Physicochemical properties, *in vitro* release and skin permeation studies of a topical formulation of standardized pomegranate rind extract

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Abstract: The aim of the present study was to develop a stable formulation containing standardized pomegranate rind extracts (SPRE) for topical use in the treatment of dermal diseases. Ellagic acid (EA) as the major active constituent of SPRE (not less than 13%) was quantified by HPLC as an indicator for studies on the stability, *in vitro* drug release, and skin penetration/retention. The formulation prepared with polyethylene glycols (PEG 400 and PEG 4000) containing 5% SPRE has been found to be stable and provide a release rate of 36.6741±5.0072 µg/cm^2/h that was best fitted to the zero-order kinetic model. EA from SPRE did not penetrate the full-thickness rat skin but the skin retention of EA was determined to be 2.22±0.16 µg/cm^2 with a total recovery of 95.14±5.51%. The results indicated that this 5% SPRE PEG ointment was of satisfactory physicochemical properties and worth further *in vivo* investigations.

Keywords: *Punica granatum*, ellagic acid, topical preparation, stability, skin retention.

INTRODUCTION

Punica granatum. L. (Punicaceae), the pomegranate, has long been used as a remedy for a variety of ailments such as aphthae and ulcers in different cultures (Lansky and Newman, 2007). Among the numerous chemical compounds identified from pomegranate, the polyphenols, mainly ellagitannins, are associated with the medical merits of pomegranate (Ismail *et al*., 2012).

Recently, an antioxidant assay-guided purification procedure for extracting pomegranate peel has been established. Ellagic acid (EA) was found to be the major antioxidant of the standardized pomegranate rind extracts (SPRE) and thus was used as an indicator for the standardization and quality control of the extraction procedure (Panichayupakaranant *et al.,* 2010a). The content of EA in SPRE must be no less than 13%. The dry powder of SPRE is stable for at least two years when kept in a well-closed container and stored at room temperature (25°C) (Panichayupakaranant *et al.,* 2010b). *In vitro* tests of SPRE have revealed its strong antioxidant and anti-inflammatory activities (Panichayupakaranant *et al.,* 2010c). Based on the physicochemical properties of SPRE and its *in vitro* pharmacological effects, it would be profitable to study the topical application of SPRE as a therapy for cutaneous inflammation. In the present study, we aimed to develop a physically and chemically stable formulation of SPRE for the topical use in skin diseases. EA was employed as a marker to evaluate the properties of the prepared formulations for stability, *in vitro* drug release and skin penetration/retention.

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MATERIALS AND METHODS

Materials

Ripe pomegranate fruits were collected from Mengzi pomegranate garden, Yunnan, China, in August 2011 and were identified by Assoc. Prof. Dr. Pharkphoom Panichayupakaranant, (Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand), and deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The fruit peels were dried at 50°C for 24 h in a hot air oven then reduced to powder using a grinder and a no. 45 sieve. Standard ellagic acid (EA), lecithin and cholesterol were from Sigma-Aldrich (St. Louis, MO, USA). Pluronic F 127 was from BASF (Lugwidshafen, Germany). Other formulation ingredients were from P.C. Drug Center Co. Ltd. (Bangkok, Thailand) and were of pharmaceutical grade. Solvents for HPLC were from Labscan (Bangkok, Thailand). Other chemicals were of analytical grade and were from Ajax Finechem Pty Ltd. (Auckland, New Zealand). Solvents for extraction were of commercial grade and were obtained from local corporation.

Animals

Male Wistar rats with weight ranging from 220-250g were used. All animals were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Food and water were given *ad libitum*. All experimental protocols were approved by the Animal Ethics Committee, Prince of Songkla University (MOE 0521.11/173, Ref. 03/2012).

