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Development and Evaluation of Topical Formulation Containing *Agrimonia pilosa* Extract

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Abstract: Natural products are promising drug candidates with various pharmacological effects. However, they can be difficult to use due to poor bioavailability or low stability. In this experiment, while developing topical formulations containing 0.1% *Agrimonia pilosa* extract, a simple and rapid method of analyzing flavonoid glucuronides, which are representative ingredients, was developed and validated, and the physicochemical properties and stability of flavonoid glucuronides were observed. As a result, an optimized cream formulation was developed. The oil phase comprised tween 60, liquid paraffin, propylene glycol, cetanol, stearyl alcohol, span 60, benzyl alcohol, and *A. pilosa* extracts, and the water phase comprised water and citric acid hydrate. Then, physicochemical and microbial stability tests of the formulation were conducted under long-term (12 months) and accelerated conditions (6 months). It was thus confirmed that both physicochemical and biological properties were stable during the test period. Consequently, an optimized formulation for the extract was developed and stability was confirmed.

Keywords: formulation stability; *Agrimonia pilosa*; cream; physicochemical; luteolin-7-O-glucuronide; flavonoid glucuronide

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1. Introduction

Natural products can be developed as medicines or used as cosmetics. And the target market is large with a high growth rate [1,2]. Natural products contain numerous ingredients that show various effects. Although, each of these components exhibits pharmacological effects, synergistic effects usually appear when they exist together in various components. However, it has been difficult to provide clarity on this point [3,4]. In ancient times, medicines were developed from natural products based on traditional knowledge. As separation, extraction, and formulation technologies have developed, the strategies for developing natural medicines have also developed systematically [5–11]. Many ingredients have shown excellent efficacy in both in vitro and in vivo experiments, but in many cases, stability is not guaranteed or bioavailability in the human body is poor. Therefore, it can be difficult to achieve the desired effect [12,13]. To reduce unexpected side effects and ensure expected effects, formulation and stability evaluation should be conducted for all products, including pharmaceuticals and cosmetics, and expiration dates must be set [14–16]. Recently, this importance has been gradually recognized and evaluations on the stability of natural products have been reported [17–19]. However, most did not analyze the ingredients and evaluate the stability.

Agrimonia pilosa is distributed throughout East Asia and Central Europe and is a widely used traditional medicinal herb owing to its anticancer, antioxidant, and anti-inflammatory

properties and beneficial effects on skin inflammation. The herb is reported to contain flavonoid, isocumarin, triterpene, and tannin components and has been used for hemostasis and to treat branch trichomoniasis and malaria [20–24]. Apigenin-7-O-glucuronide (A7OG) and luteolin-7-O-glucuronide(L7OG), a representative flavonoid glucuronides present in *A. pilosa* (Figure 1), have shown many pharmacological effects, such as anti-inflammatory properties [25–29]. Thus, *A. pilosa* exhibits potential for use as a medicine. In this study, we manufactured formulations that could be utilized and evaluated for their physicochemical properties and biological stability. The optimal formulation was selected by comparing physicochemical stability among the preparations. We selected A7OG and L7OG as an indicator ingredients because they not only possess anti-inflammatory efficacy but can also be found in a sufficient amount for analysis. The selected formulation was evaluated under long-term and accelerated stability conditions. The physicochemical stability, flavonoid glucuronide content, and biological stability were evaluated to observe the occurrence of contamination, and the stability was evaluated comprehensively. In this experiment, we aimed to validate the analysis method for flavonoid glucuronide, which is an indicator component develop an optimized formulation containing *A. pilosa* and evaluate the stability comprehensively.

