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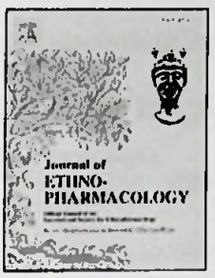

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Acute anti-inflammatory and anti-nociceptive activities of crude extracts, alkaloid fraction and evolitrine from *Acronychia pedunculata* leaves

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ABSTRACT

Ethnopharmacological relevance: *Acronychia pedunculata* (family: Rutaceae) is one of the commonly used medicinal plants in Sri Lankan traditional medicine. Different parts of this plant are used for the treatment of inflammatory conditions in the form of medicinal oils and herbal porridge.

Aim of the study: The present study aimed to evaluate the anti-nociceptive activity and anti-inflammatory activity with their mechanisms and the acute toxicity of crude extracts of the fresh leaves of *A. pedunculata* for scientific validation of the ethnopharmacological claims for this plant. Further, attention has been focused on the isolation of active compounds from active fractions of the crude extracts.

Materials and Methods: The acute anti-inflammatory effect of the aqueous (AELA) and 70 % ethanol crude extracts (EELA) and alkaloid fraction of *A. pedunculata* leaves were evaluated by the determination of inhibition of hind paw oedema induced by carrageenan in Wistar rats. Evolitrine was identified as the major alkaloid with significant bioactivities by column chromatography and NMR. The anti-nociceptive and anti-histamine activities of EELA and evolitrine were evaluated by acetic acid induced writhing and wheal formation tests respectively. In addition, *in-vitro* (2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay) and *in-vivo* (lipid peroxidation assay) anti-oxidant activity, nitric oxide inhibitory activity and acute toxicity of EELA were evaluated.

Results: Acute anti-inflammatory activity of AELA and EELA were dose-dependent. EELA was more active than AELA. The 200 mg/kg body weight (b. w.) dose of EELA was found as the minimum effective dose with maximum inhibition (78 %) of oedema at 5th hour compared to the negative control ($p < 0.05$). Evolitrine was isolated and identified as an active anti-inflammatory and analgesic compound from active alkaloid fraction of EELA. Evolitrine showed activity enhancement when compared with crude EELA. The anti-inflammatory and analgesic activities of evolitrine (50 mg/kg b. w.) were comparable to that of reference drugs indomethacin (5 mg/kg b. w) and acetylsalicylic acid (100 mg/kg b. w.). The significant ($p < 0.05$) anti-histamine activity, DPPH scavenging *in-vitro* anti-oxidant activity, *in-vivo* lipid peroxidation inhibitory activity *in-vivo*, NO inhibitory activity of EELA as compared with relevant negative controls, were identified as probable mechanisms which mediated its anti-inflammatory action. Further, EELA showed a high safety margin in the limited dose acute toxicity study.

Conclusion:

The findings of the current study rationalize the usage of leaves of *A. pedunculata* in Sri Lankan traditional medicine as an analgesic and anti-inflammatory agent. Possible mechanisms mediating this activity included anti-histamine, anti-oxidant and NO inhibitory activities. Evolitrine is the major analgesic and anti-inflammatory compound isolated from the active alkaloid fraction of EELA.

Key words: Anti-inflammatory, *Acronychia pedunculata*, Rutaceae, Evolitrine

1. Introduction

Inflammation is a protective response intended to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult (Kumar *et al.*, 2007). It is characterized by heat, redness, pain, swelling and loss of function (Chandra *et al.*, 2012).

Enzyme activation, extravasation of fluid, release of mediators, cells migration, breakdown and repair of tissue are the biochemical events which lead to inflammation. Even though inflammation is a body protective mechanism, inadequate control and inappropriate as in the case of against self-tissue, it can become the cause of injury and diseases (Kumar *et al.*, 2007). Steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are the widely used allopathic drugs in the management of inflammatory conditions. These drugs exert adverse effects such as gastric irritation which lead to the formation of gastric ulcers. Furthermore, these have shown limited success as treatments for all forms of inflammatory conditions (Arawwawala *et al.*, 2010). Hence, there is a need for continuous research focusing on the discovery of novel anti-inflammatory compounds with lesser or no adverse effects. In this context, the investigation of plant-based anti-inflammatory agents is an active area of current research.

Acronychia pedunculata (Family- Rutaceae) is one of the commonly used medicinal plants in Sri Lankan traditional systems of medicine. It is generally known as "*Ankenda*" in Sinhala, claw-flowered laurel in English and "*Kattukanni*" in Tamil (Jayaweera, 1982). Leaves, roots, barks and fruits of this plant have been used in folk medicine of different regions in the world for the treatment of diarrhoea, cough, asthma, ulcers, itchy skin, scales, pain, swellings, rheumatism and disorders with involvement of the inflammatory processes (Jayaweera, 1982). Also, they are used as anti-pyretic and antihaemorrhagic agents as well as aphrodisiacs (Han *et al.*, 2004). Various topical applications as well as orally administered drugs prepared from leaves, barks and roots of this plant, are used in Sri Lankan traditional medicine. It can also be found in countries such as Indonesia, Malaysia, Southern China, Hong Kong and Philippines (Jayaweera., 1982; Pathmasiri *et al.*, 2005; Su *et al.*, 2003).

A search of the literature on this plant revealed only a few studies on the chemical constituents and biological activities of *A. pedunculata*. Two furoquinoline alkaloids,

kokusaginine (0.1 % from the leaves) and evolitrine (0.05 % from the timber) have been reported from the Sri Lankan variety of this plant (De Silva *et al.*, 1979). However, the phytochemical screening of leaves and stem of Indian variety has shown the absence of alkaloids (Gireesha and Raju, 2016). Hence, these two phytochemicals may be tentatively considered as chemotaxonomic markers of *A. pedunculata* species of Sri Lanka.

Wu and co-workers have shown that the methanol extract of the stem and root bark of *A. pedunculata* has significant cytotoxicity in the human KB tissue culture assay. They isolated the polyphenolic dimeric acetophenone, acrovestone, as a cytotoxic principle from the extract by bioassay-directed fractionation (Wu *et al.*, 1989). Lesueur *et al.* (2008) have shown that essential oil isolated from the aerial parts, possess broad spectrum anti-microbial activity against various bacteria, particularly *Salmonella enterica* and *Staphylococcus epidemidis*. Kozaki (2014) and co-workers worked on dried leaves and twigs of this plant and they have shown the polymerase α and β inhibitory action of new acetophenone, Acronyculatin F, isolated from it. Pathmasiri *et al.* (2005) showed that acrovestenol which was isolated from a dichloromethane extract of the bark of this plant inhibited cyclooxygenase-2 (COX-2) with an IC_{50} value approximately thirteen times less than the COX-2 inhibitory reference compound (NS 398).

Although a number of acetophenone dimers (acrovestone, acrovestenol, acropyrone, acropyranol A, acropyranol B, acrofolione A, acrofolione B), an acetophenone monomer (acronyline) and furoquinoline alkaloids (dicatamine, pteleine, evolitrine, kokusaginine) have been isolated from timber, bark and leaves of *A. pedunculata*, (De Silva *et al.*, 1979; Wu *et al.*, 1989; Pathmasiri *et al.*, 2005; Kouloura *et al.*, 2012) there is a paucity of literature about the biological activity of those compounds. To the best of our knowledge, no work has been reported on *in-vivo* anti-inflammatory and anti-nociceptive activities of the extracts of this plant. The present study was focused on investigating the anti-inflammatory activity of the

aqueous and 70% ethanol crude extract of leaves of the *A. pedunculata* and carrying out activity guided separation of active compounds of extract. Since occurring of pain is a response to inflammation, anti-nociceptive activity was also evaluated. In addition, the anti-histamine, anti-oxidant (*in-vitro* and *in-vivo*) and NO scavenging activities were also investigated to explore the possible mechanisms of action by which *A. Pedunculata* exerts its anti-inflammatory effect. Although there have been no reports on toxicity in the ethnomedical usage of this plant an acute toxicity study was carried out to evaluate its safety.

2. Materials and methods

2.1 Ethical clearance

The protocol for animal experiments was approved by the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka (No. 30/14, 35/15). International guidelines and recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) were followed for the handling of animals. Assays were carried out at the Animal House and the Department of Biochemistry of University of Sri Jayewardenepura, Sri Lanka.

2.2 Animals

Healthy adult Wistar albino rats weighing 150-200 g were purchased from Medical Research Institute, Colombo 8, Sri Lanka. Male rats were used for all the experiments except the toxicity study. Rats were housed under standard conditions with a natural light-dark cycle and fed *ad libitum* with standard diet and clean fresh water. The animals were acclimatized for at least one week to the laboratory conditions before commencing experiments. Except at the period of experimental procedures the animals were handled only during cage cleaning. In all

experimental models, six animals were used in each group. All rats were deprived of food overnight (9.00 p. m to 7.00 a. m prior to conducting the experiment.

