditions (temperature: 28-31 °C, photoperiod: approximately 12 h natural light per day, relative humidity : 50-55 %) with continuous access to pelleted food (Master Feed Ltd, Colombo, Sri Lanka) and tap water. Except at the time of experimental procedures the animals were handled only during cage cleaning. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care, and guidelines and rules of the Faculty of Science, University of Colombo, for animal experimentation.

Evaluation of antinociceptive activity

Hot Plate and tail flick tests

Thirty five rats were selected and divided into four groups. Different concentrations of AISBE or vehicle (control) was administered orally in the following manner: Group 1 (n = 9) with 1 mL of

DW; Group 2 (n = 8) with 750 mg kg^{-1} of AISBE; Group 3 (n = 9) with 1500 mg kg^{-1} of AISBE and Group 4 (n = 9) with 2500 mg kg^{-1} of AISBE. Three to four hours before treatment (pre-treatment) and then at hourly intervals for 6 h post –treatment, these rats were subjected to hot plate and tail flick test.[15]

The cut off times were 20 s and 3 s for the hot plate (Model MK 35 A, Muromachi Kikai Co., Ltd., Tokyo, Japan.) and tail flick test respectively to avoid tissue damage.

Formalin test

Thirty three rats were divided into 4 groups and were orally administered with AISBE or vehicle as follows; Group 1 (n = 6) with 750 mg kg^{-1} of AISBE; Group 2 (n = 9) with 1500 mg kg^{-1} of AISBE ; Group 3 (n = 8) with 2500 mg kg^{-1} of AISBE; and Group 4 (n = 10) with 1mL vehicle. Three hours after administration, each rat was subcutaneously injected with 0.05 mL of 2.5% formaline solution (BDH Chemicals, Poole, UK) into the sub plantar surface of the left hind paw. Rats were then observed for 30 min and the numbers of licking, flinching, lifting, time spend on licking the injected paw were recorded in two phases. 1-5 min (1st phase) and 20 – 30 min (2nd phase).[16]

Mechanisms of analgesic activity

Investigation for opioid receptor mediation

Twelve rats were divided into two equal groups. Those in Group 1 were intraperitonially injected with 1.5 mg kg^{-1} of nalaxone hydrochloride (Troikaa Pharmaceuticals Ltd, Gujarat, India), an opioid receptor antagonist, and those in Group 2 with 1 mL of isotonic saline. One hour later, the rats in both groups were orally administered with 2500 mg kg^{-1} of AISBE. These rats were subjected to the hot plate test before treatment (nalaxon or saline) and 1h after AISBE treatment.[17]

Investigation for dopamine receptor mediation

Thirteen male rats were divided into two groups. Group 1 (n = 7) was orally treated with 1.5 mg kg^{-1} of metochlopramide (GlaxoSmithKline Pakistan Limited, Karachi, Pakistan), a dopamine antagonist in 1 mL of 1% methylcellulose (Griffin and George Ltd., London, UK). Group 2 (n = 6) was orally treated with 1mL of 1% methylcellulose. One hour later, both groups of rats were orally treated with 2500 mg kg^{-1} dose of AISBE and the nociception was determined before treatment and 1 h post- treatment, using the hotplate technique.[18]

Investigation for muscarinic receptor mediation

Twelve rats were divided into two equal groups. Those in Group 1 were intraperitonially injected with 2 mg kg^{-1} of atropine sulphate (Laboratorre Renaudin, Paris, France), a muscarinic receptor antagonist. Those in Group 2 were tested with 1 mL of isotonic saline. After 10 min, the rats in both groups were orally administered with 2500 mg kg^{-1} of AISBE. These rats were subjected to the hot plate test before treatment (atropine or saline) and 1h after AISBE treatment.[17]

Evaluation of effects on muscle coordination and strength

Eighteen rats were orally treated either with 2500 mg kg^{-1} of AISBE (n = 9) or vehicle (n = 9). One hour after treatment, these rats were subjected to the righting reflex test[19] bar holding test and Bridge test[20] and the respective latencies were recorded.

Evaluation of sedative activity

Eighteen rats were randomly divided into two equal groups. The rats in Group 1 were orally administered with 1mL of DW and those in Group 2 with of 2500 mg kg^{-1} of AISBE. After 1 h, each of these rats was placed on a rat hole –board apparatus and was given 7.5 min trial.[21] During this period, number of head dips, rears locomotory activity, feacal boluses were counted.

