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In-vitro anti-inflammatory and antioxidant activities of *Leea indica* (Burm.f.) Merr.

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Abstract: The aim of this study was to evaluate *in-vitro* anti-inflammatory and antioxidant properties of different solvent extracts obtained from leaves of *Leea indica* (Burulla/Gurulla) grown in Sri Lanka. Two different defatted crude extracts namely, 70% aqueous acetone and 80% aqueous methanol were prepared by steeping method. The total phenolic (TP) and total flavonoid (TF) contents of the both extracts were evaluated using Folin-Ciocalteu and aluminum chloride colorimetric methods respectively. *In-vitro* radical scavenging activity of the solvent extracts was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and antioxidant activity was determined by ferric-reducing antioxidant power assay (FRAP assay). *In-vitro* anti-inflammatory activity of extracts was determined using Human Red Blood Cell (HRBC) membrane stabilization assay. The results for the total phenolic content of the extracts were 4891.776 ± 64.965 (70% aqueous acetone) and 3413.859 ± 85.493 (80% aqueous methanol) mg Gallic acid equivalents (GAE)/100 g dry weight (DW) of leaves. Results of the total flavonoid content of the extracts were 1711.220 ± 22.829 (70% aqueous acetone) and 920.867 ± 39.833 (80% aqueous methanol) mg Catechin equivalents (CAE)/100 g dry weight (DW) of leaves. Radical scavenging activity of the extracts was determined as 13.418 ± 0.312 (70% aqueous acetone) and 9.421 ± 0.431 (80% aqueous methanol) mmol Trolox equivalents/100 g DW of leaves. Ferric reducing antioxidant power of the extracts was found as 17.796 ± 0.343 (70% aqueous acetone) and 12.422 ± 0.490 (80% aqueous methanol) mmol Fe (II) equivalents/100 g DW of the leaves. Significantly high values of TP, TF contents, radical scavenging and ferric reducing activities were exhibited by leaf extract obtained into 70% aqueous acetone. Half maximal inhibitory concentration (IC_{50}) values obtained in the membrane stabilization assay were 431.500 µg/mL and 442.100 µg/mL for 70% aq. acetone and 80% aq. methanol extracts respectively and their responses were significantly greater than the reference drug aspirin (IC_{50} 1062.000 µg/mL). Hence, it is concluded that the leaf extracts of Sri Lankan grown *Leea indica* possess marked *in vitro* anti-inflammatory and antioxidant activities which should be further investigated by *in-vivo* models.

Keywords: Anti-inflammatory, antioxidant, *Leea indica,* membrane stabilization assay

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INTRODUCTION

The recent statistics explain that most of the human diseases are originated from inflammation and it is inter-related with the oxidative stress (Yoshikawa & Naito, 2002; Biswas, 2016). Inflammation is a physiological response from body to signal the immune system to heal and repair damaged tissues and to defend against foreign organisms such as viruses and bacteria (Ahmed, 2011). Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed to treat inflammatory conditions, though they are known for causing adverse effects such as gastric ulcers, kidney failure, bleeding, hyperglycemia, and hypertension (Kumar et al., 2013). Due to the appearance of various side effects by usage of conventional drugs, medicinal plants are considered as the major reservoir for potentially new drugs. Hence, there is a rising interest in the field of natural products and drug discovery to generate safer and more efficacious novel drug therapies from sources like plants, animals, and microbes. Since medicinal plant extracts and their crude are the most important sources for new drugs and have been shown greater results in the treatment of inflammation, there are investigations through the screening of different plant extracts led to the discovery of effective and safe drugs with anti-inflammatory activity (Harvey, 2008; Kumar et al., 2013). Many research studies have been successfully carried out worldwide to investigate the anti-inflammatory potential of various medicinal plants including *Adhatoda vasica, Bacopa monnieri Linn. Achillea millefolium Linn., Ricinus communis Linn.* and *Emblica officinalis* and as well as to formulate novel anti-inflammatory drug preparations using natural biomaterials (Jena & Gupta, 2012; Kumar et al., 2013).