2.3 Chemicals

Carrageenan and histamine dihydrochloride, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid and carboxymethyl cellulose were purchased from Sigma-Aldrich Company (St. Louis MO, USA). Indomethacin, chlorpheniramine and acetyl salicylic acid were purchased from the State Pharmaceutical Corporation, Sri Lanka. All other chemicals and reagents were of analytical grade.

2.4 Plant material

Mature fresh *A. pedunculata* leaves were collected from a tree in the Colombo district (Latitude 6.8549, Longitude 79.9684), Sri Lanka. It was authenticated and a voucher specimen (KMR002) was deposited at National Herbarium, Department of National Botanic Garden, Peradeniya, Sri Lanka.

2.5 Preparation of crude leaf extracts of *A. pedunculata*

The collected fresh leaves were washed and cut into pieces. The aqueous extract of leaves of *A. pedunculata* (AELA) was prepared by refluxing 100 g of small pieces of fresh leaves with distilled water (500 mL) for 2 hours. The extract was filtered and the brownish filtrate was freeze dried. The extract yielded 2.6 g of yellowish brown coloured freeze dried powder (2.6 %). The 70 % ethanol extract of leaves of *A. pedunculata* (EELA) was made by refluxing 100 g of small pieces of fresh leaves with 70% ethanol (500 mL) for 2 hours. The extract was filtered. The dark greenish filtrate was evaporated under reduced pressure to dryness. The extract yielded 5.3 g of blackish brown semi-solid material (5.3 % w/w).

Both AELA and EELA were placed in airtight containers and stored in the refrigerator at 4 °C until used in the bioassay. The AELA and EELA were suspended in 0.5 % carboxymethyl cellulose (CMC) at a required concentration in 1 mL solution for oral administration to rats via oral feeding needles.

2.6 Phytochemical screening

The EELA was subjected to qualitative phytochemical screening for alkaloids, saponins, flavonoids, unsaturated sterols, triterpenes, leucoanthocyanins, tannins and polyphenols using standard procedures described in Fong *et al.* (1974). The EELA was tested by Wagner's and Mayer's reagents for alkaloids. The froth test, Liberman – Burchard test, Salkowski test, cyanidin test, gelatin salt reagent test and ferric chloride test were carried out to evaluate the presence of saponins, unsaturated sterols, triterpenes, flavonoids, tannins and polyphenols respectively.

2.7 Alkaloid fraction of EELA

The alkaloid fraction of EELA was extracted as follows.

The fresh leaves were dried in the oven at 40 ^{0}C to constant weight and powdered in a mechanical grinder. The dried and milled leaves (100 g) were refluxed with 70 % ethanol (500 mL) to obtain EELA as described in section 2.5. The extract yielded 7.4 g of a blackish brown semi-solid (7.4 w/w %). This was repeatedly extracted with hot HCl (2 N) until the extract no longer gave a positive result with Wagner reagent and Mayer's reagent. The combined acid extract was washed with chloroform (3 X 150 mL) using a separatory funnel. The acid extract was then basified with excess ammonia and extracted with chloroform (3 X 150 mL). The combined chloroform extract was evaporated to dryness under reduced pressure and the alkaloid fraction was obtained as a brown coloured solid (0.14 g). The

alkaloid fraction was analysis by thin layer chromatography (TLC) on silica with chloroform: ethyl acetate (9: 1 v/v) as the solvent system revealed the presence of a major alkaloid and traces of four other minor alkaloids. The visualization of alkaloids was achieved by spraying the TLC plate with the Dragendroff reagent.

2.8 Isolation and identification of evolitrine

The major alkaloid present in the alkaloid fraction of *A. pedunculata* leaves was isolated using a method described by De Silva *et al.* (1979) for the extraction of kokusaginine with some modifications. The brown coloured alkaloid fraction was recrystallized with petroleum ether (60 - 80 °C) to obtain white rod-shaped crystals. However, TLC analysis revealed the presence of two alkaloids in the crystals. The isolation of the major alkaloid from the mixture (0.103 g) was done by column chromatography on basic alumina (200 g, 56 cm X 2.5 cm) using dichloromethane as the mobile phase (flow rate 5 mL/min). The fractions (1.5 mL) were collected and TLC analysis was done to identify the presence of alkaloids. The fractions containing the major alkaloid (Fractions 38 – 60) were pooled and the solvent was evaporated. It yielded the major alkaloid as a white coloured powder (86 mg, 0.086 % w/w from dried leaves). Rechromatography of fractions 61 – 76, yielded a further 10 mg of the major alkaloid (0.01 % w/w from dried leaves). The purity of the isolated major alkaloid was checked by TLC on silica. The melting point was also determined. The molecular mass of the compound was determined using a MS 6130 attached to HPLC-1260 infinity, Quat Pump VL. The NMR spectra data were obtained from NMR Burke Ascend ™ 400 Hz. The structure of the compound established as evolitrine by comparison of its ^{1}H NMR and ^{13}C NMR with the literature (Pusset *et al.*, 1991).

2.9 Evaluation of acute anti-inflammatory activity on carrageenan-induced rat paw oedema

2.9.1 Evaluation of acute anti-inflammatory activity of AELA and EELA

Carrageenan-induced rat hind paw oedema test was used as an experimental model for the evaluation of acute anti-inflammatory effect as described by Winter *et al.* (1962) with some modifications. Wistar rats were randomly divided into six groups and baseline values of the left hind paw volume were taken at the zero hour using a plethysmometer (Letica Scientific Instruments, Barcelona, Spain). The negative and positive control groups were administered with 0.5 % (w/v) CMC and indomethacin in 0.5 % (w/v) CMC at 5 mg/kg b. w. respectively. The treated groups received two doses of AELA and EELA in 0.5 % (w/v) CMC, i.e. 100 and 500 mg/kg b. w. The volume administered was kept constant at 1 mL. After 1 hour, 0.1 mL of 1 % carrageenan suspension in 0.9 % saline solution was injected using a 27 G needle and 1 mL syringe into the subcutaneous tissue of the left hind paw of all rats under mild anesthesia. Thereafter, the volume of the injected paw of each of these rats was measured using the plethysmometer at hourly intervals up to 5 h. The degree of oedema was calculated by the paw volume increase ($V_0 - V_t$), where V_0 is the paw volume for each group before injection of carrageenan (at 0 h) and V_t is the paw volume for each group at time "t" (at 1, 2, 3, 4 and 5 h). The percentage inhibition of oedema was calculated by the following equation.

$$\text{Percentage inhibition of oedema} = \frac{[O_{NC} - O_T]}{O_{NC}} \times 100$$

Where, O_{NC} is the mean paw oedema of negative control at time "t" and O_T is the mean paw oedema in treated groups at time "t".

2.9.2 Estimation of an optimum effective dose of EELA for acute anti-inflammatory action

The method given in section 2.9.1, was used to evaluate the effective dose of EELA in acute anti-inflammatory action. The treated groups were administered with EELA in 0.5 % (w/v) CMC at the doses of 100, 200, 300 and 500 mg/kg b. w.

2.9.3 Evaluation of acute anti-inflammatory activity of alkaloid fraction of EELA

The acute anti-inflammatory activity of the alkaloid fraction of EELA (AF-EELA) was also evaluated as described in section 2.9.1. The treated groups were administered EELA and AF-EELA in 0.5 % (w/v) CMC at the dose of 100 mg/kg b. w.

2.9.4 Evaluation of acute anti-inflammatory activity of evolitrine

The method given in section 2.9.1, was also used to evaluate acute anti-inflammatory activity of evolitrine, at the doses of 25, 50, 75 and 100 mg/kg b. w. in 0.5 % (w/v) CMC. The increase in paw volume and percentage inhibition of oedema was calculated as described in section 2.9.1.