Evaluation of prostaglandin synthesis inhibition activity

The experiment was carried out according to Ratnasooriya *et a*l.,[18] using isolated dioestrous rat uteri. The spontaneous contractions of the uteri were recorded for 10 mins. The organ bath was treated in triplicate with the AISBE, so that the final concentrations of extract in the organ bath became 5, 10, 15 and 20 µg mL^{-1} and the contractions were recorded for further 10-15 min. The mean amplitude and frequency of contractions were determined.

Evaluation of membrane stabilization

The neuronal membrane stabilizing activity of *K.zeylanica*, was investigated on heat-induced haemolysis of rat erythrocyte model.[18] The concentrations of AISBE used were 0, 50, 500, 2000, 3500 and 5000 L, (n= 6). The absorbance was measured at 540 nm using a spectrophotometer (JascoV560, Jasco Corporation, Tokyo, Japan). The percentage inhibition of haemolysis with respect to control was calculated.

Evaluation of antioxidant activity

Ten µl of different concentrations of AISBE (62.5, 125, 250 µg mL^{-1}) (n = 6/concentration) and egg yolk (50 µL) were added into snap capped vials. DW (10 µL) was used as the control. Acetic

acid (20% solution, 150 µlL 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 shaken for 5 s and kept in a water bath (LCH-110 Lab Thermo Cool, Advantec, Tokyo, Japan) at 95 ^{0}C for 60 min. Butanol (1 mL) was added to each tube and again shaken for 5 s. After centrifuging at 1500 rpm for 5 min, butanol layer was separated. Absorbance values were measured at 532 nm.[22] Ascorbic acid, butylated hydroxytoluene (BHT) and vitamin E (100 µg mL^{-1}) were used as positive controls.

Antioxidant index was calculated as follows:

$$\textit{Antioxidant Index} = \left[1 - T/C\right] \times 100$$

where T = absorbance of test C = absorbance of control

TOXICOLOGICAL STUDIES

Evaluation of acute toxicity

Twelve rats were treated orally either with 2500 mg kg^{-1} AISBE (n = 6) or 1 mL of vehicle (n = 6) once a day (8:00-9:00 h) for 21 consecutive days. They were closely observed for the presence of overt signs of toxicity (salivation, diarrhoea, yellowing of fur, postural abnormalities, behavioural changes, breathing depression, marked impairments of food and water intake and body weight) stress (fur erection and exopthalmia), aversive behaviours (biting and scratching behaviour, licking at tail, paw and penis, intense grooming behavior) and Straub's tail reaction each day of treatment (6- 8 h) and on day 1 post–treatment. The rectal temperature of these rats was also determined using a clinical thermometer (TM-II, normal glass, Focal Corporation, Tokyo, Japan).

Brine shrimp leathality assay

Brine shrimp (*Artemia saline* Leach) eggs (Ocean Star International, Snowaville, USA) were placed in petri dish containing sea water for 48 h. Fifty milligrams of the freeze-dried powder of AISBE was dissolved in 100 mL of sea water to prepare stock solution of 500 µg mL^{-1}. From the stock solution 10, 2 and 0.4 mL were transferred to 50 mL volumetric flasks and made up with sea water so that the final concentrations of the solutions were 100, 20 and 4 µg mL^{-1} respectively. The controls contained 50 of sea water. To each petri dish 10 shrimp nauplii were introduced (for each concentration 6 petri dishes were prepared) and allowed to remain at room

temperature (28-30 ^{0}C) and the surviving nauplii were counted after 24 hours.[23] Percentage deaths at each dose were calculated and the LC_{50} was determined by linear regression analysis.

Evaluation of effects on haematological parameters, Serum Glutamic-Pyruvic Transaminase (SGPT), Serum Glutamic-Oxaloacetic Transaminase (SGOT), urea and creatinine levels

Twelve rats were treated either with 2500 mg kg^{-1} AISBE (n = 6) or 1 mL of vehicle (n = 6) once a day (8:00-9:00 h) for 21 consecutive days. On day 1 post-treatment, blood (1.5-2.0 mL) was collected from tail under mild ether anesthesia using aseptic precautions. The white blood cell (WBC) count, red blood cell (RBC) count and differential count (DC) of the fresh blood was determined using standard techniques.[24] Another aliquot of blood was allowed to clot at room temperature (28-30 ^{0}C) and centrifuged at 800 g for 5 min. The serum was collected and the SGOT (EC 2.6.1.1), SGPT (EC 2.6.1.2) and creatinine levels were determined using Randox kits (Randox Labotories Ltd., Co., Antrium, UK) and a spectrophotometer.

