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Determination of *In Vitro* Photoprotective Potential of Methanolic Leaf Extract of x *Citrofortunella microcarpa* (Calamondin)

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Abstract: This study focused on the evaluation of *in vitro* Sun Protection Factor (SPF) of leaf extract of x *Citrofortunella microcarpa* since it has not been investigated. Collected leaves were air-dried, powdered and macerated in methanol. The filtrate was evaporated to dryness and subjected to preliminary phytochemical analysis. The concentration series of 2.0, 1.0, 0.5, 0.25, and 0.05 mgmL^{-1} of leaf extract and a solution of 2.0 mgmL^{-1} Dermatone® were prepared in methanol. The absorbance of each sample was determined in triplicate by spectrophotometry in the range of 290–320 nm, at 5 nm intervals, using methanol as the blank. The SPF values were calculated using the Mansur-equation. Alkaloids, flavonoids, tannins, phenols, sterols, saponins, terpenoids, and glycosides were qualitatively observed. The SPF of leaf extract with respect to the concentrations 2.0, 1.0, 0.5, 0.25, 0.05 mgmL^{-1} and Dermatone® were 43.93, 42.38, 40.97, 36.63, 13.31 and 34.26 respectively. According to Pearson's correlation, a positive statically not significant relationship was observed in between SPF and concentration ($r = 0.655$, $p > 0.05$). Since the presence of profound sun screening activity, this would offer an exciting avenue for further research towards the development of herbal sunscreens of high importance especially for the people living in tropical countries.

Keywords: Calamondin, x *Citrofortunella microcarpa*, Photoprotective, Sun Protection Factor, UVB Radiation

1. Introduction

Sunlight is composed of ultraviolet and visible light. About 10% of the total light output of the sun is constituted by ultraviolet (UV) radiation. The UV region of the solar spectrum has the highest energy among other regions of the optical region (UV, visible and infrared radiation) and the wavelength is range from 10 nm to 400 nm. Among UVA, UVB and UVC radiation sunburns, photo-aging and skin cancers are attributed to UVB (290-320 nm) radiation [1-9]. Sunscreens are cosmetic products, containing UV filters of a physical or chemical nature that are applied to protect the skin from prolonged exposure to UV radiation [4, 6-7]. Sunscreen agents are also included in moisturizers, lipsticks, shampoos, makeup, hair gels, and hair mousses to protect the skin and hair and to prevent the degradation of these products on exposure to sunlight [1, 3-4, 6-8, 10]. Unlike the physical blockers, the chemical sunscreens have a specific range in which they absorb. The sun protection factor (SPF) of a compound indicates their effectiveness for protection from sunburn that is the length of time an individual can stay in the sun without damaging to the skin when it is applied a thickness of 2 mgcm^{-1} skin. An individual wearing a sunscreen

with SPF 15 can stay exposed to the sunlight fifteen times as long as if they were not wearing sunscreen without risking the skin sunburn [1, 3, 6, 8-9]. Synthetic sunscreens have been introduced as a preventive strategy for harmful effects of UV radiation on humans. With the realization of their adverse side effects such as the development of irritant dermatitis, hypersensitivity, allergies and even melanoma, the recent trend is to search for human-friendly herbal sunscreen formulations [3, 8-9].

During recent years, food, cosmetics, and pharmaceutical industries have shown a growing interest in the use of natural products obtained from sources like plants, microalgae and microorganisms because of the harmful consequences of artificial chemicals in peoples' health. Citrus plants play an important role in food, cosmetics, and pharmaceutical industries. This study focused on the evaluation of *in vitro* Sun Protection Factor (SPF) of leaf extract of x *Citrofortunella microcarpa*; Calamondin since it has not been investigated.

C. microcarpa is available year-round in the home gardens of Asian countries including; Malaysia, Indonesia, Southern China, India, Sri Lanka and in West Indies, Hawaii, Central and North America [11-12] and it has many alternate common names including calamondin orange, calamansi, calamandarin, kalamondin, kalamunding, kalamansi, Panama orange, Chinese or China orange, musk orange, Philippine lime, golden lime, scarlet lime, ma-nao-wan and acid orange [11, 13-14]. This plant is a member of the Family Rutaceae [15-18] and was formerly referred as a citrus fruit and named as *Citrus mitis* Blanco*, C. microcarpa* Bunge or *C. madurensis* Lour [11, 14, 16, 19]. But later it has been identified as an important Citrofortunella. *C. microcarpa* is an intergenetic hybrid between mandarin orange that is a member of the genus Citrus and the kumquat belonging to the genus Fortunella [12, 20-23]. Recently it has been given the hybrid name, x *Citrofortunella mitis* J. Ingram & H. E. Moore or x *Citrofortunella microcarpa* [11, 13].

