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Full Paper

Efficiency evaluation of topical emulsion of *Croton thorelii* Gagnep. extract and its related properties

Ampa Jimtaisong [1, 2, *] and Nisakorn Saewan [1, 2]

[1] School of Cosmetic Science, Mae Fah Luang University, Chiang Rai, 57100, Thailand

[2] Cosmetic and Beauty Innovations for Sustainable Development (CBIS) Research Group, Mae Fah Luang University, Chiang Rai, 57100, Thailand

* Corresponding author, e-mail: ampa@mfu.ac.th

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Abstract: The leaves of *Croton thorelii* Gagnep. were simply extracted by maceration in propylene glycol (PG), 95% ethanol and water using a sample-to-solvent ratio of 1:6 w/w for 4 hr at 50°C. The extract prepared using PG exhibits relatively higher biological activities than those using ethanol and water. It has a total phenolic content of 3.92±0.02 mg gallic acid eq./mL and the extract exhibits antioxidant activities, i.e. reducing power (103.90±0.41 mM ascorbic acid eq./mL), DPPH radical scavenging activity (81.11±8.08%), and anti-tyrosinase activity (30.30±2.50%). A topical emulsion was developed with 7% *C. thorelii* Gagnep. PG extract. The emulsion cream was classified as non-irritant after being clinically tested in 20 human volunteers. The efficacy test was performed by applying the product twice daily on the forearm for four weeks. It was found that the *C. thorelii* Gagnep. cream continually increased the skin moisture content significantly ($p<0.05$) about 2 times higher than that by the cream base. The skin melanin content also decreased significantly ($p<0.05$) and the decrease is higher (5.80%) than that by the cream base (3.80%). Additionally, the skin showed an increase in smoothness significantly ($p<0.05$) by the cream with *C. thorelii* Gagnep. extract (14.89%), which is two times higher than that by the cream base. The cream with *C. thorelii* Gagnep. also significantly decreased ($p<0.05$) the skin roughness by 11.76%. The results obtained indicate that *C. thorelii* Gagnep. can potentially be used as a skin-lightening and anti-aging ingredient in topical cosmetic preparations.

Keywords: *Croton thorelii* Gagnep. extract, moisturising cream, skin-lightening cream

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INTRODUCTION

The genus *Croton* (Euphorbiaceae) comprises about 1,300 species and about 30 *Croton* species are found in Thailand [1, 2]. *Croton* species were reported to be rich sources of diterpenoids, triterpenoids, glycosides, alkaloids and flavonoids [3]. *Croton thorelii* Gagnep. (Thai name: Plau-Ta-Wan) is currently used as traditional medicine for treatment and prevention of stomach ulcers. *C. thorelii* Gagnep. has also been reported to show an immune stimulation effect on human [4]. It was reported to contain plaunotol, an acyclic diterpene alcohol which increases the gastric mucosa blood flow [5] and possesses cytoprotective properties [6]. When applied topically, plaunotol has been found to exhibit antimicrobial activity against *Staphylococcus aureus*, a frequent cause of infection in atopic dermatitis [7]. The leaf and twig of *C. thorelii* Gagnep. extracted by ethanol and water were reported to possess antioxidant activities. The ethanol and water crude extracts exhibited IC_{50} (DPPH assay) of 125±0.11 and 190±0.11 µg/mL respectively, which indicates a lower activity than that of standard ascorbic acid (13.27±0.01 µg/mL). The extracts also contain phenolic and flavonoid compounds [8]. As mentioned earlier *C. thorelii* Gagnep. is traditionally and currently used as traditional medicine; however, there is less study on its use as cosmetic preparations. This study focuses on the preparation and use of *C. thorelii* Gagnep. extract for cosmetic preparation.

MATERIALS AND METHODS

Plant Materials and Reagents

The young leaves of *Croton thorelii* Gagnep. were collected from a certified organic farm, Rai Plau-ta-wan, Trad province, Thailand. The plant was identified and certified by the Plant Varieties Protection, Department of Agriculture, Thailand (No. 221/2548). All chemicals and reagents for activity study were of analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid and gallic acid were from Sigma-Aldrich (Germany). Folin-Ciocalteu reagent was from Sigma-Aldrich (Switzerland). Potassium ferricyanide was from Loba Chemie (India). Ascorbic acid was purchased from Kemaus (Australia). L-tyrosine was from HiMedia Laboratories (India). Mushroom tyrosinase was purchased from Sigma-Aldrich (USA). Propylene glycol (PG) was from Shell Chemicals (Singapore). Sodium lauryl sulfate was from Emery Oleochemicals (Malaysia). Kojic acid was from Rensin Chemicals (China) and deoxyarbutin was from Chemfaces (China). All spectrophotometric data were acquired using a UV-Vis spectrophotometer (Libra 522, Biochroms, UK).