Chemical analysis

Phytochemical screening of the AISBE was carried to according to Farnsworth.[25] The AISBE was subjected to column chromatography (30 cm length and 3.7 cm diameter) on reverse phase C-18 silica gel (Fluka Chemie G). 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 dichloromethane. Fractions showing similar thin layer chromatography (TLC) spots were combine after inspecting under U.V light. The combined fraction were subjected to TLC(Aldrich silica get precoated on plastic and Fluka Chemie G reverse phase C-18 pre coated glass plates). The mobile phase were 10% dichloromethane in hexane, 30% dichloromethane in hexane for normal phase TLC and 50% ethyl acetate in methanol for reverse phase TLC. TLC plates were sprayed with colour reagents specific for various classes of compounds.[26]

Statistical analysis

The data were expressed as the mean ± S.E.M. Statistical analysis was performed using Mann-Whitney *U*-test. Significant values were set at $P \leq 0.05$. Linear regression analysis was preformed to assess dose- dependencies.

RESULTS

Hot Plate and tail flick tests

As shown in the Table 1, 2500 mg kg^{-1} dose of the AISBE 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 to the control values. The 1500 mg kg^{-1} of the AISBE showed antinociceptive action from first to third hour after treatment. In contrast, the 750 mg kg^{-1}g dose of AISBE showed a significant antinociception only in the first hour. The antinociception effects at second, fourth, fifth and sixth hours were dose dependent ($r^2 = 0.94$; $P < 0.05$), ($r^2 = 0.94$; $P < 0.05$), ($r^2 = 0.94$; $P < 0.05$) respectively.

There was no significant alteration ($P > 0.05$) in the tail flick reaction time in any of the AISBE treated rats as compared to the control (data not shown).

Formalin test

Table 2 shows the results of the formaline test. 2500, 1500 and 750 mg kg^{-1} doses of AISBE significantly ($P < 0.05$) impaired most of the test parameters both in the early and late phases: high dose [(early phase: number of flinching, number of lifting , number of licking and licking time) and in the late phase (number of lifting, number of licking and licking time)] , mid dose [(early phase: number of lifting, number of licking and licking time) and in the late phase (number of lifting, number of licking and licking time)] and low dose [(early phase: number of lifting, and number of licking by 27.6%) and in the late phase (number of lifting and number of licking] compared to the control values.

Mechanisms of analgesic activity

Opioid receptor mediation

Intraperitonial administration of nalaxone did not significantly ($p \geq 0.05$) impair the prolongation of reaction time induced by 2500 mg kg^{-1} of AISBE (treatment *vs* control; 9.85 ± 0.71 s *vs* 10.09 ± 0.65 s).

Dopamine receptor mediation

The results of the metochlopramide study is depicted in Table 3. As shown metochlopramide significantly ($p \geq 0.05$) and markedly curtailed the prolongation of reaction time induced by 2500 mg kg^{-1} of AISBE.

Muscarinic receptor mediation

Intraperitonial administration of atropine did not significantly ($p > 0.05$) impair the prolongation of reaction time induced by 2500 mg kg^{-1} of AISBE (treatment vs control; 13.28 ± 1.36 s vs 10.37 ± 0.77 s).

Effects on muscle coordination and strength

None of the latencies in the Bridge, bar and righting reflex tests were significantly ($p > 0.05$) altered by the 2500 mg kg^{-1} of AISBE (data not shown).

Sedative activity

The treatment with 2500 mg kg^{-1} of AISBE significantly ($p < 0.05$) impaired locomotory activity, number of head dips and number of faecal boluses produced in the rat hole-board test as compared to control (Table 4). However, cumulative dipping time and time per head dip were not significantly (p › 0.05) altered.

Evaluation of prostaglandin synthesis inhibition activity

The 5, 10, 15 and 20 µmL-1 concentrations of AISBE failed to significantly (p › 0.05) alter either the frequency or the amplitude of spontaneous contractions in the isolated uterine preparations of rat when tested for the prostaglandin synthesis inhibition activity (data not shown).

Evaluation of membrane stabilization

All the concentration of AISBE tested failed to induce a significant (p › 0.05) change in the percentage of inhibition in the heat induced-haemolysis test of rat blood cells(data not shown).

Evaluation of antioxidant activity

As shown in Table 5, the AISBE had a mild (compared to BHT control) but dose dependent ($r^2 = 1.0$; p ‹ 0.05) antioxidant activity (See Table 5).

Toxicological studies

Evaluation of sub chronic toxicity

Subchronic treatment of the AISBE did not elicit any overt signs of toxicity, stress or aversive behaviours. There was also no change in the rectal temperature or breathing depression (data not shown). Further, Straub's tail reaction was not seen in rats treated with AISBE.

