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Original research article

Assessment of skin allergic and irritant reactions of *Delonix regia* flower extract and the finished cosmetic products

Worapapar Treesuppharat[1,2*], Wiratchanee Mahavorasirikul[1,3], Sumet Kongkiatpaiboon[1,3], Panlop Chakkavittumrong[4]

[1]*Drug Discovery and Development Center, Office of Advanced Science and Technology, Thammasat University (Rangsit Campus), Pathum Thani, Thailand, 12121*
[2]*Thammasat University Research Unit in Mechanisms of Drug Action and Molecular Imaging, Thammasat University (Rangsit Campus), Pathum Thani, Thailand, 12121*
[3]*Thammasat University Research Unit in Cannabis and Herbal Products Innovation, Thammasat University (Rangsit Campus), Pathum Thani, Thailand, 12121*
[4]*Department of Internal Medicine, Faculty of Medicine, Thammasat University (Rangsit Campus), Pathum Thani, Thailand, 12121*

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ABSTRACT

Delonix regia (Hook.) Raf. is an ornamental tree with attractive flowers, and it is a symbolic tree of Thammasat University, Thailand. Its flowers contain rutin as major component with pronounced antioxidative properties. Many studies showed the cosmeceutical potential of *D. regia* flower (DRF) extract including antioxidant, antimicrobial, and photoprotective activities. In the course of utilizing DRF extract as a cosmetic ingredient, the evaluation of the skin allergic and irritant reactions of the DRF extract and the finished cosmetic products in healthy subjects was studied. Skin compatibility of DRF extract and the finished cosmetic products was evaluated using human single application closed patch epicutaneous test. The results showed that DRF extract up to a concentration of 4% w/w did not cause any skin irritation. Weak allergic reaction was observed with 4% w/w extract in 1 of the 29 subject. Body lotion and hand cream containing 0.3% w/w DRF did not cause any allergic reaction. Liquid soap containing 0.05% w/w DRF extract caused slight erythema (grade +) in 2 subjects. Therefore, DRF extract could be used safely as topical cosmetics up to the concentration of 4% w/w.

Keywords: *Delonix regia,* human patch test, skin irritant, skin allergy, cosmetic ingredient

***Corresponding author**: woratree@tu.ac.th
https://li01.tci-thaijo.org/index.php/JBAP

1. Introduction

Delonix regia (Hook.) Raf. is a large ornamental tree with an attractive red peacock flower. It is native to Madagascar and has been introduced to many parts of the world, including Thailand. It also serves as a symbolic tree of Thammasat University, Thailand. Traditionally, *D. regia* has been used for various ailments and health conditions such as chronic fever, microbial infections, constipation, inflammation, arthritis, bronchitis, asthma, and dysmenorrhea.[1] *D. regia* contains polyphenolic compounds such as flavonols, anthocyanins, and phenolic acids as bioactive secondary metabolites that are responsible for their antioxidant activity. There are several active constituents in different parts of this plant including flowers, leaves, barks, seeds, and fruits.[1]

In the course of utilizing *D. regia* plant, its flowers have been screened for the phytochemical constituents and biological activities. Pronounced antioxidative property was observed with rutin flavonoid as a major active component. Several studies showed that antioxidants might be useful in cosmetic products because they constrain the skin aging process due to their ability to relieve oxidative stress.[2] Therefore, optimized extraction and standardization of *D. regia* flower (DRF) extract using rutin as a chemical marker was developed to provide a novel cosmetic ingredient.

In the present study, DRF extract was prepared, standardized, and prepared as a component of cosmetic ingredients [Patent pending]. The human single closed patch epicutaneous test under occlusion was used to evaluate the skin compatibility of DRF extract and the finished cosmetic products in human subjects according to the protocol developed by the European Cosmetic, Toiletry and Perfumery Association (COLIPA).[3,4]

2. Materials and Methods

2.1 Chemicals and reagents

HPLC grade methanol was obtained from Labscan (Thailand). Deionized water was purified by Ultra Clear (Siemens Water Technologies Corp.). Acetic acid was purchased from Labscan. All reagents were of analytical grade if not stated otherwise. Rutin was purchased from Tokyo Chemical Industry Co., Ltd. (Japan).

