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A Comparative Study of Anti-elastase Activity of Extract, Extract-loaded Nanoparticle, and Serum Gels of Indonesian Bilberry Leaves

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AA and KK conceptualized the study. Authors AA, ZRH and KK performed the methodology. Authors AA, ZRH and KK did the software analysis. Author KK did data validation. Author AA did the formal analysis and investigation. Authors AA and KK searched for resources. Authors AA and KK did data curation. Authors AA and ZRH wrote and prepared the original draft of the manuscript. Author KK wrote, reviewed and edited the manuscript. Authors AA and ZRH visualized the study. Author KK supervised the study. Authors AA and ZRH did the project administration. Authors AA and KK did the funding acquisition. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To do a comparative study of the anti-elastase activity of the extract, extract-loaded gelatin nanoparticles, and serum gels of Indonesian Bilberry leaves.
Methods: Extraction of the leaves used the maceration method with 70% ethanol at a pH of 2 and dried using a rotavapor and an oven. The dry extracts were phytochemical screened and tested for specific and non-specific parameters. The synthesis of gelatin nanoparticles used the desolvation method with polymer gelatin and cross-linker glutaraldehyde, and then they were characterized. The extracts and the gelatin nanoparticles were formulated into serum gels and evaluated for physical, chemical, and anti-elastase activity at 40°C for six weeks. The extracts, nanoparticles, and formulations were assayed for anti-elastase activity at 410 nm**.**
Results: The extracts met standards, while the nanoparticles showed particle sizes of 174.7 nm, polydispersity index of 0.34, zeta potential of 2.82 mV, entrapment efficiency of 38.6%, aldimine on FTIR, and shape of stacked nanoparticles. The anti-elastase activity of the extract was 49.37 ppm, while the nanoparticles were 72.92 ppm, respectively. In addition, the serum gels of F1 were 117.92 ppm, and F2 were 142.92 ppm. F1 and F2 were stable during storage. It is still possible to enhance the activity of the serum gels, such as by increasing the addition of actives.
Conclusion: The extract, gelatin nanoparticles, and serum gels of F1 and F2 have anti-elastase activity in the potent category for the extract and nanoparticles, while moderate category for F1 and F2. The serum gels are physicochemically stable during storage.

Keywords: Vaccinium varingiaefolium; serum gels; nanoparticle; extracts; anti-elastase.

1. INTRODUCTION

Elastin is about 1000 times more flexible than collagens and is a primary extracellular matrix protein. The main function is to provide elasticity and resilience to expandable tissues such as skin, aorta, and lungs. Elastin needs to be protected using a natural or synthetic anti-elastase [1].

One plant that can be a natural medicine source is Vaccinium varingiaefolium (Blume) Miq. This plant can be named "Indonesian Bilberry" because it has the same genus of Vaccinium as Vaccinium myrtillus (Bilberry) and other characteristics. These "Indonesian bilberry" leaves contain secondary metabolites, such as flavonoids, steroids, tannins, triterpenoids, saponins, and steroids. The flavonoids, antioxidants, and anthocyanins predictably have anti-elastase activity [2,3].

The Vaccinium varingiaefolium plants are growing well near volcanic craters but have very little information, while Vaccinium mystillus has been well-known worldwide. A previous study shows that the "Indonesian Bilberry" has a potent antioxidant activity (IC50 <20 ppm). The young leaves' color is red, and the old are green. In general, plant leaves with red or purple color contain anthocyanins as sources of potent antioxidants [4].

Antioxidants can counteract free radicals and safeguard the body from deteriorating diseases, including skin aging. Antioxidants may break a reaction chain of free radical formation through H-atom donations, reactive oxygen concentration reductions, free-radical reductions at the initial step, and metal catalysts of chelating transition [5].

