Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(2): 831-835
Received: 16-01-2020
Accepted: 19-02-2020

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In-vitro investigation of anti-inflammatory activity and evaluation of phytochemical profile of *Eclipta prostrata* (L.)

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Abstract
The study determined the *in-vitro* anti-inflammatory activity and the phytochemical profile of aqueous seed extract of *Eclipta prostrata* (L.). The egg albumin denaturation assay was done using aqueous seed extract of *Eclipta prostrata* (L.). The diclofenac sodium drug was used as the reference. The aqueous seeds extract of *Eclipta prostrata* (L.) showed a moderate dose dependent inhibition of egg albumin denaturation with an IC_{50} value of 1.710×10^3 µg/mL (r^2=0.9598, p<0.05) and diclofenac sodium showed a dose dependent inhibition of protein denaturation with an IC_{50} value of 794 µg/mL (r^2=0.984, p<0.05). The anti-inflammatory activity of the seed extract was less potent when compared with the reference drug. However, the extract showed a significant anti-inflammatory activity. The phytochemical profile of *Eclipta prostrata* (L.) aqueous seed extract revealed the presence of phenols, alkaloids, carbohydrates, steroid, terpenoids and tannins. The extract is likely to mediate its anti-inflammatory activity due to the presence of these phytochemicals. This novel findings scientifically justified the claim made by Sri Lankan traditional medicine in the use of seed extracts of *Eclipta prostrata* (L.) as a treatment for inflammation.

Keywords: *Eclipta prostrata* (L.), anti-inflammation, egg albumin denaturation assay, phytochemicals.

Introduction
Inflammation is the response in body to an infection, destruction or injury that is characterized by pain, redness, swelling, heat and physiological functions being disturbed (Murakami & Hirano, 2012) [13]. In protein denaturation, the secondary and tertiary structure of the protein will be lost due to external compounds or stress like strong base or acid, organics solvent, inorganic salt (concentrated) or heat. When a protein denatures, it loses its biological functions and induce the production of antigens associated with hypersensitivity type III and thereby cause inflammation. There are two types of Inflammation, acute and chronic inflammation (Murakami & Hirano, 2012) [13].

Acute inflammation is the rapid and initial response that occurs within few seconds followed by a stimuli while prolonged soreness and redness of an area that lasts from days to even years is characterised by chronic inflammation. Chronic inflammation is a cause of elongated exposure to an infection and can extend even after the infection is removed (Gabay, 2006) [4].

Inflammatory disorders occur when body's own cells are destroyed by the immune system or when the tissues undergo abnormal and uncontrolled inflammation. Most of the disorders occur when inflammation is mistakenly triggered by the immune system in the absence of infection (Murakami & Hirano, 2012) [13]. There is a huge range of inflammatory disorders such as, asthma, coeliac disease, allergy, hepatitis, transplant rejection, autoimmune disease and inflammatory bowel disease (Smedby *et al*., 2006) [16]. This rheumatoid arthritis is known to be an enfeebling chronic autoimmune disease (Kumar *et al*., 2013) [12]. Currently anti-inflammatory drugs have high demand in the modern world for the treatment of inflammation. The commonly used anti-inflammatory drugs are known as non-steroidal anti-inflammatory drugs (NSAIDs) which are recommended in orthopaedic conditions like soft-tissue injury, fractures, rheumatoid arthritis and osteoarthritis. These anti-inflammatory drugs prevent protein denaturation which induce antigens production and cause auto-immune disease (Green, 2001) [7]. NSAIDs work by inhibiting the enzyme cyclooxygenase (COX), an enzyme that produce prostaglandins (PGs). Even though NSAIDs are effective drugs, they are relatively expensive and cause harmful side effects like gastric pain, skin rashes, hypertension, dizziness, headache, fluid retention, and high bleeding risk, renal, cardiovascular and hepatic dysfunction. Apart from side effects, the discontinuation of drugs leads to the reappearance of symptoms and the toxicity after the drug discontinuation (Kumar *et al*., 2013) [12] Which

emphasise the need of an improved and a safe treatment method.

