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DSN De Silva
Department of Pharmacy, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Werahera, Sri Lanka

LL Bandara
Department of Pharmacy, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Werahera, Sri Lanka

BLC Samanmali
Department of Pharmacy, Faculty of Allied Health Sciences, General Sir John Kotelawala Defence University, Werahera, Sri Lanka

WD Ratnasooriya
Department of Basic Sciences, Faculty of Allied health Sciences, General Sir John Kotelawala Defence University, Werahera, Sri Lanka

RN Pathirana
Department of Basic Sciences, Faculty of Allied health Sciences, General Sir John Kotelawala Defence University, Werahera, Sri Lanka

WPK Abeysekara
Herbal Technology Section, Industrial Technology Institute (ITI), Malabe, Sri Lanka

Correspondence
RN Pathirana
Department of Basic Sciences, Faculty of Allied health Sciences, General Sir John Kotelawala Defence University, Werahera, Sri Lanka

Investigation of sun screening and antioxidant activity of *M. indica ver* "*Willard*"

DSN De Silva, LL Bandara, BLC Samanmali, WD Ratnasooriya, RN Pathirana and WPK Abeysekara

Abstract
Currently, there is a need and demand for the development of safe, cheap and effective topical herbal formulation as sunscreen. With this in mind, present investigation was undertaken mainly to evaluate sun protective activity of *M. indica ver* "*Willard*" *in vitro* using UV spectroscopic technique and Mansur equation. Sun Protection Factor (SPF) was evaluated for methanolic leaf extract having concentration of 2.0 mg/mL and for the methanol soluble fraction of Dermatone® (Concentration: 2.0 mg/mL), as the reference agent. The anti-oxidant activity of methanolic leaf extract of *M. indica ver* "*Willard*" was measured *in vitro* using standard procedures. The results showed that the leaf extract has high SPF value of 38.67 whilst Dermatone® showed a SPF value of 33.76. Phytochemical analysis revealed the presence of alkaloids, carbohydrates, tanins, terpenoids, phenols, glycosides, flavonoids and saponins. The methanolic leaf extract of *M. indica ver* "*Willard*" exhibited a marked anti-oxidant activity.

Keywords: *M. indica ver* "*Willard*", sun protection factor, anti-oxidant activity, potency

1. Introduction
The sun is responsible for the development and existence of life on earth. The harmful effects of solar radiation are mainly caused by the ultraviolet (UV) region of the Sun's terrestrial spectrum. The UV region is subdivided into three bands depending on the wavelength; UVA from 320nm-400nm, UVB from 290nm-320nm and UVC from 200nm-290nm [1, 2]. UVC radiation is the most biologically damaging but it is effectively filtered out by the ozone layer before reaching the Earth, whereas UVB radiation is not completely filtered out by the ozone layer. UVA radiation reaches the earth more intensively than UVB and can penetrate the deeper layers of the skin [3].
The response of the skin to exposure of UV radiation are classified as acute and chronic effects. Sunburn, tanning and vitamin D production are considered as acute effects. The chronic effects are mainly photo-aging and skin cancers [2, 3].
The level of UVB radiation reaching the surface of the earth is largely controlled by ozone, a gas in the atmosphere and the dissociation of ozone is the mechanism responsible for reducing/preventing UV radiation of short wavelengths reaching the earth's surface. Chlorofluorocarbons and other gases released by human activities lead to the depletion of stratospheric ozone thus increasing the deleterious effects on humans caused by solar radiation [2].
Human skin consist of two main defense mechanism to guard against the damaging effects of UV radiation; they are epidermal thickening and the stimulation of melanin (a natural pigment produced by oxidation of amino acid tyrosine followed by polymerization) synthesis, and proteins, lipid and nucleotides are also consider as skin's natural sun blockers [4, 5]. Deoxyribonucleic acid (a type of nucleotide) absorbs UVB radiation of about 245nm-290nm wavelength in high amplitudes thus indicating that UVB radiation is the primary mutagen as UVB induces structural DNA damage. UVA radiation is very weakly absorbed by DNA and it is responsible for indirect DNA damage mainly by generating reactive oxygen species [1, 4]. Reactive Oxygen Species (ROS) appear to causes oxidative damage and impairment of the antioxidant system resulting in reducing the protective enzyme levels and imbalances the level of total proteins, ascorbic acid (vitamin C), and other antioxidant levels on cells [6].
The solar UV index introduced by the World Health Organization (WHO) expresses the level of solar UV radiation at the earth's surface .The values range from zero to eleven and above and higher the index value, the greater the potential for damage and less time required to harm the skin and eyes. Protective measure for preventing undesirable effects of UV radiation have been introduced by the WHO and it recommends additional protective measures and the application of sunscreen with a SPF of more than 15 when the UV index is higher than two. The most popular modern strategy to prevent harmful UV radiation that penetrates the skin is