2.2 Sample Preparation

The DRF was collected from Thammasat University (Rangsit Campus), Thailand during March-May, 2020. It was dried in a hot air oven at 55°C for 72 h, ground into fine powder, and kept in an air-tight container. The dried sample was exhaustively extracted with 95% ethanol and filtered through Whatman No.1 filter paper. The solution was concentrated under vacuum using a rotary evaporator (Hei-VAP Precision, Heidolph, Germany) and dried on a water bath. The dried crude ethanolic extract was quantitatively analyzed for the content of the chemical marker rutin [Patent pending], by high-performance liquid chromatography (HPLC) and the rutin content was standardized to 1.8±0.2% w/w. DRF extract was prepared as 10% w/w stock solution in propylene glycol.

The DRF extracts for the patch test were prepared freshly. Pure white petrolatum (negative control) was of the commercial cosmetic grade. DRF extracts at concentrations of 2% and 4% w/w were prepared by diluting the 10% w/w DRF extract stock solution with pure white petrolatum.

The body lotion was prepared using the following ingredients: water, cetostearyl alcohol, isopropyl myristate, dimethicone, propylene glycol, ceteareth-6, ceteareth-25, glycerin, cetyl alcohol, vitamin B3, jojoba oil, phenoxyethanol, allantoin, fragrance, color, and DRF extract (0.3% w/w, dry weight basis). The body lotion base (vehicle control) was prepared using the same ingredients but without DRF extract.

The hand cream was prepared using the following ingredients: water, cetostearyl alcohol, cetyl alcohol, glycerin, propylene glycol, isopropyl myristate, ceteareth-6, ceteareth-25, cyclomethicone, phenoxyethanol, shea butter, fragrance, color, and DRF extract

(0.3% w/w, dry weight basis). The hand cream base (vehicle control) was prepared using the same ingredients but without DRF extract.

The liquid soap was prepared using the following ingredients: water, sodium laureth sulfate, cocamidopropyl betaine, sodium chloride, glycol distearate, DMDM hydantoin, fragrance, glycerine, propylene glycol, methylisothiazolinone, color, and DRF extract (0.05% w/w, dry weight basis). Liquid soap base was prepared with the same method but excluding DRF extract.

2.3 HPLC analysis

HPLC was performed on an Agilent 1260 Series (Agilent Technologies) equipped with a 1260 Quat pump VL quaternary pump, 1260 ALS autosampler, 1260 TCC column thermostat, and 1260 DAD VL diode array detector. The separation was done on a Hypersil BDS C18 column (4.6 × 100 mm i.d., 3.5 µm) with a C18 guard column. The elution was performed with an isocratic solvent system using a mixture of 0.5% acetic acid in water and methanol (65:35, v/v). The flow rate was set at 1.0 mL/min with a controlled temperature at 25°C. The DAD detector was set at the wavelength of 355 nm and injection volume was 10 µL.

Rutin standard solution stock was prepared by accurately weighing rutin standard and dissolving in methanol to a concentration of 1,000 µg/mL. Working standard solutions were obtained by appropriate dilution of the stock solution with methanol. Linearity was ensured using the working solution with the concentration range 3.9 – 500 µg/mL. The calibration curve was constructed from the peak area versus the concentration of the standard. Specificity was assessed by peak purity using UV spectra obtained from the diode array detector.

The dried DRF extract was accurately weighed and diluted with methanol to a concentration of 10 mg/mL using sonication. The sample was analyzed in triplicate. Prior to injection and analysis by HPLC, all extracts were filtered through 0.22 µm nylon membrane.

2.4 Ethical approval

The study protocol was approved by the Institutional Ethics Committee of Thammasat University (Approval number: 103/2564). All procedures were in compliance with applicable standards and guidelines.

2.5 Study subjects

The number of healthy volunteers used in Phase I clinical trial typically ranges from 20 to 80 to determine the safety and tolerability profiles of an investigational drug according to the US Food and Drug Administration.[5] While the ethical guideline for conducting human research in Thailand recommends that the number of volunteers should be at least 10 subjects, which is enough to interpret the results, but not more than 30 subjects in Phase I trials.[6] Thirty healthy volunteers (15 males and 15 females, aged > 20 years) were therefore recruited into the study. All were informed about the study procedures and potential unwanted effects. Written informed consents were obtained from all volunteers before study participation. Exclusion criteria were pregnancy or breastfeeding, known allergy to cosmetics and/or cosmetic ingredients, previous adverse reactions to materials used in the study (*e.g.*, plasters), skin diseases or dermatological disorders (e.g., scars, sunburn, tattoos, moles and/or irritation), concurrent administration of drugs that may interfere with the interpretation of the study results (*e.g.*, antibiotics, anti-inflammatory drugs), as well as the regular use of sunbeds which may affect the skin response.