Brine shrimp lethality assay

The AISBE was not lethal towards the brine shrimp nauplii (LC_{50} = 1790 ppm) (See Table 6).

Evaluaton of effect on haemotological parameters, serum SGPT, SGOT, urea and creatinine levels

Neither hematological parameters (RBC: control *vs* treatment: 5.5 x 106 ± 0.6 x 106 *vs* 6.9 x 106 ± 0.8 x 106 cells/mm3: WBC; 12.3 x 103 ± 9.2 x 103 *vs* 8.5 x 103 ± 3.2 x 103 cells/mm^3:

lymphocytes, 71.2 ± 2.9% *vs* 68.2 ± 3.2%; neutrophils, 33.5 ± 4.1% *vs* 29.5 ± 1.7%; monocytes, 1.5 ± 0.3% *vs* 1.8 ± 0.5%; eosinophils, 0.4 ± 0.1% *vs* 0.6 ± 0.2%; basophils, 0.00% *vs* 0.00%) nor serum enzyme levels (SGOT: control *vs* treatment 30.38 ± 0.99 vs 30.84 ± 1.34 U L^{-1} ; SGPT, 16.83 ± 4.79 *vs* 22.66 ± 0.80 U L^{-1}; urea, 16.56 ± 0.49 *vs* 17.00 ± 0.05mg dL^{-1}; creatinine, 0.55 ± 0.18 mg kg^{-1} *vs* 0.52 ± 0.02 mg dL^{-1}) was altered significantly (p › 0.05) by the AISBE.

Chemical analysis

Phytochemical screening of the AISBE showed the presence of primary, secondary and/or tertiary alkaloids. Quaternary base alkaloids were not present. AISBE also showed the presence flavanoids, tannins/polyphenols, steroids and/or terpenoids and saponins. The TLC of dichloromethane, ethyl acetate, methanol/ethyl acetate and methanol column fractions showed the presence of steroids, higher alcohol and ketones, phenols, triterpenoid glycosides. The TLC of methanol/ distilled water and the distilled water fractions showed the presence of alkaloids, triterpenoid glycosides on spraying with characteristic reagents (Table 7).

DISCUSSION

The AISBE when given orally possesses marked antinociceptive activity as evaluated from the hot plate (in terms of prolonged reaction time) and formaline test (in both early and late phases) but not in the tail flick test. Lack of motor deficiencies (in terms of Bridge, righting reflex and bar holding tests) suggest that the AISBE induced antinociception was genuine. The positive results in the hot plate test suggest that the AISBE induced antinociception is mediated centrally at supraspinal level[28] possibly acting on a descending inhibitory pathway.[29] Furthermore, it also indicates that the AISBE is effective against acute phasic nociceptive pain.[28] The AISBE induced inhibition of the early phase in the formaline test provides additional support of this inference.[16] On the other hand, impairment of the late phase of the formaline test indicates that the AISBE is effective against continuous inflammatory pain as well, possibly by inhibiting release of inflammatory mediators such as histamine , serotonin, bradykinin.[30] Furthermore, the suppression of the both phases of the formalin test indicates that the AISBE has peripherally mediated antinociceptive action as well.[16] The negative results obtained in the tail flick test[27] taken together with the positive results in the other two nociceptive tests rule out a spinally mediated antinociceptive action by AISBE inducing antinociception. However, with the test models used in this study it is not possible to judge whether the AISBE is effective against

neuropathic pain. The antinociception activity of AISBE had a rapid onset (within 1 h) and fairly long duration of action (about 5 h).The maximum pain relief was evident at 1 h.

Food restriction induces antinociception in rats[31] but such a mode of action is unlikely, as food was available throughout the study period. Stress can provoke analgesia.[32] However, AISBE was not stressogenic (as determined by absence of fur erection, exopthalmia and increase in number of faecal boluses during the hole-board test). Therefore, antinociception due to stress can be ruled out. Some sedatives induce analgesia.[33] Out of six parameters in the hole-board test, only three parameters (locomotory activity, number of head dips and number of faecal boluses) were moderately but significantly impaired by the AISBE. This suggests that the AISBE has mild sedative activity which could play an auxiliary role in the antinociceptive activity of AISBE.

