Introduction

Leucas zeylanica (L.) W.T.Aiton (Figure 1), locally called "gata-thumba" is a popular, multi-purpose medicinal plant in Sri Lanka that belongs to the family Lamiaceae. Different parts of this plant have been extensively employed in traditional medicine as a remedy for various inflammatory conditions, gout, headaches, vertigo, and also as an anthelminthic medication [1]. Besides, the leaves of the plant are consumed as a vegetable [2]. The extracts prepared from *L. zeylanica* exhibited hepatoprotective effects [3] and were found to be highly effective against *Enterobius vermicularis* infections in adults [4]. Furthermore, the lipophilic extracts have displayed potent anti-inflammatory activities by inhibiting the two enzymes; 5-lipoxygenase and microsomal prostaglandin E_2 synthase-1 involved in the synthesis of pro-inflammatory eicosanoids. These lipophilic extracts have also inhibited the activity of the xanthine oxidase enzyme and displayed moderate antimicrobial activities [1]. Moreover, *in vitro* and *in vivo* studies revealed that the extracts of *L. zeylanica* were safe and non-toxic in terms of skin irritation, cytotoxicity, and genotoxicity, thus potential utility as a pharmaceutical and cosmetics agent [5].

Fig.1: Leucas zeylanica (L.) W.T.Aiton

Although *L. zeylanica* is widely used in the treatment of skin diseases, wounds, sores, itches, etc. in traditional medicine, these claims are hardly explored and validated by scientific methods. In an early attempt, Napagoda et al. reported a strong, broad-range photoprotective activity in the aqueous methanolic extract of this plant [6]. As an extension of the above study, the current research aimed at developing skin care formulations from the aqueous methanolic extract and evaluating the photoprotective and skin whitening potential of the prepared formulations.

Method

Plant material

The whole plants of *L. zeylanica* were collected from local cultivations in Western and Sabaragamuwa provinces of Sri Lanka in 2018. The plant was authenticated by comparison with the herbarium specimens at the National Herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka. A voucher specimen (Leu-2018-4) is deposited at the Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka.

Preparation of crude extract

The whole plants of *L. zeylanica* were washed and dried in the shade (30 ^{O}C) for five days. Thereafter, dried plants were powdered and the powdered materials (11 g) were extracted in 180 mL of 70% methanol-water mixture. The extracts were evaporated to dryness with a rotary evaporator (HS-2005V-N, South Korea).

Determination of the total phenolic content of the crude extract

The total phenolic content in the plant material was estimated by the method adopted from Gangwar et al [7] with some alterations. The analysis was done using Folin- Ciocalteu method, where the ultimate response is the formation of a blue complex by the phenolic compounds present in the extract. A volume of 500 µL of extract solution (1 mg/mL) in methanol was mixed with Folin-Ciocalteu reagent (2500 µL). The resulting solution was mixed and incubated for 5 minutes. Thereafter, Na_2CO_3 (2500 µL) was added and the reaction mixture was diluted to 10 mL by adding distilled water, followed by incubation at room temperature in the dark for 2 hours. The absorbance was measured at 765 nm using a UV-visible spectrophotometer (Shimadzu, UV_1800). The gallic acid was used to prepare the standard curve. The results were expressed as mg of gallic acid equivalent (GAE)

per gram of dry plant material.

Determination of tyrosinase inhibition

The skin whitening potential of the extract was determined by following the method described by Napagoda et al [8]. This assay is based on measuring the inhibition of tyrosinase, a key enzyme in melanin biosynthesis. In brief, the plant extract was dissolved in 50 mM potassium phosphate buffer (pH 6.5) and tested for tyrosinase inhibition at a concentration of 333.3 µg/mL at the 96-microwell plate. A volume of 70 µL of the extract was mixed with tyrosinase (333 units/mL in phosphate buffer, 30 µL) and the mixture was incubated at 37 °C for 10 minutes. Thereafter L-tyrosine (110 µL) was added to each well. The reaction mixture was incubated again at 37 °C for 30 minutes. The absorbance was measured at 492 nm (Thermo Scientific-Multiskan Go Microplate spectrometer). The percentage inhibition of tyrosinase activity was calculated using the following equation.

$$\% \text{ Inhibition} = [(A - B) / A] \times 100$$

where, A = absorbance without the test sample (control), B = absorbance with the test sample. Ascorbic acid was used as a positive control. The experiments were carried out in triplicate.

