In vitro sun screening activity *Codiaeumvariegatum* and its two varieties

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Abstract- This study wasaimed at investigating the sun protective activities of leaves of three Sri Lankan croton plants, *Codiaeumvariegatum*,*C.variegatum type 1* and *C.variegatum type 2* (family :Euphorbiaceae) in vitro using UV spectroscopic technique and Mansur equation. Sun Protection Factor (SPF) values were determined (which is an index of sun protection activity) using 0.2 mg/mL of methanolic crude extract of each plant. Dermatone ® and Suncote® were used as reference agents. Phytochemical profile of *C.variegatum* was also investigated using standard protocols and TLC analysis. The results showed that crude extracts of leaves of *C.variegatum type 1* and *C.variegatum type 2* exhibit mild sun protective activity,(SPF values of 6.39± 0.08 and 8.48± 0.10 respectively) whilst *C.variegatum* has high SPF value (18.25 ± 0.22). And also reference agents Dermatone® and Suncote®had high SPF values (25.05 ± 0.33 and 27.54 ± 0.35 respectively). Flavonoid fraction of *C.variegatum* from TLC studies displayed a higher SPF value (25.92 ± 0.32) which is higher than the SPF value of its crude extract. Phytochemical screening of *C.variegatum* crude extract showed the presence of flavonoids, tannins, phenols, alkaloids and diterpenes. It is concluded that the sun protection activity of *C.variegatum*is mediated primarily by flavonoids via its antioxidant activity and this plant is a promising source to develop a cheap, consumer friendlyand efficacious sunscreen formulation.

Index Terms- *Codiaeumvariegatum*, Sun Protection Factor (SPF), Sun screen, UV-B rays, Thin Layer Chromatography (TLC)

I. INTRODUCTION

Sunlight, which is essential for life, consists of ultraviolet rays (UV-R) which are electromagnetic radiations, with wave lengths between 100 and 400nm (1, 2). Solar UV-R are of three types : UV-A (300 -400nm) ,UV-B (280 -320nm) and UV-C (200-280nm) (1,2).Short term exposure to small amount of UV light is beneficial to the well-being of humans since it is shown to facilitate skin cell regeneration , and stimulate hormone production , synthesis of vitamin D and melanin pigment (3,4). However, prolonged exposure to solar UV radiation, Particularly, UV-B radiation, commonly known as “burning rays”, is detrimental to the health and well- being of both animals and humans (1, 2, 3, 4, 5). For example, in amphibians, over exposure to UV-B rays is shown to induce severe malformation and delay their metamorphosis (5) and also been claimed as a contributory factor for their global decline (5).

Erythema (sun burn), hyperpigmentation (tanning) ,hyperplasia, thinning of skin, inflammation ,pain, development of brown and red spots, irritation and local immunosuppression are some acute effects of over exposure to UV rays (1,2,3,4,6,7,8). Fortunately, these acute effects are usually short lived and reversible (9,10). In contrast, chronic effects are much more serious and may be even life threatening (1,2,8,10). These include photo ageing/ premature ageing of the skin [rough texture, sagging, dry and leathery approach and wrinkling (both coarse and fine), degenerative change in the cells of skin, fibrous tissue and blood vessels], photo dermatotoses, actinic keratoses, inflammatory reaction of eyes, cataracts and skin cancer (1, 2, 8, 10). Moreover, a growing body of evidence suggests that long term exposure of UV radiation could suppress cell mediated immunity and thereby enhance the risk of infectious diseases and also limit the efficacy of vaccinations (11).Sadly, globally, some 12-15 million people become blind by cataract annually, of which up to 20% may be caused and enhanced by sun exposure according to WHO estimates (11).