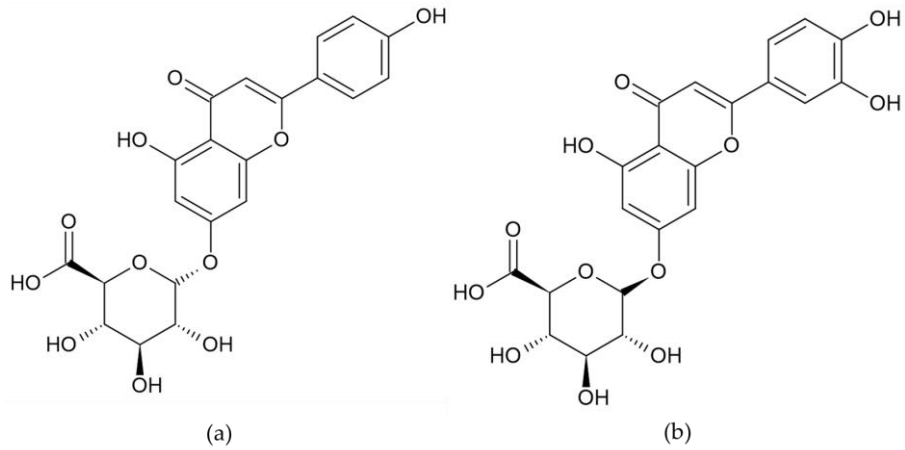

Figure 1. Structure of (**a**) apigenin-7-O-glucuronide and (**b**) luteolin-7-O-glucuronide.

2. Materials and Methods

2.1. Chemicals and Reagents

Cetanol, stearyl alcohol (Kao, Tokyo, Japan), liquid paraffin (Sonneborn, Petrolia, PA, USA), propylene glycol (Dow, Midland, MI, USA), Tween 60 and span 60 (Croda, Yorkshire, UK), and citric acid monohydrate (Shinwon, Seoul, Republic of Korea) were used to prepare cream emulsions. High-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, water, and phosphoric acid were used with the A7OG (Biopurify, Chengdu, China), L7OG (Biopurify, Chengdu, China) and baicalin (Alladin-e, Wuhan, China) standards for cream analysis. An extra pure-grade dichloromethane (J.T. Baker, Philadelphia, PA, USA) was used for sample preparation.

2.2. Preparation of A. pilosa Extract

A. pilosa derived from Mt. jiri in Korea was used. *A. pilosa* was placed in 70% ethanol at a weight ratio of 20 times and extracted under reflux for 4 h. It was concentrated using a decompression concentrator and then freeze-dried for 5 days at a temperature below −80 °C. A lyophilized powder form was prepared using a series of pretreatments. The yield was approximately 10%.

2.3. Preparation of Formulations

The formulation followed the usual manufacturing method, and the method is as follows [30]. The emulsion cream containing 0.1% *A. pilosa* extract (AE) was prepared as described below, and 10 g was packaged in an aluminum tube. Formulations were prepared by varying the composition of excipients, and the following manufacturing process was established by evaluating the stability of texture and appearance.

(1) Oil phase: Tween 60 (2%), liquid paraffin (6.8%), propylene glycol (15%), cetanol (5%), stearyl alcohol (5%), span 60 (2%), benzyl alcohol (0.5%), and AE (0.1%) were weighed. Then, they were put into a glass beaker and stirred using a paddle stirrer in a water bath at 80 °C for 10 min.

(2) Water phase: Citric acid hydrate (0.01%) was weighed in a glass beaker and dissolved in purified water in a water bath at 80 °C.

(3) Mix: The water phase was added to the oil phase in a water bath at 90 °C and stirred for 30 min with a homomixer. Then, it was cooled and stirred until a semi-solid cream was formed.

The lotion, prepared using a similar method, consisted of liquid paraffin (6.8%), propylene glycol (3%), stearyl alcohol (5%), span 60 (2%), benzyl alcohol (0.5%), and AE (0.1%). Finally, the ointment, prepared using the same method, consisted of liquid paraffin (17%), cetanol (4%), propylene glycol (4%), stearyl alcohol (2%), span 60 (2%), white petrolatum (65.4%), white wax (4%), stearic acid (1%), benzyl alcohol (0.5%), and AE (0.1%). The ointment was characterized by the lack of moisture. The composition of the components of the formulations is summarized in the table below (Table 1).

Table 1. Composition of ingredients of each formulation.