2.10 Evaluation of the anti-nociceptive activity of EELA and evolitrine

Anti-nociceptive activity was evaluated using the acetic acid-induced writhing method as described by Somchit *et al.*, 2004. Wistar rats were randomly divided into three groups (n = 6/group). The negative and positive control groups received 0.5 % (w/v) CMC and 100 mg/kg acetyl salicylic acid in 0.5 % (w/v) CMC respectively. The experimental group was given EELA in 0.5 % (w/v) CMC at the dose of 200 mg/kg b. w., the minimum effective dose with maximum inhibition of oedema as determined in the previous assay. After 1 hour of oral administration of vehicle (negative control group), standard drug (positive control group) or

plant extract (treated group), writhing was induced by the administration of 10 mL kg^{-1} b. w. of acetic acid solution (0.6% v/v) in normal saline, intraperitoneally. After two minutes of acetic acid injection, the rats were observed through a transparent box and the number of writhes was counted for a period of 20 consecutive minutes. The writhing movements were measured as a contraction of the abdominal muscles accompanied by stretching of the hind limbs. Anti-nociceptive activity was calculated compared to the respective controls by the following formula.

$$\text{Percentage anti-nociceptive activity} = \frac{W_{NC} - W_T}{W_{N.C}} \times 100$$

Where, W_{NC} is the mean number of writhes for the negative control group and W_T is the mean number of writhes for the group treated with the plant extract or the standard drug.

In order to evaluate the anti-nociceptive activity of evolitrine, a dose of 50 mg/kg b. w. evolitrine in 0.5 % (w/v) CMC was administered for the treated group and the experiment was repeated.

2.11 Evaluation of the anti-histamine activity of EELA and evolitrine

Anti-histamine activity was evaluated as described by Kumari *et al.* (2014) employing the wheal formation test. Wistar rats were randomly divided into three groups (n = 6 /group). The fur on the left lateral side of the back of each rat was shaven. After 24 hours, the negative and positive control groups received 0.5 % (w/v) CMC and chlorpheniramine in 0.5 % (w/v) CMC at the dose of 0.67 mg/kg b. w. respectively. The treated group received EELA (200 mg /kg b. w.) in 0.5 % (w/v) CMC which was found as the effective dose in acute anti-inflammatory assays. After 1 hour of oral treatment, histamine dihydrochloride in saline (50 µL of 200 µg/mL solution) was injected subcutaneously into the skin where the fur was shaven. Two minutes after the subcutaneous injection, the area of the wheal formed was

measured by drawing each wheal by placing a transparent sheet on the area of the shaven skin. Anti- histamine activity was expressed as percentage inhibition of wheal formation calculated as follows.

$$\text{Percentage inhibition of wheal formation} = \frac{A_{NC} - A_T}{A_{NC}} \times 100$$

Where, A_{NC} is the mean area of the wheal for the negative control group and A_T is the mean area of the wheal for the group treated with the EELA or standard drug.

This method was repeated to evaluate anti-histamine activity of evolitrine (50 mg/kg b. w.) in 0.5 % (w/v) in CMC by oral administration for the treated group.

2.12 Evaluation of anti-oxidant activity of EELA

2.12.1 *In-vitro* antioxidant activity of EELA by DPPH radical scavenging assay

The *in-vitro* anti-oxidant activity was assessed based on the scavenging activity of the stable DPPH free radical as described by Loizzo *et al.* (2010) An aliquot of 1.5 mL of 0.25 mM DPPH solution was mixed with 1.5 mL of EELA (3.125, 6.25, 12.5, 25 and 50 µg / mL). Similarly, a series of gallic acid (0.5, 1, 2, 3, 4 and 5 µg / mL) used as a reference substance. To avoid the interferences of absorbance by the colour of the test sample, the blank sample was prepared by mixing 1.5 mL of methanol and 1.5 mL of EELA for each concentration. The reaction mixture was allowed to reach the steady state at room temperature in the dark for 30 min after which the absorbance at 517 nm was measured. Methanol was used as the negative control. All the tests were performed in triplicate for each concentration. Anti-oxidant activity was measured in terms of radical sacvenging activity and the percentage scavenging effect was calculated using the following formula.

$$\text{Scavenging activity (\%)} = \frac{[A_0 - A_T]}{A_0} \times 100$$

Where, A_0 is the absorbance of the negative control and A_T is the absorbance of the test sample (EELA or gallic acid). The entire experiment was repeated thrice. The radical scavenging activity of test samples, was expressed as a mean of EC_{50} (µg / mL), which is defined as the mean concentration of the anti-oxidant required to lower the initial DPPH concentration by 50% in each experiment. It was determined by using the graph plotted with the mean concentration of triplicates of each test samples vs. percentage radical scavenging activity.

2.12.2 Evaluation of the *in-vivo* anti-oxidant activity of EELA by lipid peroxidation assay

The *in-vivo* anti-oxidant activity was evaluated by lipid peroxidation assay as described by Kumari *et al.* (2014) with some modifications. Wistar rats were randomly divided into two groups (n = 6/ groups). The negative control and test groups were orally administered 0.5 % (w/v) CMC and 200 mg/kg b. w. dose of EELA in 0.5 % (w/v) CMC for 28 consecutive days as a single daily dose. The volume administered was kept constant at 1 mL. On 29th day blood was drawn from rats' lateral tail vein following 12 hours fast and serum was separated to conduct the lipid peroxidation assay. Aliquots of the 200 µL of serum were deproteinized by adding 1 mL of 14 % trichloroacetic acid and 1 mL of 0.6% thiobarbituric acid (TBA). The mixture was heated in a water bath for 30 minutes to complete the reaction and then cooled on ice for 5 minutes. The absorbance of the coloured product was measured at 535 nm with a uv spectrophotometer. The concentration of malondialdehyde was calculated by using the formula of $C = A / \sum L$; where, A is the absorbance, $\sum$ is the molar extinction coefficient of the complex formed by malondialdehyde and TBA (1.56×10^5 M^{-1} cm^{-1}) (Devasagayam *et al.*, 2003), C is the concentration of malondialdehyde, L is the path length.

2.13 Evaluation of Nitric oxide radical scavenging activity of EELA

In this assay, nitric oxide generated from the sodium nitroprusside (SNP), was measured by the Greiss reaction as described by Jageta *et al.* (2004). An aliquot of 2 mL of SNP (10 mM) was mixed with 500 µL of phosphate buffered saline (50 mM) and 500 µL of EELA (50, 100, 200, 250, 300 and 500 µg/mL) or quercetin (20, 40, 80, 100 and 200 µg/mL). The same reaction mixture, without a test sample but, with an equivalent amount of ethanol served as a negative control. To avoid the interferences of absorbance by the colour of the test sample (EELA or quercetin), a blank sample was prepared by mixing 500 µL of the test sample with 2.5 mL of PBS. All samples were incubated at 25 °C for 150 min. After the incubation period, an aliquot of 1 mL was pipetted and mixed with 1 mL of freshly prepared Greiss reagent [A 1 % sulphanilamide in 5 % phosphoric acid (1.01 g sulphanilamide (assay 99 %) and 5.68 mL of phosphoric acid (assay 88 %) in 100 mL of distilled water) and 0.1 % N-(1-naphthyl)-ethylene diamine dihydrochloride (0.1 g in 100 mL of distilled water) were separately prepared and mixed together (1: 1 v/v) before the analysis]. The absorbance of the chromophore formed, was measured at 546 nm following an incubation period of 30 min. All the tests were performed in triplicate for each concentration. Anti-oxidant activity was measured in terms of nitric oxide scavenging activity and the percentage scavenging activity was calculated using the following formula.

$$\text{Scavenging activity (\%)} = \frac{[A_0 - A_T]}{A_0} \times 100$$

Where, A_0 is the absorbance of the negative control and A_T is the absorbance of the test sample.

The whole experiment was repeated thrice separately and the NO scavenging activity of test samples were expressed as a mean of EC_{50} (µg / mL), which is defined as the mean concentration of the anti-oxidant required to lower the radical concentration by 50% in each

experiment. It was determined by using the graph plotted with the mean concentration of triplicates of each test samples vs. percentage NO scavenging activity.

2.14 Limited dose acute toxicity study of EELA

The limit dose test was performed at the dose level of 5000 mg /kg b. w., in compliance with the Organization for Economic Co-operation and Development (OECD) guidelines No. 425, acute oral toxicity – fixed dose method (OECD, 2008). Female Wistar rats were randomly divided into two groups and fasted overnight with access only to water. Following the fast, control group and test group were orally administered 0.5 % (w/v) CMC and 5000 mg/ kg b. w. of EELA in 0.5 % (w/v) CMC respectively.

All the animals were observed individually for mortality and behavioural changes such as salivation, diarrhoea, loss of hair, postural abnormalities etc. for first 30 minutes, 1 h, 2 h, 4 h and 6 h after dosing and thereafter once a day for 14 consecutive days. The mortality and clinical signs, body weights and the food consumptions of rats were recorded during the period. All rats were sacrificed on the day 15 and blood samples were also collected. The separated serum was used to analyse biochemical parameters; alanine amino transferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), γ-glutamyl transferase (γ-GT), urea and creatinine.