Since ancient time, natural compounds play a crucial role in preventing and treating human diseases. The presence of plant secondary metabolites such as steroids, glycosides, terpenoids, polyphenols and alkaloids have attributed to their medicinal properties (Olaokun *et al*., 2017) [14]. In the modern world there is a great demand for anti-inflammatory drugs from natural sources as they are effective, cheap and safe (Heendeniya, Ratnasooriya and Pathirana, 2018) [9]. Medicinal plants contain hundreds of secondary metabolites that are biologically active and are extensively used in treating many diseases as pure compounds or crude material. The side effects and toxicity of NSAIDs makes the use of herbal medicines more popular (Kumar *et al*., 2013) [12].

Eclipta prostrata Linn (Sinhala name-keekirindiya, Tamil name- karippan) belongs to the Asteraceae family (therophyte herb) is usually called as false daisy. It grows in lowland and upland conditions and is distributed in damp areas, commonly as rice weeds (Gani and Devi, 2015) [5]. This medicinal plant grows throughout Sri Lanka and is highly used by traditional and ayurvedic physicians (Ediriweera, 2010) [3]. *Eclipta prostrata* Linn has some crucial pharmacological activities such as antioxidant, anti-aging, analgesic, hypolipidemic, hepatoprotective, anti-inflammatory, anti-microbial, immunomodulatory, antiviral and antivenom (Institute of Ayurveda, 2018) [10]. The flowers are specifically used due to their fungicidal, bactericidal, antispasmodic, digestive, vulnerary and analgesic properties. The entire plant is utilized as a stimulant (Gani and Devi, 2015) [5].

Materials and Methods

Collection and Authentication of plant

The seeds of *Eclipta prostrata* (L.) were collected from Homagama (Western province 6.8433˚ N, 80.0032˚E), Jaffna (Northern province 9.6615˚N, 80.0255˚E) and Tangalle (Southern province 6.0243˚N, 80.7941˚E), Sri Lanka. The selected plant was taxonomically identified and authenticated at the national herbarium by the department of National Botanic Gardens in Peradeniya, Sri Lanka.

Preparation of Aqueous extract

The collected *Eclipta prostrata* (L.) seeds were thoroughly washed in tap water and air dried in shade to remove water. The weight of the seeds were measured at regular intervals until a constant weight was attained. The dried seeds were then powdered using a blender and sealed in air tight bags.

Hundred grams of *Eclipta prostrata* (L.) powdered seeds were measured using electronic balance (Adam PW254) and was added to 1920 mL of distilled water. The solution was boiled slowly over a bunsen burner for 5 hours until the volume reduced to 240 mL and then was further concentrated for 1 hour until the final volume reached 100mL. Using a muslin cloth (double layered) the aqueous extract was filtered and stored in an air tight bottle. The extract was labelled and stored under constant temperature (4 ˚C). The prepared aqueous seed extract (ASE) of *Eclipta prostrata* was freeze dried at the Industrial Technology Institute (ITI) of Sri Lanka. The freeze dried sample of 8.7483 g was stored at-20 ˚C until it is used for the experiment.

Phytochemical analysis

The qualitative phytochemical analysis of aqueous seed extract was analysed using standard methods. (Table 1).

Table 1: Standard methods for qualitative phytochemical analysis

Phytochemicals	Type of test
Phenols	Ferric chloride test
Alkaloids	Mayers's test
Carbohydrates	Molisch test
Quinones	Alcoholic KOH test
Flavonoids	Alkaline reagent test
Steroid	Liebermann-Burchard test
Terpenoids	Salkowski Test
Tannins	Ferric chloride test
Saponin	Froth test
Coumarin	UV method

Investigation of *in vitro* anti-inflammatory activity using protein denaturation method