Leea indica (Burm.f.) Merr. is an evergreen perennial shrub or a small tree grown in Sri Lanka (Figure 1.). Almost all the parts of the plant including leaves, roots, fruits, stem bark and flowers are used for therapeutic purposes in certain formulations (Mishra et al., 2016). According to the research studies carried out, the extracts of this plant is reported to have wide range of pharmacological properties including anticancer, antioxidant, analgesic, anxiolytic, antiviral, antimicrobial, thrombolytic, anti-hyperglycemic, hypolipidemic, and phosphodiesterase inhibitory activities (Srinivasan et al., 2009; Dalu et al., 2014; Kekuda et al., 2018). This plant is frequently used as the main ingredient in "Paththu" formulations in treating bone fractures & sprains and used in treating burns, hemorrhoids, and skin diseases (warts) in Sri Lankan traditional medicine. The usage of *L. indica* plant in ayurvedic formulations for treating inflammatory ailments also convinces the potential anti-inflammatory activity of this plant. Therefore, the present research study was aimed to evaluate anti-inflammatory and antioxidant activities of leaf extracts obtained from *L. indica* (Burm.f.) Merr. (Burulla/Gurulla) which is widely used in traditional medical systems to treat various inflammatory diseases.

MATERIALS AND METHODS

Leaves of *L. indica* (Burm.f.) Merr. were collected from Gampaha district (Western province, in Sri Lanka) in 2020 and were authenticated from the National Herbarium, Peradeniya**,** Sri Lanka.

Study was conducted using the chemicals of Folin-Ciocalteu phenol reagent, hydrochloric acid**,** catechin, **s**odium carbonate, sodium hydroxide, sodium nitrite**,**

aluminum chloride, gallic acid, 2-2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), Trolox, ferric chloride, ethanol, methanol, acetone,

hexane and ferrous sulphate of analytical grade were purchased from Sigma Aldrich local agencies in Sri Lanka.

Figure 1. *Leea indica* (Burm.f.) Merr.

Preparation of the plant extracts: Two different crude extracts namely, 70% aqueous acetone and 80% aqueous methanol extracts were prepared by performing the method published (Hettihewa, 2014) with slight modifications. Briefly, coarse powder of oven dried leaves was steeped in each solvent (300 mL) separately in Scott Duran bottles with occasional shaking for 24 hours in the dark conditions at room temperature. After 24 hours, these extracts were filtered using four layers of muslin cloth and were concentrated under the vacuum using the rotary evaporator (HAHN HS-2005S-N) below the temperature of 65 ˚C. Partitioning with hexane was followed to get the defatted crude extracts and they were subjected to freeze drying (freeze dryer-BIOBASE BK-FD10PT) until gained the constant weight.

Determination of total phenolic content and total flavonoid content: The total phenolic and total flavonoid contents of the defatted crude extracts of *L. indica* leaves were determined using Folin-Ciocalteu method and the aluminum chloride colorimetric method respectively as described in the method published (Hettihewa, 2014). Total phenolic content was expressed in mg Gallic acid equivalents (GAE)/100 g dry weight (DW) of leaves and total flavonoid content was expressed in mg Catechin equivalents (CAE)/100 g dry weight of leaves.

Determination of in vitro radical scavenging activity: The radical scavenging activity of the extracts was determined using the 2,2, -diphenyl-1-picrylhydrazyl

(DPPH) assay and expressed in mmol Trolox equivalents to 100 g DW of leaves (Hettihewa, 2014).

Determination of ferric-reducing antioxidant power activity (FRAP assay): Ferric reducing antioxidant power (FRAP) assay was used to determine the antioxidant power of the extracts and the results were expressed in mmol Fe(II) equivalents/100 g DW of the leaves (Hettihewa, 2014).

Evaluation of in vitro anti-inflammatory activity using HRBC membrane stabilization assay: In-vitro anti-inflammatory activity of freeze dried plant extracts was tested by Human Red Blood Cell (HRBC) membrane stabilization assay using Heat induced Red Blood Cell (RBC) hemolysis method by following the method described by Leela Prakash & Dess in 2014 with slight modifications (Leela Prakash & Dass., 2014).

Preparation of Red blood cells suspension: Fresh human blood was collected from healthy human volunteers who had not taken any anti-inflammatory drug for 2 weeks prior to the blood withdrawal (Blood withdrawal was performed after obtaining ethical approval for the procedure). 5.00 mL of blood per one volunteer was withdrawn to EDTA (Ethylenediaminetetraacetic acid) containing tube by a trained professional under the supervision of a medical practitioner. (Healthy human volunteers were selected among the students who were between ages 20-30 years, in the Faculty of Allied Health Sciences, University of Ruhuna). The blood sample was centrifuged at 3000 rpm for 10 minutes and resulted packed cells were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted to 10% v/v suspension using normal saline.