Evaluation of UV-filtering potential of the crude extract

The UV-filtering potential of the extract was evaluated following the method described by Napagoda et al. [6] and the sun protection factor (SPF) was calculated according to the Mansur equation [9]. In brief, the UV absorption of the extract (at a concentration of 1 mg/mL) was measured between 260–400 nm at 5 nm intervals using a UV-visible spectrophotometer (Shimadzu, UV_1800). The UV absorbance values between 290–320 were substituted in the Mansur equation to calculate the SPF.

$$SPF_{spectrophootometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

where: EE(λ) – erythemal effect spectrum; I(λ) – solar intensity spectrum;

Abs(λ) – absorbance of sunscreen product; CF – correction factor (=10)

Development of herbal sunscreen formulations

Sunscreen formulations were prepared by incorporating the plant extract at different concentrations (i.e., 25%, 50%, 75%) into an aqueous cream base that comprised cetostearyl alcohol, white soft paraffin wax, and sodium lauryl sulphate.

The UV filtering capability of each formulation was determined as mentioned above. All the formulations were exposed to direct sunlight for 21 days and UV absorption was measured on 7th, 14th, and 21st day and subsequently, the SPF values were calculated. The aqueous cream base was employed as the negative control, while a commercially available sunscreen product containing benzophenone-4 and TiO_2 as active ingredients was used as the positive control.

Determination of the photostability of the formulations

The photostability of the formulations was determined following the method described by Gonzalez et al. [10] with several modifications. Each formulation (50 mg) was applied evenly on a 25 cm^2 area of a stainless-steel plate, corresponding to an area density of 2.0 mg/cm^2 [10,11]. The plates were dried for 15 min under dark conditions and thereafter exposed to natural sunlight for the same length of time from 10.00 a.m. to 3.00 p.m. Control plates that have not been exposed to sunlight were also prepared for UV absorption measurements.

The exposed and non-exposed formulations were dissolved in distilled water to reach a final concentration of 0.2 mg/mL and thereafter the UV absorbance of each sample was measured from 290 to 400 nm. A commercial sunscreen product was used as the positive control while the aqueous cream base that has been used to prepare sunscreen formulations was tested as the negative control. The experiment was performed in duplicate. The average absorbance values were used to draw a curve of absorbance versus wavelength. The area under the curve (AUC) from 290 to 400 nm as well as UV-A_1 (340–400 nm), UV-A_2 (320–340 nm), and UV-B (290–320 nm) regions were calculated for each of the exposed and non-exposed samples. The AUC

index was determined from the equation;

$$\text{AUC Index} = \frac{\text{(AUC exposed)}}{\text{(AUC Non-exposed)}}$$

According to the calculation, if the AUC Index ≥ 0.8, the sunscreen formulation was considered to be photostable [10].

Statistical analysis

All the above experiments were performed in triplicate and the values were given as mean±S.D

Results

Determination of the total phenolic content of the crude extract

The Folin-Ciocalteu reagent was used to estimate the total phenolic content of crude aqueous methanolic extract of *L. zeylanica* and expressed as gallic acid equivalents (GAE). The amount was calculated from the linear regression equation of the standard curve of gallic acid (Y=0.0041x + 0.0197, R = 0.985). The results showed that the aqueous methanolic extract of *L. zeylanica* contained a high amount of phenols (50.63 ± 18.18 mg GAE/g) suggesting that the extract is capable of absorbing UV radiation more effectively.

Determination of tyrosinase inhibition

The aqueous methanolic extract of *L. zeylanica* displayed a very mild anti-tyrosinase activity with a percentage inhibition of 25.12±4.77 at a concentration of 333.3 µg/mL. On the other hand, ascorbic acid (positive control) displayed a percentage inhibition of 100 ± 0.58 at the same concentration.

Evaluation of UV-filtering potential of the crude extract

In agreement with the findings of Napagoda et al. [6], a broad spectrum of UV absorbance was observed for the aqueous methanolic extract. This was conspicuous within the 280–340 nm range that covers both UV-B and UV-A regions of the electromagnetic spectrum (Figure 2). The subsequent calculation revealed a SPF value of 39.8±0.12 for this extract.