C. microcarpa is a moderately drought-tolerant plant and able to tolerate a wide range of soil conditions from clay-loam in the Philippines to limestone or sand in Florida [11, 23]. This tree is ranging from 2 m to 7.5 m in height. It has a straight, slender, cylindrical and densely branched stem with an extraordinarily deep taproot. The evergreen single leaflets are dark-green, alternate, aromatic, broad-oval, 4 cm to 7.5 cm long, glossy on the upper surface, yellowish-green lower surface and faintly toothed at the apex, with short and narrowly-winged petioles. Its flowers are pure-white and having five elliptic-oblong petals with rich and sweet fragrance. The showy, sweet, and edible fruits are 2 cm to 3.5 cm in diameter, nearly spherical, with very aromatic, orange, glossy, thin, and easily-removed peel. This peel is dotted with numerous small oil glands. The pulp has 6 to 10 segments and it is orange, very juicy and highly acidic. Fruit is seedless, or it has 1 to 5 small, white colour, obovoid seeds [11-12, 15-16, 24].

The folk, traditional, indigenous and ethnopharmacological uses of different parts of *C. microcarpa* by the different local communities of various countries have been documented in the literature. Among local communities in the Philippines *C. microcarpa* leaves and fruits are used to treat stomachache, cough and cold [14, 18, 23]. The air-dried leaves of *C. microcarpa* are burned and then the smoke is said to drive away the insects due to the insect repellent compounds present in the smoke [25]. Leaves also used to relieve a headache, in the treatment of skin diseases, and as a mouthwash in the treatment of a sore throat [16]. Volatile oil has carminative properties with more potency than peppermint oil [11]. The content of the volatile oil in leaves was in between 0.90% - 1.06% [11]. Sesquiterpenes hydrocarbons include hedycaryol, α-sesquiphellandrene, α-eudesmol and β-eudesmol were the most abundant phytochemicals present in leaf volatile oil [16, 26]. Crude extracts of leaves have shown antimicrobial activity against *Vibrio alginolyticus, V. parahaemolyticus, V. vulnificus, Citrobacter freundii* and *Streptococcus* sp. [27-28] and *C. microcarpa* is one of the most active antimicrobial plants found in the rural area in Terengganu, Malaysia [27]. 3/, 4/, 5, 6, 7, 8-hexamethoxyflavone isolated from the leaf extract of *C. microcarpa* has shown moderate antifungal activity against *Candida albicans* [29].

The *C. microcarpa* fruit juice is rich in vitamin C and used as an antiphlogistic (anti-inflammatory), laxative, antihypertensive, skin bleaching agent, to expel phlegm, to promote even blood circulation and normal digestion [11, 13, 16, 23]. The previous pharmacological studies revealed that the fruit juice and fruit peel extracts possessed antimicrobial [30], antioxidant [31], anti-inflammatory [32], anti-tyrosinase [33], hepatoprotective [15, 17], and anticancer [34] activities. The rind contains a wide range of medicinally beneficial phytochemicals including aldehydes, sesquiterpenes, betapinene, linalool, linalyl acetate, tannin, flavonoids, glycosides, and cyanogenetic substances [12, 15-16, 20, 26, 32, 35]. Peels are reported to be rich in limonene [16].

2. Materials and Methods

2.1. Collection and Identification of the Plant

Twigs of a matured plant of *C. microcarpa* with leaves, flowers, and fruits were collected from Horana area in Kaluthara district, Western Province of Sri Lanka (GPS 6°46'34.4"N 80°00'41.5"E), in October 2016. The plant was taxonomically identified and authenticated by a botanist at the National Herbarium, Peradeniya, Sri Lanka. The voucher specimen of the aerial part of *C. microcarpa* (specimen no; BPH/CM/2016) was deposited in the Pharmaceutical Chemistry Laboratory, Department of Pharmacy, General Sir John Kotelawala Defence University, Sri Lanka.