2.15 Statistical Analysis

All the results were subjected to descriptive statistics and expressed as mean ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance test (ANOVA) followed by Bonferroni test method for post hoc analysis, using SPSS statistic 21 to determine the significance of the difference between the experiment groups. p- values < 0.05 was considered as statistically significant.

3. Results

3.1 Phytochemical Screening

The qualitative phytochemical screening of EELA showed strong positive observations for alkaloids with Mayer's test and Wagner's test. In addition, it revealed the presence of flavonoids, unsaturated sterols, triterpenes, tannins and polyphenols.

3.2 Isolation and identification of Evolitrine

Comparison the ^{1}H NMR and ^{13}C NMR spectra (Tables 3.1 and 3.2) with published data (Pusset *et al.*, 1991) clearly established the major alkaloid isolated as described in section 2.8 as evolitrine (Figure. 3.1). The LC-MS of the compound gave a clear peak for the $[M+H]^{+}$ ion at m/z 230. Our result is at variance with the report by De Silva *et al.* (1979) that kokusaginine (Figure 3.2) is the major alkaloid in the petroleum extract of the leaves of *A. peduculata*. However, evolitrine is reported by De Silva as the major alkaloid found in the petroleum extract of the timber of *A. peduculata*. We note that the melting point of evolitrine recorded by us (129 ^{0}C) is higher than that reported by Pusset *et al.* (1991) (114 ^{0}C).

Table 3.1 ^{1}H NMR chemical shifts for evolitrine

Protons	Observed	* Reported
5	8.15 d (9.28)	8.15 d (9.3)
6	7.1 dd (9.28, 2.5)	7.09 dd (9.3, 2.6)
8	7.35 d (2.5)	7.33 d (2.6)
10	7.06 d (2.8)	7.05 d (2.8)
11	7.58 d (2.8)	7.57 d (2.8)
OMe-4	4.44 s	4.43 s
OMe-7	3.96 s	3.95 s

$CDCl_3$, TMS as internal standard,δ , ppm, coupling constant in Hz are given in parenthesis
*Pusset *et al.*, 1991

Table 3.2 ^{13}C NMR chemical shifts for evolitrine

Carbon	Observed	* Reported
1	142.6	142.5
2	161.3	161.3
3	102.0	102.2
4	157.5	157.3
5	116.9	116.8
6	123.6	123.6
7	147.1	147.7
8	105.5	106.0
9	113.3	113.6
10	104.9	104.9
11	142.5	142.5
OMe-4	59.0	59.0
OMe-7	55.4	55.5

$CDCl_3$, TMS as internal standard,δ , ppm
*Pusset *et al.*, 1991

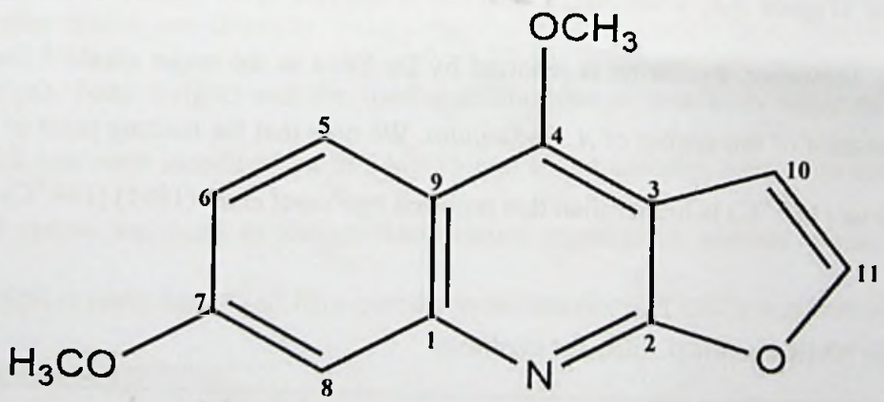

Figure 3.1 The structure of the evolitrine

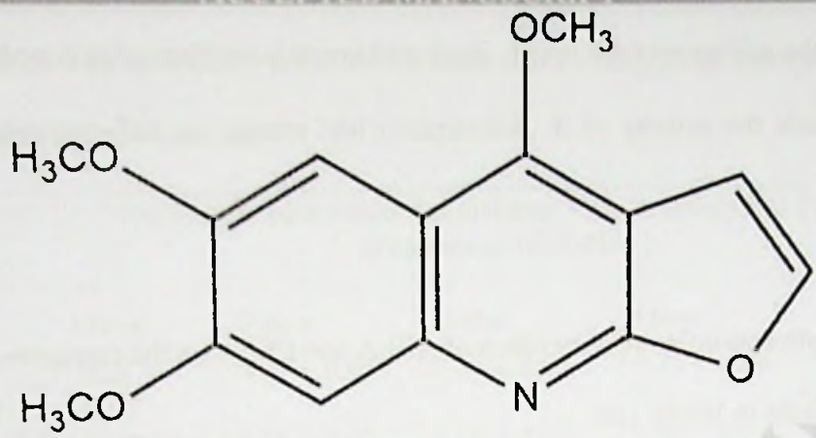

Figure 3.2 The structure of the kokusaginine

3.3 Effect of acute anti-inflammatory activity of AELA, EELA, AF-EELA and evolitrine on carrageenan-induced rat paw oedema

The subcutaneous injection of carrageenan into the hind footpad of rats produced local oedema in the following 1 h that progressed to its peak at 2 h and then began to decline. All doses of AELA and EELA showed the maximum percentage inhibition at the 5th h as did the standard drug indomethacin (Table 3.3). Both AELA and EELA at the dose of 500 mg/kg b. w., showed significant ($p < 0.05$) reductions in paw oedema as compared to the negative control. However, of the two extracts, only EELA showed a significant ($p < 0.05$) reduction at the lower dose of 100 mg/kg b. w. Hence, EELA was selected for further studies and dose response study was carried out to find out the minimum effective dose as shown in Table 3.4. All the test doses of EELA had significant acute anti-inflammatory activity as shown by significantly ($p < 0.05$) reduced paw oedema compared to the negative control. The maximum percentage inhibition on oedema was observed at the 5th hour as did indomethacin. As shown in Table 3.2, there is a significant ($p < 0.05$) increase in the inhibition of oedema, when the dose is increased from 100 to 200 mg/kg b. w. EELA. However, the differences in inhibition of oedema among the 200 mg/kg b. w. dose and the higher doses were not statistically significant ($p > 0.05$). Hence, the dose of 200 mg/kg b. w. of EELA was

identified as the minimum dose which gives maximum inhibition of paw oedema and it was used to evaluate the activity of *A. pedunculata* leaf extract on different anti-inflammatory mechanisms.

Table 3.3 Acute anti-inflammatory effect of AELA and EELA on the carrageenan-induced rat hind paw oedema in Wistar rats

Group	*Increase in paw volume at different time intervals (mL) ± SEM, (Percentage Inhibition)				
	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Group 1	0.30 ± 0.03	^{3} 0.61 ± 0.03	^{3} 0.54 ± 0.02	^{3} 0.48 ± 0.02	^{3} 0.38 ± 0.02
Group 2	0.20 ± 0.02 (33 %)	^{b} 0.32 ± 0.02 (48 %)	^{b} 0.20 ± 0.02 (63 %)	^{b} 0.10 ± 0.02 (79 %)	^{b} 0.04 ± 0.01 (90 %)
Group 3	0.24 ± 0.04 (20 %)	^{2} 0.50 ± 0.05 (18 %)	^{3} 0.42 ± 0.05 (22 %)	^{3} 0.36 ± 0.06 (25 %)	^{3} 0.28 ± 0.05 (26 %)
Group 4	0.22 ± 0.02 (26 %)	^{a} 0.43 ± 0.02 (30 %)	$^{b\,1}$ 0.35 ± 0.02 (35 %)	$^{a\,1}$ 0.28 ± 0.02 (42 %)	$^{b\,1}$ 0.20 ± 0.02 (47 %)
Group 5	0.21 ± 0.01 (30 %)	^{b} 0.35 ± 0.02 (43 %)	^{b} 0.25 ± 0.02 (54 %)	^{b} 0.16 ± 0.02 (67 %)	^{b} 0.11 ± 0.02 (71 %)
Group 6	^{a} 0.18 ± 0.01 (40 %)	^{b} 0.32 ± 0.01 (47 %)	^{b} 0.22 ± 0.02 (59 %)	^{b} 0.12 ± 0.01 (75 %)	^{b} 0.04 ± 0.01 (90 %)

