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Development of a novel herbal balm with *Leea indica* (Burm.f.) Merr (Burulla) leaf extract and *in vitro* evaluation of anti-inflammatory and radical scavenging activities

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Abstract: The demand for the utilization of herbal medicine is rising due to their high efficacy, affordability, ease of use, better adaptability with human body and lesser side effects. Leea indica (Burm.f.)Merr. (Burulla) is a medicinal plant used in Sri Lankan traditional medicine for treating many diseases. The present study was aimed to formulate a novel herbal balm with Leea indica (Burm.f.) Merr. leaf extract and evaluate in vitro anti-inflammatory and antioxidant activities. Two different defatted crude extracts namely, 70% aqueous acetone and 80% aqueous methanol were prepared and subjected to preliminary phytochemical screening. Different formulations (Fb1-Fb4) were developed by incorporating freeze dried powder of most active, defatted 70% aqueous acetone extract and tested for their physicochemical stability parameters for period of 60 days. Formulations were tested for in vitro anti-inflammatory activity (by Human Red Blood Cell membrane stabilization assay) and in vitro radical scavenging activity {(by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay)}. The phytochemical screening of tested extracts revealed the presence of alkaloids, flavonoids, phenols, tannins, saponins, terpenoids, carbohydrates and phytosterols in both extracts. Among the formulated balms, Fb4 showed the highest values of percentage inhibition (75.084 ± 0.193 % of heat induced hemolysis at the concentration of 12.5 mg/mL) and in vitro radical scavenging activity (2.892 ± 0.008 mmol Trolox per 100 g of balm). All formulations showed no remarkable variation in stability parameters during the period observed. It is concluded that the formulated balms have promising *in vitro* anti-inflammatory and antioxidant activities and Fb4 is recommended for further investigations of clinical trials.

Keywords: *Leea indica*, herbal balm, anti-inflammatory, radical scavenging activity

INTRODUCTION

Inflammation is an essential immune response which acts as a protective strategy during injury or infection in the body. Inflammation signals the immune system to remove harmful injurious agent from the body as well as rapid healing and repairing processes in

damaged tissues. Body increases production of white blood cells, other immune cells and chemical mediators as a response to an inflammatory signal (Ahmed, 2011). Inflammation is most commonly treated by non-steroidal anti-inflammatory drugs (NSAIDs) which are known for having severe adverse drug reactions including gastric ulcers, bleeding, kidney failure, hyperglycemia and hypertension (Kumar et al., 2013). Topical formulations of anti-inflammatory drugs are widely used to get both analgesic and anti-inflammatory activities in day to day life for pain and symptomatic relief of various acute and chronic conditions such as acute sprains, strains, overuse injuries, arthritis, and neuropathic pain (Makris et al., 2011).

There is a progressive demand for topical drug route over the other routes due to various advantages of topical drug delivery systems such as reduced risk of systemic adverse reactions, avoidance of first pass metabolism and gastro-intestinal tract variability, direct administration to desired site of action, non- invasiveness and prevention of drug-drug interactions. They provide a simple therapeutic regime, which is easily terminated by simple removal of the application from the surface of the skin (Mcpherson & Cimino, 2013).

Balm is a traditional or Ayurveda form of topical semi-solid, anhydrous preparation, commonly used for relieving body aches and pains. Usually, balm requires friction and must be rubbed or massaged into the skin for proper application. They are normally thicker in consistency than ointments. Balms are preferable for topical use as they are water impermeable, occlusive, moisture protecting, chemically, physically stable even without preservatives and longer acting with slow absorption (Ramesh et al., 2010).

It is reported that inflammation and oxidative stress are inter-related pathophysiological processes where occurrence of one of these two processes, stimulates the occurrence of the other one (Yoshikawa & Naito, 2002; Biswas, 2016). Oxidative stress is a state where oxidation exceeds antioxidant defense system in the body due to the loss of balance between generation of oxidants and the antioxidant defense system. Oxidative stress is generally associated with several diseases including inflammation, because it causes oxidative damage to biomolecules, disturbance of signal transduction, mutation and cell death (Yoshikawa & Naito, 2002).

Therefore, development of an agent or a formulation which can simultaneously inhibit both inflammation and oxidative stress may be very useful in treating such diseases. Many research studies have shown that medicinal plants and the herbal formulations as a good alternative to overcome the problems associated with synthetic anti-inflammatory agents (Nworu & Akah, 2015) due to their high efficacy, affordability, better adaptability with human body and lesser side effects (Harvey, 2008).