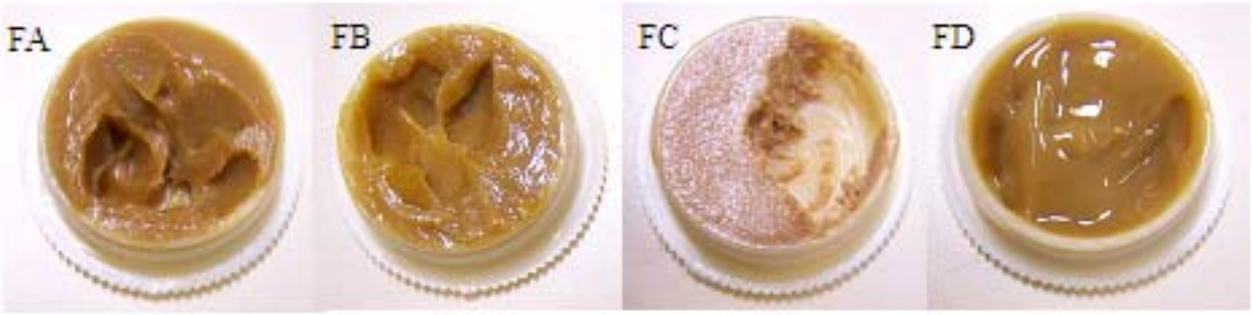

Fig. 1: Appearance of each formulation. FA: Absorption ointment, FB: Water-soluble ointment, FC: Hydrophilic ointment, FD: Pluronic lecithin organogel. All formulations contained 5% SPRE (w/w).

Methods

Preparation of SPRE

SPRE was prepared and standardized to contain 13% w/w EA by previously described method (Panichayupakaranant *et al*., 2010c). Briefly, the dried powder of pomegranate rind (0.5 kg) was extracted twice with 90 % methanol in water (v/v) (2 L) under reflux conditions for 1 h. The pooled extracts were dried *in vacuo*. The crude extract (10 g) was then suspended in 2% aqueous acetic acid and partitioned with ethyl acetate (400 ml × 4). The pooled ethyl acetate fractions were then evaporated to dry powder.

Quantitative analysis of EA

HPLC analysis was carried out using Agilent 1100 series equipped with Photodiode-array detector (PDA) and autosampler. Data were analyzed with Agilent 3D ChemStation software (Agilent, Santa Clara, CA, USA). Separation was achieved at 25°C on a 150 mm × 4.6 mm TSK-gel ODS-80Tm column. The mobile phase consisted of methanol and 2% aqueous acetic acid with gradient mode elution (0-15 min, 40-60% v/v methanol and 15-20 min, 60% v/v methanol) at a flow rate of 1 ml/min. The injection volume was 20 µL. The quantitation wavelength was set at 254 nm. The calibration curve was established with standard EA at concentrations between 3-50 µg/mL (Panichayupakaranant et al. 2010a). For determination of EA in the formulations, 1 g of formulation was diluted in 10 mL of methanol, except that the absorption ointment was diluted in 10 mL of chloroform. 1 mL of this dilution was diluted in 10 mL of HPLC methanol again. The dilution was centrifuged at 4°C, 4500 rpm for 10 minutes. The supernatant was filtered through 0.45 µm membrane filter and subjected to HPLC analysis.

Preparation of SPRE topical formulations

Test formulations

Five types of topical SPRE formulation with different water contents were prepared. The detailed compositions of blank bases of SPRE formulations are given in table 1.

Formulation A (FA): **Absorption ointment**. Stearyl alcohol, white wax, and cholesterol were melted in a suitable dish on a steam bath. The white petrolatum was added and heating was continued until a liquid melt was formed (80°C). Cool with stirring until congealed.

Formulation B (FB): **Water-soluble ointment** (polyethylene glycol ointment) The PEG 4000 was melted and the liquid PEG 400 was added. Cool with stirring until congealed.

Formulation C (FC): **Hydrophilic ointment**. Stearyl alcohol and white petrolatum were melted on steam bath and warmed to about 70°C. The water was heated to 75°C and the sodium lauryl sulfate, propylene glycol, and parabens were added. The aqueous phase was added and stirred until congealed.

Formulation D (FD): **Pluronic lecithin organogel (PLO Gel)**. Oil phase was prepared by mixing soy lecithin, propylene glycol and parabens in isopropyl palmitate. The mixture was kept overnight at room temperature (25°C) in order to dissolve constituents. Aqueous phase was prepared by dispersing Pluronic F-127 in cold water. The dispersion was stored in refrigerator overnight for effective dissolution of Pluronic F-127. The next day, the aqueous phase was slowly added into the oil phase with continuous stirring.

Formulation E (FE): **Cream**. The cetylstearyl alcohol, Cremophor A6, Cremophor A25 and liquid paraffin were melted in a suitable dish on a water bath and warmed to about 70°C. The water was heated to 75°C and the parabens and propylene glycol were added. The aqueous phase was added and stirred until congealed.