No.	Constituent	Cream	Lotion	Ointment
1	*A. pilosa* extract	0.1%	0.1%	0.1%
2	liquid paraffin	6.8%	6.8%	17.0%
3	propylene glycol	15.0%	3.0%	4.0%
4	cetanol	5.0%	-	4.0%
5	stearyl alcohol	5.0%	5.0%	2.0%
6	span 60	2.0%	2.0%	2.0%
7	benzyl alcohol	0.5%	0.5%	0.5%
8	citric acid hydrate	0.01%	-	-
9	white petrolatum	-	-	65.4%
10	white wax	-	-	4.0%
11	stearic acid	-	-	1.0%
12	water	q.s	q.s	q.s
q.s: Sufficient quantity				

2.4. Sample Preparation for Cream Formulation

First, 17 mg of A7OG or 10 mg of L7OG standard was dissolved in 100 mL of water (solution A). Then, 25 mg of baicalin standard was dissolved in 100 mL of methanol (solution B). Then, 10 mL of solution A and 12 mL of solution B were mixed with distilled water to make 100 mL of solution C (concentration of A7OG; 17.0 μg/mL;concentration of L7OG, 10.0 μg/mL). Solution C was used for the quantification of the marker. Second, 5 g of 0.1% AE cream was mixed with 20 mL of dichloromethane and 500 μL of solution B. After sufficiently dissolving the cream, the supernatant was centrifuged at 4000 rpm for 10 min at room temperature. Then, the supernatant was filtered (solution D) and analyzed

using an HPLC diode array detector (HPLC-DAD). The equation for calculating A7OG or L7OG in 0.1% AE cream is:

$$\frac{(5 \times WI.S \times S.s \times Rc)}{(Rs \times S.I \times W)},$$

where *WI.S* is the weight of the baicalin; *S.s* is the concentration of A7OG or L7OG in solution C (concentration correction required according to the purity of the standard product); *Rc* is the ratio (A7OG or L7OG peak area/baicalin peak area) of solution D; *Rs* is the ratio (A7OG or L7OG peak area/baicalin peak area) of solution C *S.I* is the concentration of the baicalin in solution C (concentration correction required according to the purity of the standard product); *W* is the weight of the cream.

2.5. Method Validation

The following method was applied to quantify A7OG and L7OG as a biomarkers in the 0.1% AE cream. And the analysis method is the same as previously implemented [31]. To summarize, an HPLC system comprising a Shimadzu SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan) was used, and the column (YoungJin Biochrom INNO-P C18 5 µm, 4.6 × 150 mm) temperature was 30 °C. The mobile phase was as follows: (A) distilled water with 0.1% phosphoric acid; (B) acetonitrile with flow rate at 1 mL/min. The following gradient was used: mobile phase B was changed from 10% to 25% between 0 and 30 min, from 25% to 10% from 30 to 40 min, and, finally, to an isocratic from 40 to 50 min. The detection wavelength for A7OG and L7OG in 0.1% AE cream was 335 nm.

Next, validation was conducted on the analysis and pretreatment methods, and validation included system suitability, specificity, straightness, precision, and stability. We already performed the validation for an A7OG assay in a previous study [31]. and the L7OG analysis method validation also proceeded in the same way. Limit of detection(LOD) and limit of quantification(LOQ) were calculated using the equation from the previous study and analyzed at similar concentrations to verify this. The criteria for the validation test were decided on by considering the concentration contained in the formulation, as suggested by the AOAC Guideline for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals [32]. As the L7OG content unit in the formulation was µg/g, the recovery rate and relative standard deviation range of the precision and accuracy were set within 90–110%, and the relative deviation of the peak area ratio of the system suitability was set within 10%.

2.6. Short-Term Evaluation of Formulations

Storage conditions for the stability test were set and proceeded in accordance with the ICH guidelines [33]. After storing the 0.1% AE cream, lotion, and ointment under long-term (storage at 25 ± 2 °C and relative humidity (RH) of 60 ± 5%) and accelerated (storage at 40 ± 2 °C and RH of 75 ± 5%) conditions for 2 weeks, the content of flavonoid glucuronides was analyzed using HPLC. The rate of change compared to the initial value was observed. Simultaneously, we observed whether the properties of the formulation remained the same; These properties included the phase separation of cream formulations, the generation of precipitates or floats in lotion, the color of the formulation, and odor changes.

2.7. Long-Term Evaluation of Selected Formulation

Following the process outlined in Section 2.6, the formulation was selected, and the next experiment was conducted. To evaluate the stability of the selected formulation for clinical trials, accelerated (storage at 40 ± 2 °C and RH of 75 ± 5%) and long-term (storage at 25 ± 2 °C and RH of 60 ± 5%) stability tests were conducted. After preparing the formulation (as per Section 2.2), it was stored in a stability chamber, and the accelerated (0, 3, and 6 months) and long-term (0, 3, 6, 9, and 12 months) stability tests were conducted. To evaluate the physicochemical stability, properties such as pH and the component content of the cream were analyzed, and microbiological tests were performed to evaluate the biological stability.