Twenty millilitre of distilled water was added to 250×10^3 µg of powdered aqueous seed extract of *Eclipta prostrata* (L.) and a stock solution of 12500 µg/mL was prepared. A serial dilution method of two folds was used to prepare a concentration series of 12500 µg/mL, 6250 µg/mL, 3125 µg/mL, 1562.5 µg/mL, 781.25 µg/mL and 390.62 µg/mL were prepared using the stock solution. A series of concentrations of 2500 µg/mL, 1250 µg/mL, 625 µg/mL, 312 µg/mL, 156.25 µg/mL and 78.125 µg/mL were prepared for diclofenac sodium as the reference drug (positive control). Reaction mixtures of samples were prepared with 2 mL of the sample from each concentration of ASE, 2.8 mL of freshly prepared Phosphate Buffer Saline (PBS) and 0.2 mL of egg albumin (extract of hen's egg). Reaction mixtures of diclofenac sodium (positive control) were prepared by replacing 2 mL of sample with diclofenac sodium from each concentration. Three negative controls were prepared by replacing 2 mL of the sample with distilled water. PBS (100 mL) of pH – 6.4 was prepared dissolving potassium chloride (0.02 g), sodium chloride (0.8 g), potassium dihydrogen phosphate (0.024 g) and disodium hydrogen phosphate (0.144g). The reaction mixtures of each concentrations of sample, diclofenac sodium and the negative control prepared were incubated at 37˚C for 15 minutes. After incubation the reaction mixtures were heated in water bath at 70 ˚C for 5 minutes. After 5 minutes the samples were cooled down to room temperature and the absorbance of the samples and diclofenac sodium at each concentration were measured using UV/VIS spectrophotometer at wavelength of 660nm.

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

Statistical analysis

The results are presented as mean ±SEM (Standard Error of Mean). The software Graphpad prism 7 was used to calculate the concentration dependencies and IC_{50} value (half maximal inhibitory concentration) by applying non-linear regression.

Results

Phytochemical analysis

The *Eclipta prostrata* (L.) seed extract showed the presence of phenols, alkaloids, carbohydrates, steroids, terpenoids, tannins and the absence of quinones, flavonoids, saponin and coumarin (Table 2).

Table 2: Phytochemical test results of aqueous seed extract of *Eclipta prostrata* (L.).

Phytochemicals	ASE of *Eclipta prostrata* (L.)
Phenols	+
Alkaloids	+
Carbohydrates	+
Quinones	-
Flavonoids	-
Steroid	+
Terpenoids	+
Tannins	+
Saponin	-
Coumarin	-

Present: +
Absent: -

***In-vitro* anti-inflammatory assay**

The results obtained with aqueous seed extract of *Eclipta prostrata* (L.) and diclofenac sodium was summarized in Table 3 and Table 4 respectively. The heat denaturation protein was inhibited by the aqueous seed extract from 60.77% to 109.10%. This effect was concentration dependent ($r^2 = 0.9598$, $p<0.05$). The IC_{50} value *of Eclipta prostrata* (L.) was 1.710×10^3 µg/mL. The results showed a positive dose dependent curve for both *Eclipta prostrata* (L.) seed extract and diclofenac sodium drug (Figure 1) The IC_{50} value of diclofenac sodium used as positive control was 794 µg/mL ($r^2 = 0.984$, $p<0.05$). The reference drug also displayed a dose response curve (Figure 2).

Table 3: Mean absorbance and percentage inhibition of heat induced denaturation of proteins by *Eclipta prostrata* (L.) seed extract.

Drug concentration (µg/ml)	Mean absorbance ±SEM	Percentage inhibition
390.62	0.9243 ± 0.031	60.77
781.25	0.626 ± 0.032	74.16
1562.5	0.605 ± 0.037	77.02
3125.0	0.2223 ± 0.034	96.44
6250.0	0.166 ± 0.014	100.85
12500.0	0.0853 ± 0.020	109.10