All blank bases were examined for their appearance and physical stability. For preparation of formulations, the SPRE amorphous powder (5% w/w) was levigated with the blank base and serially diluted using mortar and pestle.

Physical characterization of the formulations

General appearance: the general appearance was evaluated macroscopically. Parameters studied were

color, homogeneity, viscosity, spreadability and phase separation (Sanna *et al*., 2009).

Viscosity: Brookfield viscometer (RVT model, Middleboro, MA, USA) with RV-7 spindle rotating at a speed of 20 rpm was used to measure the viscosity of each formulation at 25±1°C.

Centrifugation assay: 2 g of each sample was weighed and centrifuged at 3000 rpm for 30 min (Marquele-Oliveira *et al*., 2007). The general appearance of each sample was inspected before and after centrifugation.

pH measurement: 1 g of each formulation except for the absorption ointment, which was water insoluble, was diluted in 10 mL of water and the pH value was measured with a pH meter (EZ Do 7011, Gondo Electronic Co. Ltd., Taiwan) in triplicates.

Short-term stability studies

Temperature cycle test

Samples were placed at -10°C for 24 h and then placed at 45°C for 24 h for 5 cycles. General appearance, pH, and EA content of each formulation were monitored before and after the test.

90-day stability test

Samples were stored at 25°C/AH (ambient humidity) and at 45°C/70% RH (relative humidity) for 90 days and were evaluated at 7, 14, 30, 60, 90 days after preparation for general appearance, viscosity, pH, and EA content.

In vitro drug release study

The study was performed as previously described (Tas *et al*., 2003). The amounts of EA released from different formulations through cellulose acetate dialysis membrane (Spectra/Por 3, Rancho Dominguez, CA, USA) were measured employing a modified Franz diffusion cell (Hanson Model 57-6, Hanson Research Corporation, CA, USA) with a diffusional area of 1.77 cm^2 and a receptor compartment volume of 10mL. A mixture of distilled water: methanol (1:1 v/v) was selected as the receptor fluid. The receptor compartment filled with degassed receptor fluid was stirred at a speed of 200 rpm by a magnetic stirrer (Variomag Telemodul 40S, Germany) and was thermo stated at 32±0.5°C. 1 g of each formulation was placed in the donor compartment and covered with cover glass and parafilm to prevent evaporation. At appropriate time intervals (1, 2, 3, 4, 6, 8, 10, 12 and 24 h), aliquots (500 µL) of the receiving solution was withdrawn, filtered through 0.45 µm membrane filter and subjected to HPLC analysis for EA content. The receiving compartment was replenished immediately with fresh solution. This dilution of the receiver content was taken into account when evaluating the penetration data. The cumulative amount of EA released into the receptor fluid was calculated with the following formula: $Q = V_t \cdot C_t + \sum_{i=0}^{t-1} V_s \cdot C_i$, where Q is the cumulative amount of released EA, V_t is the volume of the receptor fluid, C_t is the EA concentration at the last time of sampling, V_s is the volume of sample, and C_i is the concentration of EA at the i^{th} time of sampling. The experiment was carried out for four times and mean result was reported.

In vitro skin penetration and retention study

The protocol for the *in vitro* skin penetration study was similar to that for the *in vitro* release study except that the synthetic membrane was replaced by full-thickness abdominal skin of Wistar rat. Rats were sacrificed by overdose of pentobarbital. The epidermal hair was removed as close as possible to the skin using an electronic clipper. The skin was excised and the subcutaneous fat was carefully dissected with surgical scissors. Skin pieces were rinsed in isotonic phosphate buffered saline (IPB, pH 7.4), blotted dry with paper towel, wrapped in aluminum foil and stored at -20°C. The skin pieces were thawed and hydrated for 24 h in IPB before experiment. At the end of the in vitro penetration study, the retention, or accumulation of EA in the skin was determined. Briefly, the remaining formulation was removed from the skin surface. The skin was then washed in methanol for 30 seconds, dried with paper towel and cut into small pieces in a 2 mL centrifuge tube. 1 mL of methanol was added and the mixture was vortexed for 2 min, sonicated for 15 min, and centrifuged at 12000 rpm for 15 min. The supernatant was filtered and submitted to HPLC analysis for EA. The overall recovery was calculated as the sum of the amounts of EA in the receptor fluid (cumulative), the skin, and the remaining donor and the methanol wash of the skin.