Heat induced Red Blood Cells (RBC) hemolysis: The reaction mixture was prepared by mixing 400 µL of 10% RBC suspension and 4.00 mL of plant extract in different concentrations (2000 µg/mL, 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL) in centrifuge tubes. The reaction mixture was incubated at 56 ºC in water bath for 30 minutes. After the incubation period, the centrifuge tubes containing reaction mixtures were centrifuged at 2500 rpm for 5 minutes. Absorbance of the supernatants was taken at 560 nm using UV visible spectrophotometer. Commercially available Aspirin used as the positive control while the negative control was prepared by adding normal saline instead of test sample. The experiment was carried out in triplicates and the percent inhibition of hemolysis was calculated according to the following equation.

$$\text{Percentage inhibition of hemolysis} = \frac{(Vc - Vt) \times 100}{Vc}$$

Here,
Vc =Absorbance of negative control
Vt = Absorbance of test sample

Ethical consideration: This study was conducted after obtaining the ethical approval from the Ethics Review Committee of Faculty of Allied Health Sciences, University of Ruhuna, Sri Lanka.

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Statistical analysis Results were expressed as the mean ± SD. The results of total flavonoid content, total phenolic content, *in-vitro* antioxidant activity and *in-vitro* anti-inflammatory activity were analyzed by multiple comparison one-way ANOVA and independent sample t-test using the SPSS 25 software. At ($p < 0.05$), the values were considered significantly different at 95% level of confidence.

RESULTS

Total phenolic content and total flavonoid content of leaf extracts: The results for the total phenolic content of the extracts were significantly different ($p < 0.05$) and they were 4891.776 ± 64.965 (70% aqueous acetone) and 3413.859 ± 85.493 (80% aqueous methanol) mg Gallic acid equivalent (GAE) /100 g dry weight of leaves. Results of the total flavonoid content of the extracts were also significantly different ($p < 0.05$) and expressed as 1711.220 ± 22.829 (70% aqueous acetone) and 920.867 ± 39.833 (80% aqueous methanol) mg Catechin equivalents (CAE) /100 g dry weight of leaves. The highest concentration of total phenols and total flavonoids was obtained for 70% aqueous acetone extract.

Radical scavenging activity and ferric reducing antioxidant power of leaf extracts: In vitro radical scavenging activity of the extracts by DPPH assay was 13.418 ± 0.312 (70% aqueous acetone) and 9.421 ± 0.431 (80% aqueous methanol) mmol Trolox equivalents/100 g DW of leaves. Antioxidant activity of the extracts by FRAP assay was 17.796 ± 0.343 (70% aqueous acetone) and 12.422 ± 0.490 (80% aqueous methanol) mmol Fe (II) equivalents/100 g DW of the leaves. It was noticed that significantly high antioxidant values were exhibited by 70% aqueous acetone leaf extract compared to 80% aqueous methanol. The higher phenolic and flavonoid contents found in 70% aqueous acetone extracts would have contributed for its greater antioxidant activity.

In vitro anti-inflammatory activity of leaf extracts : In vitro anti-inflammatory activity of *L. indica* extracts was evaluated by using Human Red Blood Cell membrane stabilization method and percentage inhibition values were obtained at different concentration series of 70% aqueous acetone, 80% aqueous methanol extracts and Aspirin drug (positive control). Results obtained are expressed as % inhibition ± SD (Table 1).

Table 1. Percentage **(%)** inhibition of *L. indica* extracts and Aspirin by heat induced RBC hemolysis

Concentration µg/mL	% Inhibition ± SD		
	70% aq. Acetone	80% aq. methanol	Aspirin
125	9.724 ± 0.489^{a}	7.674 ± 0.750^{b}	10.743 ± 0.842^{a}
250	24.265 ± 0.713^{c}	20.935 ± 0.941^{d}	20.971 ± 0.588^{d}
500	52.495 ± 0.755^{e}	48.698 ± 0.713^{f}	36.410 ± 0.596^{g}
1000	75.094 ± 0.503^{h}	71.003 ± 0.391^{i}	53.840 ± 0.700^{j}
2000	85.119 ± 0.470^{k}	80.981 ± 0.710^{l}	72.305 ± 0.744^{m}

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Results are expressed as mean ± standard error. Means followed by different letters in a row are significantly different ($p<0.05$).

Both leaf extracts obtained from *L. indica* showed a significant *in vitro* anti-inflammatory activity in a concentration dependent manner. All the results were compared with standard drug aspirin at concentrations of 125, 250, 500, 1000 and 2000 µg/ml.