*Difference between measured paw volume and paw volume before carrageenan injection
Figures in parenthesis indicate percentage inhibition of oedema
^{a} $p<0.05$; ^{b} $p<0.001$ compared with negative control, ^{1} $p < 0.05$; ^{2} $p < 0.01$; ^{3} $p < 0.001$ compared with positive control
Group 1: Negative control group (0.5 % CMC), Group 2: positive control group (5 mg/kg b. w., indomethacin in 0.5 % CMC), Group 3: Treated group (100 mg/kg b. w., AELA in 0.5 % CMC), Group 4: Treated group (100 mg/kg b. w., EELA in 0.5 % CMC), Group 5: Treated group (500 mg/kg b. w., AELA in 0.5 % CMC), Group 6: Treated group (500 mg/kg b. w., EELA in 0.5 % CMC)

Table 3.4 Acute anti-inflammatory effect of different doses of EELA on carrageenan-induced rat hind paw oedema in Wistar rats

Group	* Increase in paw volume at different time intervals (mL) ± SEM, (Percentage Inhibition)				
	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Group 1	[1] 0.24 ± 0.03	[3] 0.37 ± 0.03	[3] 0.34 ± 0.05	[3] 0.25 ± 0.04	[3] 0.18 ± 0.04
Group 2	0.17 ± 0.02 (30 %)	[c] 0.18 ± 0.02 (51 %)	[c] 0.10 ± 0.01 (71 %)	[c] 0.06 ± 0.01 (76 %)	[c] 0.02 ± 0.01 (89 %)
Group 3	0.19 ± 0.04 (21 %)	[b] 0.29 ± 0.05 (22 %)	[b] 0.23 ± 0.02 (32 %)	[b 2] 0.17 ± 0.08 (32 %)	[b 1] 0.10 ±0.03 (44 %)
Group 4	[a] 0.16 ± 0.02 (33 %)	[c] 0.22 ± 0.02 (41 %)	[c] 0.12 ± 0.02 (65 %)	[c] 0.06 ± 0.01 (76 %)	[c] 0.04 ± 0.01 (78 %)
Group 5	[a] 0.16 ± 0.02 (33 %)	[c] 0.21 ± 0.03 (43 %)	[c] 0.12 ± 0.01 (65 %)	[c] 0.05 ± 0.01 (80 %)	[c] 0.03 ± 0.02 (83 %)
Group 6	[a] 0.15 ± 0.01 (38 %)	[c] 0.20 ± 0.02 (46 %)	[c] 0.11± 0.02 (68 %)	[c] 0.05 ± 0.01 (80 %)	[c] 0.03 ± 0.01 (83 %)

*Difference between measured paw volume and paw volume before carrageenan injection
Figures in parenthesis indicate percentage inhibition of oedema
[a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$ compared with negative control, [1] $p < 0.05$; [2] $p < 0.01$; [3] $p < 0.001$ compared with EELA treated group with 200 mg/kg b. w dose.
Group 1: Negative control group (0.5 % CMC), Group 2: positive control group (5 mg/kg b. w., indomethacin in 0.5 % CMC), Group 3: Treated group (100 mg/kg b. w., EELA in 0.5 % CMC), Group 4: Treated group (200 mg/kg b. w., EELA in 0.5 % CMC), Group 5: Treated group (300 mg/kg b. w., EELA in 0.5 % CMC), Group 6: Treated group (500 mg/kg b. w., EELA in 0.5 % CMC)

The carrageenan-induced paw oedema model was also used to evaluate the acute anti-inflammatory effect of AF-EELA. The results showed that the dose of 100 mg/kg b. w. of EELA treated group as well as AF-EELA treated group have significant ($p<0.05$) acute anti-inflammatory activity as compared to the negative control group (Table 3.5). Similar to the indomethacin (5 mg/kg b. w.), both groups showed the maximum inhibition of oedema at the 5th h after the injection of carrageenan. Although the treated doses of EELA and AF-EELA are higher than that of indomethacin, the maximum inhibition of oedema was less than that of

indomethacin. Hence, acute anti-inflammatory action of both EELA and AF-EELA was lower than that of indomethacin. However, the maximum inhibition of oedema by AF-EELA, was approximately twice as that of EELA. Hence, AF-EELA was identified as the active fraction in EELA and it was subjected to experiments for isolation of compounds.

Table 3.5 Acute anti-inflammatory effect of AF-EELA on carrageenan-induced rat hind paw oedema in Wistar rats

Group	* Increase in paw volume at different time intervals (mL) ± SEM, (Percentage Inhibition)				
	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Group 1	0.29 ± 0.04	^{l} 0.56 ± 0.04	^{l} 0.49 ± 0.05	^{l} 0.31 ± 0.04	^{l} 0.22 ± 0.04
Group 2	0.18 ± 0.03 (38 %)	$^{l\,c}$ 0.26 ± 0.03 (54 %)	$^{c\,l}$ 0.14 ± 0.02 (71 %)	$^{b\,l}$ 0.09 ± 0.02 (72 %)	$^{b\,l}$ 0.02 ± 0.04 (91 %)
Group 3	0.22 ± 0.03 (24 %)	^{a} 0.42 ± 0.03 (25 %)	^{a} 0.30 ± 0.05 (39 %)	^{a} 0.20 ± 0.05 (36 %)	^{a} 0.12 ± 0.04 (45 %)
Group 4	0.24 ± 0.03 (17 %)	$^{c\,l}$ 0.28 ± 0.04 (50 %)	$^{c\,l}$ 0.20 ± 0.02 (60 %)	$^{a\,l}$ 0.12 ± 0.03 (61 %)	$^{b\,l}$ 0.04 ± 0.01 (82 %)

*Difference between measured paw volume and paw volume before carrageenan injection
Figures in parenthesis indicate percentage inhibition of oedema
^{a} $p<0.05$; ^{b} $p<0.01$; ^{c} $p<0.001$ compared with negative control, ^{l} $p < 0.05$ compared with EELA treated group with 100 mg/kg b. w dose
Group 1: Negative control group (0.5 % (w/v) CMC), Group 2: Positive control group (5 mg/kg b. w., indomethacin in 0.5 % (w/v) CMC), Group 3: Treated group (100 mg/kg b. w., EELA in 0.5 % (w/v) CMC), Group 4: Treated group (100 mg/kg b. w., AF-EELA in 0.5 % (w/v) CMC)

Evolitrine was identified as the major alkaloid present in the active alkaloid fraction of EELA and the optimally effective anti-inflammatory dose of evolitrine was determined by

carrageenan paw oedema model. The results revealed that all the test doses of evolitrine significantly ($p < 0.05$) reduced paw oedema as compared to the negative control (Table 3.6). Hence, all the test doses of evolitrine have significantly higher acute anti-inflammatory activity than the negative control. Further, all evolitrine doses showed their maximum percentage inhibition of oedema at the 5th h as did the indomethacin. Hence, the results from this study strongly indicate the NSAIDs' like activity of evolitrine.

In addition, there is a significant difference ($p < 0.05$) in oedema between the highest (100 mg/kg b. w.) and lowest (25 mg/kg b. w.) doses of evolitrine. However, the difference between the doses of 50 mg/kg b. w. and 100 mg/kg b. w. was not significant ($p > 0.05$). Hence, the dose of 50 mg/kg b. w. of evolitrine was found as the minimum effective dose with maximum inhibition of paw oedema. It is interesting to note that percentage inhibition of oedema by effective dose of evolitrine throughout the period of study, was comparable to indomethacin which was the positive control.

Table 3.6 Acute anti-inflammatory effect of the evolitrine on the carrageenan-induced rat hind paw oedema in Wistar rats

Group	* Increase in paw volume at different time intervals (mL) ± SEM, (Percentage Inhibition)				
	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
Group 1	[1] 0.25 ± 0.02	[3] 0.50 ± 0.02	[3] 0.48 ± 0.02	[3] 0.41 ± 0.02	[3] 0.35 ± 0.01
Group 2	[a] 0.17 ± 0.02 (32 %)	[c] 0.25 ± 0.03 (50 %)	[c] 0.16 ± 0.02 (67 %)	[c] 0.10 ± 0.02 (76 %)	[c] 0.05 ± 0.02 (86 %)
Group 3	0.20 ± 0.01 (20 %)	[c 2] 0.32 ± 0.02 (36 %)	[c 2] 0.22 ± 0.02 (54 %)	[c 2] 0.15 ± 0.02 (63 %)	[c] 0.09 ± 0.02 (74 %)
Group 4	[c] 0.16 ± 0.01 (36 %)	[c] 0.24 ± 0.01 (52 %)	[c] 0.18 ± 0.01 (62 %)	[c] 0.10 ± 0.01 (76 %)	[c] 0.04 ± 0.01 (89 %)
Group 5	[c] 0.14 ± 0.01 (44 %)	[c] 0.21 ± 0.01 (58 %)	[c] 0.14 ± 0.01 (71 %)	[c] 0.07 ± 0.01 (83 %)	[c] 0.03 ± 0.01 (91 %)
Group 6	[b] 0.15 ± 0.01 (40 %)	[c] 0.20 ± 0.01 (60 %)	[c] 0.11 ± 0.01 (77 %)	[c] 0.05 ± 0.01 (88 %)	[c] 0.02 ± 0.01 (94 %)