The AISBE did not inhibit heat induced haemolysis of rat erythrocytes *in vitro*. Thus, the antinociception is unlikely to be due to membrane stabilizing effect and/or raising of nociception threshold as reported with some herbal drugs and local anaesthetics.[34,35] The AISBE did not induce a marked breathing depression or Straub's tail reaction. Further, the antinociceptive activity of AISBE was not blocked by naloxone, an opioid receptor antagonists.[36] Collectively, these data indicate that the antinociception is unlikely to be mediated *via* opioid mechanisms. On the other hand, the impairment of the numbers of licking, flinching, lifting, time spend on licking in the formaline test at both initial and late phases could arise from the presence of phenolic constituents[37] and steroidal constituents[38] as was present in the this AISBE.

Antinociception can be induced *via* cholinergic mechanisms.[39] However, AISBE-evoked antinociception was not impaired by atropine, a muscarinic receptor antagonist,[37] indicating that such a mode of action is unlikely to be operative here. The inability of the AISBE to induce overt clinical signs characteristic of cholinergic receptor stimulation lends further support to this notion. Prostaglandins induce pain. Prostaglandin synthesis inhibitors are potent analgesics.[32] However, antinociception in this study is unlikely to be mediated *via* prostaglandin synthesis inhibition as the AISBE failed to suppress contractions of isolated diestrous uterine preparations of rat. In contrast, dopamine receptor antagonist, metochloropromide,[36] markedly attenuated the antinociception induced by the AISBE. This suggests that the antinociception is mediated via dopaminergic mechanisms; dopamine is a neurotransmitter in the descending pain inhibition pathway in brain. Furhtermore, dopamine agonists are shown to act as analgesics.[32] Dopamine

agonists such as apomorphine, selegiline, entracaporn possess tertiary nitrogen[40] which could exist as quaternary salts in combination with plant acids. Some known dopamine agonist also possess phenolic groups.[18] The AISBE showed the presence of primary, secondary, tertiary alkaloids as well as phenolic constituents.

Oxygen free radicals are implicated with pain.[41] The AISBE had a mild antioxidant activity which could play an auxiliary role in inducing of antinociception. Subchronic treatment of the AISBE was well tolerated.; neither produced overt signs of clinical toxicity (in terms of salivation, diarrhea, body temperature, yellowing of hair, loss of hair, postural abnormalities, behavioral changes impairment of food and water intake and body weight), nor hepatotoxicity (in terms of SGOT , SGPT levels), nor nephrotoxicity (in terms of serum creatinine and, urea levels), nor haematotoxicity (as judged by standard haematological tests). Further, the AISBE had LC_{50} value of 1790 µg mL^{-1} in brine shrimp lethality assay, indicating that it is non toxic. (values less than 1000 µg mL^{-1} is considered as toxic).[42] These findings suggest that the AISBE has potent and safe antinociceptive activity when given orally, which could be used in the rationalization of the ethnobotanical use of *K. zeylanica.*

In conclusion, the findings of this study provide first scientific evidence for potent and safe, oral antinociceptive effect of Sri Lanka endemic plant, *K. zeylanica*, justifying its ethno medical use. The antinociceptive activity was primarily mediated via doperminergic mechanisms through its steroidal, alkaloidal and phenolic constituents. In addition, sedative and anti oxidant activities may play an auxiliary role in inducing antinociception.

Table 1 : The effect of oral administration of different doses of aqueous extract of *Kokoona zeylanica* inner stem bark (AISBE) on the hot plate reaction time of rats

Treatment AISBE (mg kg^{-1})	Hot plate reaction time (s) mean ± S.E.M						
	Pre treatment	Post treatment					
		1st hour	2nd hour	3rd hour	4th hour	5th hour	6th hour
750 (n = 8)	8.25 ± 0.76	12.15 ± 1.41*	8.99 ± 1.07	10.48 ± 1.82	9.86 ± 1.33	8.42 ± 1.02	8.45 ± 1.19
1500 (n = 9)	8.94 ± 0.57	13.03 ± 1.12*	11.38 ± 0.89*	12.41 ± 0.87*	9.32 ± 0.63	9.59 ± 0.52	10.83 ± 0.69
2500 (n = 9)	8.77 ± 0.46	12.36 ± 0.76*	12.31 ± 0.90*	11.67 ± 0.93*	11.31 ± 1.29*	12.24 ± 1.76*	11.66 ± 1.27
Control (n = 9)	8.26 ± 0.62	8.86 ± 0.90	8.64 ± 0.75	8.78 ± 0.63	8.15 ± 0.76	8.52 ± 0.91	8.51 ± 0.83

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

Table 2 : The effect of oral administration of different doses of aqueous extract of *Kokoona zeylanica* inner stem bark extract (AISBE) on formaline test (mean ± S.E.M).

