ACUTE DERMAL AND OCULAR IRRITATION TESTING OF GRAPEFRUIT SEED EXTRACT IN NEW ZEALAND WHITE RABBITS

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ABSTRACT

We investigated the acute dermal and ocular irritation potentials of grapefruit seed extract (GSE) in New Zealand white rabbits. In the dermal irritation test, erythema, eschar, and edema formation were observed after 24 h of GSE treatment, and the skin returned to normal after 72 h. The dermal primary irritation index of GSE was 0.25; therefore, it was classified as a mild irritant. In the ocular irritation test, there were no clinical signs related to the application of GSE; therefore, it was classified as a non-irritant according to the Draize scoring system. These results suggest that GSE is mildly or not irritating to the skin and eye, indicating that it can be used in cosmetic and pharmaceutical applications with no serious toxic effects at moderate doses.

Key words: Grapefruit seed extract (GSE), Dermal irritation test, Ocular irritation test.

INTRODUCTION

Grapefruit (*Citrus paradisi Macf*. Rutaceae) seed extract (GSE) has been reported to be a natural antimicrobial agent due to the presence of citric acid, naringin, limonoid, kaempferol, quercetin, and other compounds (Cvetnić and Vladimir-Knežević 2004; Jang *et al*., 2011). GSE has been found to exert effective broad-spectrum antimicrobial activity against *Escherichia coli*, *Salmonella*, *Candida*, other fungi (Krajewska-Kulak *et al*., 2003), and skin and oral pathogens (Ha *et al*., 2009; Lee *et al*., 2009). Therefore, GSE has been investigated for its efficacy as a sanitizer in hand wash, soap, and dentifrice to reduce populations of human bacterial pathogens (Ha *et al.*, 2009; Lee *et al.*, 2009).

GSE also has been used to preserve food and beverages such as fruit, including strawberries (Jang *et al*., 2011) and grapes (Xu *et al*., 2007), vegetables (Xu *et al*., 2007; Lee *et al*., 1998), fishery products (Cho *et al*., 1990b; Corbo *et al*., 2008); pork (Hong *et al*., 2009); chewing gum (Jin *et al*., 2003) and Korean rice wine Makgeolli (Choi *et al*., 2014). Furthermore, the effects of GSE have been examined in dermal and pharmaceutical products (Kelly and Grotkin 2014).

GSE is regarded as environmentally safe without toxicity to humans or animals at effective concentrations (Heggers *et al*., 2002; Ionescu *et al*., 1991). However, little informations regarding its *in vivo* dermal and eye irritation activity as an antifungal and antimicrobial agent are available for dermal and pharmaceutical formulations (Kelly and Grotkin 2014). Therefore, we examined the potential dermal toxicological and ocular irritation effects of GSE in New Zealand white (NZW) rabbits.

MATERIALS AND METHODS

Preparation of reagents: GSE (DF-100) was purchased from Chemie Research and Manufacturing Co., Inc. (Casselberry, FL, USA; glycerin, 30%; naringin, 2.19%). All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) as high-performance liquid chromatography-grade reagents. All solutions were prepared using ultra-pure deionized water (Millipore, Billerica, MA, USA).

Acute dermal toxicity test of GSE: Six specific-pathogen-free NZW rabbits (body weight, 2.16–2.45 kg) were purchased from Samtako Bio Korea Ltd. (Osan, South Korea). The rabbits were housed individually in stainless steel cages (width, 405 mm; length, 605 mm; height, 365 mm) and acclimated to an environmentally controlled room with a temperature of 22 ± 1°C, relative humidity of 60 ± 5%, ventilation of 10–20 air changes/h, light level of 150–300 Lux, and a 12-h light/dark cycle. The animals were provided water and laboratory rabbit pellet food (Agribrands Purina Korea, Seongnam, South Korea) *ad libitum*. The rabbit dermal toxicity test for GSE was approved by the Institutional Animal Care and Use Committee (IACUC) of Silla University (Busan, South Korea; no. SUACUC-2016-011). The application of GSE to intact and abraded skin at treatment and control sites was performed according to the methods of Draize *et al*., (1944) and Choi *et al*., (2015). The day of application was designated Day 0. The rabbits underwent

one 24-h GSE treatment. The amount of GSE was set at 0.5 mL/square (2.5 x 2.5 cm^2) (Draize *et al*., 1944; Choi *et al*., 2015). Clinical signs were observed at least once per day throughout the experimental period. Body weight was measured on the day the animals were received and on Days 0, 1, and 3. The patches were removed 24 h after treatment and any remaining GSE was washed away using a physiological saline solution. Macroscopic grading of erythema, edema, bleeding, and eschar formation was performed 30 min after removing the patches and 72 h after the initial application. Edema was evaluated using the grading criteria of Draize *et al*., (1944). The erythema/eschar and edema scores at 24 and 72 h were added and divided by six (the number of animals), and the sum of these means was divided by four, the product of the number of application sites and the number of observations used to calculate the primary irritation index (PII). The dermal irritation potential was evaluated using the PII, and the irritancy of GSE was classified as follows: non-irritant (0.0–0.5), mild (0.6–2.0), moderate (2.1–5.0), and severe (5.1–8.0). In addition, any clinical signs observed in the animals after GSE treatment were considered (OECD 1981).