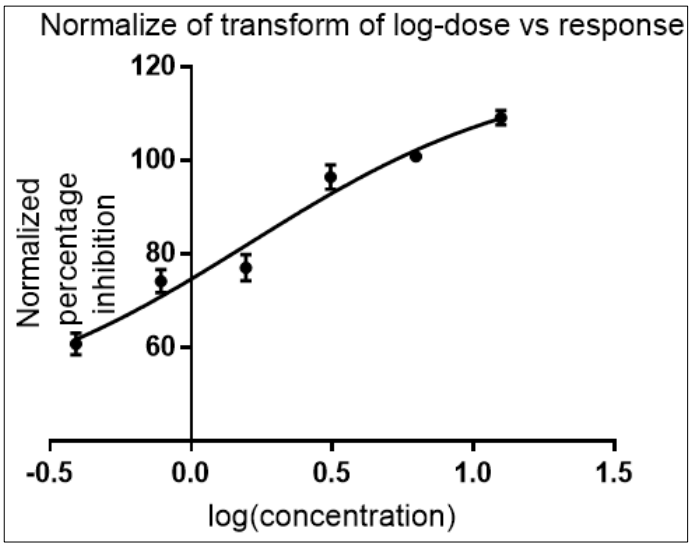

Fig 1: Concentration response curve for anti-inflammatory properties exhibited by *Eclipta prostrata* (L.)

Table 4: Mean absorbance and percentage inhibition of diclofenac sodium used as anti-inflammatory compound.

Drug concentration (µg/ml)	Mean absorbance ±SEM	Percentage inhibition
78.13	2.0 ± 0.056	19.58
156.25	1.752 ± 0.045	31.28
312.00	1.807 ± 0.046	29.50
625.00	1.596 ± 0.048	47.59
1250.00	0.743 ± 0.074	104.80
2500.00	0.475 ± 0.026	113.30

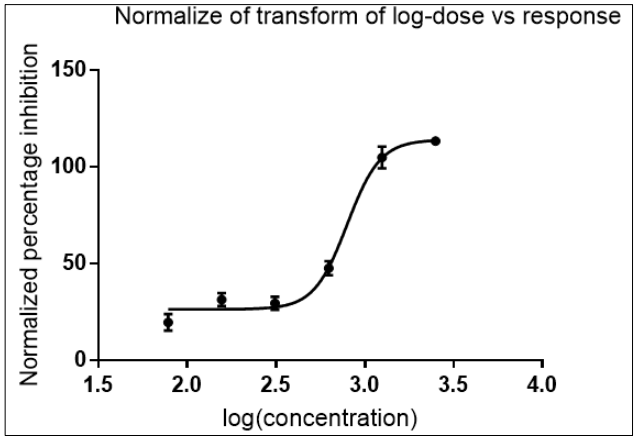

Fig 2: Concentration response curve for anti-inflammatory properties exhibited by diclofenac sodium.

Discussion

The study was done to assess the anti-inflammatory properties of *Eclipta prostrata* (L.) seeds that are being used in the treatment of inflammation in Sri Lankan traditional medicine (Institute of Ayurveda, 2018) [10]. The study was performed using water extract as in Sri Lanka traditional medicine uses decoction (water extract) to treat inflammation (Jayawikrama *et al*., 2018) [11]. The anti-inflammatory activities of the plant was tested *in- vitro* by egg albumin denaturation assay. This assay is a reliable, validated, quick and sensitive technique used to detect anti-inflammatory properties in natural products (Chandra *et al*., 2012) [2]. In order to avoid the use of living animals and avoid ethical problems, *in-vitro* method was selected for the study (Jayawikrama *et al*., 2018) [11]. The anti-inflammatory activity was quantified based on protein denaturation in this assay. Protein denaturation is a considerable cause for inflammation (Sangeetha and Vidhya, 2016) [15]. Protein denaturation induce the production of antigens that is associated with hypersensitivity type III and cause inflammation. Protein denaturation can also induce auto-antigen production in rheumatic diseases. Inhibition of denaturation process is a property of an anti-inflammatory agent. The anti-inflammatory activity is greater when the degree of inhibition is high (Heendeniya, Ratnasooriya and Pathirana, 2018) [9].