Leea indica (Burm.f.) Merr. (Burulla/Gurulla) is well known medicinal plant used in Sri Lankan traditional Ayurvedic medicine for many therapeutic purposes. Almost all parts of the *L. indica* are reported to use in traditional medical systems especially in Asian countries for many years and no serious side effects have been reported from topical applications (Kekuda et al., 2018). *L. indica* leaves are commonly used in Sri Lankan traditional Ayurvedic medicine as main ingredient of "Paththu" formulations for treating bone fractures and sprains and also used in treating burns, hemorrhoids and skin diseases. Therefore, this research study was aimed to formulate a novel herbal balm enriched with

anti-inflammatory and radical scavenging activities using leaf extract of *L. indica* (Burm.f.) Merr. grown in Sri Lanka.

MATERIAL 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.

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). 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) using the temperature below 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.

Preliminary phytochemical screening: The phytochemical screening tests were carried out to detect the presence of following phytochemicals in the plant extracts (Kumar et al., 2012; Chaudhary & Kumar, 2014).

Tests for alkaloids: Mayer's test: extracts were acidified with concentrated hydrochloric acid and few drops of Mayer's reagent were added. A white or yellow color precipitate indicated the presence of alkaloids. Dragendroff's test: Extracts were acidified with conc. hydrochloric acid and a few drops of Dragendroff's reagent were added. Formation of orange or orange-red color precipitate indicated the presence of alkaloids. Wagner's test: Extracts were acidified with conc. hydrochloric acid and a few drops of Wagner's reagent were added. A brown/reddish color precipitate indicated the presence of alkaloids. Hager's test: Extracts were acidified with conc. hydrochloric acid and a few drops of Hager's reagent were added. A yellow color precipitate indicated the presence of alkaloids.

Test for phenolic compounds: Ferric chloride test: Extracts were treated with few drops of 10% neutral ferric chloride solution. Formation of dark blue or green color indicated the presence of phenols or polyphenols. Test for flavonoids (alkaline reagent test) extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicated the presence of flavonoids. Lead acetate test: xtracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicated the presence of flavonoids.

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Test for Saponins (froth test): Extracts were diluted with distilled water to 20 mL and were shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponins.

Test for terpenoids (Salkowski test): Extracts were dissolved in (2 mL) chloroform and H_2SO_4 acid was added slowly along the wall of test tube to form a layer. Formation of reddish brown ring in the interface indicated the presence of terpenoids.

Test for tannins (gelatin test): 1% gelatin solution containing sodium chloride was added to the extracts. Formation of white precipitate indicated the presence of tannins.

Tests for carbohydrates (Molisch's test): Extracts were treated with 2 drops of alcoholic α-naphthol solution and con. H_2SO_4. Formation of violet ring at the junction indicated the presence of carbohydrates. Benedict's test: Extracts were treated with Benedict's reagent and heated gently in a water bath for 5 minutes. Orange red precipitate indicated the presence of reducing sugar. Fehling's test: Extracts were hydrolyzed with dil. HCL, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicated the presence of reducing sugars.

Test for sterols and triterpenoids (Libermann-burchard's test): Extracts were treated with few drops of acetic anhydride and heated to boiling. They were cooled and conc. H_2SO_4 was transferred along the wall of test tube. A brown ring at the junction of two layers and the upper layer turning green indicated the presence of sterols while formation of deep red color indicated the presence of triterpenoids.

Formulations of herbal balm: The different types of herbal balm formulations were developed according to the compositions given in the Table 1 by following the method described (Ramesh et al., 2010) with slight modifications.

Table 1: Composition of formulated herbal balms

Ingredient	Formulations (g)				
	Base	Fb1	Fb2	Fb3	Fb4
Freeze dried aq. acetone leaf extract	-	-	0.0625	0.125	0.25
Bees wax	2.280	2.280	2.280	2.280	2.280
Castor oil	0.650	0.650	0.650	0.650	0.650
Menthol	0.620	0.620	0.620	0.620	0.620
Eucalyptus oil	-	0.500	0.500	0.500	0.500
Camphor	-	0.500	0.500	0.500	0.500
Methyl salicylate	-	0.450	0.450	0.450	0.450

Evaluation of physicochemical stability parameters: Final formulations were evaluated for their physicochemical stability parameters such as pH, appearance, odor, and homogeneity and phase separation for 60 days at room temperature.

Evaluation of in vitro anti-inflammatory activity of the formulations: In vitro anti-inflammatory activity of final formulations was tested by Human Red Blood Cell (HRBC) membrane stabilization method (Heat induced RBC hemolysis method) as described (Leelaprakash & Dass, 2011) with some minor modifications.

Preparation of red blood cells suspension: Fresh human blood (20.00 mL) 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 balm sample 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 two herbal balm formulations were used as the positive controls 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

Evaluation of in vitro radical scavenging activity of the formulations: In vitro radical scavenging activities of the formulations developed were determined by using 2,2,-diphenyl-1-picrylhydrazyl (DPPH) assay with compared to commercially available two herbal balm formulations as positive controls (Hettihewa, 2014). The radical scavenging activity was calculated using the standard calibration curve of Trolox and expressed as mmol Trolox equivalents to 100 g balm.