Preparation of Extract

The leaves of *C. thorelii* Gagnep. were dried in a solar green house at 40 °C for 2 days and then ground to powder (80-micron). The powder was separately extracted with PG, 95% ethanol (EtOH) and deionised (DI) water using a sample-to-solvent ratio of 1:6 w/w. The mixture was stirred at 50°C for 4 hr. The extract solution was then filtered using filter paper (Whatman no.1) and was kept in the refrigerator for further use.

DPPH Radical Scavenging Assay

The scavenging activity of the extracts against DPPH radicals was evaluated according to the previously reported methods [9, 10] with some modifications. A solution of DPPH in absolute ethanol (0.1 mM, 3 mL) was added to the extract (1 mL). The reaction was allowed to continue at

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37°C for 30 min. in the dark and then the absorbance (Abs) was measured at 517 nm. Per cent scavenging activity (SA) on DPPH radicals was calculated as

$$\%SA = \{Abs_{control} - Abs_{sample})/Abs_{control}\} \times 100$$

where Abs_{sample} is absorbance in the presence of sample (sample dilution+DPPH solution) and $Abs_{control}$ is absorbance of control (sample solvent+DPPH solution). The results were expressed as mean ± SD (n = 3).

Determination of Reducing Power

The reducing power was determined by the method of Oyaizu [11] with some modifications. The extract (diluted to 10%, 1 mL) was mixed with 2.5 mL of phosphate buffer (0.2M, pH 6.66) and 2.5 mL of 1% potassium ferricyanide. The mixtures were incubated at 50°C for 20 min., after which 2.5 mL of 10% trichloroacetic acid was added, followed by centrifugation at 5000 rpm for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% ferric chloride. The absorbance of the mixture was measured at 700 nm. Ascorbic acid (0.078, 0.156, 0.313, 0.625, 1.250 mM) was used as reference standard. The increase in absorbance of the reaction mixture indicated the reducing power of the natural reducing agent. The result was reported as ascorbic acid equivalent (AAE) (mM AAE/mL extract).

Determination of Total Phenolic Content

The total phenolic content of the extracts was determined by Folin-Ciocalteu total phenolic assay [12]. Gallic acid (GA) was used as standard and a range of concentrations (50, 100, 250, 500, and 1000 mg/L) was used to create a standard curve. Deionised water (1.58 mL) and Folin-Ciocalteu reagent (100 µL) were added to the extract sample, the standard, or blank (20 µL). The reaction mixture was incubated at room temperature for 5 min. Sodium carbonate solution (10% 300 µL) was then mixed with it and incubated for 90 min. The absorbance of the mixture was measured spectrophotometrically at 765 nm. The total phenolic content was calculated from a standard curve and expressed as gallic acid equivalent (GAE) (mg GAE/mL extract). Determinations were made in triplicate.

Tyrosinase Inhibition Test

Tyrosinase inhibitory activity is generally determined with a spectrophotometer. The procedure was performed according to the previous reported method [13] with modifications. A reaction mixture consisting of the extract (0.9 mL) and L-tyrosine solution (0.244 mM) in aqueous phosphate buffer (pH 6.8) (2 mL) was prepared; for control sample, an equivalent volume of 50% methanol solution was used in place of the inhibitor solution. The oxidation of L-tyrosine was initiated by introducing 0.1 mL of aqueous mushroom tyrosinase solution (0.1 mg/mL). The test mixture and control mixture were incubated for 10 min. at 37°C. Dopachrome appearance was monitored spectrophotometrically at 475 nm. Ascorbic acid, kojic acid and deoxyarbutin were used as reference standards for comparison. The tyrosinase inhibition (%) was calculated by the following equation.

$$\text{Tyrosinase inhibition (\%)} (= [(A_{control}-A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is absorbance of the control system and A_{sample} is absorbance of the sample or standard.

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Preparation of Cosmetic Emulsion

An oil-in-water (O/W) emulsion cream was prepared with the formulation shown in Table 1. The preparation without the *C. thorelii* extract was used as base formula. At first, the aqueous phase and lipid phase were separately heated up to 70°C. Then an O/W cream was prepared by adding the lipid phase to the aqueous phase. The mixture was homogenised at 3000 rpm for 5 min. and was cooled to 45°C. Then the preservative, vitamin E acetate, farnesol (and) linalool and *C. thorelii* extract were added to the emulsion. The suitable amount of *C. thorelii* extract in the preparation was studied based on the tested activity and physiochemical properties. The product with cosmetically acceptable characteristics was selected for efficiency evaluation.

