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Original Article

FORMULATION, ANTIOXIDANT, AND ANTI-AGING ACTIVITY OF *RUBUS FRAXINIFOLIUS* FRACTION

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ABSTRACT

Objective: The aim of this research was to evaluate the antioxidant activity and elastase inhibition of the Rubus gel fraction as well as its physical stability for 12 w. *Rubus fraxinifolius* leaves were reported to have strong antioxidant and elastase enzyme inhibitory activity.

Methods: Water fraction from ultrasonic-assisted extraction (UAE) of old Rubus leaves was used and formulated into face gel preparations. Antioxidant activity 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and 2,2'-azino-bisphenol S (2,2'-ABPS) (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), elastase inhibition, and stability test during 12 w of storage, both at room temperature (30±2 °C) or accelerated temperature (40±2 °C) were performed.

Results: The anti-aging gel showed better antioxidant activity and elastase inhibition on F1 (3%) at both conditions compared to F2 (4%) and F3 (5%). The physical stability test met the requirements. However, there was a slight decrease in antioxidant activity and elastase inhibition after 12 w of storage at 30±2 °C and 40±2 °C (F1, F2, and F3).

Conclusion: The F1 met the standards and was relatively stable at 30±2 °C during 12 w of storage.

Keywords: Antioxidant, Gel, *Rubus fraxinifolius leaves*, Elastase inhibition

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INTRODUCTION

In 2022, IQAir released a global air pollution report based on data from over 30,000 official air quality monitoring stations. The report highlighted Indonesia as having the 26th poorest air quality worldwide, particularly concerning PM 2.5 (2.5-micron particle pollution). Within Southeast Asia, Indonesia stood out as having the highest air pollution levels compared to neighboring countries [1]. Air pollution remains a prominent environmental concern, posing health risks, with elevated Reactive Oxygen Species (ROS) in air pollution contributing to premature aging.

Premature aging is a complex biological process influenced by air pollution and external factors such as diet, smoking, alcohol, and environmental conditions, contributing significantly to one's appearance [2]. This process manifests through the skin that appears older than its actual age, showing premature signs of aging like wrinkles and early dark spots [3].

Given the rising demand for safe and natural products, there's a growing interest in herbal-based formulations. Plants used in cosmetic products have various benefits, such as antioxidant, anti-inflammatory, antiseptic, and antibacterial properties. Herbal cosmetics are believed to avoid the common side effects associated with synthetic ingredients [4]. Natural extracts can shield the skin through various mechanisms, such as reducing ROS reactivity, inhibiting oxidation, absorbing UV rays, suppressing enzyme activity, minimizing wrinkle formation, and safeguarding against aging [5, 6]. This protective function is attributed to the suspected presence of phenolic compounds, flavonoids, and triterpenoids from natural sources. As factors that accelerate aging continue to increase, prevention strategies and formulation advancements are crucial, leveraging the potential of natural extracts. One of the natural materials that can be developed is *Rubus fraxinifolius* leaves, which are reported to have very strong antioxidant activity and elastase enzyme inhibitory activity due to the presence of phenolic compounds, flavonoids, and triterpenoids [7, 8].

Gels are semisolid colloidal systems characterized by their unique structure, which consists of a liquid component and a solid phase known as a gelling agent. This gelling agent forms aggregates, immobilizing the gel's liquid phase [9]. Gel preparations have several advantages, including high viscosity and adhesion value, so they do not easily flow on the skin's surface. In addition, the gel is easy to spread and does not leave marks, only a thin layer when used [10]. The attractive physical appearance of gel preparations is one of the attractions for consumers to use these gel preparations.

This study aims to formulate an anti-aging gel preparation, *Rubus fraxinifolius,* which has good characteristics and stability during storage and has an anti-aging activity, which includes antioxidant activity and elastase enzyme inhibitory activity [11].

MATERIALS AND METHODS

Plant material

Mature leaves of *Rubus fraxinifolius* were selected as the plant source. These leaves were collected from Sindangjaya, Cibodas, located in West Java, Indonesia (GPS coordinates: latitude-6.74238°, longitude 107.006648°). To confirm their leaves authenticity, were verified at the Herbarium Bogoriensis, under the supervision of BRIN Cibinong, Indonesia, with the reference code B-2454/II.6.2/DI.05.07/7/2022.

Chemical and reagents

The chemical substances used in the study included Trizma base, PPE (porcine pancreas elastase) code E1250, SANA (N-Succinyl-Ala-Ala-Ala-p-nitroanilide) code S4760, TPTZ (2,4,6-tris(2-pyridyl)-triazine) reagent from Sigma Aldrich, Germany; DPPH (2,2-diphenyl-1-picrylhydrazyl) from TCI, Japan; quercetin, ascorbic acid, potassium dihydrogen phosphate, sodium hydroxide, sodium acetate, iron (III) chloride hexahydrate, ethanol pa, methanol pa, and iron (II) sulfate heptahydrate from Merck, Germany; water pro injection (Pharmaceutical laboratories, Indonesia).

Methods

Extraction and fractionation

The mature *Rubus fraxinifolius* leaves were subjected to extraction using the UAE (ultrasonic-assisted extraction) method. This involved

adding 150 ml of solvent to 15 g of leaf powder, maintaining a ratio of 1:10 between leaf powder and solvent. The extraction process occurred over 30 min, utilizing an ultrasonic bath operating at 40 kHz and a temperature of 40 °C [12].