the application of topical sunscreen products that contain UV absorbing, reflecting or scattering sunscreen agent [7].

Synthetic Sunscreen agents although safe they exert several undesirable health effects. Sensitivity although rare, can occur such as photo allergic reactions including contact dermatitis, allergies. Melanomas and skin cancers [8, 13]. Most of the organic substances used in sunscreen products have not been established as safe for long term human use yet [5, 9].

The use of Herbal products are being implementing in treating many complicated diseases nowadays. Due to the lack of associated adverse effects, synthetic sunscreen products are rapidly being replaced by the herbal sunscreen products. Using natural ingredients in skin care products is possible due to the fact that plants have the ability to protect themselves from solar UV radiation. Natural antioxidants are capable of scavenging free radicals (mainly ROS) which is the main destroying factor in the skin and can also stimulate skin repair and maintenance [5, 9]. There is a slight improvement over in using antioxidant substances in sunscreen products to provide supplemental photo-protection activity. Antioxidants from natural sources provide new possibilities for the treatment and prevention of UV mediated Disease [10].

The growing awareness of the harmful effects generated by the solar UV radiation and the subsequently developing market for sunscreen influences the sun care segment of the cosmetic industry and therefore the research. Consequently there is a need and a great demand for development of novel plant based natural sunscreen which is relatively cheap and user friendly [5].

In this study, we report for the first time, *in vitro* sunscreen activity of leaves of *M. indica ver* "*Willard*" by evaluating the SPF values using spectroscopic technique and Mansur equation. Mango (*Mangifera indica* L.) is a juicy stone fruit belonging to the family of Anacardiaceae in the order of Sapindales and is grown in many parts of the world, particularly in tropical countries [11]. This plant was selected since we assume that it may have marked sun protection activity as it grows massively and provide good harvest mostly in dry zone under highly exposed to continuous solar radiation. Mango has been used as a herb in Ayurveda medicine and indigenous medical systems for hundreds of years. Different parts of the mango tree possess different medicinal properties. Studies have revealed that it carries antioxidant, anti-inflammatory, antimicrobial, anti-diabetic, hypotensive and cardio tonic properties [12].

2. Materials and Methods

2.1 Plant material collection and identification

The matured leaves of *M. indica ver* "*Willard*" was collected from Mirusuvil (geographical coordinates; 9º 40' 00" North, 80º 14' 00" East) Northern Province, Sri Lanka in February, 2017. The plant was identified by K.G.S Seneviratne, Assistant Director of Agriculture (Research), Fruit crops research and development station, Department of Agriculture, Gannoruwa, Peradeniya, Sri Lanka. Voucher specimens (SL/Pharm/01) were deposited in the pharmaceutical chemistry laboratory, Department of Pharmacy, Faculty of Allied Health Sciences, General Sir John Kotelawala Defense University, Werahera, Sri Lanka.

2.2 Preparation of methanolic extract of *M. indica ver* "*Willard*"

The leaves of *M. indica ver* "*Willard*" were thoroughly cleaned by running tap water and were then oven dried at 40 ^{0}C until a constant weight was obtained. The dried leaves were then crushed using a domestic grinder to a coarse powder. A quantity of 10 grams of coarse powder was macerated for seven days in 100mL of methanol (99.8%, Sigma-Aldrich Company, St Louis, USA). The resulting extract was filtered through a double layered muslin cloth and the filtrate was evaporated to dryness. The dry product was stored in an air-tight container at 4 ^{0}C until use.