Table 1. Emulsion cream formulation

Ingredient	%w/w	Function
DI water	qs to 100	Diluent, solvent
Isoprene glycol	5.0	Humectant
Stearic acid	3.5	Cream consistency, viscosity enhancer
Behenyl alcohol	3.0	Cream consistency, viscosity enhancer
Jojoba oil	4.0	Emollient
Phytosqualane	4.0	Emollient
Dimethicone	1.0	Emollient
Glyceryl stearate	2.0	Emulsifier
Arachidyl alcohol, behenyl alcohol & arachidyl glucoside	2.0	Emulsifier
Hydroxyethyl acrylate/sodium acryloyldimethyl taurate copolymer, isohexadecane & polysorbate 60	2.0	Viscosity enhancer, emulsifier
Methylparaben/phenoxyethanol	1.0	Preservative
Vitamin E acetate	0.5	Antioxidant
Farnesol (and) linalool	0.5	Odour-neutralising
C. thorelii extract	qs	Active ingredient

Clinical Study

All of the study in human volunteers was carried out in accordance with the Declaration of Helsinki [14, 15]. Thai healthy volunteers, both male and female aged between 20-60 years, were enrolled in the study. The study was approved (approval no. REH-62158) by the ethical committee of Mae Fah Luang University prior to enrollment. All participants were informed about the study and signed a consent form and were free to discontinue their participation at any time during the study without any consequences.

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Primary skin irritation test

The potential skin irritation test was performed on the *C. thorelii* extract and cream by closed-patch test method for 24 hr using Finn chambers® (8 mm, Smart Practice, USA). Sodium lauryl sulfate (0.5%) was used as positive control and DI water as negative control. Observation was undertaken 30 min., 24, 48 and 72 hr following patch removal. Mean irritation index (MII) was then calculated from the sum of irritation grade per total number of subjects [16-18].

Skin efficacy test

Skin efficacy test was performed with enrolled 20 volunteers by applying 2 mg/cm^2 of creams (cream base and extract cream) on an inner forearm twice a day for 4 weeks. During the testing period, the volunteers were not allowed to use any skin-care products on the forearm where the product would be used. Then skin hydration was measured by Corneometer CM825 (Courage and Khazaka, Germany). The scaliness, roughness, smoothness and wrinkles of the skin were obtained by Skin Visioscan VC98 (Courage and Khazaka, Germany). Melanin content was analysed by Mexameter (Courage and Khazaka, Germany) at week 0 and week 4. The comparison of all parameters was accomplished by SPSS version 21 (SPSS Inc, Chicago, USA). Significant differences between means were determined by pair t-test. P values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

DPPH Radical Scavenging Activity, Reducing Power, Total Phenolic Content and Tyrosinase Inhibition Activity

DPPH radical scavenging activities of *C. thorelii* solution extract (diluted to 5%) are shown in Table 2. It can be seen that the PG extract has the highest activity (81.11±8.08%), followed by the EtOH and water extracts. When compared with standard ascorbic acid, the PG, EtOH and water extracts exhibit 44.40±0.23, 32.60±0.26 and 23.80±0.32 mM AAE/mL respectively. Both the reducing power and the total phenolic content of the PG extract are also highest compared to the EtOH and water extracts (Table 2).

The PG extract also exhibits higher tyrosinase inhibition than the ethanol and water extracts (Table 2). When compared with the well-known whitening agents, i.e. kojic acid (12.5 µg/mL) and deoxyarbutin (12.5 µg/mL), its inhibition activity is 3 times lower. However, the PG extract exhibits only about 20% less inhibition when compared with that by ascorbic acid (12.5 µg/mL).

In line with the presence of a higher amount of total phenolic content, extraction using PG as solvent gives an extract with higher activities than those obtained by using ethanol or water. This result is similar to that of the previously reported extraction of *Camellia oleifera* seed dregs [19]. PG is an ingredient generally used in cosmetic preparation. It is allowed to be incorporated into the formulation at a concentration of up to 50% [20]. The use of PG as extraction solvent allows for an easy and simple preparation method. The resulting extract is ready for use in cosmetic preparations. Because PG is moisture-retaining and nontoxic, the extract can be directly added to the formulation after simple filtration. However, PG has a high boiling point (188.2°C), so its removal to obtain a concentrated extract is not possible, which may result in an extract with relatively low activity for compounds with a low bioactivity.