2.6 Patch testing

The human single closed patch test under occlusion was performed using an allergEAZE® patch test chamber (SmartPractice Canada, AB T2E8V1, Canada). One patch consists of ten chambers (8 x 8 mm). A webril cotton (8 x 8 mm) was placed onto each chamber and saturated with sufficient amount of the test material to cover the surface of the pad. Ten test materials, as listed in Table 1, were placed on the patch

and occluded on the upper backs of all subjects with a hypoallergenic micropore tape (3M Health Care, MN, USA). After 48 h of skin contact, the patches were removed. Signs of allergic reactions were observed at 48 and 72 h following exposure and results interpreted according to International Contact Dermatitis Research Group (ICDRG) criteria[7] (Table 2). Skin irritation reactions were evaluated at 48 h after exposure according to Cosmetic, Toiletry and Fragrance Association (CTFA) criteria[8] (Table 3).

2.7 Data Analysis

Data are presented as the number and frequency of each grade of skin reaction. For the cosmetic products, the raw scores of skin irritant responses were calculated according to the formula[9]:

$$raw\ score = \frac{\sum(grade \times n\ of\ responders)}{4(maximum\ grade) \times N(total\ subjects)} \times 100 \times 1/2$$

The raw scores were converted to standardized z-scores using the mean and standard deviation of the skin irritant response of cosmetic products as follows[9] **:**

$$z\text{–}score = \frac{(X - \mu)}{\sigma}$$

Where X is the raw score, μ is the mean of the response of cosmetic products, and σ is the standard deviation of the response**.**

The primary irritation index[9] was classified into four levels based on the severity of skin irritation reactions (z-scores), as shown in Table 4. The index is useful for determining the primary irritation of human skin in the field of cosmetic products**.**

Table 1. List of test materials

Number	Test materials
1	Pure white petrolatum
2	2% w/w of *Delonix regia* flower extract in petrolatum (2% w/w DRF extract)
3	4% w/w of *Delonix regia* flower extract in petrolatum (4% w/w DRF extract)
4	40% w/w of Propylene glycol in petrolatum (40% w/w PG)
5	Body lotion with 0.3% w/w of DRF extract
6	Hand cream with 0.3% w/w of DRF extract
7	1% dilution, Liquid soap with 0.05% w/w of DRF extract
8	Body lotion base
9	Hand cream base
10	1% dilution, Liquid soap base

Table 2. Evaluation criteria of the International Contact Dermatitis Research Group (ICDRG) for the signs of allergic reactions[7]

Symbol	Morphology	Assessment
–	No reaction	Negative reaction
?+	Faint erythema only	Doubtful reaction
+	Erythema, infiltration, possibly papules	Weak positive reaction
++	Erythema, infiltration, papules, vesicles	Strong positive reaction
+++	Intense erythema, infiltration, coalescing vesicles	Extreme positive reaction
IR	Various morphologies, *e.g.*, soap effect, bulla, necrosis	Irritant reaction

Table 3. Evaluation criteria of the Cosmetic, Toiletry and Fragrance Association (CTFA) for skin irritant reactions[8]

Symbol	Grade	Clinical description
–	0	Negative reaction
+	1	Slight erythema, either spotty or diffuse
++	2	Moderate uniform erythema
+++	3	Intense erythema with edema
++++	4	Intense erythema with edema and vesicles

Table 4. Primary irritation index for cosmetic products by human patch test[9]

Range of z-score	Severity of skin irritant
$0.0 \leq z < 0.5$	Slight
$0.5 \leq z < 2.0$	Mild
$2.0 \leq z < 3.0$	Moderate
$3.0 \leq z$	Severe

3. Results

The standardized DRF extract was prepared and subjected to HPLC analysis. HPLC chromatogram is shown in Fig.1. It was found that DRF extract had a rutin content of 1.88 ± 0.04% w/w (average ± SD, triplicate analysis) which conformed to the specification.