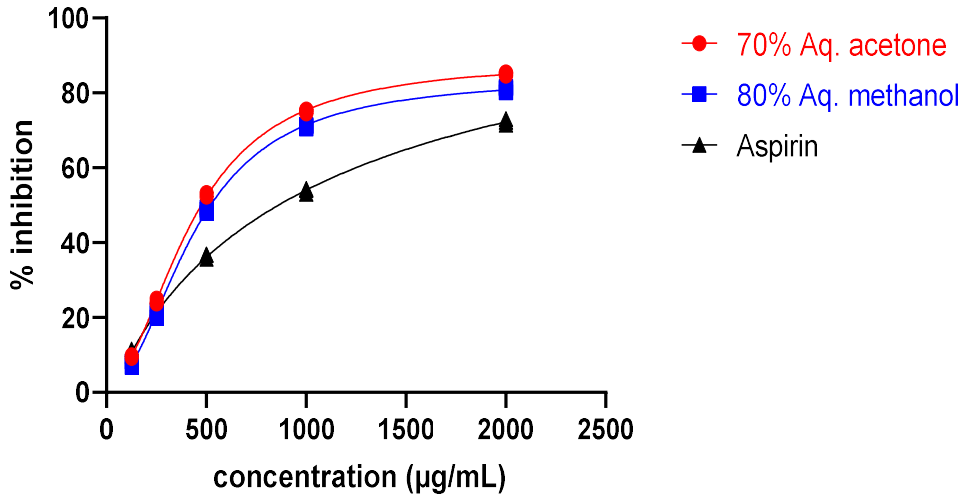

Figure 2. Dose response curves of % inhibition of two different solvent extracts from *L. indica* and Aspirin

Both extracts exhibited greater percentage inhibition of hemolysis compared to aspirin (Positive control) and 70% aq. acetone extract was more effective in inhibiting heat induced red blood cell hemolysis than 80 % methanol extract. However, the effect of aspirin was less when compared to both extracts obtained from *L. indica*. This was further confirmed by comparing their half maximal inhibitory concentration (IC_{50}) values calculated using the plot of Log concentration Vs % inhibition (Figure 3.).

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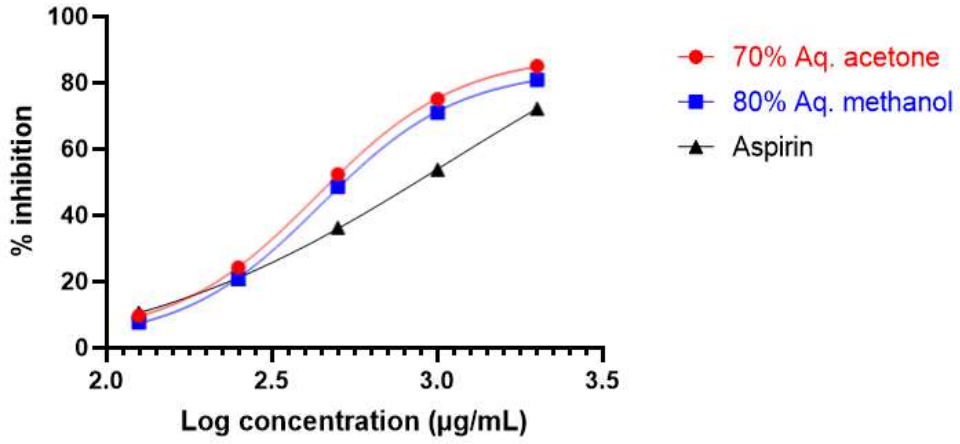

Figure 3. Comparison of *L. indica* extracts and aspirin for effect on inhibition of hemolysis

Both *L. indica* leaf extracts showed lower IC_{50} values ($IC_{50\ acetone}$ =431.500 µg/mL, $IC_{50\ methanol}$= 442.100 µg/mL) compared to aspirin ($IC_{50\ Aspirin}$ 1062.000 µg/mL). The lowest IC_{50} value of 431.500 µg/mL was found in 70% aq. acetone extract exhibiting the highest *in vitro* anti-inflammatory activity.