STATISTICAL ANALYSIS

The data obtained were expressed as mean ±SD. Data of stability tests and skin retention were analyzed by t-test. Data of the *in vitro* drug release study were analyzed by one-way ANOVA followed by Tukey's post-hoc test. A significant difference was considered at $p<0.05$.

RESULTS

Stability studies

Physical properties

Five types of blank base were formed and all the bases were viscous, smooth and spread able (table 1). All blank bases were white except the blank base of FD was light yellow due to the presence of lecithin. The blank bases of FA, FB, FC and FD were compatible with SPRE and formed light brown colored, odorless, homogenous and very viscous ointments upon adding SPRE amorphous powder (fig. 1). The viscosities of FA, FB, FC, and FD all

exceeded 8,000,000 cP. In the case of FE, phase separation occurred when SPRE was added thus FE was not studied further. In the centrifuge tests, FA, FB, FC, and FD with 5% SPRE were stable and showed no phase separation, color change or viscosity decrease as inspected macroscopically. For solubility in water, FA is insoluble in water but FB is soluble in water (approximately 1 g/mL, 25°C). FC is oil in water ointment base that insoluble in water but water can be incorporated to the base. FD is insoluble in water but water can be incorporated to the base. FE is hydrophilic cream that insoluble in water but water can be incorporated to the base.

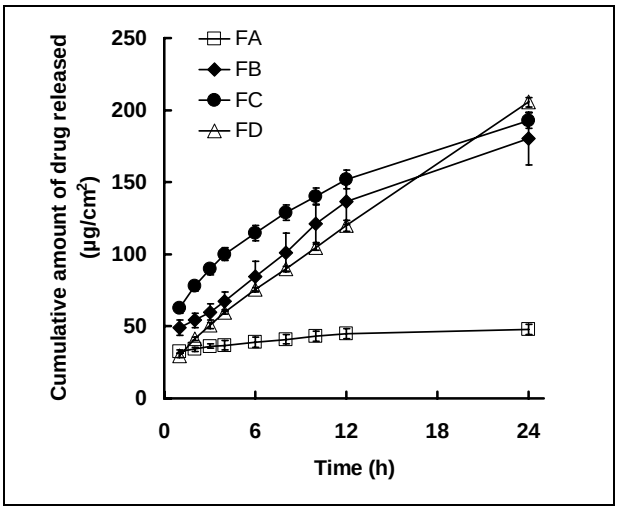

Fig. 2: Drug release profile of the tested formulations. Data are expressed as mean±SD (n=4). EA was quantified at each time point for all tested formulations by HPLC as the indicator of chemical stability of the formulation.

Temperature cycle test

In this test, the physical properties of FA, FB, FC, and FD were stable, showing no phase separation, color change or viscosity decrease. The pH of FB, FC and FD did not change (table 2). However, FB, FC and FD darkened in color after the temperature cycles. When EA was quantified by HPLC as the marker compound to evaluate the chemical stability of each formulation (table 3), FC and FD showed a decrease in EA content after the temperature cycles so these two formulations were withdrawn from the 90 days storage test.

90 days storage test

FA and FB, which were stable in the temperature cycle test, were stored at 25°C /AH and at 45°C /70% RH for 90 days. The pH of FB was stable (table 4) under both conditions during 90 days, but color darkening of FB was observed from the first inspection day (day 7) when stored at 45°C /70% RH. As for the chemical stability of FA and FB, the EA content in both FA and FB did not change during 90 days under both conditions (table 5).

In vitro drug release study of the tested formulations

All formulations were subjected to *in vitro* drug release study. EA was used as an indicator for the drug release profile of the formulations (fig. 2). The drug release profile of each formulation was unique. The cumulative amount of EA (µg/cm^2) released from each tested formulation at the end of the 24 h period was in the following order: FD (205.47±3.08 µg/cm^2)>FC (192.87±5.32 µg/cm^2)>FB (180.33±18.53 µg/cm^2)>FA (47.97±3.65 µg/cm^2) (fig. 3). Three kinetic models were applied to the drug release data of the 0-12 hour period to obtain the best fit curve and kinetic parameters. These kinetic models were as follows:

a. Zero-order kinetics: $Q=k_0t + b_0$

b. First-order kinetics: $\ln Q=k_1t + b_1$

c. Higuchi model: $Q=k_h\sqrt{t}$

Q is the cumulative amount of EA released per unit area of synthetic membrane in time, t is time and k_0, k_1, or k_h are the estimated release rate of the zero-order kinetic model, the first-order model, and Higuchi model respectively. The zero-order model means the drug release rate is independent of its concentration in the formulation (Najib and Suleiman, 1985), while in the first-order model the drug release rate depends on the drug concentration (Desai *et al.*, 1966). The Higuchi model describes systems in which base matrix dose not interact with reception medium and drug release rate is independent of its concentration (Higuchi, 1963). The *in vitro* drug release data of FA and FC followed Higuchi model while FB and FD followed the zero-order model (table 6).