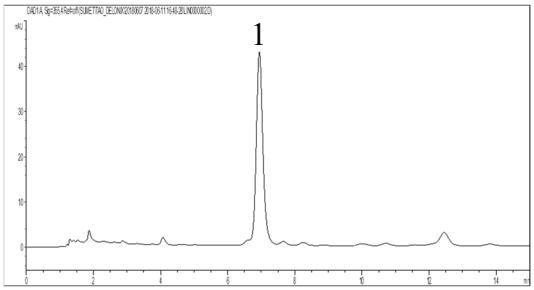

Fig. 1. HPLC chromatogram of DRF extract detected at 355 nm. Peak identification: (1) rutin

Representative cosmetic products containing DRF extract, i.e., body lotion, hand cream, and liquid soap were prepared. The final dose of DRF extract in body lotion and hand cream was 0.3% w/w while the dose in liquid soap was 0.05% w/w. Corresponding lotion base, hand cream base, and liquid soap base were prepared using the same ingredients but without DRF extract which served as a vehicle control.

All 30 healthy subjects were enrolled in this study. One subject developed an erythema with all test materials as well as a pure white petrolatum (negative control) at 48 h exposure. This erythema may have resulted from the skin occlusion itself but not from the test materials. This subject was excluded, and the remaining 29 subjects (14 males and 15 females) were further evaluated for skin reaction. The mean age of the volunteers was 31.03±6.54 years (range 20-47 years). The skin reactions, including allergy and irritation after applying the patch for 48 h are summarized in Tables 5 and 6, respectively. The results showed that pure white petrolatum (negative control) and 40% w/w PG (vehicle control for DRF extract) did not cause skin allergy or irritant reactions in all subjects. The allergic responses to DRF extract at both concentrations (2% and 4% w/w) that occurred at 48 h in two subjects were unclear (grade ?+). One subject developed a weak positive allergic reaction (grade +) to only 4% w/w DRF extract at 72 h. No skin irritation reaction was observed in any subject at all concentrations of the DRF extract.

The finished cosmetic products containing DRF extract under investigation

in this study included body lotion, hand cream, and liquid soap. The corresponding base without DRF extract was used as a vehicle control for each cosmetic product. Skin allergic reactions at 48 h of exposure to the bases were found in 11, 7, and 13 subjects for body lotion base, hand cream base, and liquid soap base, respectively. All disappeared or were reduced in severity at 72 h after exposure. On the other hand, all finished cosmetic products containing DRF extract, except liquid soap, caused a relatively lower incidence of skin allergic reactions compared with their bases. The most common skin allergic reactions at 72 h of exposure were caused by liquid soap containing DRF extract (13 subjects), body lotion base (9 subjects), and liquid soap base (7 subjects) (Table 5). All finished cosmetic products containing DRF extract and their corresponding bases, except liquid soap containing DRF extract, did not cause skin irritation reactions in any subject at 48 h of exposure (Table 6). Slight erythema, either spotty or diffuse, occurred in 2 subjects after exposure to liquid soap containing DRF extract. As shown in Table 7, the z-score of the liquid soap-containing DRF extract was 3.08 and its primary irritation index was classified as a severe skin irritant. Nevertheless, this z-score was less than the safety zone of soap products (3.98)[9], suggesting the safety of this cosmetic product.

Table 5. Number of volunteers and classification of the level of skin allergic reactions to test materials at 48 and 72 h after patch application

Test materials	Number of volunteers											
	48 h						72 h					
	Total	?+	+	++	+++	IR	Total	?+	+	++	+++	IR
Pure white petrolatum	0	0	0	0	0	0	0	0	0	0	0	0
2% w/w DRF extract	2	2	0	0	0	0	0	0	0	0	0	0
4% w/w DRF extract	2	2	0	0	0	0	1	0	1	0	0	0
40% w/w PG	0	0	0	0	0	0	0	0	0	0	0	0
Body lotion with 0.3% w/w DRF extract	1	1	0	0	0	0	0	0	0	0	0	0
Hand cream with 0.3% w/w DRF extract	4	4	0	0	0	0	1	0	1	0	0	0
Liquid soap with 0.05 % w/w DRF extract	12	9	3	0	0	0	13	9	4	0	0	0
Body lotion base	11	9	2	0	0	0	9	6	3	0	0	0
Hand cream base	7	6	1	0	0	0	3	3	0	0	0	0
Liquid soap base	13	10	3	0	0	0	7	6	1	0	0	0