Treatment AISBE (mg kg^{-1})	Early phase (0 – 5 minutes)				Late phase (20 – 30 minutes)			
	Number of flinching	Number of lifting	Number of licking	Linking time (s)	Number of flinching	Number of lifting	Number of licking	Linking time (s)
750	5.85 ±1.76	4.14 ±0.7*	10.43 ±1.06*	80.28 ±8.73	0.57. ±43	4.86±.88*	7.71±1.57*	40.23±
1500	3.14±1.03	6.14±1.14*	7.14±1.08*	53.57±5.89*	0.71±0.36	2.71±0.81*	7.43±1.21*	32.28±
2500	3.00±0.87*	1.14±0.46*	8.42±0.87*	62.71±6.61*	1.00±0.84	2.85±1.03*	7.00±0.93*	38.85±
Control	5.6±1.0	8.9 ±0.96	14.4 ±1.43	80.7±7.57	0.7 ±0.52	9.4 ±2.5	13.5 ±2.4	56.8±

* Values are significant at p≤ 0.05 vs. control

Table 3 : The effect of metochlopramide on the hot plate reaction time of aqueous extract of *Kokoona zeylanica* inner stem bark (AISBE) .

Treatment	Hot plate reaction time (s) mean ± S.E.M	
	Pre treatment	Post treatment (at 1st hour)
Metochlopramide + AISBE (n = 7)	7.68 ± 0.60	7.63 ± 0.41*
1% Methylcellulose + AISBE (n = 6)	7.91 ± 0.85	11.36 ± 1.47

* Value is significant at $p \leq 0.05$ vs. Control

Table 4 : The effect of oral administration of aqueous extract of *Kokoona zeylanica* inner stem bark extract(AISBE) on parameters of the hole board test (mean ± S.E.M).

Treatment	Number of Crossings	Number of rearings	Number of head dips	Cumulative dipping time (s)	Time/head dip (s)	Fecal boluses
Control (n = 9)	18.11±3.36	14.33±2.10	6.78±0.81	9.23±2.32	1.23±0.22	1.55±0.42
2500 mg kg^{-1} of AISBE (n = 9)	8.33±2.11*	11.78±1.86	3.55±0.94*	7.31±3.60	1.92±0.87	0.55± 0.24*

* Values are significant at p≤ 0.05 vs. control

Table 5 : Antioxidant index of different concentrations of *Kokoona* zeylanica inner stem bark extract (AISBE) (mean ± S.E.M)

Concentration (µg mL^{-1})	Antioxidant index*
AISBE 62.5	21.46 ± 5.58
AISBE 125	38.27 ± 6.01
AISBE 250	40.14 ± 7.16
BHT 100	69.36 ± 2.23
Ascorbic acid 100	73.34 ± 1.05
Vitamine E 100	57.95 ± 2.66

* *Antioxidant Index* $= \left[1 - \frac{T}{C}\right] \times 100$

where T = absorbance of test C = absorbance of control

Table 6 : Effect of *Kokoona zeylanica* inner stem bark extract (AISBE) on Brine shrimp lethality assay (mean ± S.E.M)

Concentration of AISBE (ppm)	Percentage death of nauplii
4	6.66 ± 3.33
20	10.0 ± 5.16
100	10.0 ± 5.16

Table 7 R_f values of the natural products present in the chromatographed aqueous inner stem bark extact (ASIBE) of *k. zeylanica*

Class of compound	**R_f values**
Steroids (from dichloromethane, ethyl acetate, methanol/ethyl acetate, methanol fractions)	0.66, 0.18
Higher alcohol and ketones (from dichloromethane, ethyl acetate, methanol/ethyl acetate and methanol fractions)	0.75, 0.67
Phenols (from dichloromethane, ethyl acetate, methanol/ethyl acetate and methanol fractions)	0.80, 0.75
Triterpenoid glycosides (from dichloromethane, ethyl acetate, methanol/ethyl acetate and methanol fractions)	0.69
Alkaloids (from the methanol /water fraction)	0.88, 0.82, 0.71
Triterpenoid glycosides (from the methanol /water fraction)	0.75,0.18