Ocular toxicity test of GSE: Nine specific-pathogen-free NZW rabbits (body weight, 2.29–2.56 kg) were purchased from Samtako Bio Korea Ltd. (Osan, South Korea). The rabbits were bred as described previously. The ocular toxicity test for GSE was approved by the IACUC of Silla University (Busan, South Korea; no. SUACUC-2016-010). The animals were divided into two groups: an eye-washed group (n = 3) and an unwashed group (n = 6). The animals' eyes were examined 24 h before GSE application, and all animals' eye were normal. The amount of GSE was set to one application of 0.1 mL/eye (Draize *et al*., 1944). The application was performed according to the methods of Draize *et al*., (1944) and Choi *et al*., (2015). Clinical signs were observed at least once per day throughout the experimental period. The day of application was designated Day 0. Body weight was measured on Days 0, 1, 4, and 7. The eyes, the area around the eyes and eyeballs, and the behavior of the animals were observed on Days 1, 2, 3, 4, and 7. The findings were graded using the Draize scoring system (Draize *et al*., 1944). The observations were evaluated based on the severity of the ocular response, and the irritation potential of GSE was classified based on the index of acute ocular irritation (IAOI), mean index of ocular irritation (MIOI), and the individual ocular irritation index (IOII) on Day 7.

RESULTS

Acute dermal irritation due to GSE: There were no significant changes in mortality, clinical signs, or body weight during the experimental period (data not shown). At the intact skin GSE treatment sites of the six animals, very slight erythema (score = 1 in three animals) was observed after 24 h of treatment. In all abraded skin, very slight erythema (score = 1 in three animals) was observed after 24 h of treatment. The very slight erythema observed at 24h and disappeared at 48h in all skin sites (data not shown). No edema was observed in the abraded or intact skin at the treatment sites of any animals after 24 h. After 72 h, no erythema, eschar, or edema was observed in the abraded and intact skin of the treatment sites of any animals. The PII of GSE was determined to be 0.25; therefore, it was classified as a mild irritant in this acute dermal irritation test. At the control site, no erythema, eschar, or edema was observed in any animal after 24 and 72 h (Fig. 1, Table 1).

Ocular irritation due to GSE: There were no significant changes in mortality, clinical signs, or body weight during the experimental period (data not shown). In the unwashed group, redness was observed in the conjunctiva of all six animals after 24 h of treatment. In three animals, redness lasted 48 h, and in two animals it lasted 72 h. After 4 days, no redness was observed in any animal of the unwashed group. In the eye-washed group, redness was observed in all three animals after 24 h of treatment. In one animal, redness lasted for 48 h. After 72 h, no redness was observed in any animals of the eye-washed group. No cornea, iris, edema, or lacrima except redness was observed in the unwashed and eye-washed groups. The IOII and the MIOI were 0 in the eye-washed group and unwashed group after 7 days. Therefore, these results indicate that GSE is a non-irritant for eyes (Table 2).