2.7.1. Physicochemical Stability

By applying the method outlined in Section 2.3, the stability of the flavonoid glucuronides was evaluated, and the occurrence of deterioration, indicated by the color and odor of the cream, was visually evaluated. Finally, the rate of pH change was observed using a 931 pH meter (Metrohm, Barendrecht, The Netherlands).

2.7.2. Microbial Stability

Microbial stability was evaluated to evaluate whether the formulation would not cause contamination during the storage period. The test was completed according to the method described in the USP (United States Pharmacopeia) [34]. The tests are outlined below. The evaluation was not conducted between 3 and 9 months because it was judged that there would be no change over that time.

Total Aerobic Bacterial Count

First, 10 g of the formulation was added to 90 mL of tryptone soya broth, and 0.1 mL of the stirred solution was collected. Then, this solution was diluted in 100 mL of peptone sodium chloride buffer and filtered before being placed on soybean-casein-digested agar medium and incubated for 3–5 days.

Total Yeast Count

First, 10 g of the formulation was added to 90 mL of tryptone soya broth, and 0.1 mL of the stirred solution was collected. Then, this solution was diluted in 100 mL of peptone sodium chloride buffer and filtered. Two sheets of the filtered membrane filter were placed onto Saburo glucose agar medium and incubated (20–25 °C) for 5–7 days.

Pathogen Tests

To test for Escherichia coli, 10 g of the formulation was added to 90 mL of tryptone soya broth and incubated (30–35 °C, 18–24 h). Then, 1 mL of this solution was inoculated into MacConkey broth and cultured (42–44 °C, 24–48 h). Next, the cultured medium was transferred to MacConkey agar medium and cultured. The test was considered positive if there was colony proliferation.

To test for Staphylococcus aureus, 10 g of the formulation was added to 90 mL of tryptone soya broth and incubated (30–35 °C, 18–24 h). Then, this solution was plated on mannitol salt agar medium and cultured (30–35 °C, 18–72 h). The test was considered positive when there were yellow or white colonies surrounded by a yellow band.

To test for Salmonella spp., 10 g of the formulation was added to 90 mL of tryptone soya broth and incubated (30–35 °C, 18–24 h). After inoculating 0.1 mL of the culture medium into the Rappaport-Vassilliadis Salmonella enrichment broth, it was incubated (30–35 °C, 18–24 h). Then, the specimen was transferred to xylose lysine deoxycholate agar medium and cultured (30–35 °C, 18–48 h). The test was considered positive when there were red colonies after sufficient proliferation.

To test for Pseudomonas aeruginosa, 10 g of the formulation was added to 90 mL of tryptone soya broth and incubated (30–35 °C, 18–24 h). The specimen was transferred to cetrimide agar medium and cultured (30–35 °C, 18–72 h). The test was considered positive if there was no colony proliferation.

3. Results

3.1. Summary of Validation

We previously conducted and reported validation for A7OG [31], and this time we successfully analyzed and validated L7OG (Table 1). Therefore, a method of quantifying A7OG and L7OG with low amounts in the formulation as indicator components was established. In Table 2, is the mean of the slopes, and σ is the standard deviation of the intercepts

Table 2. Validation results.

Parameter		Result	Acceptance Criteria
System Suitability		0.6%	%RSD $\leq$ 10%
Specificity		Yes	No interference
Linearity (μg/mL)		6.0–14.0	
Slope		36,641	
Intercept		−10300	
Regression coefficient (R^2)		0.9998	$R^2 \geq 0.995$
%Recovery	60%	106.4 ± 2.2%	Between 90% and 110%
	100%	101.2 ± 2.8%	
	140%	96.4 ± 1.8%	
Precision (%RSD)		1.7%	%RSD $\leq$ 10%
Intermediate Precision (%RSD)		3.0%	%RSD $\leq$ 10%
LOD (μg/mL)		0.9	S/N > 10
LOQ (μg/mL)		2.9	S/N > 3