For fractionation, the active constituents of *Rubus fraxinifolius* leaves were extracted using a separating funnel employing a liquid-liquid partition technique-the extraction procedure involved using a ratio of 1:10 between the extracted material and the solvent. Three distinct solvents were utilized: a non-polar solvent (n-hexane), a semi-polar solvent (ethyl acetate), and a polar solvent (purified water). The resulting liquid fractions obtained from pure water (residue) phases were subjected to evaporation to obtain dry fractions. These dry fractions were then formulated and evaluated to determine their activity [13].

The detailed extraction and fractionation procedures were conducted following the methodologies outlined in the prior research [8].

Determination of active ingredient by densitometry TLC

The sample was hydrolyzed first to obtain sugar-free compounds by adding 100 ml of 1M HCl to 1 g of the sample and then heating it in a water bath for 30 minutes. The mixture was withdrawn with ethyl acetate and evaporated. Test and standard solutions were spotted on the plate and eluted using mobile phase chloroform: methanol (9:1). Identification was made by observing spots of the test solution and standard solution under UV light and then calculating the Rf value [13, 14].

Purified water fraction gel anti-aging formulation of *Rubus fraxinifolius* leaves

The Anti-aging gel was formulated in three variations: Formula 1 (F1) contained a fraction concentration of 3% (30,000 µg/ml), Formula 2 (F2) featured a fraction concentration of 4% (40,000 µg/ml), and Formula 3 (F3) comprised a fraction concentration of 5% (50,000 µg/ml). The concentration determination in the formula is based on a literature study from previous research. Formulations used active substances from other Rubus genus [15, 16].

Preparation of gel anti-aging

The anti-aging gel formulation comprised the purified water fraction extracted from *Rubus fraxinifolius* leaves and all other components specified in table 1. The listed ingredients were weighed according to the provided formula. The process involved the following steps: Carbomer was dispersed within water until a uniform mass was achieved. Subsequently, glycerin was added and stirred consistently until a homogeneous gel mass (mass I) was performed. Sodium Metabisulfite and EDTA were dissolved in water for injection (WFI) until fully dissolved (creating mass II). Mass II was gradually added into mass I and homogenized, resulting in mass III. According to predetermined concentration variations, the purified water fraction of Rubus leaves was added to mass III and stirred until homogeneity was achieved. Phenoxyethanol was added and mixed thoroughly until mass IV, a gel-like consistency, formed. Finally, TEA (triethanolamine) was gradually added until the gel mass reached the desired final pH, forming the gel aligned with the specified requirements.

Table 1: Gel anti-aging formula (% w/w)

Ingredient	F1 (%)	F2 (%)	F3 (%)	Function
Purified water fraction of Rubus	3	4	5	Active substance
Carbopol	0.7	0.7	0.7	Gelling agent
Triethanolamine	1	1	1	Alkalizing agent
Glycerin	10	10	10	Humectants
Ethylenediaminetetraacetic acid	0.1	0.1	0.1	Chelating agent
Sodium metabisulfite	0.1	0.1	0.1	Antioxidant
Phenoxyethanol	0.6	0.6	0.6	Preservative
Purified water	ad. 100 ml	ad. 100 ml	ad. 100 ml	Solvent

The prepared anti-aging gel was transferred into a final container. Subsequently, the final assessment of the gel was conducted. In comparison, the control preparation shared similarities with the anti-aging gel, except for the absence of the purified water fraction extracted from *Rubus fraxinifolius* leaves.

Preparation evaluation

Organoleptic

The anti-aging gel's appearance, color, and fragrance were scrutinized during the organoleptic evaluation. This assessment was carried out through direct visual observation.

Homogeneity test

The gel was spread into one glass slide and covered with another to determine the presence of coarse particles or any lack of homogeneity. The evaluation was conducted by observing the sample under a light source. This process allowed for identifying any irregularities in texture or uniformity within the gel [18].

pH stability test

A one g of gel was measured and then dissolved in 100 ml of purified water. Following this, a pH test was carried out. The pH meter (Ohaus ST3100-F, USA) was calibrated before the sample test using standard buffers at pH 4, 7, and 10. The pH electrode was immersed into the mixture gel, and the resulting pH value was recorded at room temperature. The observed pH values should ideally fall within the pH range of 4.5 to 6.5, aligning with the pH range suitable for skin applications.

Testing of viscosity and flow properties

The viscosity and flow characteristics were assessed using a viscometer (Cole Parmer Rotational Viscometer, USA). The following procedure was undertaken. First, the prepared anti-aging gel was placed in a measuring cup. An appropriate spindle, such as spindle l4, was selected and attached to the viscometer's tool. The spindle was immersed in the preparation until its limit was reached. The desired spindle type and rotational speed were chosen according to the guidance provided in the manual. To initiate viscosity measurement, the 'on' button was pressed. The spindle rotated for approximately five revolutions until it reached a stable state. The viscosity value (measured in Cp or mPa. s units) was read from the screen. It was confirmed that the percentage scale displayed on the screen fell within the range of greater than 15% but less than 100% [19].

Gel spreadability test

A 0.5 g sample was positioned between two circular glass plates at the center. A load of 50 g was applied to the upper plate for 1 minute. Following this, the diameter of the sample's spread was observed. Subsequently, a load of 100 g was placed on the upper plate for another minute, and the diameter of the sample's spread was again observed. This procedure was conducted to assess how varying loads influence changes in the diameter of the gel's distribution [20].