2.2. Preparation of Methanolic Leaf Extracts of C. Microcarpa

The leaves of *C. microcarpa* were removed from the plant and thoroughly washed in running tap water, secondly in distilled water. They were air-dried at room temperature in shade about 4 days until a constant weight was obtained. Air-dried leaves were cut into small pieces using a razor blade.

Fifty grams of cut pieces of leaves were blended and the powder was macerated for 4 days in 300 mL of distilled methanol (ACS reagent, 99.8% purity from Sigma-Aldrich). The resulted extract was filtered through double-layered muslin cloth and then through Whatmann filter paper (No.1) and the filtrate was evaporated to dryness (powder form) using the rotary evaporator. The yield obtained was 10.4% w/w. The dried crude methanol extract was inserted into an air-tied universal bottle covering with an aluminium foil and stored securely at 4°C for later use.

2.3. Phytochemical Analysis of Methanolic Leaf Extract

The methanol crude extract was subjected to qualitative analysis for alkaloids, reducing sugars, saponins, flavonoids, phenols, sterols, glycosides and terpenoids using standard procedures.

2.4. Determination of in Vitro Sun Protection Factor

The crude solid product of *C. microcarpa* was re-dissolved in distilled methanol (ACS reagent, 99.8% purity from Sigma-Aldrich) to prepare concentration series of 2.0 $mgmL^{-1}$, 1.0 $mgmL^{-1}$, 0.5 $mgmL^{-1}$, 0.25 $mgmL^{-1}$, and 0.05 $mgmL^{-1}$. A commercially available sunscreen cream with SPF 35 was purchased from the market (Dermatone®) as the reference. Dermatone® was dissolved in methanol to obtain a solution of 2.0 $mgmL^{-1}$.

The absorbance of UV radiation by each dilution of *C. microcarpa* and Dermatone® were determined at 23°C with an equilibration time of 1 hour, in 1 cm quartz cells, in triplicate, using a UV-visible spectrophotometer (GENESYS 10S UV-VIS spectrophotometer, Thermo SCIENTIFIC) from 290 to 320 nm, at 5 nm intervals taking methanol as the blank.

The *in vitro* SPF values for each dilution of *C. microcarpa* and Dermatone® were then calculated using the Mansur equation given below. Where CF-Correction factor (=10), EE–Erythemal effect spectrum; I-Solar intensity spectrum; Abs-Absorbance of the sunscreen product. The values of EE x I are predetermined constants [1-2, 8-10, 36].

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

2.5. Statistical Analysis

The data were statistically analyzed using SPSS 21.0. The data were expressed as mean absorbance ± Standard Error of the Mean (SEM). Pearson's product-moment correlation and simple linear regression were run to assess the relationship between the concentration of the methanolic leaf extract and the *in vitro* sun protection activity (SPF). The significance level was set at $p < 0.05$.

3. Results

3.1. Preliminary Phytochemical Screening

As shown in Table 1, preliminary phytochemical screening of the methanolic leaf extract of *C. microcarpa* exhibited the presence of alkaloids, reducing sugars, sterols, terpenoids, flavonoids, phenols, glycosides, and saponins.

Table 1. *Preliminary phytochemical screening of the methanolic extract.*

Phytochemicals	Preliminary Screening Tests	Results
Alkaloids	Mayer's test	+
	Wagner's test	+
	Hager's test	-
Reducing sugars	Fehling's test	+
Sterols	Salkowski test	+
	Liebermann-Burchard test	+
Terpenoids	Salkowski test	+
Flavonoids	Ammonium test	+++
	Aluminum Chloride test	+++
Phenols	Ellagic Acid test	+
Glycosides	Keller-Kiliani test	+
	Concentrate H_2SO_4 test	+
Saponins	Foam test	+

(+) => presence of the phytochemical (-) => absence of the phytochemical.

3.2. In Vitro Sun Protection Factor (SPF)

As shown in Table 2, all concentrations of leaf extracts and the reference agent (Dermatone®) exhibited markedly high absorbance values range from 1.2 to 4.9. As indicated in Table 3, the computed SPF values for each concentration of methanolic leaf extract and Dermatone® were higher than SPF 13.

3.3. Relationship Between the Concentration of the Extract and in Vitro SPF

As shown in Table 4, there was a strong positive statically not significant correlation between the concentration of the methanolic leaf extract and the in vitro SPF ($r = 0.655$, $p > 0.05$) with concentration explaining 42.9% of the variation in SPF. But the correlation between log-concentration of the methanolic leaf extract and the in vitro SPF was strong, positive and statically significant ($r = 0.930$, $p < 0.05$) with log-concentration explaining 86.5% of the variation in SPF. Half-maximal effective concentration (EC_{50}) of this extract was 0.036 ± 0.075 mg/mL.