3.2. Short-Term Stability Evaluation of Formulations

For the ointment, the AE powder was not mixed with the excipient and irregularly dispersed. Therefore, the ointment type was immediately excluded. The content of flavonoid glucuronide in the lotion formulation tended to decrease over time (Figure 2), and this decrease was more pronounced under accelerated testing than under long-term testing. Therefore, it seems that the higher the temperature, the faster the A7OG and L7OG decomposition. During the storage period, there were no sediments or floating material in the solution, and the sample color was slightly darker than that in the initial stage of the accelerated conditions. Conversely, the AE cream showed no change in the content of A7OG and L7OG. Therefore, the AE cream was selected for the next step.

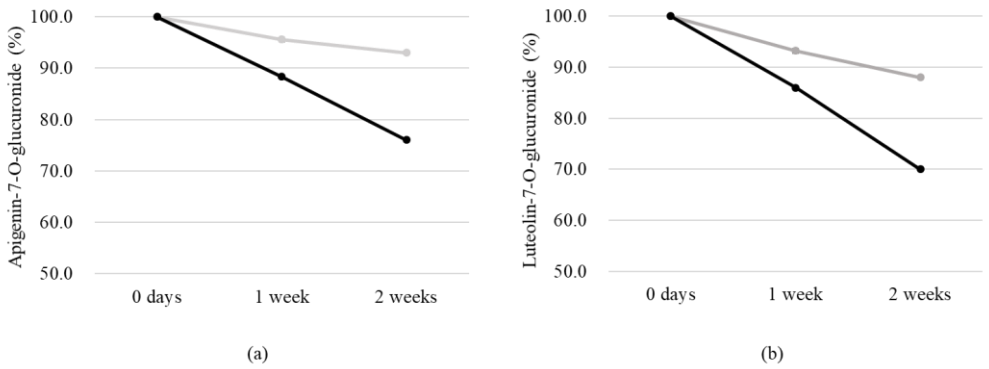

Figure 2. Change rate (%) of apigenin-7-O-glucuronide (**a**) and luteolin-7-O-glucuronide (**b**) contained in 0.1% AE solution from 0 to 2 weeks. The grey line indicates the A7OG content from the long-term stability test, and the black line indicates the result of the accelerated test.

3.3. Stability of Cream Formulation

The experiment was conducted using three batches of the AE cream formulation (Figure 3a,b). The chromatogram was analyzed after the pretreatment of the AE cream (Figure 4). The physicochemical and biological stability of each batch of 0.1% AE cream was evaluated. First, the characteristics and biological stability of 0.1% AE cream were stable and met the criteria for both long-term preservation and accelerated test conditions (Tables 3 and 4). The flavonoid glucuronide content in the cream formulation was also stable. Therefore, it appears that there was no reaction between the excipients, A7OG and L7OG. However, in the accelerated test, the contents tended to increase slightly (Figure 5). This may be because the moisture evaporates under accelerated conditions and the content increases relatively, but there is no change in the mass of the cream mold. Because these amounts are different from each other, it is possible that the content of the ingredients may

have been affected by a slight change in the mass of the cream. Therefore, it is necessary to conduct additional tests using other packages for 0.1% AE cream.

Figure 3. (**a**) 0.1% AE cream packaged in an aluminum tube, (**b**) 0.1% AE cream.

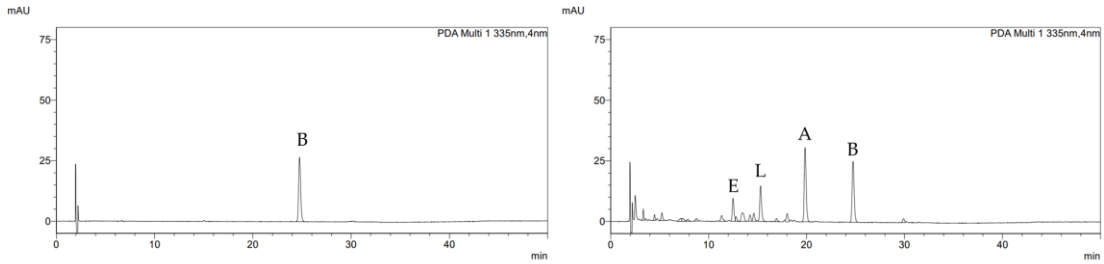

Figure 4. Chromatogram of (**a**) placebo cream and (**b**) 0.1% AE cream. E is the peak of ellagic acid, L is the peak of luteolin-7-O-glucuronide, A is the peak of apigenin-7-O-glucuronide and B is the peak of baicalin as internal standard.