Temperature stability test

Cycling test

The anti-aging gel was tested by cycling test. It was subjected to a

cycle where it was stored at a temperature of 4 °C for 24 h and then transferred to an oven set at 40 °C for another 24 h. Each of these temperature cycles constituted one complete iteration. This testing cycle was repeated six times in total. After completing these six cycles, organoleptic observations were conducted to evaluate any changes or effects on the gel's properties [18].

Stability test at room temperature

The anti-aging gel was stored at a consistent room temperature of 30±2 °C for 12 w. Throughout this period, a series of assessments were conducted at specific intervals at w 0, 2, 4, 8, and 12. Organoleptic observations were performed, examining changes in color, odor, and the presence or absence of syneresis (the separation of liquid from a gel-like substance). pH measurements were taken at the same time intervals to monitor any alterations in the pH level of the gel. The homogeneity of the gel was also evaluated to ensure that its composition remained uniform throughout the storage period [21].

Accelerated stability test

Gel anti-aging was stored at an accelerated temperature (40±2 °C) for 12 w. Organoleptic observations (change in color, odor, and presence or absence of syneresis), pH, and homogeneity were carried out at w 0, 2, 4, 8, and 12 [21].

Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) method

The antioxidant activity test for gel preparations was conducted using the DPPH method, following the same procedure used for testing the extracts and fractions in previous studies [8, 21].

Blank preparations and F1, F2, and F3 formulations were initially prepared. The process proceeded: 1 g from each formulation was precisely weighed and dissolved in 10 ml of methanol. The samples were centrifugated for 30 minutes at 4500 rpm (Hettich Zentrifugen, Germany). Following centrifugation, the supernatant portion of each sample was retained for subsequent analysis. The concentrations for F1, F2, and F3 formulations were adjusted as follows: F1 concentration was elevated to 3.000 g/ml, F2 was increased to 4.000 g/ml, and F3 concentration was raised to 5.000 g/ml. Afterward, these concentrations were further modified to achieve 10, 20, and 30 µg/ml concentrations for each formulation.

FRAP (ferric reducing antioxidant power) method

The antioxidant activity test for the gel preparations was conducted using the FRAP method, following the same procedure used for testing the extracts and fractions in previous studies [23].

The procedure began with preparing blanks, F1, F2, and F3 formulations. The steps involved were as follows: approximately 1 g of each formulation was weighed, and the measured amount was then dissolved in 10 ml of methanol p. a. (pro-analysis grade methanol). The samples were subsequently centrifugated (Hettich Zentrifugen, Germany), operating at 4500 rpm for 30 minutes. The analysis was conducted using the supernatant portion of each sample. The concentrations for F1, F2, and F3 formulations were adjusted to the following levels: F1 concentration was set to 3.000 µg/ml, F2 concentration was established at 4.000 µg/ml, and F3 concentration was determined as 5.000 µg/ml. Subsequently, a concentration series with a starting concentration of 10 µg/ml was created. This concentration series ultimately reached a final concentration of 12.5 µg/ml within the designated well.

ABTS (2,2'-azino-bisphenol S (2,2'-ABPS) (3-ethylbenzothiazoline-6-sulphonic acid) method

The antioxidant activity test for the gel preparations was conducted using the ABTS method, following the same procedure used for testing the extracts and fractions in previous studies [20].

First, the preparations (blanks, F1, F2, and F3) were made. Weigh 1 g of the preparation and dissolve it in 10 ml of ethanol p. a. After that, it was centrifuged for 30 minutes at 4500 rpm (Hettich Zentrifugen, Germany). The supernatant portion was used for the analysis. F1 concentration was increased to 3.000 g/ml, F2 to 4.000 g/ml, and F3 to 5.000 g/ml. Then, concentrations were adjusted to 5, 10, and 15 µg/ml.

Elastase inhibition

The testing approach was based on earlier research with minor adjustments. Before assessing the elastase enzyme inhibition, an initial sample was performed in the gel anti-aging preparation. A 1 g sample was weighed, and 10 ml of Tris buffer pH 8.0 was added. The mixture was centrifuged (Hettich Zentrifugen, Germany) for 30 minutes at 4500 rpm. The supernatant part was analyzed. F1 concentration was increased to 3.000 g/ml, F2 concentration to 4.000 g/ml, and F3 concentration to 5.000 g/ml.

RESULTS AND DISCUSSION

Cycling test

The cycling test method serves as a stability assessment by simulating extreme temperature fluctuations, involving the storage of the preparation at a low temperature of 4 °C for 24 h, followed by exposure to a high temperature of 40 °C. This procedure aims to subject the product and its packaging to varying stresses. The test involves six cycles, each consisting of 24 h at low temperature followed by 24 h at high temperature.

Upon concluding the 6th cycle, organoleptic observations were conducted. As depicted in fig. 1, the outcomes indicated no significant differences compared to organoleptic observations in the initial conditions. The results showed that the gel maintained its brown color and distinct aromatic scent. Notably, the preparation exhibited no syneresis (separation of liquid), remained unchanged in color, shape, and odor, and did not form crystals. Consequently, it can be deduced that the preparation exhibits favorable stability under these conditions.