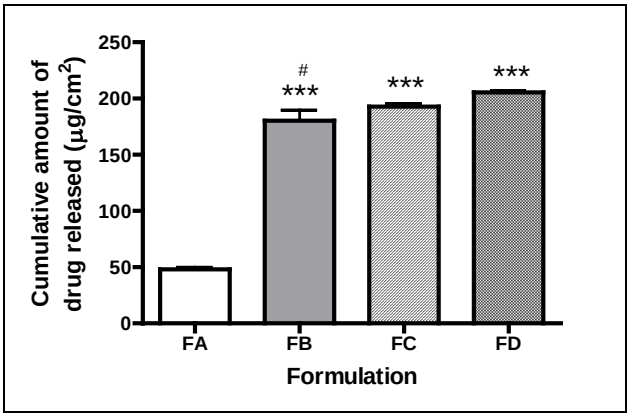

Fig. 3: Cumulative amount of EA released from the tested formulations at the end of the 24 h period. Data are expressed as mean ±SD (n=4). *** $p<0.001$ vs. FA, # $p<0.05$ vs. FD, one-way ANOVA followed by Tukey's post-hoc test.

In vitro skin permeation/retention study

Based on the results of stability and *in vitro* drug release studies, FB was selected for the *in vitro* skin permeation study. FB containing 0.65% pure EA, which was equivalent to its amount in 5% SPRE formulation, was prepared and studied in parallel with FB of 5% SPRE. At

the end of a period of 24 h, the amount of EA that permeated through the full-thickness rat abdominal skin into the reception medium was lower than the detection limit of the HPLC method (1 μg/mL). However, the skin retention of EA from both 5% SPRE formulation and 0.65% EA formulation were determined though no significant difference between these two formulations was observed (table 7).

Table 1: Compositions of blank bases of SPRE formulation

Component	Formulations (% w/w)				
	A	B	C	D	E
Propylene glycol	-	-	12	10	8
Lecithin	-	-	-	10	-
Cholesterol	3	-	-	-	-
Isopropyl palmitate	-	-	-	10	-
Pluronic F 127	-	-	-	14	-
Parabens*	-	-	0.2	0.2	0.2
Sodium lauryl sulfate	-	-	1	-	-
Stearyl alcohol	3	-	25	-	-
White petrolatum	86	-	25	-	-
Cetylstearyl alcohol	-	-	-	-	7
Cremophor A6	-	-	-	-	1.5
Cremophor A25	-	-	-	-	1.5
Liquid paraffin	-	-	-	-	12
White wax	8	-	-	-	-
Polyethylene glycol 4000	-	40	-	-	-
Polyethylene glycol 400	-	60	-	-	-
Distilled water	-	-	36.8	55.8	69.8

* Parabens consist of 10% methyparaben and 2% propylparaben in propylene glycol (w/v).

DISCUSSION

In the present study, different topical formulations of SPRE have been investigated. The 5% SPRE PEG ointment has been found to be stable and provide a good *in vitro* release profile.

Formulation bases of different water content were chosen since water content is an important factor that affects the physical properties and stability of a topical formulation. Another reason for choosing bases with different water content was the consideration of the solubility of SPRE. The solubility test for SPRE revealed that this mixture is soluble in methanol (35 mL/g, 25°C) and propylene glycol (80 mL/g, 25°C), but only slightly soluble in water (700 mL/g, 25°C) and is insoluble in hexane and chloroform (Issuriya, 2008; Jansuk, 2007). FA is an oily ointment containing no water. But the presence of cholesterol as surfactant in this formulation enables a small amount of water such as water soluble drug or exudate from inflamed skin to be incorporated. FB also contains no water but it is water soluble as it consists of hydrophilic polymers. FC is an o/w ointment of 37% water. FD and FE are o/w creams with increasing water contents (55.5% and 69.5%, respectively). FE failed to form stable formulation with SPRE, indicating that cream base of high water content was not suitable for SPRE. But FD formed a physically stable formulation with SPRE though it did have high water content. The reason may lie in the higher emulsifying effect of soy lecithin compared to Cremophor A6 and Cremophor A25.