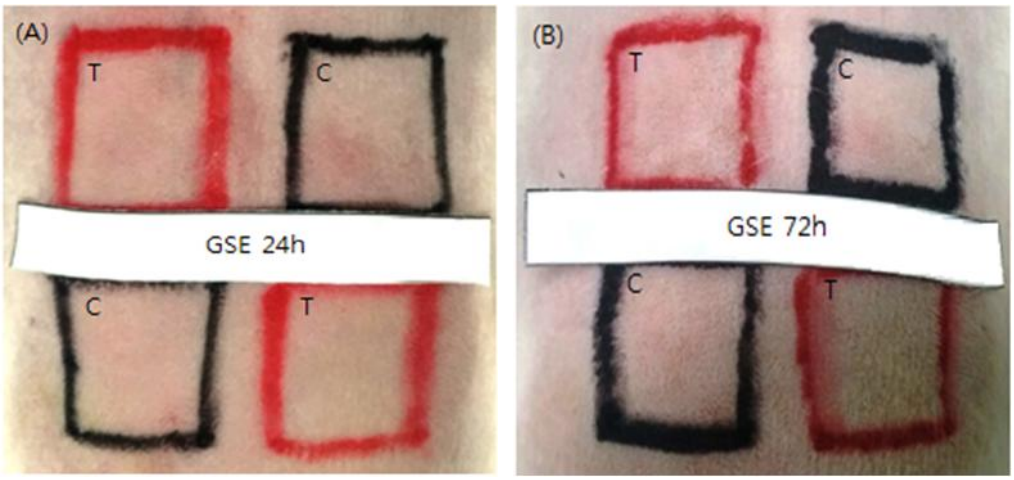

Fig. 1. Skin reaction after dermal administration of GSE (0.5 mL/square) on specific-pathogen-free New Zealand white rabbits. Each square is 2.5 x 2.5 cm^2. A: Skin reaction 24 h after treatment. B: Skin reaction 72 h after treatment, T: GSE-treated skin, C: control (distilled water) skin.

Table 1. Skin reaction grades and indices with grapefruit seed extract (GSE) application.

		Evaluation of irritation															
		Control								Treatment							
	Site	Intact				Abraded				Intact				Abraded			
ID	Response	Erythema (Es) [b]		Edema		Erythema (Es)		Edema		Erythema (Es)		Edema		Erythema (Es)		Edema	
	Time[a]	24	72	24	72	24	72	24	72	24	72	24	72	24	72	24	72
1		0	0	0	0	0	0	0	0	1	0 (Es)	0	0	1	0 (Es)	0	0
2		0	0	0	0	0	0	0	0	1	0 (Es)	0	0	1	0 (Es)	0	0
3		0	0	0	0	0	0	0	0	0	0 (Es)	0	0	0	0 (Es)	0	0
4		0	0	0	0	0	0	0	0	0	0 (Es)	0	0	0	0 (Es)	0	0
5		0	0	0	0	0	0	0	0	1	0 (Es)	0	0	1	0 (Es)	0	0
6		0	0	0	0	0	0	0	0	0	0 (Es)	0	0	0	0 (Es)	0	0
Total		0	0	0	0	0	0	0	0	3	0	0	0	3	0	0	0
Mean		0	0	0	0	0	0	0	0	0.5	0	0	0	0.5	0	0	0
Sum of Means		0								1.0							
PII [c]		0								0.25							

[a] Hours after treatment, [b] (Es): Eschar formation, [c] Primary Irritation Index (PII) = Sum/4.

Table 2. Evaluation of ocular irritation with grapefruit seed extract (GSE) application.

Group			None washed						Washed		
Animal number			1	2	3	4	5	6	1	2	3
Cornea	Degree of opacity (A)	1-4 days	0	0	0	0	0	0	0	0	0
		7 day	0	0	0	0	0	0	0	0	0
	Diffuse areas of opacity (B)	1-4 days	0	0	0	0	0	0	0	0	0
		7 day	0	0	0	0	0	0	0	0	0
Iris (C)		1-4 days	0	0	0	0	0	0	0	0	0
		7 day	0	0	0	0	0	0	0	0	0
Conjunctiva	Redness (D)	1 day	2	1	1	2	1	1	0	1	1
		2 day	1	1	0	1	0	0	0	1	0
		3 day	1	0	0	1	0	0	0	0	0
		4 day	0	0	0	0	0	0	0	0	0
		7 day	0	0	0	0	0	0	0	0	0
	Edema (E)	1-4 days	0	0	0	0	0	0	0	0	0

	7 day	0	0	0	0	0	0	0	0	0
Lacrima (F)	1-4 days	0	0	0	0	0	0	0	0	0
	7 day	0	0	0	0	0	0	0	0	0
IIOI	1 day	4	2	2	4	2	2	0	2	2
	2 day	2	2	0	2	0	0	0	2	0
	3 day	2	0	0	2	0	0	0	0	0
	4 day	0	0	0	0	0	0	0	0	0
	7 day	0	0	0	0	0	0	0	0	0
MIOI	1 day				2.67				1.33	
	2 day				1.00				0.67	
	3 day				0.67				0.00	
	4 day				0.00				0.00	
	7 day				0.00				0.00	

IIOI, individual index of ocular irritation = (A × B × 5) + (C × 5) + (D + E + F) × 2
MIOI, mean index of ocular irritation = IIOI / number of animals used

DISCUSSION

GSE is a commercial product derived from the seeds and pulp of grapefruit that is an effective broad-spectrum bactericide (Cho *et al*., 1990a; Choi *et al*., 2000; Reagor *et al*., 2002; Cvetnić *et al*., 2004), and fungicide (Heggers *et al*., 2002). In addition, it is environmentally safe with no toxicity to humans or animals at effective concentrations (Ionescu *et al*., 1991; Heggers *et al*., 2002). Recently, GSE has been added to cosmetic formulations to impart antifungal and other antimicrobial properties (Kelly and Grotkin 2014).