2.3 *In-vitro* evaluation of sun protection factor of methanolic leaf extract of *M. indica ver* "*Willard*"

The product obtained from *M. indica ver* "*Willard*" was re-dissolved in methanol (99.8 % purity, Sigma-Aldrich Company, St Louis, USA) to prepare a sample solution of 2.0mg/mL. Simultaneously, Dermatone® was dissolved in methanol to obtain a solution of 2.0mg/mL to use as the reference agent. The absorbance of UV radiation by the prepared methanol extracts of *M. indica ver* "*Willard*" and Dermatone® were measured in micro plate, in triplicate using "SPECTRA max. PLUS^{384}" Microplate spectrophotometer from 290- 320nm at 5nm intervals using methanol as the blank. The SPF values were calculated by using the Mansur equation, [1, 3, 13] given below.

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

Where CF- Correction Factor (=10): EE- Erythermal Effect Spectrum: I -Solar Intensity Spectrum: Abs- Absorbance. The values of EE×I are constants and predetermined

2.4 Phytochemical analysis of methanolic leaf extract of *M. indica ver* "*Willard*"

The methanolic leaf extract of *M. indica ver* "*Willard*" was subjected to qualitative analysis for alkaloids (using Mayor's test, Wagners test, Dragondraff test), carbohydrates (Molish test, Fahlings test), saponins (Foam test, Heamolysis test), flavonoids (Amonium test, Alluminium Chloride test), tannins (Ferric Chloride test, Lead Sub Acetate test), phenols (Ellargic Acid test), sterols (Salkoski test, Libermann Burcherd test), Terpenoids (Salkoski test) and glycosides (Keller- Kiliani test, Conc. Sulphuric Acid test) [14].

2.5 Evaluation of the antioxidant activity of methanolic leaf extract of *M. indica ver* "*Willard*" *in vitro*

The methanolic leaf extract of *M. indica ver* "*Willard*" was studied for its antioxidant activity.

2.5.1 Total polyphenolic content assay (TPC)

The total polyphenolic content (TPC) of leaf extract was determined by the Folin-Ciocalteu reagent using 96- well micro-plates. Twenty microliters of 1 mg/mL and 0.5 mg/mL of *M. indica ver* "*Willard*" leaf extract were added to 110 µl of ten times diluted freshly prepared Folin-Ciocalteu reagent. Then 70µl of 10 % sodium carbonate solution was added to the mixture and was incubated for 30 minutes in room temperature (30±2 °C). The absorbance was read at 765 nm. Five different concentrations of gallic acid (1, 0.5, 0.25, 0.12 and 0.06 mg/mL) were used to construct the standard curve. TPC of leaf extract was expressed as mg of gallic acid equivalents per gram of extract of leaf [15].

2.5.2 Total flavonoid content assay (TFC)

The total flavonoid content of leaf extract was determined by the aluminium chloride method using 96-well micro-plates. One hundred microliters of 2% aluminium chloride in methanol solution was added to 100µl of 0.5 mg/mL and 0.25

mg/mL of *M. indica ver* "*Willard*" leaf extracts in methanol. The mixture was incubated for 10 minutes in room temperature (30±2 °C). and the absorbance was read at 367 nm. Pre-plate reading was recorded before adding the aluminium chloride solution. Five different concentrations of quercetin (125, 62.5, 31.2, 15.625, 7.812 µg/mL) were used construct the calibration curve. TFC of leaf extract was expressed as mg quercetin per gram of extract of leaf [15].

2.5.3 DPPH radical scavenging assay

Reaction volumes of 200 µl, containing 125 µM of 2,2-dipenyl-1-picrylhydrazyl (DPPH) radical and 50 µl of different concentrations of *M. indica ver* "*Willard*" leaf extract (250, 125, 62.5, 31.25, 15.625µg/mL) were incubated for 15 minutes at room temperature (30±2 °C) and the absorbance was read at 517 nm. Five different concentrations of Trolox (12.5, 6.25, 3.12, 1.56, 0.78µg/mL) were used to construct the standard curve. The results were expressed as mg of Trolox equivalents per gram of extract of leaf [15].