Table 2: pH of tested formulations before and after temperature cycles

Formulation	pH	
	Before	After
FB	3.62±0.05	3.58±0.04
FC	3.27±0.03	3.23±0.05
FD	3.55±0.06	3.43±0.05

Data are expressed as mean±SD, n=3. No significant difference of pH values was observed for any formulation before and after this test (paired t-test, $p>0.05$).

Table 3: EA content of tested formulations before and after temperature cycles

Formulation	EA content (%)	
	Before	After
FA	100	102.1± .2
FB	100	101.9±1.6
FC	100	74.9±0.1 *
FD	100	88.8±3.3 *

Data are expressed as mean ±SD, n=3. The EA content before the test accounted for 100%. * $p<0.05$, paired t-test.

Table 4: pH of FB during 90 days storage at different temperature

Day	pH	
	25°C /AH	45°C /70% RH
0	3.57±0.02	
7	3.59±0.04	3.57±0.02
14	3.58±0.01	3.60±0.04
30	3.58±0.01	3.57±0.01
60	3.60±0.02	3.61±0.02
90	3.58±0.03	3.57±0.02

Data are expressed as mean ±SD (n=3). No significant difference of pH values was observed during 90 days for both conditions (paired t-test, $p>0.05$).

It is known that a herbal extract usually has many constituents, mostly uncharacterized, so it can be difficult to establish a marker compound to evaluate the consistency of the raw extract or the quality of the

Table 5: EA content of FA and FB during 90 days storage in different conditions

	EA content (%)			
Day	FA		FB	
	25°C /AH	45°C /70% RH	25°C /AH	45°C /70% RH
0	100.00		100.00	
7	102.98±5.87	102.08±3.79	97.12±3.04	101.87±0.82
14	102.10±1.69	102.21±1.53	100.19±8.60	105.09±2.54
30	96.81±1.31	99.00±2.62	98.25±0.75	100.11±0.76
60	101.16±0.44	101.29±1.03	101.95±0.64	103.00±1.55
90	103.53±5.56	103.66±2.46	98.87±0.53	102.05±0.77

Data are expressed as mean±SD (n=3). EA content did not change for FA and FB (paired t-test, $p>0.05$). The EA content on day 0 was designed as 100%.

Table 6: Kinetic parameters of drug release from tested formulations

Formulation	Zero-order model		First-order model		Higuchi model	
	k_0 (μg/cm^2/h)	r^2	k_1 (1/h)	r^2	k_h (μg/cm^2/h$^{1/2}$)	r^2
FA	1.1598±0.1608	0.9846	0.0299±0.0030	0.9727	5.6509±0.3904	0.9941
FB	8.2208±1.1125*	0.9950	0.0961±0.0040*	0.9931	36.6741±5.0072*	0.9534
FC	7.7771±0.3464*	0.9752	0.0740±0.0016*	0.9212	35.8848±1.5932*	0.9997
FD	8.0023±0.1831*	0.9959	0.1177±0.0033*	0.9348	36.3949±0.8033*	0.9918

Data are expressed as mean±SD (n=4), r^2 is the correlation coefficient, k_0, k_1, or k_h are the estimated release rates of the zero-order kinetic model, the first-order model, and Higuchi model respectively. * $p<0.05$ vs. FA, one-way ANOVA followed by Tukey's post-hoc test.

formulations developed (Marquele-Oliveira *et al*., 2007). In this study, the pomegranate rind extract was standardized to contain 13% w/w EA as its major active constituent (Panichayupakaranant *et al*., 2010c), thus EA is qualified to serve as an indicator for the chemical stability of the SPRE formulations. After five cycles of drastic temperature change, the EA content in the water containing formulations (FC and FD) decreased significantly. This means that application of the EA-rich SPRE in water containing bases such as cream and hydrogel should be carried out carefully. As it has been reported that the ester group of EA tends to be hydrolyzed to hexahydroxyphenic acid in aqueous, acid and base solution (Panichayupakaranant *et al*., 2010b), the instability of EA in aqueous formulations is probably due to its susceptibility to hydrolysis. However, the darkening of the SPRE formulations after temperature cycles was not attributable to EA decomposition because the darkening was also observed for FB when stored at 45°C/70% RH, in which EA was stable through 90 days. Oxidation of some chromogenic constituents in the extract is possibly responsible for the color change. The color of FB did not change at 25°C/AH through 90 days, thus high temperature may accelerate the oxidation, suggesting the necessity of keeping SPRE product in cool environment.