Stationary and mobile phase are described in the Methodology section

REFERENCES

1. Ganashan P., Balendira S., Dassanayaka MD., Country report to the FAO international technical conference on plant genetic resources. FAO Sri Lanka, 1996.
2. Jayaweera D.M.A., Medicinal Plants (Indigenous and Exotic) Used in Ceylon, with taxonomic updating by Lilani K Senaratne. Colombo, National Science Council of Sri Lanka 2006; Vol. 2: 26-7
3. Gunatilaka, A.A.L., "Triterpenoid quinonemethides and related compounds (celastroids). Chemistry of Organic Natural Products", In Hertz W, Kirby GW, Moore RE, Steglich W. Tamm Ch. New York, Springer 1996; 1–123
4. Dayananda P. D., Yapa W.B., Ratnasooriya W.D., **"**The effect of aqueous stem bark of *Kokoona zeylanica* Thw. on the blood level of mice", Pharmacognosy Magazine, 2008; Vol. 4: 308-14.
5. De León L., Beltrán B., Moujir L.M., "Antimicrobial activity of 6-oxophenolic triterpenoids", Planta Medica, 2005; Vol. 71: 313–19.
6. Jo M., Nakamura N., Kakiuchi N., Komatsu K., Qui M., Shimotohno K *et al*., "Inhibitory effect of Yunnan traditional medicines of hepatitis C viral polymerase", J Nat Med 2006; Vol. 60: 217–224.
7. Bai, J.P., Shi Y.L., Fang X., Shi Q.X., "Effects of demethylzeylasteral and celastrol on spermatogenic cell Ca^{2+} channels and progesterone-induced sperm acrosome reaction", European Journal of Pharmacology, 2003; Vol. 464: 9–15.
8. Ushiro S., Nakayama J., Fujiwara T., Komatsu Y., Sugimachi K *et al*., "New nortriterpenoid isolated from anti-rheumatoid arthritic plant, *Tripeterygium wilfordii,* modulated tumor growth and neovascularization", International Journal of Cancer 1997; Vol. 72: 657–663.
9. Tamaki T., Morota T., Kawamura H., Maruyama H., Kanekos A., Nunome S. *et al.*, "Immunosuppressive and anti-inflammatory effects of phenolic nortriterpenoids, demethylzeylasteral, from *Tripterygium wilfordii*", Natural Medicine, 1997; Vol. 51: 98–104.
10. Lin Z., Yang C., Zhang Y., Wang G., Xu X., Lu Q. *et al*, "Effect of demethylzeylasteral on the transformation of mouse spleen cell blast and the rejection of rat transplanted kidney", Chinese Journal of New Drugs, 2003; Vol. 12: 186–8.
11. De León L., Moujir L.M., "Activity and mechanism of the action of zeylasterone against *Bacillus subtilis*" , Journal of Applied Microbiology, 2008; Vol. 104(5): 1266–74

12. Fubacher T.R. and Gunatilaka A.A., "Cata;ytic inhibition of topoisomeras II alpha by demethylzeylasterone, a 6-oxophenolic triterpenoid from *Kokoona zeylanica*", Journal of Natural Products, 2001; Vol. 64:1294-6
13. Jayasinghe D.M., Ayurveda Pharmacopoeia. Sri Lanka, Department of Ayurveda, Colombo, 1979
14. Dhawan B.N., Srimal R.C., Laboratory Manual for Pharmacological Evaluation of Natural Products. International Center for Science and High Technology, Trieste, Italy 2000; 7-9.
15. Langerman L., Zakouski M.I, Piskoun B., Gran G.J., "Hot plate versus tail flick; evaluation of acute tolerance to continuous morphine infusion in the rat model", Journal of Pharmacology and Toxicology Methods, 1995; Vol. 34: 23-7.
16. Farsam H.M., Jahaniani F., "Anti-inflammatory and analgesic activity of *Biebersteinia multifida* DC root extract", Joutnal of Ethnopharmacology 2000; Vol. 71: 443-7.
17. Ratnasooriya W.D.,Dharmasiri M.G.,"Water extracts of *Psychotria sarmentosa* has analgesic and antihyperalgesic activities in rats",Medical Science Research,1999;Vol.27: 715-8.
18. Ratnasooriya W.D., Deraniyagala S.A., Bathige S.D.N.K., Goodnasekara C.L., Jayakody J.R.A.C., "Antinociceptive action of aqueous extract of the leaf of *Ixora coccinea* in rats', Acta Biologica, 2005; Vol.56: 21-34.
19. Mortein W.J., Lai N.K., Patrick S.L., "Antinociceptive actions of Cannabinoids following intraventricular administration in rats", Brain Research, 1993; Vol. 629: 300-304.
20. Plaznik A., Palejko W., Kotawski W., „The role of accumbence GABA-B receptors in the regulation of rat behavior", Neuroscience Research Communication, 1993; Vol. 12: 23-30.
21. File S.E, Wardill A.G., "Validity of head-dipping as a measure of exploration in modified hole-board", Psychopharmacology, 1975; Vol. 44: 53-7 .
22. Dorman H.J.D., Deans S.G., Nob R.C., Surai P., "Evaluation *in vitro* of plant essential oils as natural antioxidant", Journal of Essential Oil Research, 1995; Vol. 7: 645 – 51.
23. Ghai C.L., A Textbook of Practical Physiology, New Delhi, Jaypee Brothers Medical Publishers Ltd, New 1993; 119-202.
24. Mackeen M.M., Ali A.M., Lajis N.H., Kawazu K., Hassan Z., Amran M. Antimicrobial, antioxidant, antitumor promoting and cytotxic activities of different plant part extracts of *Garcinia atrviridis* Giff. Ex T. Anders, Journal of Ethnopharmacology, 2000; Vol. 72: 395-402.