Table 3. Physicochemical and microbial evaluation in the accelerated stability test (40 ± 2 °C; 75 ± 5% RH; three batches).

		Criteria	**0 Months**	**3 Months**	**6 Months**
Physicochemical stability	Sensory	Light yellow, odorless, and homogeneous	Conforms	Conforms	Conforms
	A7OG (μg/g)	-	12.2 ± 0.1	12.4 ± 0.2	12.7 ± 0.0
	L7OG (μg/g)		8.1 ± 0.1	8.2 ± 0.1	8.3 ± 0.1
	pH	4.5–5.5	5.0	5.1	4.9
Microbial stability	Total aerobic bacterial count	$<10^5$ CFU/g	<1 CFU/g	-	<1 CFU/g
	Total yeast count	$<10^2$ CFU/g	<1 CFU/g	-	<1 CFU/g
	Pathogens	Not detected	Not detected	-	Not detected

Table 4. Physicochemical and microbial evaluation of 0.1% AE cream in the long-term stability test (25 ± 2 °C; 60 ± 5% RH; three batches).

		Criteria	**0 Months**	**3 Months**	**6 Months**	**9 Months**	**12 Months**
Physicochemical stability	Sensory	Light yellow, odorless, homogeneous	Conforms	Conforms	Conforms	Conforms	Conforms
	A7OG (μg/g)	-	12.2 ± 0.1	12.4 ± 0.2	12.2 ± 0.1	12.2 ± 0.0	12.5 ± 0.2
	L7OG (μg/g)	-	8.1 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	8.2 ± 0.1
	pH	4.5–5.5	5.0	5.1	4.9	4.8	4.8

Table 4. *Cont.*

		Criteria	0 Months	3 Months	6 Months	9 Months	12 Months
Microbial stability	Total aerobic bacterial count	$<10^5$ CFU/g	<1 CFU/g	-	<1 CFU/g	-	<1 CFU/g
	Total yeast count	$<10^2$ CFU/g	<1 CFU/g	-	<1 CFU/g	-	<1 CFU/g
	Pathogens	Negative	Not detected	-	Not detected	-	Not detected

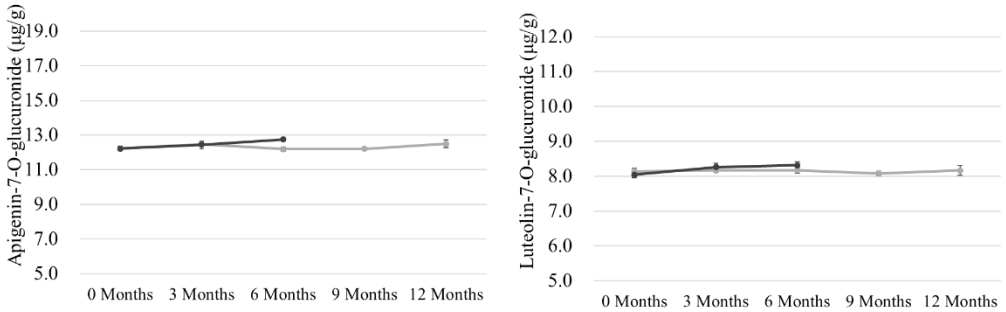

Figure 5. (**a**) Content of apigenin-7-O-glucuronide (A7OG) in 0.1% AE cream. (**b**) Content of luteolin-7-O-glucuronide (L7OG) in 0.1% AE cream. The grey line indicates the content from the long-term stability test, and the black line indicates the result of the accelerated test.