Most organizations (12) and dermatologists (12,13) now strongly recommend to liberally apply a topical sun screen formation having a sun protection factor (SPF) value between 15-20, probably ,year round ,to protect the human skin against harmful UV rays, especially, UV-B rays(8,12,13). However , sun screen use began in the early 20th century (14).And ,now several sunscreen formations are available in the market in the form of oil ,ointments, creams, gels, balms , waxes, lotions(1,2,6,8,12). Some of these sun screen formulations act as physical sunscreens and (reflect UV rays) contains synthetic ingredients (such as zinc oxide ,titanium dioxide, avobenzone) (12,13,15).These are fast acting ,efficacious and provide broad spectrum protection of solar UV rays (8,12,13,15). However , overall ,this type of sunscreens are relatively expensive and cosmetically unacceptable because of their opaque quality ,occtusiveness, comeogenicity and tendency to stain cloths (6,8,12,16).Furthermore ,their safety is doubted since they induce contact and/or irritant dermatitis ,hypersensitivity ,allergies, whitening ,vitamin D deficiencies and skin cancers (6,8,12,16). Another point of concern is that some are found to be phototoxic (becoming toxic when exposure to UV rays) (4) and their uses are more likely to give birth to underweight babies if applied during pregnancy period (7). Alternatively, sunscreens containing natural herbal ingredients (such as flavonoids, phenols, tannins) are cheaper and affordable, safer efficacious and appears to be more acceptable /consumer friendly (7,12,15,16). Clearly, there is a large demand and need, more than ever before, for development of novel sunscreens for herbal origin fulfilling these features.

Accordingly, we have initiated a programme of research to investigate the sun screen potential of selected Sri Lankan plants. So far, we have accessed the *in vitro* sun screen potential of Sri Lankan orthodox black tea made from *Camellia sinensis*leaves (17) and salt marshy plants (*Suaedamonica*, *Suaedamaritima* , *Halosarciaindica*and *Salicornia brachiata*) (18,19).

This study investigates the *in vitro* sunscreen potential of three selected ornamental plant types (Family ; Euphorbiaceae), namely *Codiaeumvariegatum*, *C.variegatum variety 1*,*C.variegtum variety 2* .These are evergreen ornamental shrubs with a maximum height about 6 meters with large, simple thick leathery and shiny multi colored leaves 5-30 cm long and 0.5 – 8 cm broad which are alternatively arranged. The young leaves are usually green, yellow, red or brown in color and the color changes to gold, maroon, scarlet, purple, brown, cream or white as they mature (20). They are now wide spread in tropical regions of old and new world but were originally found in South Eastern Asian countries like Indonesia, Malaysia, Philippines, Thailand, India, and Sri Lanka (20,21). These plants only survive in outdoors in dry environments where the ambient temperature does not normally drop below 10^0C (22). Cold temperatures cause loss of leaves (21,22).

Croton species not only have ornamental value but also used in the ethnomedicineof several countries. In African, Asian and South American countries they are used in the treatment of cancer, constipation, diabetes, gastric ulcers ,digestive problems, dysentery,malaria, fever, intestinal worms, hypertension, hypercholesterolaemia ,hyperlipidaemia, inflammation ,pain (especially tooth),amoebiasis ,,gonorrhea, wounds and weight loss (20,21,22,23).In addition, they are used as a wound healer, purgative and sedative (20).Moreover, interestingly ,leaf extraction of*C.variegatum* is shown to possess strong molluscicidal activity (24).

II. Materials and Methods

Collection and identification of*C.variegatum*,*C.variegatum type 1* and *C.vriegatum type2* .

Mature leaves of all the three plants types were collected from the garden of the Department of Chemistry ,University of Ruhuna ,Matara,Sri Lanka .(Geographical coordinates ; $5^0$56' 25.8" N, $80^0$34' 34.9" E). Plants were identified by Mr. N.P.TGunawardena, National Herbarium, Department of National Botanic Garden, Peradeniya, Sri Lanka and Dr. (Mrs) K.Fernado , Former Director of the Seed Certification and Plant Protection Center of the Agriculture Department , Peradeniya, Sri Lanka.

Voucherspecimens of leaves of *C.variegatum variety* 1(ASD/chem/1) , *C.variegatum variety 2*(ASD/chem/2) and *C.variegatum* (ASD/chem/3) were deposited in the Chemistry lab, Department of Chemistry ,University of Ruhuna ,Sri Lanka.

Preparation of extracts of*C.variegatum variety 1*, C.variegatum *variety 2* and *C.variegatum*

Mature leaves of each plant type were firstly washed in running tap water and secondly in distilled water. These leaves were then air dried at room temperature (28-30 ^{0}C) for three days until a constant weight was reached .Each type of leaves were then cut into small pieces using razor blade. Five hundred grams of cut pieces of each type of leaves was separatelymalevated in 500 mL of methanol (GPR grade, Merck Chemicals) for 4 days. The resulting extracts were filtered separately through cotton wool followed by Whatmann filter paper (No:1). The filtrates were evaporated to dryness using a rotaryevaporator;yeild (g/100g); *C.variegatum type 1 6.0*;*C.variegatum type 2*4.0 and;*C.variegatum*4.5. In addition to methanolicextracts, with *C.variegatum type 1* water extract (yield: 5.0g /100g) and diluteHCl extract (yield: 7.5g/100g) and withC.variegatumacidified methanolic extract (5.5g/100g) were also made .