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Table 2. Properties of *C. thorelii* extracts

Extract/Substance	DPPH radical scavenging activity (%)	Reducing power (mM AAE/mL)	Total phenolic content (mgGAE/mL)	Tyrosinase inhibition activity (%)
PG extract	81.11±8.08	103.90± 0.41	3.90±0.02	30.30±2.50
EtOH extract	60.25±9.08	33.27±0.08	1.19±0.01	21.21±3.25
Water extract	44.79±11.38	26.60 ± 0.12	0.53±0.01	12.10±2.32
Ascorbic acid (12.5 µg/mL)	-	-	-	38.38±4.63
Kojic acid (12.5 µg/mL)	-	-	-	81.82±8.02
Deoxyarbutin (12.5µg/mL)	-	-	-	91.92±1.75

Preparation of Cosmetic Emulsion

An O/W emulsion base was prepared and an opaque white cream with light texture was obtained (Figure 1). *C. thorelii* PG extract was incorporated into the emulsion at 3, 5, 7 and 10%. The amount is based on the DPPH activity results, as at 5% solution extract it exhibits about 81% DPPH radical scavenging activity. It was found that a higher amount leads to a stronger colour of greenish-yellow cream. The emulsion cream with 7% *C. thorelii* PG extract exhibits cosmetically acceptable characteristics and was selected for efficiency evaluation. It possesses a pH of 4.5, which is suitable for topical application [21]. The cream with 7% *C. thorelii* PG extract was experimentally found to exhibit 88.36±0.26% DPPH radical scavenging activity.

Figure 1. Appearance of emulsion cream base (a) and cream with 7% *C. thorelii* extract (b)

Clinical Study

The potential skin irritation of products was investigated by closed patch test method. The patch test was removed at the end of the 24-hr test period. The score of skin irritation was graded according to Table 3 [16-18]. The MII was then calculated from the sum of irritation grade per total number of subjects and the interpretation was according to Table 4 [16-18]. Calculated MII values of products are tabulated in Table 5, which indicates that the *C. thorelii* PG extract and its emulsion cream are non-irritating products [18].

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Table 3. Grading scale for scoring irritation test performed on subjects using patches

Score	Skin reaction
0	No visible erythema
0.5	Doubtful reaction, pinkish, mottled
1	Slight diffuse redness
2	Moderate uniform redness
3	Intense redness
4	Very intense redness with oedema or epidermal lesions

Table 4. Interpretation of MII obtained from irritation test performed on subjects using patches

MII value	Interpretation
MII < 0.2	Non-irritant
0.2 ≤ MII < 0.5	Slight irritant
0.5 ≤ MII < 1.0	Moderate irritant
MII ≥ 1	Irritant

Table 5. Calculated MII values of tested products

Substance	MII value	Interpretation
PG (7%)	0.75	Non-irritant
C. thorelii PG extract (7%)	0	Non-irritant
Emulsion cream base	0	Non-irritant
Emulsion cream with 7% *C. thorelii* extract	0	Non-irritant
Negative control (DI water)	0	Non-irritant
Positive control (0.5% sodium lauryl sulfate)	1.425	Irritant

In the skin efficacy test the moisturising effect of the product was measured in terms of hydration level using Corneometer® (CM 825 Courage and Khazaka, Germany). This parameter indicates the hydration level of the epidermis and dermis, expressed in an arbitrary unit from 0 to 130 (<30 very dry, 30-40 dry, >40 sufficiently moisturised) [22]. Compared to cream base, the *C. thorelii* emulsion cream continually increases the skin moisture content significantly ($p<0.05$) from 38.13±7.39 to 42.37±7.63 (Table 6): an increase of 11.76%, which gives sufficient hydration of the skin. The result compares well to the cosmetic formulation with *Lithospermum erythrorthizon* root extract previously reported, which showed 11.77% increase in skin humidity of volunteers [23].

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Melanin index of skin was measured to study the effect of the cream as a skin-lightening product. The skin melanin content decreases significantly ($p<0.05$) from 211.67±51.55 to 199.38±49.57 and the decrease is higher (12.29) than that by the cream base (8.15) (Table 6). Traditionally, commercial skin whitening agents for suppressing tyrosinase activity used in cosmetics include arbutin, kojic acid, ascorbic acid, ellagic acid and tranexamic acid [24]. In addition, there are several plant extracts that have been shown to be effective agents for suppressing the overproduction of melanin or regulating melanin synthesis, such as *Cassia fistula* flowers [25] and *Etlingera elatior* [26]. In this study the skin melanin content decreases significantly ($p<0.05$) at 5.80%. It has been reported that the melanin reduction activity of cream containing 1% *Etlingera elatior* crude extract was at 6.67% after testing in volunteers for 4 weeks [26]. The cream with *C. thorelii* PG extract thus also seems to have a good skin-lightening effect on human volunteers.