In previous research (Ko *et al*., 1995), the effects of GSE manufactured in the laboratory using Brazilian grapefruit seeds on lethality and primary skin irritation, as well as the acute eye irritation potential, were evaluated in both rats and rabbits. According to the results, the acute oral LD_{50} of GSE for Sprague-Dawley rats is 3.75 g/kg with 95% confidence limits of 2.55 and 5.52 g/kg. A patch application of GSE to intact and abraded skin of male white rabbits led to mild to moderate erythema and no or mild edema. Application to the eyes of rabbits led to severe corneal opacity, iritis, conjunctival erythema, edema, and discharge. These results were indicative of mild skin irritation and severe eye irritation.

Major constituents of GSE (DF-100) include naringin, limonoid, kaempferol, quercetin, citric acid, and other compounds (Cvetnić and Vladimir-Knežević 2004; Jang *et al*., 2011). Plants generally exhibit variation in their cellular chemical composition and biological activity according to season, habitat, and life stage (Larcher 2003). For example, the naringin contents of commercially available GSE from Chemie Research and Manufacturing Co., Inc. (Casselberry, FL, USA; naringin, 1.97%) and Quinabra - Quimica Natural Brasileira Ltda. (Eldorado, Brazil; naringin, 0.48%) differ based on the source of the grapefruit (Choi *et al*., 2014).

In this study, after 72 h of GSE treatment, no erythema, eschar, or edema was observed in the abraded or intact skin of the treatment sites of all animals tested (Fig. 1), and the dermal PII of GSE was 0.25; therefore, it was classified as a mild irritant (Table 1). According to previous research (Ko *et al*., 1995), 7-day GSE application to intact and abraded skin of male white rabbits led to mild to moderate erythema and no or mild edema. Regarding ocular irritation, we determined the IAOI of GSE at Day 7 to be 0, classifying it as a non-irritant according to the Draize scoring system (Draize *et al*., 1944) (Table 2). However, Ko *et al*., (1995) observed severe corneal opacity, iritis, conjunctival erythema, edema, and discharge after 13 days, revealing mild skin irritation and severe eye irritation. Comparing these acute dermal and ocular irritation test results of GSE, the GSE used in this study (purchased from Chemie Research and Manufacturing Co.) and the GSE used by Ko *et al*. (1995) (manufactured in the laboratory using Brazilian grapefruit seed) exhibited very different degrees of dermal and ocular irritation, with lower dermal and ocular irritation levels observed in this study than by Ko *et al*., (1995). This may have been due to differences in the manufacturing process of GSE or natural variations in the chemical composition of the grapefruit seeds used (Larcher 2003).

In a study by Djerrou *et al*. (2013) the repeated administration of mastic gum (*Pistacia lentiscus*) for 4 weeks initially increased the edema and erythematic reaction, however, these symptoms resumed to normal before the end of the experiment. Mastic gum is currently considered as safe and is used as a raw material in toothpaste, gum, cosmetics, lotions, and perfumes. According to Lee *et al.* (2012), red ginseng which is extensively used as an ingredient in cosmetics and food industry, causes erythema in intact skin during a skin sensitization test.

The skin penetrating medicinal products do not cause skin irritation reactions. However, skin cosmetic or related food products could be associated with some skin irritation problems. If such reactions are reversible, the materials are considered to be safe to some extent. The

majority of skin cosmetics or related food products cause irritation to the eye; therefore, their safety depends on the reversibility of the reactions. As the skin irritation triggered by red ginseng or mastic gum disintegrate over time (Djerrou *et al*., 2013; Lee *et al*. 2012), we propose that GSE is not harmful to the eye as well as skin.

It has been suggested that GSE may be incorporated into dermal and pharmaceutical formulations to confer antimicrobial activity to prevent product spoilage and aid in the preservation of dermal and pharmaceutical formulations (Kelly and Grotkin 2014). Since the irritation level of GSE may differ depending on the content of its major compounds, dermal and ocular irritation testing may be necessary before incorporating GSE into dermal and pharmaceutical formulations.

In conclusion, the sensitization level of GSE used in this study in rabbits was classified as moderate for skin and non-irritating for eye. These dermal and ocular sensitization results indicate that GSE does not irritate the skin and eye, suggesting that it can be used in dermal and pharmaceutical applications with no serious toxic effects at moderate doses.

Conflict of interest: The authors declare no conflict of interest.

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