Table 6. Number of volunteers and classification of the level of skin irritation reactions to test materials at 48 h after patch application

Test materials	Number of volunteers				
	–	+	++	+++	++++
Pure white petrolatum	29	0	0	0	0
2% w/w DRF extract	29	0	0	0	0
4% w/w DRF extract	29	0	0	0	0
40% w/w PG	29	0	0	0	0
Body lotion with 0.3% w/w DRF extract	29	0	0	0	0
Hand cream with 0.3% w/w DRF extract	29	0	0	0	0
Liquid soap with 0.05% w/w DRF extract	27	2	0	0	0
Body lotion base	29	0	0	0	0
Hand cream base	29	0	0	0	0
Liquid soap base	29	0	0	0	0

Table 7. Skin irritation assessment based on z-score of the cosmetic products containing DRF extract and their corresponding bases in comparison with the safety zone[9]

Cosmetic product category	Test materials	Z-score	Severity of irritation	Safety zone
Leave-on	Body lotion with 0.3% w/w DRF extract	0.00	Slight	0.94
	Hand cream with 0.3% w/w DRF extract	0.00	Slight	0.94
Leave-on	Body lotion base	0.00	Slight	0.94
	Hand cream base	0.00	Slight	0.94
Wash-off	Liquid soap with 0.05% w/w DRF extract	3.08	Severe	3.98
	Liquid soap base	0.00	Slight	3.98

4. Discussion

Medicinal plants have been used in Thailand from the past to the present, and nowadays they are popular to be developed into products in various forms such as medicines, dietary supplements, cosmeceuticals, and cosmetics. Consumer behavior is increasingly turning to natural products to avoid the side effects of synthetic chemicals. Many plants in nature are rich in compounds that can fight free radicals and inhibit the formation of melanin pigment effectively. *D. regia* plant contains polyphenolic compounds such as flavonols, anthocyanins, and phenolic acids as bioactive secondary metabolites that are responsible for their antioxidant activity.[1]

The antioxidant activity of 70% ethanolic DRF extract has been found in terms of the total phenolic content of 34.44 mg catechol equivalent/ g extract and the flavonoidal content of 30. 45 mg quercetin equivalent/g extract.[10] Vivek *et al.* reported the antioxidant activity of DRF extract with an IC_{50} of 24. 88 µg/ mL by using ABTS radical scavenging method.[11] We also tested for the antioxidant activity of our crude ethanolic DRF extract using DPPH and ABTS methods. The IC_{50} values were found to be 4.70 and 13.73 µg/mL, respectively.

There has been no evidence for the traditional use of DRF extracts as cosmetics, food, or drug in humans. The present study is the first to evaluate the safety profiles (skin allergic and irritant reactions) of DRF and the skin products containing DRF in human subjects. Human single closed patch test under occlusion was used to evaluate the

skin allergic and irritation reactions of DRF cosmetic ingredients and its finished cosmetic products. This method could define a threshold concentration for skin irritation reactions to prevent unacceptable severe reactions.[3,4]

Patch testing is a biological provocation test that has evolved into a standardized way of investigating type IV hypersensitivity reactions. Variability in testing results could be due to the test system, test material, biological or functional status of the volunteer being tested, and the person evaluating the results.[12] High variation in skin reactivity in the grading system ranging from negative reaction (−) to 3+/4+ is due to intra-[13] and inter-[14] individual variability, as well as other environmental factors in human skin responses such as seasonal variation.[15] High incidence of skin irritations to test chemicals has been reported in male compared with female subjects. In addition, the severity of skin irritation has been reported in old-aged (56-74 years of age) compared with young-aged subjects.[16]

The standardized DRF extract was tested at concentrations of 4% and 2% w/w based on dry weight (containing rutin 0.072 and 0.036% w/w, respectively). The highest tested concentration was obtained according to the physical stability of the finished cosmetic formulation. Optimized doses in the finished cosmetic products were obtained from the physical appearance, viscosity, compatibility, customer satisfaction, and product stability during the formulation development trials. The final dose of DRF extract in body lotion and hand cream was 0.3% w/w while the dose in liquid soap was 0.05% w/w.