*Difference between measured paw volume and paw volume before carrageenan injection
Figures in parenthesis indicate percentage inhibition of oedema
[a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$ compared with negative control, [1] $p < 0.05$; [2] $p < 0.01$; [3] $p < 0.001$ compared with evolitrine treated group with 100 mg/kg b. w dose.
Group 1: Negative control group (0.5 % CMC), Group 2: Positive control group (5 mg/kg b. w., indomethacin in 0.5 % CMC), Group 3: Treated group (25 mg/kg b. w., evolitrine in 0.5 % CMC), Group 4: Treated group (50 mg/kg b. w., evolitrine in 0.5 % CMC), Group 5: Treated group (75 mg/kg b. w., evolitrine in 0.5 % CMC), Group 6: Treated group (100 mg/kg b. w., evolitrine in 0.5 % CMC)

3.5 Effect anti-nociceptive activity of EELA and evolitrine

As shown in the Table 3.7 and Table 3.8, the EELA and evolitrine restrained the writhing reflux induced by acetic acid with an inhibition percentage 25 % and 63 % respectively. This inhibition was compared with the positive control group which was received acetyl salicylic acid. There was a significant ($p < 0.05$) difference between rat groups treated with EELA and acetyl salicylic acid indicating that the anti-nociceptive activity of EELA was less than acetyl salicylic acid.

Evolitrine inhibits writhes by 63 % compared with 25 % by EELA at four times the dose of evolitrine. Thus, there is a considerable enhancement of activity in evolitrine when compared with EELA. Hence, it was identified as the major analgesic compound present in the *A. pedunculata* leaves. In contrast to the results of EELA, there was no significant ($p > 0.05$) difference between the positive control and evolitrine treated group. Further, acetyl salicylic acid inhibits writhes by 55 % by a dose at two times the dose of evolitrine. Thus, evolitrine has a higher anti-nociceptive activity than the acetyl salicylic acid.

Table 3.7 Anti-nociceptive effect of EELA on acetic acid induced writhing

Group	Number of writhes ± SEM (cm^2)	% Inhibition
Group 1	$^{\#\#}61 \pm 4$	-
Group 2	$^{**}29 \pm 2$	52
Group 3	$^{\#*}46 \pm 3$	25

Values for area are expressed as mean ± SEM (n=6/group)

* $P < 0.01$, ** $P < 0.001$ compared with negative control; $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.001$ compared with the positive control

Group 1: Negative control group (0.5 % (w/v) CMC), Group 2: positive control group (100 mg/kg b. w., acetyl salicylic acid in 0.5 % (w/v) CMC), Group 3: Treated group (200 mg/kg b. w., EELA in 0.5 % (w/v) CMC)

Table 3.8 Anti-nociceptive effect of evolitrine on acetic acid induced writhing

Group	Number of writhes ± SEM	% Inhibition
Group 1	$^{\#}$67 ± 4	-
Group 2	*30 ± 2	55
Group 3	* 25 ± 2	63

Values for area are expressed as mean ± SEM (n=6/group)
*$p < 0.001$ compared with negative control; $^{\#}p<0.001$ compared with the positive control
Group 1: Negative control group (0.5 % CMC), Group 2: positive control group (100 mg/kg b. w., acetyl salicylic acid in 0.5 % CMC), Group 3: Treated group (50 mg/kg b. w., evolitrine in 0.5 % CMC)

3.6 Effect anti-histamine activity of EELA and evolitrine

The EELA and evolitrine significantly ($p < 0.001$) inhibited wheal formation on the skin of the rat after the injection of histamine with 29 % and 35 % inhibition respectively as compared with the negative control group which received 0.5 % (w/v) CMC. However, both of EELA and evolitrine showed less anti-histamine activity as compared to the standard drug, chlorpheniramine (Table 3.9 and 3.10).

Table 3.9 Anti-histamine effect of EELA on wheal formation

Group	Area of wheal ± SEM (cm^2)	% Inhibition
Group 1	^{b}2.1 ± 0.2	-
Group 2	**0.7 ± 0.1	67
Group 3	^{a}* 1.5 ± 0.2	29

Values for area are expressed as mean ± SEM (n=6/group)
*$p < 0.05$, ** $p < 0.001$ compared with negative control; $^{a}p<0.01$, $^{b}p<0.001$ compared with the positive control

Group 1: Negative control group (0.5 % (w/v) CMC), Group 2: positive control group (0.67 mg/kg b. w., chlorpheniramine in 0.5 % (w/v) CMC), Group 3: Treated group (200 mg/kg b. w., EELA in 0.5 % (w/v) CMC)

Table 3.10 Anti-histamine effect of evolitrine on wheal formation

Group	Area of wheal ± SEM (cm^2)	% Inhibition
Group 1	[b] 2.0 ± 1.3	-
Group 2	** 0.9 ± 0.1	55
Group 3	[a] * 1.3 ± 0.1	35

Values for area are expressed as mean ± SEM (n=6/group)
*p < 0.01, ** p < 0.001 compared with negative control; [a] p<0.05, [b] p<0.001 compared with the positive control
Group 1: Negative control group (0.5 % (w/v) CMC), Group 2: positive control group (0.67 mg/kg b. w., chlorpheniramine in 0.5 % (w/v) CMC), Group 3: Treated group (50 mg/kg b. w., evolitrine 0.5 % (w/v) CMC)

3.7 The DPPH radical scavenging assay

The EELA exhibited dose dependent DPPH scavenging activity with an EC_{50} value of 33.4 ± 0.1 µg/mL. This activity is less than standard anti-oxidant, gallic acid, which has a EC_{50} of 2.4 ± 0.1 µg/mL.

3.8 *In-vivo* antioxidant activity by lipid peroxidation assay

The results have shown that serum MDA value of rats fed with EELA (1.1 ± 0.3 µmol / L) was significantly ($p < 0.05$) lower than that of the negative control group (1.8 ± 0.2 µmol / L).

3.9 Nitric oxide radical scavenging assay

The EELA exhibited dose-dependent nitric oxide scavenging activity with EC_{50} value of 209.7 ± 1.2 µg/mL and it was 108.6 ± 0.6 µg/mL for quercetin.

3.10 Limited dose acute toxicity study EELA

In the limited dose acute toxicity test, none of the rats in control and treated groups showed mortality and behavioural changes during the period of observation (14 days). Further, both

rat groups showed body weight gains and average food consumption throughout the study period. Table 3.11 shows the clinical chemistry profiles of each rat groups and there was no significant difference ($p > 0.05$) between EELA treated groups and healthy control group.

Table 3.11 Clinical chemistry data of Wistar rats in the limited dose acute oral toxicity study

Parameters	Group 1	Group 2
ALP (IU/L)	117.0 ± 2.7	121.4 ± 1.2
ALT (IU/L)	45.8 ± 2.3	48.6 ± 2.2
AST ((IU/L)	74.7 ± 1.8	74.7 ± 2.9
Creatinine (mg/dL)	0.7 ± 0.1	0.6 ± 0.1
γ- GT (IU/L)	22.6 ± 1.1	21.5 ± 1.3
Urea (mg/dL)	3.5 ± 0.2	3.9 ± 0.2

Values for clinical chemistry data are expressed as mean ± SEM (n=6/group).
*$p<0.05$ compared with healthy control
Group 1: Healthy control group (Distilled water), Group 2: Treated group (5000 mg/kg b. w., EELA in 0.5 % (w/v) CMC)

4. Discussion

In the present study, an attempt has been made to evaluate the anti-inflammatory effect of 70 % ethanol extract (EELA) and the aqueous extract (AELA) of *A. pedunculata* using the carrageenan-induced rat hind paw oedema model. Further, this model was also used to identify the active fractions and active compounds present in the EELA.