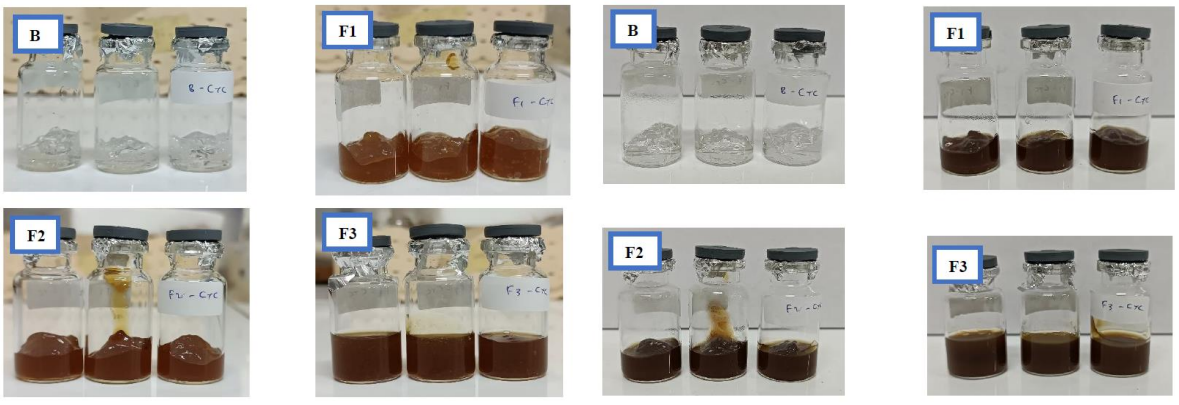
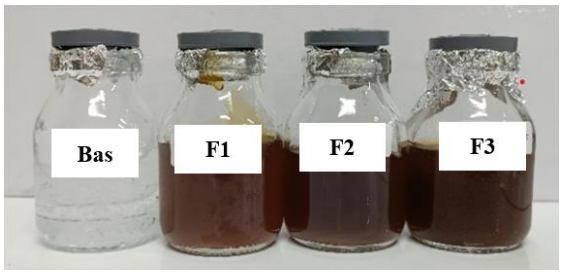


Fig. 1: The results of the cycling test for control (B), Formula 1 (F1), Formula 2 (F2), and Formula 3 (F3): (A) before and (B) after six cycles

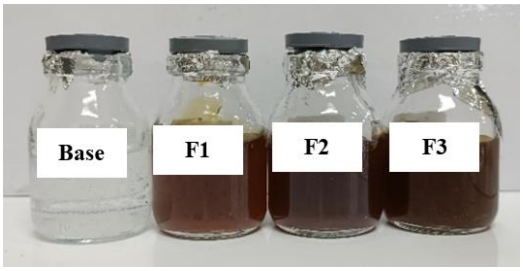
Organoleptic

Based on the results obtained from organoleptic observations conducted at intervals of 0, 2, 4, 8, and 12 w, there was a change in gel preparation, particularly in terms of its color, especially when stored at a temperature of 40 °C, as depicted in fig. 2. Initially exhibiting a red-brown color, the gel preparation transitioned to a slightly darker brown by the 8th w of storage, predominantly under 40 °C. In contrast, at a storage temperature of 30 °C, the color of the gel largely remained unchanged.

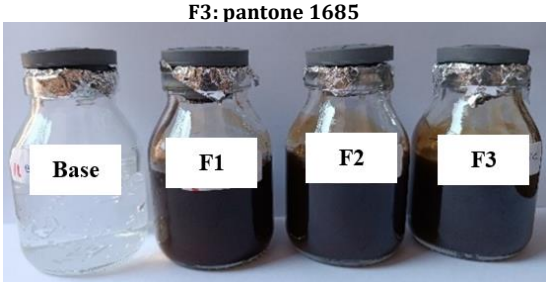
This transformation in color can be attributed to the potential degradation of active ingredients and excipients within the gel formulation. For instance, ingredients like sodium metabisulfite, which functions as an antioxidant, and the Rubus fraction itself could undergo degradation. Additionally, the gel's texture alteration from a gel-like structure to a more liquid state could be attributed to a rise in acidity as indicated by the pH of the gel. This observation is further supported by the findings from the viscosity tests, which demonstrated a consistent decline in viscosity over time. The decrease in viscosity aligns with the change in pH and the subsequent transformation in the physical properties of the gel preparation.



F1: pantone 160 F2: pantone 1685 F3: pantone 1685

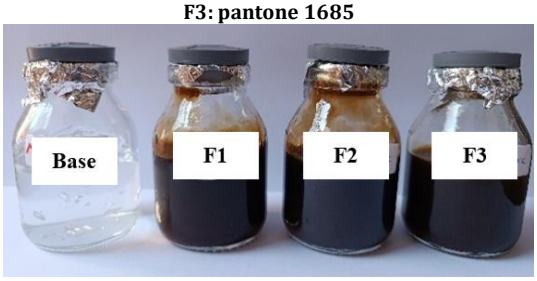


F1: pantone 160 F2: pantone 1685 F3: pantone 1685



F1: pantone 160 F2: pantone 1685 F3: pantone 1685

A



F1: pantone 1615 F2: pantone 161 F3: pantone 168

B

Fig. 2: The results of the organoleptic test (A) Before and after 12 w in temperature 30 °C and (B) Before and after 12 w in temperature 40 °C

Homogeneity

The homogeneity test was performed to determine the uniformity of the particles in the gel dosage form. This evaluation is essential for ensuring optimal quality when the gel is used. Homogeneity stands as a crucial parameter impacting the quality of the gel formulation. The examination of homogeneity across all gel formulations yielded consistent outcomes, as depicted in fig. 3. This illustration demonstrates that the particles within the gel were uniformly distributed without clustering or clumping.