Phytochemical analysis

The methanolic extract of *C.variegatum* were initially subjected to qualitative analysis foralkolides(using Meyer's test, Wagner's test and Dragendroff's test) tannins and phenols ($FeCl_3$ test),flavonoids (HCl and magnesium turnings test), glycosides (kellar – killiani test) and diterpenes(Cu $(CH_3COO)_2$ test), sterols (Liberman - Brurchard test) and diterpens (Salkowski test) (25).

***In vitro* evaluation of sun protection factor (SPF).**

Five milligrams of the dried extracts of the three croton plants were separately redissolved in 25 mL of methanol to prepare solution of 0.2 mg/mL. In addition, 0.2 mg/mL methanolic solutions of two reference sun protective agents, namelyDermatone ® and Suncote ®were also made. Absorbance of UV radiation by the methanolic extracts of three croton types, water and dilute HCl extract of *C.variegatum type 1*, and acidifiedmethonolic extract of *C.variegatum* were determined in triplicate (at 23^0C with an equilibrium time of 1hr) in 1cm quartz cells, using a UH 53000 Hitachi Spectrophotometer from 290 to 320nm, at 5 min. intervals timing, methanol as the blank. Sun protection factor (SPF) value was determined using Mansur equation (10,17,25) given below.

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

Where EE – erythemal effect spectrum: I – Solar intensity spectrum: Abs – Absorbance of sunscreen product: CF – correction factor (=10). The value of EE × Iisa constant and predetermined.

Following TCL analysis , the absorption spectra for the phytochemicals obtained from the characteristics 5 bands : Band 1 (diterpenes), Band 2 (flavonoids), Band 3 (alkaloids), Band 4(tannins) and Band 5(phenols) were obtained and SPF values were determined using Mansur equation (10,17,25).

Statistical Analysis

The results are given as mean ± SEM. Statistical comparisons were made using X^2 test. Significance was set at $P < 0.05$.

III. Results

Absorption spectrum profile of *C.veriegatum*methonolic extract is shown in Figure 1. As shown absorbancepeaks were evident in all three regions of the UV spectrum: UVC (2.9); UVB(2.1);

UVA(2.4).