Table 2. *Mean absorbances of each concentration of leaf extract and Dermatone®.*

Wave length	EE x I	Mean absorbance ± SEM of *C. microcarpa* leaf extract					Dermatone®
		2.00 mg/mL	1.00 mg/mL	0.50 mg/mL	0.25 mg/mL	0.05 mg/mL	2.00 mg/mL
290	0.0150	4.338±0.028	4.393±0.028	4.272±0.025	4.005±0.012	1.514±0.000	3.179±0.017
295	0.0817	4.315±0.027	4.426±0.024	4.333±0.033	3.911±0.019	1.415±0.000	3.338±0.035
300	0.2847	4.284±0.016	4.527±0.100	4.297±0.005	3.743±0.010	1.338±0.000	3.134±0.017
305	0.3278	4.886±0.025	3.990±0.021	3.921±0.050	3.563±0.023	1.299±0.000	3.618±0.085
310	0.1864	3.933±0.031	4.224±0.010	4.083±0.011	3.624±0.005	1.311±0.000	3.563±0.100

Wave length	EE x I	Mean absorbance ± SEM of *C. microcarpa* leaf extract					Dermatone® 2.00 mg/mL
		2.00 mg/mL	1.00 mg/mL	0.50 mg/mL	0.25 mg/mL	0.05 mg/mL	
315	0.0839	4.139±0.023	4.160±0.038	3.998±0.024	3.666±0.013	1.368±0.000	3.478±0.041
320	0.0180	4.099±0.009	4.277±0.005	4.088±0.019	3.746±0.036	1.437±0.000	3.639±0.124

EE-Erythemal effect spectrum; I-Solar intensity spectrum; SEM-Standard Error of Mean.

Table 3. *In vitro SPF values of each concentration of extract.*

	C. microcarpa leaf extract					Dermatone® 2.00
Concentration (mg/mL)	**2.00**	**1.00**	**0.5**	**0.25**	**0.05**	
SPF	43.93	42.38	40.97	36.63	13.31	34.26

Table 4. *Correlation between concentration of the methanolic leaf extract and the in vitro SPF.*

	Pearson Correlation Coefficient (*r*)	*p*	r^2	Adjusted r^2
Concentration Vs SPF	0.655	0.230	0.429	0.239
Log-Concentration Vs SPF	0.930	0.022	0.865	0.820