According to the results obtained the plant *Eclipta prostrata* (L.) displayed *in-vitro* anti-inflammatory activity that is dose dependent with an IC_{50} value of 1710 µg/ml. The reference drug diclofenac sodium also displayed a dose dependent anti-inflammatory activity with an IC_{50} value of 794 µg/ml. When compared to the reference drug *Eclipta prostrata* (L.) seeds displayed 2.15 times less potent anti-inflammatory activity. The seed extract displayed a moderate anti-inflammatory activity when compared with the reference drug diclofenac sodium. The diclofenac sodium used in the experiment was a pure drug whereas the plant extracts used were crude extracts. This could be a significant reason for the differences in the potency. An isolated pure active ingredient of the plant extract could possess a more potent anti-inflammatory activity than in the current study. When the percentage inhibition varies with the concentration, it is known as concentration or dose dependency. *Eclipta prostrata* (L.) showed a dose dependent anti-inflammatory activity with an r^2 value 0.9598 as the value is closer to 1. Diclofenac sodium showed a dose dependent anti-inflammatory activity with an r^2 value of 0.984. The observed dose dependent effect is an indication that the phenomenon is not random but is specific, causal and genuine (Jayawikrama *et al*., 2018) [11].

For the plant extract, 390.62 µg/mL was the lowest concentration used as the spectrophotometer had less sensitivity. The highest concentration used was 12500.0 µg/ml because of the colour interference. As diclofenac sodium is a pure drug the absorbance can be measured at lower concentrations. During the heating of the assay, the sample mixture was first incubated at 37 ˚C and then the temperature was increased to 70 ˚C by heating in a water bath. The sample was gradually heated to prevent protein coagulation that could result in the formation of clumps (Heendeniya, Ratnasooriya and Pathirana, 2018) [11]. Diclofenac sodium used as reference drug displayed a dose dependent anti-inflammatory property *in-vitro* as previous findings.

The IC50 value of the reference drug found was 794 µg/ml which was different from other reported investigations. According to Chandra *et al*. (2012) [2], the IC50 value of diclofenac sodium was 625 µg/ml and according to Kariawasam *et al*. (2017) the IC50 value was 379.375 µg/ml. This difference could be due to the use of different branded drugs that have different bio availabilities (Ameri *et al*., 2012) [1]. The anti-inflammatory activity of the plant extract was less at lower concentrations when compared with the reference drug. Even though diclofenac sodium showed potent anti-inflammatory properties, it is a NSAIDs with certain side effects such as certain gastrointestinal problems, allergies and central nervous system problems (Todd and Sorkin, 1988) [17]. *Eclipta prostrata* (L.) is a medicinal plant that contains natural compounds which may not induce serious side effects as NSAIDs.

Identification of phytochemicals helps to screen the biological activities possessed by a plant. This is useful in the discovery of new drugs. Phytochemicals are known as secondary metabolites of a plant. Qualitative phytochemical analysis was done using the seed extract and according to the results the phytochemicals present in *Eclipta prostrata* (L.) seeds were phenols, alkaloids, carbohydrates, steroid and terpenoids.

Conclusion

In conclusion, the results obtained showed that the seeds of Sri Lankan *Eclipta prostrata* (L.) possessed anti-inflammatory activity. It justified the claim made by Sri Lankan traditional medicine in the treatment of inflammatory disorders. *Eclipta prostrata* (L.) seeds showed a moderate potency *in vitro* anti-inflammatory properties mediated by phenols, alkaloids, terpenoids and tannins.

Acknowledgement

I would like to start off by thanking S. Kannadas and K. Nerangeni who sponsored and supported me in completing my research. A special gratitude I give to my supervisors professor W.D. Ratnasooriya and professor R.N. Pathirana, whose contribution in inspiring and encouraging me, helped me to coordinate my project especially in writing this report. My Deepest appreciation and sincere thanks to my coordinator Miss. Surangi Fernando of British College of Applied Studies (BCAS) for giving me this amazing opportunity to conduct my research project at a reputable place like Kothalawala Defence University (KDU). My sincere appreciation and gratitude to the rest of the lecturers for molding my knowledge and raising me to this level of education. Last but not least, many thanks to my family and my friends for helping me in abundant ways to complete my research project, for which without them this wouldn't have been possible.

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