A

B

Fig. 3: The results of homogeneity test, A Before and after 12 w in temperature 30 °C, B Before and after 12 w in temperature 40 °C

pH stability

pH measurements were conducted before and after the fraction was formulated in gel preparation. The initial measurement of the fraction's pH aimed to assess the active component's acidity level and determine whether it might impact the stability of the preparation once the fraction was added [24]. To conduct this measurement, 1 g of the fraction was dissolved in 100 ml of purified water. The pH measurement was performed three times, resulting in a pH value of 4.15.

The relatively low pH of the fraction is attributed to the presence of acidic groups within the fraction, originating from the phenolic content of the leaves. These phenolic compounds act as hydrogen ions (H+) donors, contributing to the solution's acidity.

The purpose of measuring the pH of the gel is to determine whether the gel formulation has the potential to cause skin irritation [25]. The pH observations conducted over 12 w, as depicted in table 2, aimed at assessing the pH changes in the gel preparation stored under both conditions and accelerated temperature during the stability test.

The findings revealed a reduction in the pH of the gel preparation over the storage period. This decrease in pH was more visible in gels stored under accelerated temperature conditions, while those stored at room temperature showed little decline in pH. These observations underline the influence of temperature on the gel's quality during storage. The decrease in gel pH was possible because the phenolic compounds came out of the gel matrix and increased the acidity of the preparation [26]. When correlated with organoleptic observations, these results directly correspond to color changes, particularly during high-temperature storage.

Table 2: The result of pH stability in 30 °C and 40 °C

Week observation	30 °C			40 °C		
	FI	F2	F3	FI	F2	F3
Week 0	6.50±0.00^{bcde}	6.10±0.00^{bcde}	5.88±0.00^{bcde}	6.50±0.00^{bcde}	6.10±0.00^{bcde}	5.88±0.00^{bcde}
Week 2	6.34±0.00^{acde}	6.02±0.00^{acde}	5.86±0.00^{acde}	6.34±0.00^{acde}	6.04±0.00^{acde}	5.86±0.00^{acde}
Week 4	6.17±0.00^{abde}	5.97±0.00^{abde}	5.73±0.00^{abde}	5.93±0.00^{abde}	5.72±0.00^{abde}	5.62±0.00^{abde}
Week 8	6.05±0.00^{abce}	5.72±0.00^{abce}	5.62±0.00^{abce}	5.80±0.00^{abce}	5.42±0.00^{abce}	5.30±0.00^{abce}
Week 12	5.93±0.01^{abcd}	5.59±0.00^{abcd}	5.47±0.00^{abcd}	5.53±0.01^{abcd}	5.28±0.00^{abcd}	5.16±0.00^{abcd}

All data was shown as the mean±SD in which each treatment was repeated three times. ^{a,b,c,d,e}Indicates a significant difference between week observation of each formula with Tukey analysis ($p<0.05$, n=3).

The pH value is essential for cosmetic preparations to determine the acidity level of the preparation and its potential to cause skin irritation. The pH of cosmetic formulations should ideally align with the physiological pH of the skin's "acid mantle." This ensures that the cosmetic product is gentle and compatible with the skin's natural pH environment.

The observations conducted over 12 w, encompassing 30 °C and 40 °C storage conditions, demonstrated that the gel preparation's pH remained within the range corresponding to the skin's pH. This finding indicates that the preparation retained its compatibility with the skin's acid mantle, emphasizing its potential for skin-friendly use.

Spreadability of gel

The spreadability test is an essential requirement for gel preparations. A high spreadability characteristic indicates that the gel's application area is expanded, resulting in a more even distribution of the active substances. This, in turn, enhances the effectiveness of therapeutic outcomes [18].

From the observations for 12 w in table 3 and table 4, it can be seen that the diameter of the gel in the spreadability test experienced a slight increase, especially at accelerated temperature storage. Observations are more visible in formula three storage at 40 °C, where the consistency of the gel is increasingly liquid as indicated by the larger diameter value both at the addition of 50 g or 100 g of load.

Table 3: The result of the spreadability test of 50 and 100 g load in 30 °C

Week observation	30 °C					
	F1 (50 g)	F1 (100 g)	F2 (50 g)	F2 (100 g)	F3 (50 g)	F3 (100 g)
week 0	4.6±0.06	5.1±0.06	4.9±0.06	5.1±0.06^{bcde}	5.9±0.10^{bcde}	6.9±0.06^{bcde}
week 2	4.6±0.06	5.0±0.06	5.0±0.06	5.4±0.10^{a}	6.2±0.15^{a}	7.0±0.06^{acde}
week 4	4.6±0.06	5.0±0.00	4.9±0.12	5.4±0.06^{a}	6.2±0.06^{a}	7.2±0.10^{ab}
week 8	4.5±0.06	5.0±0.10	5.0±0.00	5.5±0.06^{a}	6.3±0.06^{a}	7.3±0.06^{ab}
week 12	4.5±0.00	5.0±0.06	5.0±0.06	5.5±0.06^{a}	6.3±0.00^{a}	7.3±0.00^{ab}

All data was shown as the mean±SD in which each treatment was repeated three times. ^{a,b,c,d,e} indicates significant differences between week observation of each formula with Tukey analysis ($p<0.05$, n=3).