2.5.4 ABTS radical scavenging assay

Reaction volume of 200 µl, containing 40 µM of 2,2´-azino-bis (ABTS^{+}) radical and 50 µl of different concentration of *M. indica ver* "*Willard*" leaf extract (6.25, 3.12, 1.56, 0.78, 3.9 µg/mL) were incubated for 10 minutes at 25±2 and the absorbance was read at 734 nm. Five different concentrations of Trolox (12.5, 6.25, 3.12, 1.56, 0.78µg/mL) were used to construct the standard curve. The results were expressed as Trolox equivalents antioxidant capacity in mg of Trolox equivalents per gram of extract of leaf [15].

2.5.5 Ferrous reducing antioxidant power assay (FRAP)

The working FRAP reagent prepared by mixing 300 mM acetate buffer, 20 mM ferric chloride solution and 10 mM TPTZ solutions in a ratio of 10:1:1 just before use and heated up to 37°C. Reaction volume of 200 µl of containing, 150 µl working FRAP reagent, 30 µl acetate acetate buffer and 20 µl of (0.25, 0.125 mg/mL) were incubated for 8 minutes at room temperature (30±2 °C) and the absorbance was read at 600 nm. The results were expressed as mg of ferrous sulphate per gram of extract of leaf [15].

2.5.6 Oxygen radical absorbance capacity (ORAC)

Fluorescein solution (4.8 µM) and AAPH (40 mg/mL) solution were prepared prior to use in phosphate buffer (75mM, P^{H} 7.4). Reaction volume of 200 µl, containing 100 µl of 4.8µm fluorescein and *M. indica ver* "*Willard*" leaf extract (6.25 g/mL) were pre-incubated for 10 minutes at 37°C followed by the addition of 50µl of AAPH (40mg/mL) to each well to initiate the reaction. The plate was placed on the florescent microplate reader (SPECTRA MAX GEMINI EM) set with excitation and emission at 49nm and 535nm and the decay of fluorescein was recorded in 1minuteintervals for 35 minutes. TROLOX was used as a standard antioxidant. The ORAC activities of the samples were calculated by comparing the net area under the curve of ffluorescein decay between the blank and the samples. The results were expressed as ORAC values in mg of Trolox per gram of extract of leaf [15].

Data analysis - The Softmax Pro5.2v software of the micro-plate reader (SPECTRAmaxPLUS384 Molecular Devices, Inc, USA) was used to calculate the TPC, TFC, FRAP value, percentage of radical scavenging activities of DPPH and ABTS assays, and IC50 values of methanolic leaf extract of of *M. indica ver* "*Willard*". The Softmax Pro5.4.1v software of the fluorescent micro-plate reader (SPECTRAmax- Gemini EM, Molecular Devices Inc, USA) was used to calculate the ORAC values of methanolic leaf extract of of *M. indica ver* "*Willard*". For each sample of each test, three replicates were used.

2.6 Determination of potency of the leaf extract of *M. indica ver* "*Willard*"

The product obtained from *M. indica ver* "*Willard*" leaves was re-dissolved in methanol to prepare sample solutions of 2mg/mL, 1mg/mL, 0.8mg/mL, 0.4mg/mL, 0.2mg/mL, 0.1mg/mL, 0.05mg/mL. Sample solutions of similar concentrations was prepared using Dermatone® as the reference agent using methanol as the solvent. The absorbance of UV radiation by the methanol extracts of *M. indica ver* "*Willard*" and Dermatone® were measured in micro plates, in triplicate using SPECTRA max. PLUS^{384}" Microplate spectrophotometer from 290- 320nm at 5nm intervals using methanol as the blank. The SPF values were calculated using the Mansur equation. The dose- response curves were plotted. The EC_{50} values were calculated using AAT Bioquest® – EC_{50} calculator.

3. Results

The results obtained and computed are shown in Tables 01, 02 and 03. As shown in table 01, 2.0mg/mL methanolic leaf extract of *M. indica ver* "*Willard*" displayed high absorbance values (range: 3.6 to 3.9) as the reference agent, Dermatone® (range: 2.9 to 3.5). As shown in table 02, calculated SPF values of the methanolic extract of *M. indica ver* "*Willard*" leaves and Dermatone® are 38.67 and 33. 76, respectively. The data are expressed as Mean ± SEM (n=3).

Phytochemical screening of the methanolic leaf extract of *M. indica ver* "*Willard*" revealed the presence of alkaloids, saponins, flavonoids, tannins, phenols, glycosides, carbohydrates and terpenoids.