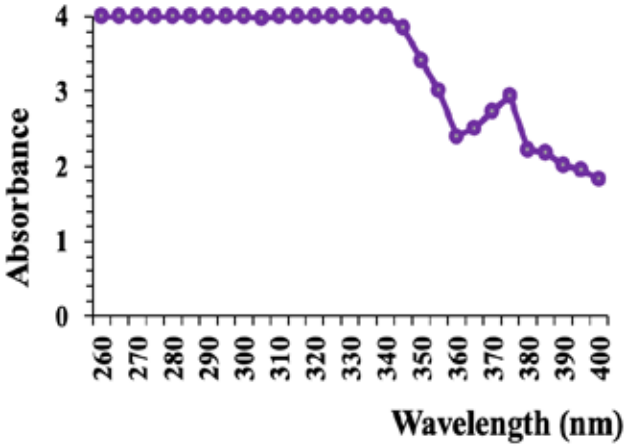

Fig.2: Absorption of UV radiation between 260–400 nm by the aqueous methanolic extract

The average absorbance values (n=3) observed for the plant extract are plotted against the wavelength.

Development of herbal sunscreens and evaluation of UV-filtering potential

An ideal sunscreen should possess high SPF value, a broad spectrum of UV-protection, and photostability [12]. Therefore, the sunscreen formulations prepared in this study were evaluated using the above parameters.

As indicated in Figure 3, a broad spectrum of UV absorbance was observed for all the formulations prepared from *L. zeylanica* extract. In comparison to the two counterparts, more noticeable UV absorbance was detected in the formulation comprised of 75% of the extract (Figure 3). This formulation (Figure 4) displayed its maximum absorbance at 290 nm while retaining high absorbance values in the range of 280–350 nm covering both UV-B and UV-A regions. The UV absorption potency of the commercial sunscreen product (positive control) was lower than that of the herbal formulations. The UV absorbance was insignificant in the negative control (not shown in Figure 3) which was used as the cream base to prepare the sunscreen formulations. This indicates a negligible contribution of the cream base to the observed high UV absorbance in the formulations prepared using it.

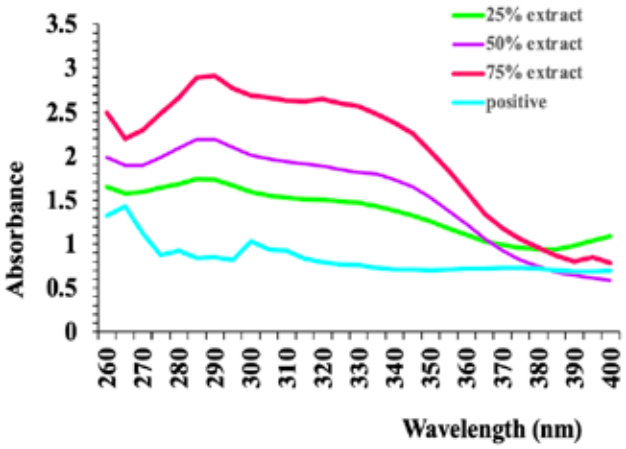

Fig.3: Absorption of UV radiation by different formulations

The average absorbance values (n=3) observed for the three sunscreen formulations and the positive control (commercial sunscreen) are plotted against the wavelength.

Fig 4: Formulation comprised of 75% of the aqueous-methanolic extract of *L. zeylanica*

Figure 5 illustrates the variation of SPF values with time upon exposure to direct sunlight. The initial SPF values of the formulations consisting of 25%, 50%, and 75% of *L. zeylanica* were observed as 15.67±0.39, 19.83±2.49, and 26.76±3.82 respectively. Interestingly the SPF values of all three formulations hardly changed by storing in direct sunlight. The SPF value of the commercial sunscreen was found as 5.03±0.32.

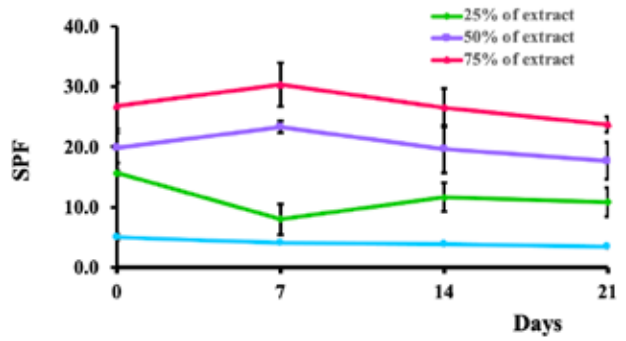

Fig.5: Variation of SPF in different formulations after exposure to direct sunlight

The sunscreen formulations and the positive control were stored under direct sunlight for 21 days. The SPF values were calculated on 7th , 14th and 21st day using the UV-absorbance data. The variation of SPF value at different time intervals is shown in this figure (n=3).