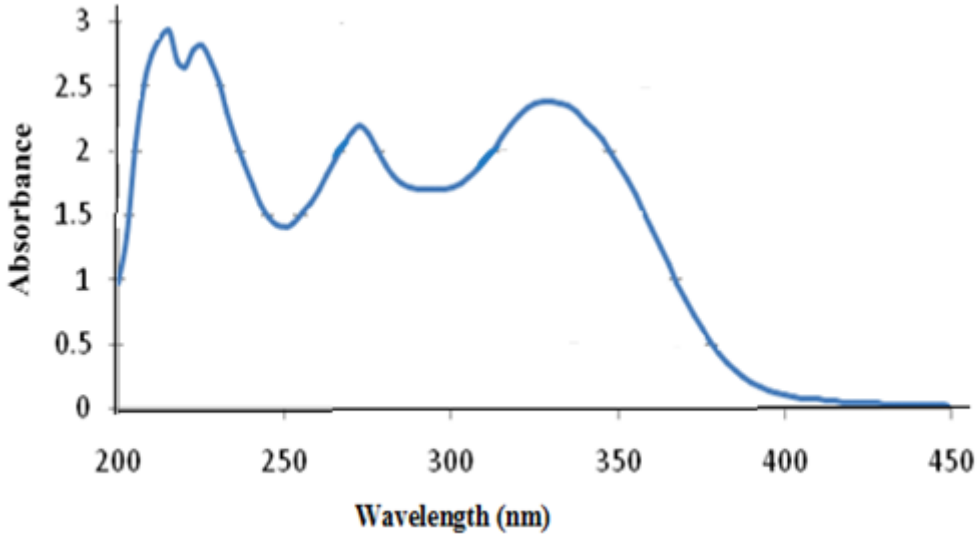

Figure 1. Absorption spectrum of methanolic extract of *Codiaemvariegatum*

The results of the SPF evaluations of the crudeextracts are given in Table 1. As shown, the two reference agents exhibited high SPF values. Further, these two SPF values of the reference agent were comparable and not significantly (P>0.05) different. The lowest SPF value was shown in dilute HCl extract of *C.variegatum type1*, whilst its water extract and methonolic extracts had moderately high SPF values. However, these SPF values were significantly (P<0.05) lower than the two reference agents. In contrast, methonolic extract of *C.variegatum*showed a high SPF value, although, significantly lower (P<0.05) than the two reference agents. Furthermore, methonolic extract of *C.variegatum* extract showed high absorbance values ranging from 1.7 to 2.2 between the wave lengths tested (290-320nm) .Acidified extract of *C.variegatum*, on the other hand, exhibited a mild SPF value.

Type of plant	Nature of extract / and reference agent	Sun protection factor (SPF) value
C.variegatum	Methanolic	18.25 ± 0.22
C.variegatum	Acidified methanolic	7.34 ± 0.09
C.variegatum type 1	Methanolic	6.39 ± 0.08
C.variegatum type 1	Aqueous	7.11 ± 0.09
C.variegatum type 1	Acidic (Dilute HCl)	3.80 ± 0.05
Dermatone®	Methanolic	25.05 ± 0.33
Suncote®	Methanolic	27.54 ± 0.35

Table1. Sun protection factor (SPF) values of 0.2 mg/mL of *Codiaemvariegatum* extracts, and reference agents Dermatone® and Suncote® (Mean ± SEM).

The SPF values obtained with different phytochemical fractions of the methonolic extract of *C.variegatum* are shown in Table 2. As shown, flavonoid fraction had the highest SPF value which was almost identical to the SPF values of the two reference agents and the lowest SPF values were evident with diterpene and phenol fractions.

Class of phytochemical	Sun protection factor (SPF) value
Diterpenes (Band 1)	2.41 ± 0.03
Flevonoids (Band 2)	25.92 ± 0.32
Alkaloids (Band 3)	7.00 ± 0.09
Tannins (Band 4)	4.87 ± 0.06
Phenols (Band 5)	2.40 ± 0.03

Table2. Sun protection factor (SPF) value of different phytochemical fractions of methanolic extracts of *Codiaeumvariegatum* (mean ± SEM).

As shown in Table 3, initial phytochemical analysis of methonolic extract of *C.variegatum*showed the presence of alkolides, tannins and phenols, flavonoids or diterpenes. The thin layer chromatography (TLC) conducted with butanol ; acetic

acid ;water (3.5:1.4) solvent system (after trying several solvents of solvent system) revealed 5 spots (major compounds) (R_f =0.42 ;R_f =0.62; R_f =0.79; R_f =0.81 and R_f = 0.95) when visualizedunder UV light (254 nm). Compound/s from each band was then extracted in methanol and a secondTLC was carried out using the previous solvent system. The results showed 5 distinctive band with single spots indicating that there are no contaminations (Band 1, R_f =0.81 ;Band 2 ,R_f= 0.75 ;Band 3, R_f= 0.70;Band 4, R_f =0.68 and Band 5, R_f =0.46).

Phytochemical analysis showed that the Band 1 contains: diterpenes; Band 2 flavonoids; Band 3 alkolides; Band 4 tannins and Band 5 phenols.

Phytochemical class	Presence /absence
Alkaloids	+++
Tannins and phenols	+++
Flavonoids	+++
Diterpenes	+++

+ = small amount; ++ = moderate amount; +++ = large amount

Table3. Phytochemical screening of methanolic extract of *Codiaeumvariegatum*.

IV. Discussion

This study assessed the *in vitro* sunscreen activities (in term of SPF) of three varieties of Sri Lankan croton plants, namely,*C.variegatum, C.variegatumtype 1* and *C.variegatum type 2*. The efficiency of a sunscreen is generally expressed by its SPF value (8,26,27).The higher SPF value ,the more effective the agent as a sun screen (8,26,27).The SPF value of the synthetic and natural products /formulations can be investigated by using both *in vivo* and *in vitro* techniques (8,17,26,27).

