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Extraction of *Coccinia grandis* leaf for development of insect bites cream formulation

Thamonwan Bubchaiya[1], Rassamichan Kanavan[1], Chawalinee Asawahame[2], and Aranya Jutiviboonsuk[3*]
[1]Undergraduate student, Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University, Samutprakarn, Thailand
[2]Department of Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University, Samutprakarn, Thailand
[3]Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Huachiew Chalermprakiet University, Samutprakarn, Thailand
*Corresponding Author's Email: ongsri@yahoo.com Telephone: 0816182424

Rationale

Coccinia grandis (L.) Voigt is known as Ivy gourd. It is a member of the Cucurbitaceae family in the genus Coccinia. The fresh leaf is used as a medicinal plant in Thailand for relieving inflammation from insect bites. Several pharmacological activities of the plant extract have been reported such as antioxidant, antihyperglycemic, anti-ulcer, antimicrobial, and anti-inflammatory activities (Pekamwar, 2013, p.54-58, Amir Hossain, 2014, p.65-71, Varuna, 2018, p.188-200). *β*-sitosterol is a phytosterol compound which was found in many parts of ivy gourd, including leaf (Alagarraja, 2017, p.54-58). The strong anti-inflammatory activity of *β*-sitosterol was observed by membrane stabilization of human red blood cell and inhibition of protein denaturation methods which indicated that *β*-sitosterol promoted the neutralizing lysosomal enzymes or stabilizing the lysosomal membrane (Ushir Yogrsh, 2013, p.85-89). According to Thai traditional medicine, fresh leaves of C. grandis are crushed and applied on affected area which is inconvenient. Therefore, Ivy gourd extract was formulated as cream for ease of use and promoting the use of Thai herb.

Research Objectives

The objectives of the study are to evaluate the in-vitro anti-inflammatory activity of the leaf extracts of C. grandis and to formulate a topical cream for insect bites treatment.

Methodology

Plant material:

Fresh *C. grandis* was purchased from Thipgasorn market, the local market at Bang Phli district, Samut Prakan province, Thailand. The size of fresh leaves used in the study was 6-13 cm wide and 4-10 cm long. The fresh leaves were washed with tap water and dried at 50°c using hot air oven and ground into coarse powder by blender.

Preparation of plant extracts for qualitative analysis:

The plant powder (3 g each) was extracted with 60 mL hexane, dichloromethane, absolute ethanol, ethanol-water (9:1), or water using ultrasonic bath for 30 minutes at room temperature, then each extract was filtered and dried in vacuum rotary evaporator to obtain CGHe, CGDi, CGEt, CGEW, and CGWa extracts, respectively. Each extract was redissolved to 10 mL volume with its solvent and subjected to qualitative analysis using thin-layer chromatography. TLC analysis was performed on 10 x 10 cm aluminum plates precoated with 0.2 mm layers of silica gel 60 F254 (E. Merck, Germany). Sample and standard solutions, each 10 µL, were applied on the plates as 2 mm wide bands. The mobile phase was dichloromethane-ethyl acetate 9:1 (v/v). The developed plates were detected with color reaction using anisaldehyde-sulfuric acid reagent, the color reaction occurred when heat at 105°c for 15 minutes. *β*-sitosterol (1.25 mg/mL) was used as reference standard.

Preparation of plant extracts for cream formulation:

The plant powder (640 g) was extracted with 14.7 L ethanol-water (9:1) using ultrasonic bath for 2 hours at room temperature, then the extract was filtered. 8.7 L of the filtrate was dried in vacuum rotary evaporator to obtain CGEW-F. Another 6 L of the filtrate was sent to liquid-liquid extraction with hexane/water to obtain CGH-F (hexane phase) and CGW-F (aqueous phase).

Albumin denaturation inhibition assay:

Protein denaturation has been correlated with the inflammatory disorders. Several non-steroidal anti-inflammatory drugs (NSAIDs) enhanced the ability to prevent heat denaturation of bovine serum albumin (BSA) at pathological pH 6.2-6.5 (Grant, 1970, p.715-722). The reaction mixture consisted of 0.9 mL of 5% bovine serum albumin, 2.8 mL of phosphate buffer saline pH 6.3, and 2.0 mL of 100 µg/mL of the extract. Diclofenac sodium was used as reference. The mixture was incubated at 37°C for 15 minutes then heated at 70°C for 5 minutes. Absorbance of the mixture was measured at 660 nm. The percentage inhibition of albumin denaturation was calculated using the equation as shown below.

$$\%\text{inhibition} = [1 - (\text{absorbance of sample}/\text{absorbance of control})] \times 100$$

Preparation of *C. grandis* cream:

Five different cream bases were prepared. The oil phase consisted of stearic acid, cetyl alcohol, stearyl alcohol, liquid paraffin, isopropyl palmitate, and Span 60 was melted at 75°C and stirred. The water phase consisted of propylene glycol, Tween 60, Tween 80, sorbitol, and distilled water was heated at 78°C. The water phase was poured into the oil phase with continuously stirring until the mixture congealed. Then preservative was added, and the mixture was stirred to become homogenous cream. The physical properties (color, odor, consistency, washability, and pH) of these cream bases and their stability using centrifugation test were evaluated. The cream base with good properties and stability was chosen to prepare *C. grandis* cream. *C. grandis* extract was dissolved in liquid paraffin before added into the cream base to produce two concentrations (1% and 5%) of *C. grandis* cream.