Table 4: The result of the spreadability test of 50 and 100 g load in 40 °C

Week observation	40 °C					
	F1 (50 g)	F1 (100 g)	F2 (50 g)	F2 (100 g)	F3 (50 g)	F3 (100 g)
week 0	4.6±0.06	5.1±0.06	4.9±0.06	5.1±0.06^{bcde}	5.9±0.10^{bcde}	6.9±0.06^{cde}
week 2	4.6±0.06	5.0±0.06	4.9±0.10^{e}	5.4±0.06^{ade}	6.3±0.06^{a}	6.9±0.06^{cde}
week 4	4.6±0.06	5.0±0.06	4.9±0.06	5.5±0.06^{ade}	6.4±0.06^{a}	7.4±0.10^{abe}
week 8	4.6±0.10	5.1±0.10	5.1±0.10^{b}	5.7±0.06^{abc}	6.5±0.10^{a}	7.5±0.10^{ab}
week 12	4.6±0.06	5.1±0.10	5.1±0.06^{b}	5.8±0.00^{abc}	6.5±0.00^{a}	7.6±0.06^{abc}

All data was shown as the mean±SD in which each treatment was repeated three times. ^{a,b,c,d,e} indicates significant differences between week observation of each formula with Tukey analysis ($p<0.05$, n=3).

This observation underscores how temperature fluctuations, especially at accelerated conditions, can influence the physical properties and consistency of the gel preparation, potentially affecting its spreadability characteristics.

Viscosity and flow properties

Viscosity testing is an essential requirement for gel preparations. Higher viscosity in a preparation corresponds to a thicker dosage form. In this study, viscosity measurements were conducted using a Cole Parmer viscometer. These measurements were taken at intervals of 0, 2, 4, 8, and 12 w.

During these measurements, spindle number 4 was utilized (except for formula 3, where spindle number 3 was used) at a rotational speed of 30 rpm. It was observed that the addition of the fraction concentration led to a reduction in viscosity. In other words, as more fractions were incorporated, the viscosity decreased. The rheograms in week 0 generated for the three formulas and their respective bases indicated a pseudoplastic type of fluid viscosity. This pseudoplastic behavior suggests that the viscosity of the gel preparations decreases as shear stress is applied. In simpler terms, the gel becomes thinner and more fluid-like under shear stress, a common characteristic of many gels.

Table 5: Viscosity measurement

Week	Viscosity (Cp) at 30 rpm, spindle l4, 30 °C			Viscosity (Cp) at 30 rpm, spindle l4, 40 °C		
	FI	F2	F3 (L3)	F1	F2	F3 (L3)
0	11917±1.00	11059±3.51	3264±1.79	11917±1.00	11059±3.51	3264±1.79
2	11892±2.52	8675±1.76	1676±2.58	11848±8.25	8714±10.09	1551±1.52
4	10765±11,93	7814±2.13	1563±1.64	11703±11.73	7028±4.70	1418±10.67
8	10297±11.02	7317±1.66	1624±9.86	10593±11.28	7030±10.35	1396±2.12
12	9694±11.16	7543±11.65	1460±2.86	9720±4.72	7099±11.86	1247±1.64

All data was shown as the mean±SD in which each treatment was repeated three times. [a,b,c,d,e] indicates significant differences between week observation of each formula with Tukey analysis ($p<0.05$, n=3).

The results of viscosity observations carried out for 12 w in table 5 showed that the viscosity decreased during storage in Formula 1, 2, and 3. This is suitable with the pseudoplastic flow type, where the gel viscosity will decrease with increasing shear rate. As the voltage increases, the disordered molecules start to align in the flow direction with the solvent released from the gel matrix.

At a temperature of 40 °C, the decrease in viscosity is higher compared to a temperature of 30 °C, where the consistency of the gel looks more liquid. This is possible because, during high-temperature storage, ingredients are degraded in the formula, thus affecting the viscosity of the preparation.

Gel anti-aging antioxidant activity

DPPH method

The DPPH method is a common technique used to measure the antioxidant capacity of substances by assessing their ability to counteract oxidative stress through electron donation. Measurement of antioxidants using the DPPH method involves evaluating the reduction of DPPH free radicals. This reduction occurs due to donating of electrons from antioxidants, which neutralizes these free radical molecules. Initially, the DPPH radical appears purple in color. However, as antioxidants donate electrons to the DPPH radicals, the radical loses its color and transforms into a pale yellow hue [26, 27].

The assessment of antioxidant activity using the DPPH method within Formula 1 (F1), Formula 2 (F2), and Formula 3 (F3) revealed a strong antioxidant activity across all three formulations, as illustrated in table 6 throughout storage at 0, 4, and 12 w. Over the storage period, the antioxidant activity of the anti-aging gel demonstrated a decrease in the IC50 value under both temperature storage conditions, 30±2 °C and 40±2 °C. Examining the observation activity for each formula at a temperature of 30 °C, it becomes apparent that in formulas with higher fractions (F2 and F3), the antioxidant activity tended to be unstable during the 12 w storage period. At the elevated storage temperature of 40 °C, the antioxidant activity displayed a more significant decline, especially pronounced in the case of F3. This points to the fact that elevated temperatures during storage can indeed impact antioxidant activity, primarily due to the intrinsic instability of the antioxidants. These observations underscore the importance of storage conditions in maintaining the intended antioxidant efficacy of the formulations.