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.

Statistical analysis: All experimental measurements were carried out in triplicates and the results were expressed as the mean ± SD. The results were analyzed using the SPSS 25 software. At ($p < 0.05$), the values were considered significantly different at 95% level of confidence.

RESULTS

Percentages of yields of the extracts and preliminary phytochemical screening: The significantly high dry yield was recorded for 70% aqueous acetone extract (12.919 ± 0.233), compared to dry yield of 80% aqueous methanol extract (9.348 ± 0.367) obtained from *L. indica* leaves. The results of the phytochemical screening test revealed that both defatted crude leaf extracts obtained from *L. indica* plant have alkaloids, phenolics, saponins, flavonoids, tannins, carbohydrates, terpenoids and phytosterols. Our research findings are supported by the literature reported in 2017 by Ghagane et al. In their study, they have found that various phytochemicals including phenols, flavonoids, alkaloids and glycosides present in this plant (Ghagane et al., 2017).

Evaluation of the formulations developed: All formulations of herbal balm were found to be semi-solid, homogenous, pleasant in odor, good in appearance with no phase separation and pH in the range of 6 to 7. Physicochemical stability parameters of all formulated balms showed no remarkable variation during the period of 60 days tested. The percentage inhibitions of balm formulations by membrane stabilization of erythrocytes were evaluated at 12.5 mg/ml concentration and all the formulations showed high *in vitro* anti-inflammatory activities compared to positive controls (Commercial balms) (Table 2). Herbal balm formulations (Fb2, Fb3 & Fb4) which contained 70% aq. acetone crude extracts exhibited promising *in vitro* anti-inflammatory activity in a concentration dependent manner. Balm base and Fb1 which contained some known anti-inflammatory ingredients except plant extract also resulted lower level of percentage inhibition compared to others. Many research studies have been conducted worldwide to develop anti-inflammatory herbal formulations using medicinal plant extracts as the key ingredient (Padmanabhan & Jangle, 2012) and improved efficacy had been found and it may be due to the fact that herbal drugs contain different molecules which act synergistically on the same target through different pathways while most of the modern allopathic drugs contains single active ingredient that targets only one specific pathway (Kumar et al., 2013).

Table 2: The percentage inhibitions of balm formulations

Formulation	% Inhibition ± SD	Radical Scavenging activity (mmol Trolox/100 g balm)
Base	14.352± 0.443	0.053 ± 0.005
Fb1	42.845±0.981	0.422 ±0.004
Fb2	57.786± 0.405	0.934 ± 0.003
Fb3	62.037± 0.318	1.967 ± 0.010
Fb4	75.084± 0.193	2.892 ± 0.008
Commercial Balm 1	59.806±0.740	1.559 ± 0.006
Commercial Balm 2	54.040± 0.875	1.676 ± 0.004

The *in vitro* radical scavenging activity of final formulations was evaluated by using (2, 2 diphenyl-1-picrylhydrazyl) DPPH assay compared to commercial balms at 50 mg/mL concentration of samples, with reference to the standard curve equation: y = 0.2635x, r2 = 0.9966. The results were expressed as mmol Trolox equivalents/100 g of formulation (Table 2). Fb3 and Fb4 herbal balm formulations exhibited higher *in vitro* radical scavenging activity in DPPH assay compared to positive controls.

The presence of the phytochemicals in preliminary phytochemical screening further support the promising anti-inflammatory and antioxidant activities shown by *L. indica* leaf extracts as the active ingredient in found in these herbal formulations (Hettihewa & Srilal unpublished data). It is reported that different groups of naturally occurring antioxidant phytochemicals such as polyphenols account for most of the antioxidant activity of plants (Tsao & Deng, 2004). According to the research studies flavonoids, phenols, saponins, alkaloids and terpenoids are considered as the major phytochemicals accounts for anti-inflammatory activity of plants. Among them, flavonoids are reported to exert greater role as an anti-inflammatory agent than other phytochemicals (Kumar et al., 2013).