Carrageenan is the sulphated polysaccharide obtained from a sea-weed (Necas and Bartosikova, 2013), which is a widely used phlogistic agent which shows signs and symptoms of inflammation (Birada, 2010). Development of oedema in the paw of the rat following injection of carrageenan is a multimediated phenomenon that liberates diverse mediators. It is believed to be a biphasic event (Vinegar *et al.*, 1969). The initial phase

observed during the first two hours is attributed to the release of histamine and serotonin (Vasudevan *et al.*, 2006). The late phase of oedema is linked to the eicosanoid release, neutrophil infiltration and release of neutrophil-derived mediators and production of free radicals (Cuzzocrea *et al.*, 1998). The chemical substances produced in both phases, cause an increase in vascular permeability and thereby promote accumulation of fluid in tissues that accounts for oedema (Umukoro *et al.*, 2006). The results of the present study revealed that two crude extracts (AELA and EELA) and the alkaloid fraction of *A. pedunculata* leaves as well as evolitrine isolated from AF-EELA possess anti- oedema activity in the early and in late phases of carrageenan induced paw oedema model. These activities clearly indicate that all test extracts and evolitrine may have inhibitory effects on chemical mediators involved in both phases.

In this study, indomethacin was used as the positive control at a dose of 5 mg/kg b. w for the comparison of anti-oedema effect. Indomethacin, a cyclooxygenase inhibitor, exerted the maximum anti-oedema effect during the second phase of oedema due to the reduction of prostaglandins which are the second phase mediators. Further, it has been reported that the second phase of oedema is sensitive to most clinically effective anti-inflammatory agents (Della *et al.*, 1968). It is interesting to note that all tested fractions / compounds (AELA, EELA, AF-EELA and evolitrine) showed maximum inhibition of oedema at the fifth hour as did the indomethacin. These results may provide some evidence for the inhibitory activity of prostaglandin by *A. pedunculata* leaves.

The current results showed that EELA is more active than the AELA in the acute anti-inflammatory action. This activity increment could be due to the possibility of median polar active compounds in the EELA is more soluble in ethanol than water. As EELA showed more

activity, the carrageenan-induced rat paw oedema model was used to find the optimal anti-inflammatory dose of EELA. The dose of EELA (200 mg/kg b. w.) was found as the minimum effective dose with maximum inhibition of paw oedema (78 %). Although no literature is available on *in-vivo* anti-inflammatory activity of *A. pedunculata* leaves several other Rutaceae members have demonstrated similar activity i. e. 200 mg/ kg b. w of ethanol extract of *Pleiospermium alatum* (53 %; Suky *et al.*, 2011), 400 mg/ kg b. w. of ethanol extract *Aegle marmelosa* (30 %; Raju *et al.*, 2016), 200 mg/ kg b. w. of methanol extract *Chloroxylon sweitenia* (52 %; Sivakumar *et al.*, 2005), 250 mg/ kg b. w. of ethanol extract *Murraya koenigii* (52% ; Darvekar *et al.*, 2011), 20 mg/kg b. w. of methanol extract *Ruta graveolens* (91 %; Ratheesh and Helen, 2007) etc. According to these literature reports, a comparatively stronger anti-inflammatory activity is evident in leaves of *A. pedunculata* (except the *Ruta graveolens*) among the Rutaceae members. Thus, these data provide scientific evidence for the comparatively strong anti-inflammatory activity of leaves of *A. pedunculata* commonly used in Sri Lankan traditional medicinal system.

Many beneficial medicinal effects of plant materials typically result from the combinations of phytochemicals which are derived as secondary products of the plants. Different plants are rich in different secondary products such as alkaloids, flavonoids, sterols, terpenoids, phenolic acids and many others. The abundance of scientific evidence indicates that such compounds have biological effects such as anti-microbial activity, antioxidant activity, anti-inflammatory activity, modulation of detoxification of enzymes, modulation of hormones, stimulation of immune system etc. (Nyamai *et al.*, 2016). Hence, in the process of active compound isolation, phytochemical screening provides important information to conduct the study forward. The strong positive observations of EELA with Mayer's test and Wagner's test indicated that it is rich with alkaloids. As, various types of alkaloids isolated from

medicinal plants of family Rutaceae have shown anti-inflammatory activity (Iman *et al.*, 2017; Kuo *et al.*, 2004; Ratheesh *et al.*, 2013) and as our test extract also is rich in alkaloids, the study was focused on evaluating the anti-inflammatory activity of the alkaloid fraction of EELA. As the alkaloid fraction showed the anti-inflammatory activity enhancement at the 5th hour on carrageenan-induced oedema it was identified as a fraction containing the active compounds. Subsequently, evolitrine was identified as the major alkaloid compound present in EELA. The minimum effective dose with maximum inhibition of oedema of evolitrine (50 mg/kg b. w.) is comparable with the acute anti-inflammatory activity of doses of 100 mg/kg b. w. of the crude EELA and its alkaloid fraction, as well as with the standard drug indomethacin (5 mg/kg b. w.) at the 5th hour of the experiment. Although the dose of evolitrine (50 mg/kg b. w.) is lower than that of EELA (100 mg/kg b. w.) and the AF-EELA (100 mg/kg b. w.), it shows more inhibition of oedema. Hence, evolitrine from *A. pedunculata* leaves was identified as an active compound with anti-inflammatory properties.

Evolitrine has previously been isolated from the bark and leaves of *E. litoris*, timber of *Acronychia pedunculata*, bark of *E. belahe ballion*, stem barks of *Dutaillyea drepacea*, leaves of *Melicope indica*, wood of *Esenbeckia* species, root bark and aerial parts of *Evodia lunu-ankenda* (Lal *et al.* (2005). Further, Lal and co-workers (2005), isolated evolitrine from dichloromethane extract of twigs of *E. lunu-ankenda* and acute anti-inflammatory activity was evaluated on carrageenan-induced rat hind paw oedema model by only measuring the paw volume at 3rd h after the injection of carrageenan. As they have shown, evolitrine reduced the rat paw oedema in a dose-dependent manner and showed the maximum percentage inhibition (78%) at the 3rd hour for the highest tested dose of 60 mg/kg b. w. Although we have measured the paw volume of rats at 1 h intervals up to 5 h, our results at 3rd hour were comparable with the reported anti-inflammatory activity of evolitrine by Lal *et*

al. (2005). In the present study, 50 mg/kg b. w. of evolitrine showed 62 % paw oedema inhibition at 3rd hour.

In addition to the anti-inflammatory activity, the peripheral analgesic activity was also evaluated using the acetic acid-induced writhing test. Acetic acid is responsible for an increased level of prostaglandin-E_2, prostaglandin-F_2, serotonin and histamine in peritoneal fluid, hence causing inflammatory pain by inducing capillary permeability (Adeapo and Orherhe, 2013). Further local peritoneal receptors are postulated to be partly involved in the abdominal constriction response. The percentage of inhibition on the number of writhes obtained with an effective dose of EELA and evolitrine indicate that they possess peripheral analgesic effect.

The anti-nociceptive activity of evolitrine was comparable with reference drug acetylsalicylic acid. The acetylsalicylic acid can inhibit cyclo-oxygenase in peritoneal tissues thus interfering with mechanism transduction in primary afferent nociceptors. The responsive results of acetic acid-induced writhing suggest the mechanism of evolitrine may be linked partly to the blockade or release of endogenous inflammatory substances.

Histamine is one of the most common and powerful inflammatory mediators in the first phase of the carrageenan-induced paw oedema model. It causes symptoms of allergic reactions that are mostly involved in acute inflammation mediated by the histamine H_1 receptor. The histamine H_1 receptor mainly contributes to vasodilation, increased of vascular permeability and pain at the cellular level (Yong *et al.*, 2013). As histamine is one of the mediators involved in the initial phase of carrageenan-induced oedema, the anti-histamine activity could contribute to the impairment of the early phase of carrageenan induced paw oedema. Hence,

the observed low anti-histamine activity of EELA and evolitrine may still contribute to their anti-inflammatory activity.

The oxygen free radicals can be generated enzymatically during the later phase, by migrated leukocytes, macrophages and damaged endothelial cells at the site of inflammation (Boughton-Smith *et al.*, 1993). These play a major role in virtually every step of the development of inflammation. Vascular permeability, cellular infiltration and tissue damage can be increased by oxygen free radicals (Boughton-Smith *et al.*, 1993; Guzik *et al.*, 2003) which play a key role in the regulation of immune responses (Reddy and Urooj, 2013). The plant extracts which are having free radical scavenging properties, act as anti-oxidants and thereby act as anti-inflammatory agents. In the present study, antioxidant activity of EELA was evaluated using *in-vitro* DPPH free radical scavenging activity and *in-vivo* lipid peroxidation assay to get more insight into the mechanism of action. As shown in the results EELA has significant ($p < 0.05$) *in-vitro* free radical scavenging activity. This indicates that the EELA possesses significant anti-oxidant effect, which may have contributed to the inhibition of inflammation during the late phase. This activity may be mediated by compounds such as flavonoids, tannins and polyphenols present in the EELA, as revealed by the phytochemical screening of the extract.