The analysis for antioxidant activity revealed that methanolic leaf extracts of *M. indica ver* "*Willard*" has a marked antioxidant activity as shown in Table 03.

The calculated EC_{50} values of the methanolic leaf extract of *M. indica ver "Willard"* and Dermatone® were 0.473 mg/ml and 0.700 mg/ml respectively.

Table 1: Absorbance of 2.0 mg/mL methanolic extract of *M. indica ver* "*Willard*" leaves and the reference agent Dermatone®

		Absorbance ± SEM	
Wavelength (nm)	**EE×I**	***M.indica ver* "*Willard*"**	**Dermatone®**
290	0.0150	3.9430±0.0110	3.5277±0.0416
295	0.0817	3.9340±0.0064	3.5630±0.0738
300	0.2874	3.9370±0.0037	3.5060±0.0602
305	0.3278	3.8680±0.0265	3.3710±0.0602
310	0.1864	3.8053±0.0511	3.2487±0.0676
315	0.0839	3.7217±0.0665	3.1080±0.0664
320	0.0180	3.6407±0.0865	2.9670±0.0644

EE-Erythemal effect spectrum I-Solar intensity spectrum SEM-Standard Error Median

Table 2: Sun protection factor of 2.0 mg/mL methanolic leaf extract of *M. indica ver* "*Willard*" and the reference agent Dermatone®

	SPF
M.indica ver "*Willard*"	38.67
Dermatone®	33.76

Table 3: *In vitro* Anti-oxidant activities of methanolic leaf extracts of *Mangifera indica ver* "*Willard*" in different assays.

TPC (mg gallic acid equivalents/g of Willard leaf)	TFC (mg quercetin equivalents/g of Willard leaf)	FRAP (mg FeSO4/g of Willard leaf)	DPPH (mg Trolox equivalents/g of Willard leaf)	ABTS (mg Trolox equivalents/g of Willard leaf)	ORAC (mg Trolox equivalents/g of Willard leaf)
298.38± 5.99	77.62±1.03	132.81±0.41	416.64±11.08	26.38±1.95	410.28±7.39

Data represented as mean ± SEM (n=3)

4. Discussion

Measurement of SPF is the ultimate way to determine effectiveness of sunscreen formulation. The higher the SPF value the more protection a sunscreen offers against UV radiation [1]. In this study SPF values were evaluated using the UV absorption spectroscopy technique and the Mansur equation. Evaluation of SPF values *in vitro* is a simple, reliable, quick, inexpensive, and a validated technique compared to *in vivo* methods. Moreover, *in vitro* assays bypass the variability and ethical issues related with *in vivo* methods [13, 16].

The results showed that the 2.0 mg/mL methanolic leaf extract of *M. indica ver* "*Willard*" leaves has a high SPF value of 38.67 as SPF values 2-12, 12-30 and ≥30 are considered as having respectively minimum, moderate and high sun protective activity [16]. The skin cancer foundation recommends a SPF value of 15 or higher as acceptable UVB protection for normal everyday activity and SPF 30 or higher acceptable for intense outdoor exposure [11] and also WHO recommends to use sunscreen products of SPF value higher than 15 where the UV index is higher than two.

The calculated EC_{50} values for the leaf extract of *M. indica ver* "*Willard*" (0.473 mg/ml) showed that the EC_{50} values are lower than the reference agent Dermatone® (0.700 mg/ml), thus suggesting that the potency of that the leaf extracts is higher than the reference agent.

This is a novel finding for the Sri Lankan variety of *M. indica ver* "*Willard*" leaves with marked antioxidant activity, which indicates their potential as prophylactic and therapeutic agent against several diseases. The prevalence of flavonoids and phenols is shown by the high values for TPC and TFC antioxidant assays and also by the phytochemical screening in this study. It is well known that the antioxidant activity of plants is mediated via flavonoids, phenols and tanins, and antioxidants can scavenge free radicals while suppressing the UV induced skin damage, thereby conferring sun protection [16].

5. Conclusion

The leaf extracts of *M. indica ver* "*Willard"* possesses strong sun protection activity (38.67) and marked antioxidant activity *in vitro* thus displaying immense potential to be developed as a safe, cheap and effective topical sun screen.

6. Reference

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