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Formulation of a novel anti-inflammatory balm using *Neolitsea cassia* (L.)

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Abstract
Inflammation is a pathological condition that may lead to various chronic diseases. This study evaluates the anti-inflammatory effect of *Neolitsea cassia* plant leaves, screens its preliminary phytochemistry, and the plant material is subsequently used in a herbal balm formulation. The *in vitro* anti-inflammatory activity of 70% acetone extract of the plant was assessed using the human red blood cell membrane stabilization method, specifically against heat-induced haemolysis. Aspirin was used as the positive standard of the assay. The results demonstrated that the test plant contains various phytochemicals such as phenolics, flavonoids, alkaloids, and saponins but no carbohydrates. The extract exhibits significant *in vitro* activities ($p<0.05$) in doses ranging from 125 µg/mL to 2000 µg/mL compared to normal saline control. The bioactivity was found to be concentration-dependent and 2000 µg/mL concentration of plant extract showed the highest inhibition percentage of *in vitro* haemolysis (70.35±0.01%). The balm formulations developed with different doses of *N. cassia* extracts showed consistent physical stability over two months of period from the manufacture. Therefore, it is concluded that *Neolitsea cassia* plant possesses significant anti-inflammatory activity, thus it is useful in the development of stable anti-inflammatory herbal balm formulations.

Keywords: Lauraceae, herbal balm, anti-inflammatory, cell membrane stability, *in vitro*

Introduction
Inflammation is a protective strategy that has evolved in animals to respond to harmful conditions in the body. It allows the host to remove stimuli that damage body tissue through various immune responses [1]. Anti-inflammatory agents block certain biomolecules in the body which cause inflammation and thereby ease the pain, swelling, and generalized fever. They may also prevent or slow the progression of chronic inflammatory diseases [2]. There is a growing need and demand for the development of novel, affordable, effective, and safe anti-inflammatory drugs, preferably derived from natural sources recently due to the comparative advantages persisting in herbal medicines. Secondary metabolites from plants have been an important source of medication in mankind and a number of medicines with natural origin are in allopathic use even today [3].
Neolitsea cassia (L.) Kosterm, also known as "Dawul Kurundu" in Sinhala, is native to Sri Lanka and belongs to the Lauraceae family. The plant is found in higher altitude understory, even at rainforests. It is a perennial tree which maximum height is about 20 m. This tree has thick grayish bark and the wood is hard, lightweight and pale orange in colour [4]. This plant is particularly popular in some areas because its gummy leaves are used in the preparation of traditional food as well as various types of herbal remedies. A water-soluble arabinoxylan has been isolated from the leaves of *N. cassia* (L) where small proportions of other sugars have also been found in previous investigations [5]. Further analysis of phytochemicals has revealed carbohydrates, tannins, flavonoids, and alkaloids in dried mucilaginous material. Also, phytochemical investigation on stem and leaves have led to the isolation of novel phytochemical compounds [6, 7]. Although there are some previous studies done on the evaluation of physicochemical characteristics of the mucilage and the plant material, there is no recent scientific evidence for its anti-inflammatory activity. Hence, the present study was aimed to scientifically investigate the anti-inflammatory potential of *N. cassia* leaves *in vitro* by human red blood cells membrane stabilization assay and to formulate a stable topical balm preparation with the acetone plant extract.

Materials and Methods
Chemicals and equipment
All chemicals were in analytical grade and acetone and hexane were used for the extraction of the plant. Normal saline was used for the preparation of blood suspension. UV-visible spectrophotometer (JENWAY 6305), analytical balance (RADWAG AS 220.R2), hot air sterilizer (GEMMY 888), and centrifuge apparatus (GEMMYCO PLC-025) were used.

Ethical approval
Ethical approval for the protocols involving human participants (for blood collection) was obtained from the Ethics Review Committee, CINEC Campus, Malabe, Sri Lanka.

Authentication and preparation of plant extract
Neolitsea cassia (L.) plants and leaves were collected from Gampaha District, Sri Lanka and the plant was taxonomically identified by the National Herbarium, Peradeniya, Sri Lanka. Collected leaves samples were oven dried at 55 °C until a constant weight was obtained and then the sample was milled into a coarse powder. The 70% acetone crude extract was prepared by macerating the powdered plant sample while shaking for 24 hours. Chlorophyll in the acetone extract was removed by using hexane in a separatory funnel. Finally, the resulting extract was freeze-dried and the solid powder was stored at 2 - 4 °C.

Phytochemical screening

Preliminary phytochemical screening was done by well-established methods. In brief, Wagner's test for alkaloids, ferric chloride test for phenolics, frothing test for saponins, Molisch's test for carbohydrates, cyanidin test for flavonoids were performed [8].