Inflammation causes lysis of lysosomal membranes which releases their enzymes producing various inflammatory diseases. It finally leads to tissue injury due to the damage of macromolecules and free radical induced lipid peroxidation. The release of lysosomal constituents such as bacterial enzymes and proteases in lysosomes of activated neutrophils produces further tissue damage and inflammation (Leelaprakash & Dass, 2011). Therefore, the inflammatory conditions can be controlled by inhibiting the release of lysosomal enzymes or by stabilizing the lysosomal membrane. Since human red blood cell membrane is similar to lysosomal membrane, stabilization of red blood cell membrane by inhibiting heat induced hemolysis, in which heat is the injurious factor, can be taken as a measure of anti-inflammatory activity of a test sample. Since red blood cell membrane is analogues to lysosomal membrane, stabilization of the erythrocyte membrane during injurious conditions implies that agent can as well as stabilize lysosomal membranes during similar inflammatory conditions (Sarveswaran et al., 2017). Free radicals cause oxidative stress in the body. Therefore, radical scavenging activity is a good indicator of antioxidant activity present in a test sample (Biswas, 2016).

CONCLUSIONS

The preliminary phytochemical analysis demonstrated the presence of various phytochemicals including alkaloids, phenols, flavonoids, tannins, terpenoids, saponins in the both extracts. It is concluded that the formulated herbal balms have promising *in vitro* anti-inflammatory and radical scavenging activities compared to the positive controls tested and formulation Fb4 can be recommended for further investigations of clinical trials.

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DECLARATION OF CONFLICT OF INTEREST

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

REFERENCES

Ahmed A U (2011) An overview of inflammation: Mechanism and consequences. Front. Biol., 6(4): 274–281.

Biswas S K (2016) Does the Interdependence between Oxidative Stress and Inflammation Explain the Antioxidant Paradox ? Oxidative Medicine and Cellular Longevity. *2016*, 1–9.

Chaudhary S, Kumar A (2014) Phytochemical analysis and assessment of in-vitro anthelmintic activity of *Cassia auriculata Linn* leaves. American Journal of Phytomedicine and Clinical Therapeutics. 2(2): 161–167.

Ghagane S C, Puranik S I, Kumbar V M, Nerli R B, Jalalpure S S, Hiremath M B, Aladakatti R (2017) *In vitro* antioxidant and anticancer activity of *Leea indica* leaf extracts on human prostate cancer cell lines. Integrative Medicine Research. *6*(1), 79–87.

Harvey A L (2008) Natural products in drug discovery. Drug Discovery Today. 13(19/20): 894–901.

Hettihewa S K (2014) Extraction, characterization and *in vitro* testing of flavonoids rich fractions obtained from *Actinidia macrosperma* fruit. PhD Thesis, University of Auckland, New Zealand.

Kekuda P T R, Raghavendra H L, Bharadwaj N A, Akhilesha S (2018) Traditional uses, chemistry and pharmacological activities of *Leea indica* (Burm.f.) Merr. (Vitaceae):A comprehensive review. International Journal of Green Pharmacy. 12(1): 71–80.

Kumar A, Jha, K K, Kumar D, Agrawal A, Gupta A (2012) Preliminary Phytochemical Analysis of Leaf and Bark (Mixture) Extract of *Ficus Infectoria* Plant. The Pharma Innovation. 1(5): 71–76.

Kumar S, Bajwa B S, Kuldeep S, & Kalia A N (2013) Anti-Inflammatory Activity of Herbal Plants : A Review. International Journal of Advances in Pharmacy, Biology and Chemistry. 2(2): 272–281.

Leelaprakash G, & Dass S M (2011) *In vitro* anti-inflammatory activity of methanol extract of *Enicostemma axillare*. International Journal of Drug Development & Research. *3*(3): 189–196.

UE Makris M D, M J Kohler M D, L Fraenkel M D M P H (2011) Adverse effects of topical NSAIDs in older adults with osteoarthritis:a systematic review of the the literature. NIH Public Access,J Rheumatol, 37(6):1236–1243.

Mcpherson M L, & Cimino N M (2013). Topical NSAID Formulations. Pain Medicine. 14: 35–39.

Nworu C S, & Akah P A (2015) Anti-inflammatory medicinal plants and the molecular mechanisms underlying their activities. African Journal of Traditional, Complementary and Alternative Medicines. 12(Suppl.): 52–61.

Padmanabhan P, Jangle S N (2012) Evaluation of *in-vitro* anti-inflammatory activity of herbal preparation:A combination of four. International Journal of Basic and Applied Medical Sciences. 2(1): 109–116.

Ramesh S, Venkanna B U, Rangesh P, & Udyavara B R (2010) Herbal balm composition and methods of preparation thereof. World Intellectual Property Organization International Bureau. 2(12).

Sarveswaran R, Jayasuriya W J A B, & Suresh T S (2017) *In vitro* assays to investigate the anti-inflammatory activity of herbal extracts: A review. World Journal of Pharmaceutical Research. 6(17): 131–141.

Tsao R, & Deng Z (2004) Separation procedures for naturally occurring antioxidant phytochemicals. Journal of Chromatography B. 812(2004):85–99.

Yoshikawa T, NaitoY (2002) What Is Oxidative Stress ? JMAJ. 45(7): 271–276.