Evaluation of physical properties and stability of *C. grandis* cream:

Physical appearance of cream was evaluated by observing its color, odor, and consistency. Dye test was used to evaluate type of emulsion. The viscosity was determined using Brookfield viscometer model LVDV-II+Pro with T-bar spindle at speed of 12 rpm and 25°C for 1 minute. pH was also evaluated. Centrifugation test was used for the accelerated stability study of cream. The cream was centrifuged for 30 minutes at 3,500 rpm and inspected for signs of creaming. Freeze-thaw stability study of cream was also determined. The cream was stored in airtight glass containers and stability tests were performed on 4 cycles of temperature testing, -20°C (48 hours) and 45°C (48 hours). Then physical properties, viscosity and pH of cream were evaluated.

Results and Discussion

The extraction of *C. grandis* leaves using 5 different solvents obtained CGHe, CGDi, CGEt, CGEW, and CGWa extracts. TLC chromatograms (fig. 1) showed that *β*-sitosterol was found in CGHe, CGDi, CGEt, and CGEW extracts, except in CGWa extract. *β*-sitosterol, a phytosterol found in many parts of *C. grandis* such as root, aerial parts, fruit, stem, and leaf (Alagarraja, 2017, p.54-58) was reported as a potent anti-inflammatory activity in rodents using reversed passive Arthus reaction in the rat and mouse ear edema assay (Paniagua-Pérez, 2017, p.123-130). The chemical composition of CGEW extract was greater than that of any other extract. In addition, the solubility of *β*-sitosterol in ethanol was higher than that in n-hexane (Wei, 2010, p.2917-2919). As a result, the plant powder (640 g) was extracted with 14.7 L of 90% ethanol to yield 124.95 g of CGEW-F extract (19.52% w/w of plant powder).

The CGEW-F extract (50 g) was then liquid-liquid extracted with hexane/water to yield 20.64 g of CGW-F extract (7.79%w/w of plant powder) and 14.19 g of CGH-F extract (5.09% w/w of plant powder). Anti-inflammatory activities of CGEW-F, CGW-F, and CGH-F extracts were evaluated by albumin denaturation inhibition assay. At the concentration of 100 µg/mL, CGH-F extract presented 87.44% inhibition of albumin denaturation which was greater than diclofenac sodium (27.23%). Whereas CGEW-F and CGW-F extracts showed 6.39% and 21.06% inhibition, respectively.

Cream base which demonstrated good properties and stability was composed of cetyl alcohol (7%), stearyl alcohol (5%), liquid paraffin (5%), propylene glycol (10%), Tween 80 (5%), and distilled water (q.s. to 100%). The CGH-F extract was subjected to produce 1% and 5% *C. grandis* creams. The dye test revealed that both creams were o/w type, and their physical properties are presented in table 1. The results showed that increasing of the extract concentration caused the increasing of pH and viscosity of cream. The centrifugation test indicated the stability of *C. grandis* creams and cream base. The color, odor, consistency, pH, and washability of 1% and 5% *C. grandis* creams did not change after the 4 cycles of freeze-thaw stability study. Nevertheless, the increasing of viscosity of both creams was observed. The viscosity of 1% *C. grandis* cream increased from 872,000 to 948,733 centipoise while viscosity of 5% *C. grandis* cream increased from 978,000 to 1,145,000 centipoise. Accordingly, the extract of *C. grandis* leaf can be formulated as 1% and 5% creams with good physical properties and good stability.

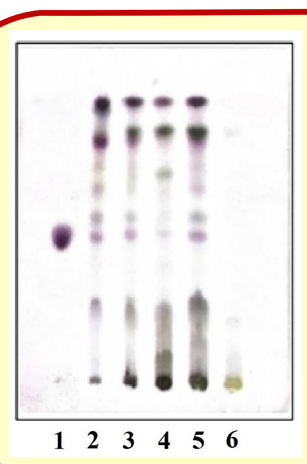
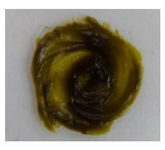


Figure 1 TLC chromatogram of *C. grandis* extracts.
(1) *β* - sitosterol,
(2) CGHe extract
(3) CGDi extract
(4) CGEt extract
(5) CGEW extract
(6) CGWa extracts.

Table 1 Physical properties of *Coccinia grandis* creams

Physical properties	*C. grandis* cream (1%)	*C. grandis* cream (5%)	Cream base
Color	Green brown 	Dark green brown 	White
Odor	Characteristic	Characteristic	Odorless
Consistency	Smooth and non-grease	Smooth and non-grease	Smooth and non-grease
pH	6.15	7.09	5.13
Washability	Good	Good	Good
Viscosity (centipoise)	872,000	978,000	863,000
Centrifugation test (phase separation)	No	No	No

Conclusion

The hexane fraction which was extracted from the 90% ethanolic extract of *C. grandis* leaves, contained the anti-inflammatory compound, *β*-sitosterol. It presented 87.44% inhibition of albumin denaturation at the concentration of 100 µg/mL. The formulation of 1% and 5% *C. grandis* creams produced good physical property creams with good stability under 4 cycles of freeze-thaw study.

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