Determination of the photostability of the formulations

Table 1 shows the AUC index values of the prepared sunscreen formulations along with positive control and negative control. According to this, all the formulations displayed photostability by giving AUC index values higher than 0.80 within the total spectrum as well as UV-B, UV-A_1, and UV-A_2 regions. Moreover, the positive control was also photostable but its photostability was lower than the photostability of the sunscreen formulations which were under investigation. The negative control lacked photostability due to the AUC index values far below 0.80. This suggested the unavailability of any UV-protective molecule within the cream base. The 50% extract incorporated formulation displayed the highest photostability among all the formulations by giving high AUC index values. Furthermore, all the formulations displayed promising photostability in UV-A than in UV-B, as these formulations have high AUC index values in UV-A region. Therefore, improvement of the prepared sunscreens in commercial scale would give higher protection to consumers as commercially available sunscreens are highly susceptible to UV-A mediated photodegradation.

Table 1: AUC index values for the investigated sunscreen formulations

Sample	AUC Index			
	Total spectrum	UV-B	UV-A_1	UV-A_2
25% of extract	1.20	1.12	1.34	1.21
50% of extract	1.27	1.23	1.36	1.29

Discussion

Skin is the outermost barrier of the body that is constantly being challenged by various factors such as solar ultraviolet radiation, humidity, allergens, microbes, pollutants, etc. Therefore, a diverse range of skin care products including

sunscreens, moisturizers, anti-aging, anti-acne as well as skin whitening formulations have been introduced and become highly popular [13]. The topical application of sunscreens enriched with UV absorbing, reflecting, or scattering molecules reduces the penetration of harmful UV radiation whereas the skin whiteners usually interfere with the synthesis of melanin, a photoprotective molecule responsible for the pigmentation of human skin [6, 8]. The UV-A and UV-B components of solar ultraviolet radiation are responsible for immediate and delayed tanning [14] while Asian skin in particular is highly vulnerable to solar radiation-induced irregular and hyperpigmentation [15]. Thus, many sunscreen products contain UV-absorbing aromatic compounds like salicylic acid, para-aminobenzoic acid, and benzoic acid, which have been identified as tyrosinase inhibitors interfering with the production of melanin, hence preventing the sun tanning of the skin. However, it is speculated that despite the effective filtering of UV radiation, the inhibition of melanin biosynthesis by such UV-filters might influence the malignant melanoma incidence upon solar exposure [16]. In this respect, the very mild anti-tyrosinase activity of the aqueous methanolic extract of *L. zeylanica* extract would make our sunscreen formulations distinctive from most of commercial sunscreens.

Since many commercial sunscreens are capable of providing protection against UV-B only, the development of broad spectrum sunscreens with both UV-B and UV-A radiation barring potential is a timely need. The sunscreen formulations developed from the aqueous methanolic extract of *L. zeylanica* could be considered as a solution to this issue. Herbal formulations prepared in this work were found to be superior to the commercial sunscreen that was used as the positive control with respect to the SPF value and photostability. The protection offered by the sunscreen formulation from UV radiation increases with the extract concentration, thus the highest absorbance and the highest SPF were observed with the formulation consisting of 75% of the extract. This sunscreen formulation is of great significance not only due to its high SPF value but also due to its photostability and broad spectrum sunscreen activity. Normally, sunscreen products are categorized according to their SPF values as “minimal” (SPF < 12), “moderate” (SPF 12-30), and “high” (SPF ≥ 30) sun protection products [17].

Our observations demonstrated that the formulation comprised of 75% extract has the potency of a “moderate” sun protection product due to its SPF value of 26.76±3.82. This formulation displayed a broad spectrum of sun protection by efficiently absorbing the UV radiation between 280–350 nm which covers UV-B and UV-A regions of the solar electromagneticspectrum. Based on these findings, experiments are underway to enhance the UV filtering capacity of these formulations using a nanotechnological approach to develop herbal skin care agents in a commercial scale.

Conclusion

The findings of this study revealed that the aqueous methanolic extract of the whole plant of *L. zeylanica* was a promising candidate for the development of herbal sunscreen formulations. The presence of a high amount of phenols in this extract might be responsible for the effective absorption of UV radiation. In general, all formulations prepared from this extract exhibited good SPF values and photostability, yet, the formulation containing 75% of the extract was found to be superior with respect to the broad spectrum UV-filtering ability, high SPF, and photostability. These results offer great promise for the development of herbal skin care products of high commercial value from the aqueousmethanolic extract of *L. zeylanica.*

Acknowledgment

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Conflicts of interest

The authors declare that they have no competing conflict of interest.

Ethical Approval
Not applicable

Reference

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