***In vitro* anti-inflammatory activity**

Human red blood cell membrane (HRBC) stabilization in heat-induced haemolytic conditions method described in literature was used to assess the anti-inflammatory activity of the crude plant [9]. The assay was carried out for different concentrations (2000 µg/mL, 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL) of test plant extract samples. Aspirin was used as the positive control, whereas normal saline was used as the negative control. Percentage inhibition of heat-induced haemolysis was then calculated using the UV-visible absorbance data and the IC_{50} values were determined by the dose-response curves. All the tests were triplicated and statistical analysis was done by ANOVA test.

Formulation of anti-inflammatory herbal balm

Different formulations of herbal balm incorporated with *N. cassia* acetone extract were developed by altering the composition as given in Table 1. Each of the excipient components was measured using analytical balance and were added into a container at 75 ^{0}C. The mixture was stirred well until all the materials were molten and a homogenous balm base was formulated. Then the container was taken out, let to cool, and different amounts of the plant extract were added portion-wise to the blend. Stirring was continued until the final balm formulations (Figure 1) were obtained at room temperature (28 ^{0}C).

Table 1: Composition of the formulated anti-inflammatory herbal balm

Ingredient	Weight of the ingredient (g)			
	Balm base	Formulation 1	Formulation 2	Formulation 3
Beeswax	2.56	2.56	2.56	2.56
Castor oil	4.60	4.60	4.60	4.60
Menthol	1.24	1.24	1.24	1.24
Eucalyptus oil	1.00	1.00	1.00	1.00
Camphor	1.00	1.00	1.00	1.00
Liquid paraffin	0.90	0.90	0.90	0.90
Glycerine	1.20	1.20	1.20	1.20
Plant extract	0.00	0.03	0.05	0.30

Evaluation of physicochemical stability parameters

The formulated balm was stored in well-stoppered glass vials at room temperature (28±3 ^{0}C) for 60 days. On manufacture day and after the stability testing period, appearance, odour, homogeneity, and phase separation in the balm were evaluated as physical stability parameters.

Fig 1: Anti-inflammatory herbal balms formulated with different *Neolitsea cassia* doses

Results and Discussion

The cold maceration extraction method employed in this study yields in majority of the thermolabile compounds in plant samples in their intact form, due to less thermal energy supply to the material [10]. The 70% acetone extract was further purified by solvent-extraction using hexane to remove chlorophyll. This step is important in the subsequent phytochemical analysis and HRBC membrane stabilization assay, since both of these procedures depend on the colour absorbance of the test samples. Green colour resulting from chlorophyll can be eliminated by extracting the plant material with a non-polar solvent, such as hexane [11]. In the present study, 70% acetone extracts of *Neolitsea cassia* showed positive results for saponins, alkaloids, phenolics, and flavonoids (Table 2) however, the Molisch's test led to a negative result indicating the absence of carbohydrates in the sample.

Table 2: Results of phytochemical screening of *Neolitsea cassia* 70% acetone extract

Phytochemical group	Test method	Results
Phenols	Ferric chloride test	Positive
Saponin	Frothing test	Positive
Carbohydrate	Molisch test	Negative
Alkaloids	Wagner's test	Positive
Flavonoids	Cyanidin test	Positive

Inflammation causes the lysis of cellular lysosomal membranes which releases enzymes that exacerbates tissue damage by destroying macromolecules and triggering lipid peroxidation. [12]. the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization suggests that test extract may as well stabilize lysosomal membranes to mitigate inflammatory responses [13]. A dose-dependent and significant red blood cell membrane stabilizing activity ($p<0.05$) was shown by the test leaf extracts at various concentrations compared to the control (Figure 2). IC_{50} value of the plant extract was determined to be 935.41 µg/mL whereas the positive control, aspirin resulted in an IC_{50} of 234.96 µg/mL in the same assay.

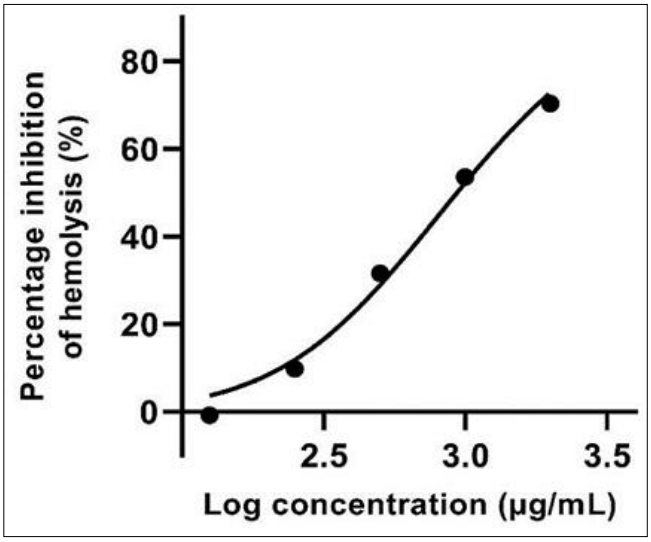

Fig 2: *In vitro* anti-inflammatory activity of *Neolitsea cassia* 70% acetone extract