Further, reactive oxygen species induced lipid peroxidative tissue damage has been implicated in the pathogenesis of various chronic inflammatory diseases including arthritis (John and Shobana, 2012). Lipid peroxidation is assessed indirectly by the measurement of the secondary products such as malondialdehyde (MDA) which is a spontaneous breakdown product of peroxides that can be produced from the free radical attack on polyunsaturated fatty acids (Tukozkan *et al.*, 2006). Our study reports the reduction of MDA level in rat

serum by the treatment of EELA, indicating that it contains significant ($p<0.05$) *in-vivo* anti-oxidant activity. This results further confirmed the antioxidant effect, which may have probably contributed to the inhibition of inflammation during the late phase.

Although nitric oxide (NO) acts as an important physiological messenger and effector molecule in many biological systems where it is present in low concentrations, high concentrations of NO causes adverse effects mainly pro-inflammatory actions (Antosova *et al.*, 2012). Among the different types of nitric oxide synthase (NOS), Type II or inducible NOS is the major type of NOS which plays a significant role in inflammation. The iNOS which present mainly in macrophages and endothelial cells produce NO that has an important role in various inflammatory processes and the inhibition of NO can also be identified as one of the anti-inflammatory mechanisms. Hence, significant ($p<0.05$) inhibition of NO level also may contribute to the anti-inflammatory activity of EELA which was clearly observed in this study.

As all the above activities i.e. anti-histamine, anti-oxidant, lipid peroxidation and NO scavenging, contribute to the anti-inflammatory activity, these findings suggest possible modes of action to explain the anti-inflammatory action of tested extracts and the isolated compound, evolitrine. The prostaglandin E_2 (PGE_2) and cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6), which are pro-inflammatory mediators, contribute to induction of inflammation and pain by acting on specific ion channels and receptors (Wang *et al.*, 2014). Previous studies (Fahmy *et al.*, 2017; El Shoubaky *et al.*, 2016; Al-Sayed and Abdel-Daim, 2018) have shown that there were significant increases ($p < 0.001$) in the levels of PGE_2, TNF-α, IL-1 and IL-6 in the carrageenan treated animals compared to the normal groups. Eldahshan and Abdel-Daim

(2015) also showed that there were increased levels of PGE_2 and TNF-α in paw tissue exudates and PGE_2, TNF-α, IL-1 and IL-6 levels in plasma that had been collected after 5 hours of 1% carrageenan injection.

The tested crude extract of *A. pedunculata* leaves and isolated compound, evolitrine, had marked anti-inflammatory and anti-nociceptive activities as shown by a reduction of the oedema in carrageenan injected paws and the number of writhes induced by 0.6 % acetic acid. As the role of pro-inflammatory cytokines in inflammatory model has been well clarified in many previous studies, in order to better understand the anti-inflammatory and anti-nociceptive mechanisms of tested crude extract and evolitrine, it is important to study their effects on these inflammatory mediators. The studies on crude EEAL have shown that it causes a significant ($P<0.05$) reduction in plasma PGE_2 level in the adjuvant induced arthritis rat model (Ratnayake, 2018). As PGE_2 is responsible for the oedema formation and also for the pain that accompanies the inflammatory reactions, the anti-inflammatory and anti-nociceptive effect of A. *pedunculate* leaves could be attributed in part to the inhibition of PGE_2 (Eldahshan and Abdel-Daim , 2015; Williams and Peck, 1997) synthesis.

Although cytokines are not constitutively produced under normal physiological conditions, the inflammatory stimuli induce the gene expression of cytokines. Further, there is evidence that free radicals could activate oxidative stress sensitive transcription factors, such as nuclear factor kB (NF-kB) (Verri *et al.*, 2012). The NF-kB is known as a pro-inflammatory transcription factor that induces the production of COX-2, cytokines, and other pro-inflammatory molecules. As our tested crude extract showed significant ($p<0.05$) *in-vitro* and *in-vivo* anti-oxidant activity, it could have inhibitory action on production of those pro-inflammatory mediators which could contributed to its anti-inflammatory action. Hence,

further studies on cytokine levels of *in-vivo* models are recommended in order to investigate the detailed anti-inflammatory and analgesic mechanisms of the extract of *A. pedunculata* and evolitrine.

In acute toxicity study, none of the rats showed mortality or body weight reduction during the study period. According to Allan *et al.* (2012), toxic doses generally cause 10% or more reduction in body weight. Hence, an absence of body weight reduction in EELA treated group, indicates the non-toxic nature of *A. pedunculata* leaf extracts at the tested doses. Further, EELA may not have adversely affected normal metabolism. Besides, there was no significant difference ($p > 0.05$) in food consumption between EELA treated and healthy control groups. The average food consumption was constant throughout the study period in all rats. As food consumption also is one of the most sensitive indicators of toxicity (Bhardwaj and Gupta, 2012), the comparability of food consumption in control and treated groups provide evidence for the safety of *A. pedunculata.*

Further, clinical chemistry profiles of EELA treated rat group also showed that there were no significant differences ($p > 0.05$) in the tested parameters when compared with healthy control group. As the elevation of serum concentrations of AST and ALT are indicators for possible hepatic and/or cardiac damage, the absence of elevation of these enzymes provide evidence for safety of EELA on the liver and heart which are vital organs in the body. Further, there were no significant changes in urea and creatinine levels in treated groups. Hence, there is no possible toxicity effect on kidneys too. As the dose of 5000 mg/kg b. w. is a very high dose, it provides evidence for the high safety margin of *A. pedunculata* leaves.

5. Conclusion

In conclusion, the EELA has the potential to control inflammatory conditions in Wistar rats by means of multiple mechanisms such as anti-histamine, anti-oxidant and nitric oxide inhibition. Also, it has analgesic activity. These observations provide some valuable scientific support for the therapeutic application of leaves of *A. pedunculata* claimed in the traditional medicine system in Sri Lanka.

Further, EELA does not exert any acute toxic effects in Wistar rats for a high dose and this provides scientific support for the high safety margin of this plant. In the present study, evolitrine was identified as the major anti-inflammatory and analgesic compound present in the leaves of *A. pedunculata*. Hence, evolitrine has the potential to be incorporated into novel treatment agents against inflammatory and pain conditions.

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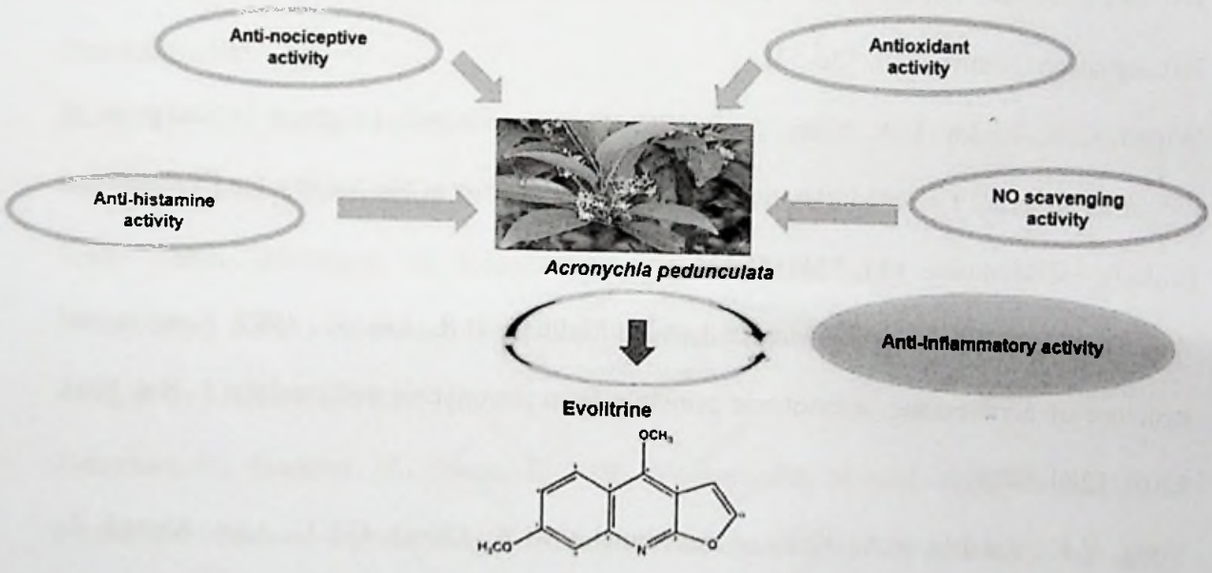
Anti-nociceptive activity
Antioxidant activity
Anti-histamine activity
NO scavenging activity
Acronychia pedunculata
Anti-inflammatory activity
Evoiitrine
OCH3
H3CO
N
O