FRAP method

The FRAP (ferric reducing antioxidant power) assay is an electron transfer-based method based on the reduction reaction principle. This method involves the reduction of a yellow Fe^{3+} complex to a blue-green Fe^{2+} complex in an acidic environment. This reduction process is facilitated by electron donors originating from antioxidant compounds in the tested sample. The measurement of absorbance at 593 nm allows for the estimation of the quantity of reduced iron, and this measurement can be correlated with the concentration of antioxidants present in the sample [27, 28].

Table 6 highlights that antioxidant activity exhibits a slight decrease when stored at 40 °C compared to storage at 30 °C. This decline can be attributed to the potential degradation of ingredients within the formulation at higher temperatures, leading to a diminished capacity to capture free radicals-moreover, prolonged storage, whether at 30 °C or 40 °C, reduces inhibitory activity.

When analyzing the inhibition values between storage at 30 °C and 40 °C, it showed that inhibitory activity decreased during the 12 w when stored at 40 °C. Comparing the antioxidant activity among the formulas presented in table 6, it was observable that higher concentrations of fractions within the formula correspond to lower antioxidant activity. This trend could be attributed to the acidic nature of the fraction, wherein a higher fraction content may impact the fraction's stability and other formula components. This, in turn, contributes to the reduction in antioxidant activity.

ABTS method

The test is based on the capacity of antioxidants to reduce the ABTS•+radical, which exhibits a bluish-green chromophore. This reduction process converts the radical into ABTS, resulting in a decolorization effect. This principle is used to assess the antioxidant activity of a given material. The alteration in color can be quantified by measuring light absorption at around 750 nm or 734 nm wavelengths. The ABTS decolorization method is suitable for evaluating the antioxidant activity of both hydrophilic and lipophilic antioxidants. This versatility makes it a valuable tool for gauging the overall antioxidant potential of various substances [27].

Based on table 6, described that antioxidant activity tends to be unstable during storage. When compared to the inhibition values at 30 °C and 40 °C, the inhibition activity decreased during 12 w of storage at 40 °C storage. Moreover, the antioxidant activity between the formulas showed that the higher the number of fractions in the formula, the lower the antioxidant activity. This is possible due to the fraction's acidic nature, so the more significant fraction's content will affect the fraction's stability and other ingredients in the formula, thereby reducing antioxidant activity. At 40 °C storage temperature, antioxidant activity decreases in formulas with higher fraction content. This is possible at higher temperatures and will result in the degradation of the fraction itself.

Gel anti-aging elastase inhibitory activity

Elastase is an enzyme that plays a significant role in premature skin aging. This enzyme can catalyze the breakdown of the extracellular matrix, which provides elasticity and flexibility to various body tissues and organs [29]. The inhibition of elastase activity is centered around a reaction involving N-succinyl-(Ala)3-nitroanilide (SANA) acting as a substrate and porcine pancreas elastase serving as an enzyme. This reaction leads to the formation of p-nitroaniline, which imparts a yellow color. The concentration of p-nitroaniline can be measured through absorbance readings at 405 nm after a reaction time of 50 minutes at 25 °C [30].

In the case of Formula 1, Formula 2, and Formula 3, as written in table 6, storage at both 30 °C and 40 °C reduced the percentage of enzyme inhibition compared to the initial measurements at week 0. The decline in enzyme inhibition percentages at w 4 and 8 could be attributed to variations in the conditions of the enzymes and substrates throughout the storage period.

Moreover, the decrease in elastase inhibitory activity might also be from the degradation of specific ingredients in the preparation, particularly those compounds with anti-elastase properties. As these components degrade over time, their capacity to inhibit elastase activity could diminish, leading to reduced inhibition percentages during storage. At a storage temperature of 30 °C, the elastase inhibition activity decreased notably in formulas with higher fractions.

This trend might be explained by the increasingly acidic nature of the fractions as their concentration rises. This heightened acidity could affect the overall stability of the formulation's ingredients, subsequently impacting their elastase inhibitory activity.

Enzyme testing entails careful consideration of several critical factors, including temperature, pH, ionic strength, and the appropriate concentrations of essential components like substrates and enzymes. Ensuring the blank value remains consistent throughout the testing duration is equally crucial. Deviations in blank values, even minor ones, can notably impact variations and the overall test outcomes. Furthermore, various external factors can influence the test results. Spontaneous side reactions, oxidative processes, component instability, turbidity, or other occurrences within the test mixture can all introduce variables that affect the results. When such situations arise, it's imperative to identify the root cause and take steps to eliminate it. Unaddressed reactions can significantly alter the test mixture, especially if the sample is stored for an extended period, as is often the case in comprehensive testing scenarios [31].