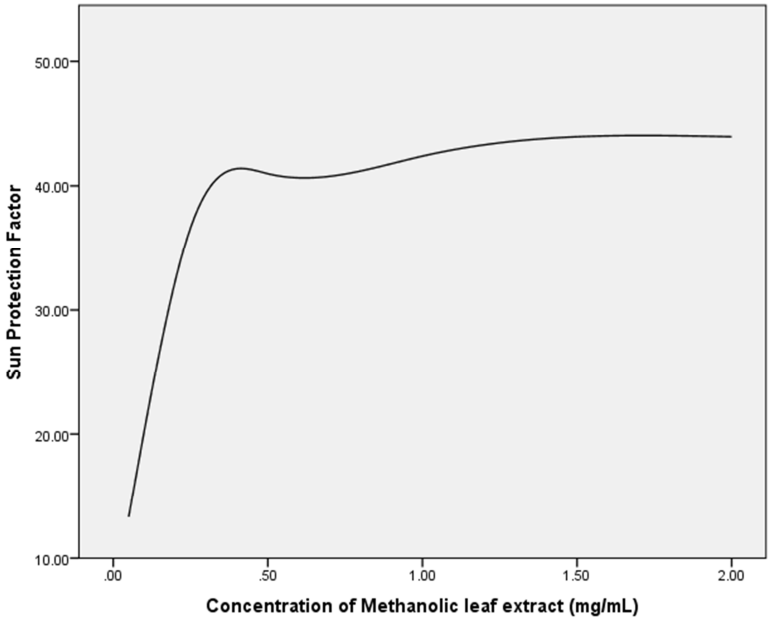

Figure 1. *Concentration Vs SPF.*

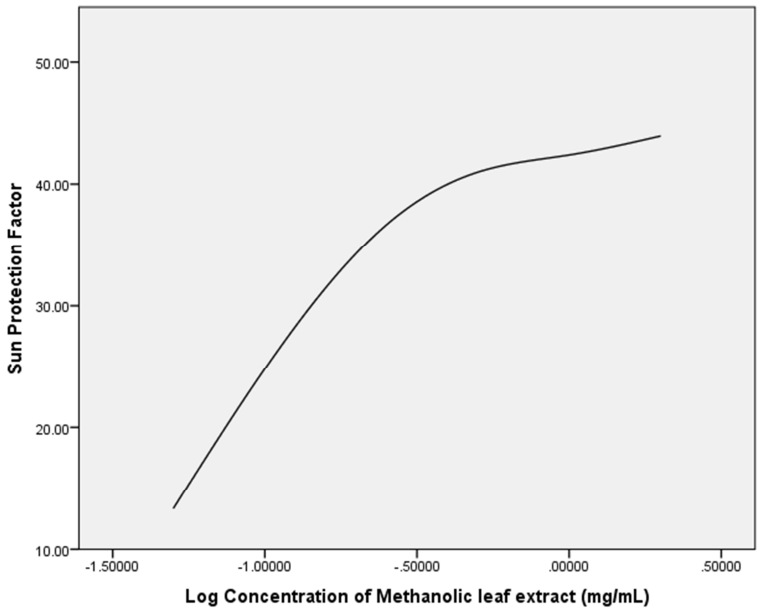

Figure 2. *Log-concentration Vs SPF.*

4. Discussion

Sri Lanka lies within the equatorial belt, a region which receives ample amount of solar radiation. The UV index in Sri Lanka varies from 6 to 12 throughout the year that falls within the "high", "very high" and "extreme" risk categories. The UV index gives an idea about the strength of sunburn producing UV radiation at a particular place and a time. The UV Index is defined as the amount of skin-damaging UV radiation expected to reach the earth's surface at the time when the sun is highest in the sky [37]. Usage of sunscreens with SPF ≥ 30 is highly recommended for the people living in tropical countries such as Sri Lanka.

Sunscreen formulations are categorized into three groups based on their SPF values as minimal (SPF < 12), moderate (SPF 12–30) and high (SPF ≥ 30) photo-protective products [8]. Further, the protection percentage from UVB radiation is different according to the SPF value of the sunscreen formulation. Sunscreens with SPF 15 provides about 93% protection against UVB and SPF +30 provides about 97% protection against UVB radiation [4, 8].

The present investigation reveals that all concentrations of *C. microcarpa* leaf extracts have high sun screening potentials that fall within "moderate" and "high" sun protective categories. 2.0 $mgmL^{-1}$, 1.0 $mgmL^{-1}$, 0.5 $mgmL^{-1}$ and 0.25 $mgmL^{-1}$ extracts have shown high sun protective properties and about 97% protection against UVB radiation. Their SPF values were higher than the reference agent Dermatone®. 0.05 $mgmL^{-1}$ extract has shown minimal sun protective properties and about 93% protection against UVB radiation.

The phytochemicals such as flavonoid and phenolic compounds are widely distributed in the plant kingdom [2]. The chemistry of the *C. microcarpa* leaves has received only moderate attention. Sesquiterpene hydrocarbons include hedycaryol (19.0%), sesquiphellandrene (18.3%), eudesmol (14.4%) and eudesmol (8.6%) have been reported as the most abundant in the leaves of *C. microcarpa* [16]. Hesperidin, Hesperetin, luteolin, naringenin, narirutin, naringin, neohesperidin, nobiletin and tangeretin are some of the main flavonoids found in various citrus fruits [38]. Phenolics and flavonoids (Quercetin, apigenin and rutin) are reported to be effective in UVA and UVB range and good in preventing UV-induced oxygen free radical generation and lipid peroxidation, which involved in photoaging and skin cancer [2-4]. So, the plant extracts rich in these UV light absorbing phytochemicals are possible to use in the formulation of sunscreens with high SPF value.

5. Conclusions

The methanolic leaf extract of *C. microcarpa* showed good photoprotective activity against UVB radiation. These effects can be attributed to phytochemicals such as flavonoids, phenolics present in the plant. Therefore, a sunscreen formulation developed by this plant extract would be of high importance especially for the people living in tropical countries lied within the equatorial belt, a region which receives ample amount of solar radiation.

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