25. Fransworth N.R., Photochemical Screening. University of Illinois.. USA, Chicago College of Pharmacy, 1996; 32-65.
26. Stahl E., Thin Layer Chromatography: A Laboratory Handbook, New York, Academic Press, 1965; 483-502.
27. Bluman A.G., Elementary Statistics. California, U.S.A, Addison Wesley Longmann, 1988; 599-623.
28. Wong C.H., Day P., Yarmush J., Zbuzek U.K., "Nifedipine-induced analgesic after epidural injections in rats", Anesthesia. Analgesia, 1994; Vol. 79: 303-6.
29. Richardson J.D., Anaosen L., Hargreaves K.M., "Antihyperalgesic effect of spinal connabinoids", European Journal of Pharmacology, 1998; Vol. 345: 145-153.
30. Tjolsen A., Berge O., Hunskaar S, Rosland J.H., Hole K.. "The formalin test: An evaluation of the method", Pain, 1992; Vol. 51: 5-17.
31. McGivern R., Berka C., Berntson G.G., Walker J.M., Sandman C.A., **"**Effect of naloxone on analgesia induced by food deprivation", Life Science, 1979; Vol.25: 885-888.
32. Rang H.P., Dale M.M., Ritter J.M., Moore P.K., Pharmacology 5th edition, Edinburgh, Churchill Livingstone, 2003; 352-366.
33. Anonymous. British National Formulary. The British Medical Association and the Royal Pharmaceutical Society of Great Britain, London, UK, 2003; 613.
34. Dharmasiri M.G., Ratnasooriya W.D., Thabrew M.I., "Water extract of leaves and stem of pre flowering but not flowering plants of *Anisomeles indica* possesses analgesic and antihyperalgesic activity in rats", Journal of Pharmaceutical Biology, 2003; Vol. 41: 37-44.
35. Ratnasooriya W.D., Galhena G., Liyanage S.S.P., Jayakody J.R.A.C., Ediriweera E.R.H.S.S., "Analgesic and antihyperalgesic activity of decoction of Sri Lankan *Scoparia dulcia* in rats", Journal of Tropical Medicinal Plants, 2003; Vol.4: 63-9.
36. Bennett P.N., Brown M.J., Clinical Pharmacology. USA New York, Churchill Livingstone, 2003; 342- 635.
37. de Campos R.O.P., Santos A.R.S., Vaz Z.R., Pinheiro T.R., Pizzolatti M.G., Filho V.C. *et al*., "Antinociceptive properties of the hydroalcoholic extract and preliminary study of a xanthone isolated from *Polygala cyparissias",* Life Science, 1997; Vol. 61: 1619-30.

38. Miguel O.G., Calixto J.B, Santos A.R., Messanna I., Ferrari F., Filho V.C., *et al.,* "Chemical and preliminary analgesic evaluation of Geraniin and Furosin isolated from *Phyllanthus sellowianus",* Planta Medica, 1996; Vol. 62: 146-9.
39. Anonymous. British National Formulary. The British Medical Association and the Royal Pharmaceutical Society of Great Britain, London, UK, 2000; 571.
40. Kaseda S., Nomoto M., Iwata S., "Effect of selegilline on dopamine concentration in the striatum of a primate", Brain Research, 1999; Vol. 81 :44-50.
41. Halliwell B., "Free radical antioxidants in human disease: Curiosity, cause or consequence", Lancet, 1994; Vol. 344: 721-4.
42. Meyer B.N., Ferrigni N.R., Putnum J.E., Jacobsen L.B, Nichols D.E., "Brine shrimp: A convenient general bioassay for active plant constituents", Planta Medica, 1982; Vol. 4: 31-4.