In vivo techniques are cumbersome, expensive, time consuming, produce variable results (depending on the subjects used) and also involves ethical issues (27). On the other hand,*in vitro* techniques are simple, quick, inexpensive, validated, more reliable and well established (2,6,10,16,17,25). Hence, we employed an *in vitro* technique using UV absorption spectroscopy (290-320nm) and Mansur equation (10,17,25).which is widely used in evaluating sun protecting potential of natural products (2,4,6,7,10,17). Since, sun protective value is known to vary with several factors such as the nature of the solvent, concentration of the tested material, duration and the temperature equilibration ,type of cuvette used and the quality of the spectrophotometer (6,17,27), methonolic extracts of croton varieties having a concentration of 0.2 mg/mL , an equilibrate time of 1hr, an ambient temperature of 23^0C , and high quality 1cm quartz cells and well calibrated spectrophotometer were used as done previously by us (17,18,19) and other investigators (6,7,27). Hence, the results obtained are valid, reliable and meaningfully compared and interpreted with all of other workers.

The results clearly demonstrated that at 0.2 mg/mL concentration (a low concentration) of methanolicextracts of*C.variegatum variety 1* and *C.variegatum variety 2* have mild sun protective activity whilst C.variegatumhas profound sun protective activity *in vitro*. In contrast, water extract, HCl extract or acidified methanolic extracts had poor sun protection activity. This is a novel finding for any croton species worldwide. Further, the results indicate the high potential of developing cheap and a promising herbal sun protective formulation based on *C.variegatum*; in SPF ranking, SPF values 2-12, 12-30 and >30 are regarded as having mild, medium and maximum sun protective activity (8), and the SPF value of 18.25 suggest that this extract is capable of protecting the skin against 93% of normal solar UV-B rays (28). Nevertheless, SPF value of*C.variegatum* was 27-33% lower than the two reference agents tested ,yet ,it passes the threshold SPF value of a good sunscreen : most organizations and dermatologists recommended to liberally apply topical sunscreen formulations having SPF values 15 or more 15-30min before exposure to sun to minimize normal effects of suns UV.B rays (12,13). Another plus point for *C.variegatum* to be developed as a potential sunscreen is its high absorbance values (1.6 to 2.2) exhibited over a wide range of absorbance (between 290-320) : it is claimed that wider the range of absorbance of a photoprotective formulation higher would be its effectiveness in preventing sunburns resulting from solar UV B rays (27).

Large number of studies have shown that UV-B rays trigger the production of free radicals such as $^{\bullet}O_{2,\ HOO}{}^{\bullet}$, $^{\bullet}OH$ in the human skin (1, 2, 10, 16). Free radicals are now implicated with UV-induced photodamage in the skin (1, 2). Further, sunscreen activity of many effective herbal formulations are attributed mainly to their antioxidant activity (ability to quench free radicals) (1,2,10) and nowadays, many commercially available sunscreens are enriched with antioxidants like vitamin E (2,15,28). Powerful antioxidant activity has been shown in*C.variegatum* extracts (29), and, thus, a strong possibility exist that sun protective activity evident in this study, particularly, with the methanolic extract of *C.variegatum* is mediated through its antioxidant activity. Qualitative photochemical analysis and TLC studies revealed the presence of phenols, tannins, flavonoids, diterpenes and alkaloids. Ofthese, it is well established that flavonoids (mainly), phenolics and tannins are strong antioxidants (26, 29, 30). Further, the SPF value obtained with flavonoid fraction of *C.variegatum* was significantly increased (upto 25.92± 0.32) from the SPF value (18.25 ± 0.22) of its crude methanolic extract whilst the SPF value obtained for diterpenes (2.41 ± 0.03), alkaloids (7.0± 0.09), tannins (4.87 ± 0.06) and phenols (2.40 ± 0.03) were significantly lowered. These observations clearly indicate that the sun protective activity of *C.variegatum* is mediated via itsflavonoids. Interestingly, UV absorbing other constituents are also reported in plants growing in arid environments (31). In addition, an increase in UV absorbing constituents has been demonstrated when leaves are artificially exposed to UV rays (31). Possibility exists that these phytoconstituents are also present in croton species as well and contribute, at least partly, to the sun

protective activity of the plants examined in this study. Further studies are wanted to confirm these notions.

In toto the present study established methanolic extract of the*C.variegatum,C.variegatum type 1* and

*C. variegatumtype 2*possesses marked sun protective activity in vitro which is mediated, primarily by flavonoids via their antioxidant actions. A strong possibility exists to develop a safe, cheap, consumer friendly and readily available sun protective topical formation from *C.variegatum* leaves.

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