Table 6. Results of skin moisturisation and melanin content tests

Week	Skin water content		Melanin index	
	Cream base	*C. thorelii* cream	Cream base	*C. thorelii* cream
0	37.52±6.44	38.13±7.39	214.40±46.61	211.67±51.55
4	40.17±5.95	42.37±7.63*	206.25±43.66*	199.38±49.57*

* Statistically significant difference when compared to baseline ($p<0.05$)

Additionally, when the skin surface profile was measured, the skin scaliness, roughness, skin wrinkles and smoothness were obtained. The scaliness values of the skin treated with cream base and *C. thorelii* cream decrease significantly ($p<0.05$) at about the same extent (Figure 2). The roughness values decrease in both cases but at a much greater extent (11.76%) by the *C. thorelii* cream compared with that by cream base (1.09%). As for skin smoothness, it shows an increase in value significantly ($p<0.05$) by the *C. thorelii* cream (14.89%), 2.3 times higher than that by cream base (6.57%). The increase in skin smoothness and skin water content is related with the decrease in skin scaliness and roughness [27]. Finally, the skin wrinkle parameter decreases for both the cream base and the *C. thorelii* cream, but the comparative values are not statistically significant. A longer treatment time may be needed in order to observe the long-term effect of the product [28, 29].

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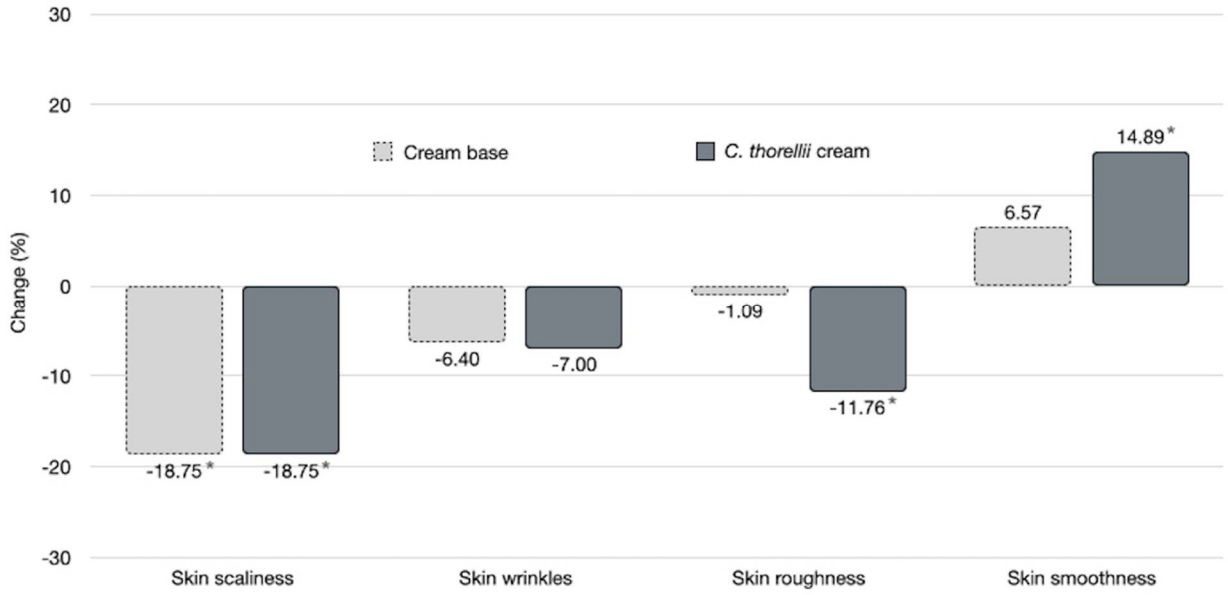

Figure 2. Skin surface profile obtained using Visioscan®. Data are in percentage of change when compared to baseline; negative values indicate decrease while positive values indicate increase. Values with * indicate statistically significant difference compared to baseline ($p<0.05$).

CONCLUSIONS

The PG extract of *C. thorelii* Gagnep. exhibits higher antioxidant activity, reducing power, total phenolic content and tyrosinase inhibition activity than do ethanol and water extracts. The daily use of topical cosmetic cream with *C. thorelii* PG extract is safe. The product also helps decrease skin melanin content, smoothen the skin and increase skin hydration. The *C. thorelii* PG extract thus seems to have good potential as a natural lightening and anti-aging ingredient in cosmetic products.

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