4. Discussion

It is important to manage and maintain the active ingredients in formulations containing natural products. Moreover, the physicochemical properties during the initial drug development should be maintained, and microbiological contamination should be avoided. However, the evaluation of many natural products is limited to the extent of the appearance and mechanical characteristics. The reason is that it is generally difficult to quantify the component content, and a separate pretreatment technology must be developed. And there are numerous components that cannot be analyzed at once. Therefore, it is reasonable to determine and manage the representative ingredients that can be analyzed. Methods for analyzing these components should be established and continuously evaluated, and various techniques for analyzing ingredients have been studied and developed [35]. An analysis method was developed for one A7OG component and validation was conducted previously [31]. In this study, L7OG was quantified using the same analysis method, and validation was also successfully conducted. This is the first time that L7OG in the formulation has been quantified and validated. In addition, it was expected that the peak of ellagic acid content could be analyzed and quantified, but it was difficult to quantify due to the distribution of each solvent during the pretreatment process. Therefore, this component was excluded as an indicator component. A method of simultaneously analyzing and quantifying low-concentration components was established using a quick and simple pretreatment process. AE has numerous other components, but the amount in this formulation was too low for a UV detector, making it difficult to quantify or not distributed during the pretreatment process. Therefore, A7OG and L7OG were selected as indicators that represent the ingredients in the formulation and managed.

Later, various formulations (cream, lotion, and ointment) were prepared to determine the most efficient way to use AE. Topical formulations were selected because they can avoid the first-pass effect in the body [36]. In addition, excipients such as liquid paraffin, propylene glycol, stearyl alcohol, and span 60 were used, which are widely used in topical formulations. The formulations were designed to be manufactured at an economical

cost by simplifying the manufacturing method. Before manufacturing the formulation, the compatibility study between the excipient and the AE was briefly conducted using HPLC-DAD for two weeks. Since most of the excipients form an oil phase, they were hardly mixed with AE, and that there was no significant change in the peak pattern during the test period. Later cream, lotion, and ointment were classified according to differences in moisture and the composition of ingredients. If the active components are vulnerable to moisture, they can be expected to stabilize in ointments, which have little moisture. However, the composition of the ointment selected in this experiment did not sufficiently dissolve the 0.1% concentration of AE. This may be because the moisture content was insufficient to dissolve the powder, so further manufacturing processes need to be developed. Furthermore, the preference for ointments is lower than that for other topical formulations because of their sticky texture and low spreadability. Thus, the ointment was excluded at the short-term stability stage. The lotion formulation was also excluded because the contents of flavonoid glucuronides was decreased. This may be because it was hydrolyzed by water and decomposed into glucuronide and aglycone. Furthermore, the color darkened, and the physicochemical properties changed under the accelerated conditions. Conversely, there was no change in the content and physicochemical properties in the cream formulation. The lotion and cream formulations in this experiment differed in terms of water content, propylene glycol content, and the presence of cetanol as an absorbent. Based on our results, the stability of flavonoid glucuronides was affected by these differences. Propylene glycol and cetanol play a role in maintaining moisture. They are excipients widely used in topical formulations and have the ability to suppress the flow of formulations by absorbing moisture [37,38]. Accordingly, liquidity is suppressed making it difficult for water molecules to move freely and react with other components, including A7OG and L7OG. By appropriately utilizing them, stability was increased. On the contrary, the lotion formulation with low viscosity was thought to cause frequent reactions at high temperatures due to the lower levels of absorbents and higher levels of water. The cream formulation was stable under long-term stability (12 months) and accelerated (6 months) tests. This indicates that flavonoid glucuronides were stable in the cream formulation and were suitable for use with other excipients. However, in the accelerated test, the contents increased slightly. Further experiments using a packaging container with finer airtightness are required. And finally in the microbiological evaluation results, there was no contamination in the cream formulation.

Considering the results of this experiment, it is evident that the cream formulation is the optimal form for the topical use of AE. We successfully manufactured a cream formulation capable of ensuring the stability of AE. Therefore, a cream formulation was developed that can apply the pharmacological efficacy of AE. We plan to evaluate not only its pharmacological effect on the human body but also its longer-term the longer term stability using this formulation.

5. Conclusions

An optimized cream formulation for *A. pilosa* extract was developed that could comprehensively guarantee not only the properties and biological stability but also the chemical stability of the ingredients. The stability of this formulation is planned to be further studied in various aspects in the long term. In addition, *A. pilosa* cream was developed to treat atopic dermatitis, and its effectiveness will be evaluated in through clinical trials evaluating atopic dermatitis, inflammation and itching.

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