Aspirin is used in pharmacotherapeutics to relieve inflammations and this study shows that aspirin is about four-fold potent than the *N. cassia* 70% acetone test extract. According to Figure 3, the highest percentage inhibition was given at 2000 µg/ml of both plant extract (70.35±0.01%) and aspirin (61.15±0.00%). The plant extract produced a greater inhibition of haemolysis than aspirin at 2000 µg/ml concentration however, in lesser concentrations, the percentage inhibition by plant extract was significantly lower than aspirin ($p<0.05$).

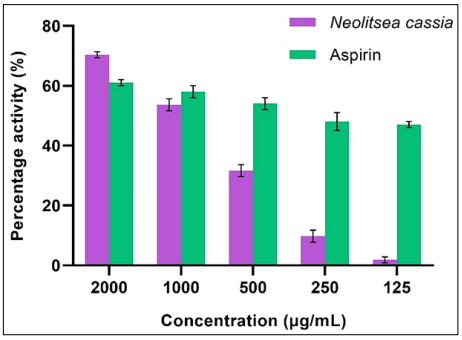

Fig 3: Comparison of anti-inflammatory activity of *Neolitsea cassia* 70% acetone extract and aspirin in heat-induced haemolysis assay

The involvement of *N. cassia* carbohydrates for cell membrane stabilizing mechanism would be minimal according to the findings of this study, as such molecules were absent in the phytochemical screening. In a previous study, gum-like arabinoxylan compounds have been isolated from *N. cassia*. It holds water molecules inside its structure, thereby functions as a thickening agent to be used as an additive in pharmaceutical preparations [5]. Along with that, coccinine, daibucarboline, linderane, epicatechin, and several other compounds have also been isolated from this plant. Therefore, anti-inflammatory activity of the test extract is assumed to be exerted through these secondary metabolites evidenced in literature [7].

The formulated herbal balm would alleviate inflammation and provide relief from pain and swelling, offering a natural alternative to synthetic anti-inflammatory medications. *Costus afer*, *Zingiber officinale*, *Vitex negundo*, *Syzygium aromaticum*, and *Cinnamomum camphora* are some of the plants that had been used to formulate herbal balms [14, 16]. In this study different excipients were used for different pharmaceutical functions. Beeswax acts as a thickening agent and emulsifier. It provides structure and stability to the balm, giving it a solid yet spreadable consistency. Castor oil is known for its moisturizing and emollient properties. Menthol and camphor give a cooling sensation and may also act as mild analgesics or counter-irritants. Glycerine attracts moisture from the environment into the skin, keeps the skin hydrated and enhances the absorption of the balm [16, 17]. The formulated herbal balm showed physical stability during the short-term investigation, since all the testing parameters were almost similar on manufacture date as well as after 60 days (Table 3).

Table 3: Stability assessment of *Neolitsea cassia* incorporated herbal balm

Parameter	Formulation 1		Formulation 2		Formulation 3	
	Day 1	Day 60	Day 1	Day 60	Day 1	Day 60
Colour	Yellow	Yellow	Yellow	Yellow	Brown	Brown
Odour	Significant herbal odour	No change	Significant herbal odour	No change	Significant herbal odour	Slightly changed
Homogeneity	Yes	Yes	Yes	Yes	Yes	Yes
Phase separation	No	No	No	No	No	No

The slight change of odour in formulation 3 may be due to the degradation of pharmaceuticals or any microbial activity. To minimize these unfavourable effects in the formulation, antioxidants or preservatives (such as vitamin E, benzalkonium, etc.) can be used in a future study [18, 19]. Furthermore, conducting extended stability testing under various environmental conditions will ensure long-term efficacy of the balm. Incorporating advanced delivery systems such as nano-encapsulation can facilitate the transdermal penetration and release of active ingredients from the balm [20].

Conclusions

Stabilization of the HRBC membrane in heat-induced haemolysis was studied to establish the mechanism of anti-inflammatory action of *Neolitsea cassia* plant. The presence of active phytochemicals such as alkaloids, flavonoids, saponin, and related phenolics may be

responsible for the *in vitro* activity evidenced in the present study. The herbal balm formulated with the test extract was physically stable for two months of period from the manufacturing, however, further modifications and investigations are required for its optimization.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

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