Table 6: Antioxidant activity and elastase inhibition

Formula	W	DPPH (IC_{50})	FRAP (Activity)	ABTS (IC_{50})	Elastase (% Inhibition)
F1 (30 °C)	Week 0	17.33 ± 0.07^{bc}	73.78 ± 0.49^{bc}	10.09 ± 0.11^{bc}	43.25±1.27
	Week 4	16.72 ± 0.07^{ac}	74.80 ± 0.39^{a}	8.76 ± 0.07^{ac}	40.47±3.02
	Week 12	15.99 ± 0.22^{ab}	74.84 ± 0.19^{a}	9.67 ± 0.11^{ab}	37.87±3.86
F2 (30 °C)	Week 0	15.26 ± 0.15^{bc}	74.39±0.99	12.55 ± 0.08^{bc}	42.76±3.67
	Week 4	17.15 ± 0.07^{ac}	74.18±1.27	10.88 ± 0.09^{ac}	39.10±2.66
	Week 12	13.97 ± 0.15^{ab}	74.35±1.27	9.07 ± 0.07^{ab}	34.31±2.30
F3 (30 °C)	Week 0	14.56 ± 0.09^{bc}	74.63±0.98	13.56 ± 0.13^{bc}	43.87 ± 3.89^{c}
	Week 4	15.96 ± 0.11^{ac}	74.51±0.63	11.59 ± 0.07^{ac}	38.91±2.30
	Week 12	13.76 ± 0.12^{ab}	74.80±0.92	11.32 ± 0.07^{ab}	33.31 ± 2.64^{a}
F1 (40 °C)	Week 0	17.33 ± 0.07^{bc}	73.78±0.49	10.09 ± 0.11^{bc}	43.25±1.27
	Week 4	18.47 ± 0.14^{a}	74.80±0.98	8.13 ± 0.08^{ac}	41.17±1.36
	Week 12	18.32 ± 0.11^{a}	74.84±0.19	8.83 ± 0.27^{ab}	36.93±2.36
F2 (40 °C)	Week 0	15.26 ± 0.15^{bc}	74.39±0.99	12.55 ± 0.08^{bc}	42.76 ± 3.67^{c}
	Week 4	19.41 ± 0.18^{ac}	74.63±0.56	10.41 ± 0.08^{ac}	40.55±3.29
	Week 12	17.81 ± 0.06^{ab}	74.88±0.69	9.98 ± 0.08^{ab}	36.39 ± 2.87^{a}
F3 (40 °C)	Week 0	14.56 ± 0.09^{bc}	74.63±0.98	13.56 ± 0.13^{bc}	43.87±3.89
	Week 4	18.36 ± 0.10^{ac}	74.80±0.98	10.86 ± 0.09^{ac}	38.12±3.87
	Week 12	25.59 ± 0.05^{ab}	74.63±1.41	10.39 ± 0.10^{ab}	35.56±2.04

All data was shown as the mean±SD in which each treatment was repeated three times. ^{a,b,c}indicates a significant difference between the week of observation of each formula with Tukey analysis ($p<0.05$, n=3).

Determination of active Ingredient by densitometry TLC

Determination of ursolic acid content in the gel was carried out after the TLC-densitometry method had been validated. The samples whose levels will be measured are first subjected to a hydrolysis process using 1 M HCl, and then the active compound is withdrawn using the solvent ethyl acetate. The best mobile phase for determining active substance levels uses chloroform: methanol (9:1), with the standard used being ursolic acid. The plate used was a Silica Gel 60 F254 TLC plate with a sample volume of 25 µl.

Table 7: Determination of active ingredient

Week observation	30 °C			40 °C		
	FI	F2	F3	FI	F2	F3
Week 0	327.0 ± 0.00^{bcde}	317.6 ± 0.00^{bcde}	368.7 ± 0.00^{bcde}	327.0 ± 0.00^{bcde}	317.6 ± 0.00^{bcde}	368.7 ± 0.00^{bcde}
Week 12	319.9 ± 0.01^{abcd}	309.4 ± 0.00^{abcd}	372.2 ± 0.00^{abcd}	230.3 ± 0.01^{abcd}	208.6 ± 0.00^{abcd}	293.9 ± 0.00^{abcd}

All data was shown as the mean±SD in which each treatment was repeated three times. ^{a,b,c,d,e}indicates a significant difference between week observation of each formula with Tukey analysis ($p<0.05$, n=3).

The observations conducted in both week 0 and week 12 provide valuable insights, as indicated in table 7. The data showcases a trend where the levels of active substances tend to decrease more significantly at a temperature of 40 °C compared to 30 °C. This phenomenon suggests that elevated temperatures may lead to the degradation of the formula's ingredients, subsequently impacting the levels of ursolic acid within the gel preparation.

The degradation process under higher temperatures can considerably impact the stability and composition, potentially leading to a decline in the concentration of ursolic acid and other active components. Such temperature-induced degradation is a crucial consideration in cosmetic formulation and stability testing, as it directly influences their efficacy and overall quality [32].

CONCLUSION

Based on the optimization results of previous research, it is known that old *Rubus fraxinifolius* leaves extracted using the UAE method provide better performance results than younger leaves in terms of antioxidant activity and elastase enzyme inhibitory activity. The results of multilevel fractionation using the solvents n-hexane, ethyl acetate, and distilled water gave the results of the most active distilled water fraction in terms of antioxidant activity and elastase enzyme inhibitory activity; then the formulation was carried out using varying levels of the active substance of the *Rubus fraxinifolius* fraction at levels of 3%, 4%, and 5%.

From the optimization of formulation, the facial gel formulation containing a 3% distilled water fraction of *Rubus fraxinifolius* leaves has good preparation characteristics, antioxidant activity, and inhibition of the elastase enzyme. This formula also tends to be physically stable for 12 w, especially at a storage temperature of 30 °C.

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AUTHORS CONTRIBUTIONS

Sulistyowati-literature review, data curation and writing, original draft; Berna Elya-conceptualization, methodology, review and editing, supervision; Syamsu Nur-conceptualization, literature review, data curation; Raditya Iswandana-review and editing, supervision, evaluation, visualization.

CONFLICT OF INTERESTS

There are no conflicts of interest listed by the authors.

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