DRF extract and its finished cosmetic products caused only doubtful and/or weak skin allergic reactions in healthy subjects during a period of 72 h of application. The highest concentration of DRF extract (4% w/w) caused a weak allergy in one subject (1/29, 3.45%). The incidence rates of subjects with doubtful and/ or weak allergy to the cosmetic products containing DRF extract and their corresponding bases were as follows: liquid soap with DRF extract (13/ 29, 44.83%) > body lotion base (9/29, 31.03%) > liquid soap base (7/29, 24.14%) > hand cream base (3/29, 10.34%) > hand cream with DRF extract (1/29, 3.45%). No allergic reaction was observed with body lotion containing DRF extract. According to ICDRG, contact dermatitis is an inflammatory skin reaction caused by direct contact with hazardous agents and its mechanism may involve allergy, and/ or irritation. [7] This allergic reaction is a type IV hypersensitivity. It affects T cell-mediated immune responses against an offending chemical or antigen that contacts the skin.[17] This causes the skin to release pro-inflammatory cytokines resulting in a localized inflammation response. Anti-inflammatory agents that can reduce itchiness and inflammation are used for treatment of allergic contact dermatitis such as topical steroids. Interestingly, the leave-on cosmetic products (body lotion and hand cream) containing DRF extract caused apparently lower incidence of allergic reactions than their corresponding bases. This could be explained by the anti-inflammatory activity of DRF extract. Significant anti-inflammatory activity was reported in carrageenan-induced paw edema model in rats following oral administration of 70% ethanolic DRF extract at a dose of 300 mg/ kg body weight. [18] Furthermore, in carrageenan-induced paw edema and formalin-induced paw edema models in mice, more potent anti-inflammatory activity was also reported with the methanolic extract of DRF at a dose of 400 mg/kg body weight given orally for seven consecutive days, compared with the aqueous extract of DRF and a control.[19] The potency of anti-inflammatory activity of the methanolic extract was comparable to diclofenac sodium (positive control). There is evidence showing that some non-steroidal anti-inflammatory drugs (NSAIDs) potentially cause skin adverse events including rash and pruritus, which may involve both immunological and non-immunological mechanisms.[20] These skin hypersensitivity reactions have been

associated with the inhibitory effects of NSAIDs on cyclooxygenase-1 (COX-1) enzyme.[21] As several mediators and signaling molecules are involved in inflammation and skin hypersensitivity reactions, the anti-inflammatory activity of the DRF extract could at least in part contribute to a lower risk of allergic reactions. Further studies are needed to investigate the molecular mechanism on anti-inflammatory activity of the DRF extract. Skin irritation was not observed in all subjects after applying DRF extract and the finished cosmetic products for 48 h. An exception was liquid soap containing DRF extract: its primary irritation index was classified as a severe skin irritation in two subjects (2/29 or 6.90%). It is noted that the wash-off products are expected to cause higher incidence and severity of skin irritation than leave-on products. This is a limitation of the occlusive patch test for the wash-off products such as facial cleansers, shampoo, and soap. Other tests such as the controlled use test may be more appropriate than the occlusive patch test for evaluation of skin reactivity of the wash-off products.[22] According to the primary irritation index and safety zone of cosmetic products by analysis of skin patch tests in 7440 Korean women over 12 years,[9] the primary irritation index of the liquid soap containing DRF extract (3.08) was less than the safety zone of soap products (3.98). This indicates that the liquid soap containing DRF extract was safe for human skin. The efficacy of the cosmetic products containing DRF extract such as moisturizing and anti-wrinkle properties is being further studied.

5. Conclusion

The DRF extract and its finished cosmetic products caused only doubtful and/or weak skin allergy but no skin irritation in healthy volunteers. Therefore, the DRF extract could be used as a safe cosmetic ingredient at a concentration threshold of up to 4% w/w. The finished cosmetic products containing DRF extract up to 4% w/w may have a potential for application as safe topical cosmetics for human skin.

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