DISCUSSION

The present study revealed the presence of remarkable *in-vitro* anti-inflammatory activity and antioxidant activity in both solvent extracts namely 70% aqueous acetone and 80% aqueous methanol extracts of *L. indica* leaves. By considering the values obtained for total phenolic, total flavonoid, *in-vitro* antioxidant activity and *in-vitro* anti-inflammatory activity of the two different solvent extracts, 70% aqueous acetone was identified as the most effective solvent for the extraction of bioactive compounds in the study. The highest *in-vitro* anti-inflammatory and antioxidant properties were exhibited by 70% aqueous acetone extract and it may be due to the presence of larger contents of phenols and flavonoids in this extract than 80% methanol extract, which are usually responsible for these bioactivities. Both plant extracts showed greater ability to stabilize red blood cell membrane in heat induced hemolysis assay in a concentration dependent manner and these responses were comparably higher than the response exhibited by positive control, Aspirin. Therefore, these results provide strong scientific evidences to use this plant as a good source of anti-inflammatory agent as well as antioxidant agent and present study also justifies the use of this plant in Sri Lankan traditional medicine in treating various inflammatory conditions.

Our research findings are in the concurrent with the study conducted by Ghagane et al. in 2017 for different solvent extracts namely, methanol, ethanol and aqueous extract of *L. indica* leaves using Folin Ciocalteu reagent method and expressed as (65.20 ± 0.15), (60.97 ±0.23), (53.04 ±0.15) and (84.59 ±0.52) mg/GAE of plant extract respectively (Ghagane et al., 2017). According to the research study by Rahaman et al. in 2013, total phenolic content of ethanol extract of *L. indica* leaves was found as 24.00± 0.81g GAE/100g of plant extract for Folin Ciocalteu assay (Rahaman et al. 2013). Reddy et al. had evaluated the total phenolic content of aqueous, ethanol, ethyl acetate and hexane extract of *L. indica* leaves and the highest amount was found in the fractionated aqueous extract (37.29 mg of GAEs/g of extract). A strong correlation between phenolic content and antioxidant activity had been found showing that the phenolic compounds mainly contributes for the antioxidant activity of the plant extracts (Reddy et al., 2012).

CONCLUSION

Significantly high values of total phenolic, total flavonoid contents, radical scavenging, ferric reducing activities, and *in-vitro* anti-inflammatory activity were exhibited by leaf extract obtained into 70% aqueous acetone. Half maximal inhibitory concentration (IC_{50}) values obtained in both extracts were significantly greater than the reference drug, aspirin. Hence, it is concluded that the leaf extracts of Sri Lankan grown *L. indica* possess marked *in vitro* anti-inflammatory and antioxidant activities which should be further investigated by *in-vivo* models.

ACKNOWLEDGMENTS

Authors would like to express sincere gratitude to Ms. Harshani Erangika and staff members of the Department of Pharmacy, Faculty of Allied Health Sciences, University of Ruhuna, Galle, Sri Lanka.

DECLARATION OF CONFLICT OF INTEREST

We do hereby declare that the study does not encompass any conflict of interest.

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Annexures

Annexure 01: Letter of certification of the botanical identifaction

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ජාතික උද්භිද උද්‍යාන දෙපාර්තමේන්තුව, පේරාදෙණිය
தேசிய தாவரவியல் பூங்கா திணைக்களம்
National Herbarium
Department of National Botanic Gardens

තැ. පෙ. 14, පේරාදෙණිය, ශ්‍රී ලංකාව
த.பெ.14, பேராதனை, இலங்கை
P.O. Box 14, Peradeniya, Sri Lanka

My No: NH/BOT/4/2019- 20 Your No.: Date: 2020. 02. 26

Dr. Sujeewa K. Hettihewa,
Head,
Department of Pharmacy,
Faculty of Allied Health Sciences,
University of Ruhuna,
Galle.

Request to get approval for the authentication of plant materials for the research project

This refers to your letter dated 06th of February 2020 regarding the above matter.

This is to inform you that plant specimen submitted to the National Herbarium by Ms. T. L. I. Srilal (MD/ PH/ 2015/ 127), an undergraduate Pharmacy student at Department of Pharmacy, Faculty of Allied Health Sciences, University of Ruhuna was identified as follows.

Tag no.	Family	Species
01	Vitaceae	*Leea indica* (Burm.f.) Merr.

Dr. (Mrs.) R. A. S. W. Ranasinghe
Deputy Director

Dr. R.A.S.W. RANASINGHE
Deputy Director - National Herbarium
Department of National Botanic Gardens
Peradeniya

නියෝජ්‍ය අධ්‍යක්ෂ
ජාතික ශාකාගාරය.
Deputy Director
National Herbarium

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Annexure 02 : Degree & Designation of Authors

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  Faculty of Allied Health Sciences, University of Ruhuna, Sri Lanka

- **Thotawaththage Loshadhi Indunika Srilal,** (B.Pharm), Department of Pharmacy, Faculty of Allied Health Sciences, University of Ruhuna, Sri Lanka