Drug release from formulation was thought to be controlled by the interaction between the drug molecules and the base components through ionic and hydrogen bonding (Ahmed *et al*., 2011). The finding that formulation with a faster drug release rate was characterized by high water content (FD) or hydrophilicity (FB) indicated that an interaction between SPRE and the hydrophilic groups such as hydroxyls which are abundant in PEG and Pluronic F-127 would facilitate the movement of drug molecules in formulation. This is supported by the good solubility of SPRE in polar solvent like propylene glycol and also by the result that *in vitro* drug release of FB, FD and FC followed the zero-order kinetic model and Higuchi model, respectively, which means drug release rates of these formulation do not depend on the drug concentrations but on the compositions of the formulations. It has been reported that the weak interaction, indicated by the poor solubility, between the drug and the oily base might serve as a driving force for faster drug release (Hashem *et al*., 2011). Therefore, considering the practical insolubility of SPRE in non-polar solvent such as chloroform (>10,000 mL/g, 25°C). FA (absorption ointment) was expected to provide a good drug release rate. However, our results showed that FA had the lowest drug release rate. Besides the driving force provided by the interaction between drug and base, another important factor affecting release rate is the diffusion of the drug in the base. The slow drug diffusion from the internal phase of the base due to the extraordinarily low solubility of a drug may retard the drug release because only the drug molecules around the interface of the base and reception medium would be released quickly (Gendy *et al*., 2002; Konur Hekimoglu *et al*., 1983). Although the rheological properties are believed to play an important role in the drug release from

semi-solid vehicles (Gendy *et al*., 2002; Tas *et al*., 2003), our results did not relate the drug release profile to the viscosity of the formulation as much, since all tested formulations had a viscosity that exceeded the testing range of the instrument.

Table 7: Skin retention and overall recovery of 5% SPRE and 0.65% EA formulations

Formulation	Retention (μg/cm^2)	Recovery (%)
5% SPRE FB	2.22±0.16	95.14±5.51
0.65% FB	2.38±0.22	94.91±5.24

Data are expressed as mean±SD (n=4). EA was quantified at the end of a period of 24 h for all tested formulations by HPLC as the indicator of skin permeation/retention.

Through the stability and *in vitro* drug release studies, only FB containing 5% SPRE presented both stability and satisfactory drug release rate so it was subjected to the *in vitro* skin penetration/retention study. EA was used again as an indicator for this study. It has been reported in a recent research that EA alone could penetrate into the epidermis of human skin but the percutaneous absorption of EA was not detected (Junyaprasert *et al*., 2012). In our previous study, a logarithm of the partition coefficient (Log P) of 2.44±0.374 (25°C), indicating high lipophilicity, has been determined for SPRE (Issuriya, 2008). Deduced from the Log P (> 2), SPRE would preferably localize in the lipophilic layers of the skin (the *stratum corneum*) and its partition into the more aqueous viable epidermis and dermis would be limited. As expected in this study, the permeation of SPRE into the receptor fluid was considerably low (under the detection limit). As it was questionable that whether the presence of many constituents other than the marker compound EA would influence the skin permeation of EA. FB with 0.65% pure EA was studied in parallel with FB with 5% SPRE. EA from either formulation was not detected in the receptor fluid. However, skin retentions of EA from both formulations were determined although there was no significant difference between these two formulations. The results thus indicated that EA from SPRE was able to penetrate into the full-thickness rat abdominal skin due to the lipophilicity of SPRE but the existence of many constituents in SPRE did not affect the percutaneous absorption of EA into the receptor fluid.

CONCLUSION

The results obtained from the present study demonstrated that a topical PEG ointment of SPRE has been successfully developed. This topical formulation of SPRE maintained its physical and chemical stability when stored at 25°C/AH for 90 days and showed the best release rate compared to other formulations. The *in vitro* skin permeation/retention study revealed that a considerable amount of EA from this formulation was accumulated in the rat skin. This formulation thus is worthy for further studies in *in vivo* models.

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