Nanoparticle technology has been used widely as carriers for the drug delivery of chemicals, biomolecular, and small and big molecules. Natural biomolecules, like gelatin, are an attractive alternative to synthetic polymers because of their toxicity. In general, gelatin nanoparticles provide many benefits, like compatible and degradable biologically. Also, the synthesis of gelatin nanoparticles and the encapsulation process involved moderate conditions and no toxic chemical or organic solvent uses. Gelatin nanoparticles can be synthesized using proteins and prepared using the emulsion, electrospray, and desolvation methods [6].

The active ingredient released from gelatin nanoparticles can be managed effectively by the cross-linking degree control of the gelatin molecule. For example, a sustained release containing growth factors for tissue regeneration can heal a wound. These nanoparticles delivered effectively drugs and spheroid cultures or cell

sheets because they prevent cell death caused by hypoxia [7].

Cosmetic products have a role that is rapidly evolving in our society. Their increased uses are a needed contribution to achieving personal wellness. To date, nanotechnology uses are to improve cosmetic performances in many different manners, like increasing the active ingredient entrapment efficiency and skin penetration, controlling drug delivery, enhancing physical stability, improving moisturizing capacity, and providing better UV inhibition. In using semisolid formulations on skin penetration issues, attention should be paid, especially to the adverse effects of nanoparticles [8].

This study aimed to do a comparative study of anti-elastase activity of extract, extract-loaded gelatin nanoparticles, and serum gels of Indonesian Bilberry leaves.

2.1 MATERIALS AND METHODS

2.1 Materials

Vaccinium varingiaefolium leaves were from Mount Tangkuban Parahu, North Bandung, West Java Province, Indonesia. Plant identification was at the Herbarium Depokensis (UIDEP), Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, and other materials were of pharmaceutical or analytical grades.

2.2 Methods

2.2.1 The extract preparation and characterization

The extraction of plant leaf used the maceration method for 24 hours, with the ratio of dry powder simplicia to ethanol 1 to 10 at a pH of 3. The ethanolic extract is filtered and dried in two steps at 45˚C (rotavapor) and 40˚C (oven). The assays of dry extract include specific and non-specific parameters, such as organoleptic (appearance, color, and odor), pH, solubility, moisture content, phytochemistry, heavy metal content, and antioxidant activity [2,9,10,11].

2.2.2 Gelatin nanoparticle synthesis and characterization

Synthesis of gelatin nanoparticle formulation used the below compositions: Gelatin (200 mg), purified water to dissolve gelatin (25 mL), extract (100 mg), 96% ethanol (10 mL), dimethylsulfoxide (7 mL), purified water to dissolve extract (5 mL), poloxamer 188 (300 mg), glutaraldehyde (0.3 mL), HCl solution to adjust pH, and acetone (37 mL). Under stirring at 500 rpm, the extract was placed in a co-solvent of 96% ethanol, dimethylsulfoxide, and purified water until dissolved. The gelatin was dissolved in purified water at 40°C until completely dissolved. The two solutions of gelatin and extract were mixed with the addition of poloxamer to make a clear and homogeneous solution. The solution pH was measured and adjusted to pH 3.00 by adding an HCl solution. Then, acetone was added dropwise until complete, stirred for 10 minutes, and added glutaraldehyde for 3 hours. After the nanoparticles formed, they were purified using a dialysis tubing method for 24 hours, in triplicate, freeze-dried to obtain dry nanoparticles, and then stored for further characterization. Nanoparticle characterizations include organoleptic (color, odor, shape), particle size, polydispersity index, zeta potential, moisture content, entrapment efficiency, and FTIR [7,12,13,14].

2.2.3 Serum gel formulations

Serum gel formulations contained no extracts or no gelatin nanoparticles (F0), the extracts (F1), and the extract-loaded gelatin nanoparticles (F2), respectively. The F1 and F2 contained 100 times its IC50, respectively. Other excipients in weight percent were carbomer-940 (0.5), propylene glycol (15), triethanolamine (0.5), potassium sorbate (0.1), sodium metabisulfite (0.1), green tea oil (qs), and purified water (to make 100). A step-by-step serum gel formulations were prepared using carbomer 940 (20:1), spread into pure water little by little, and left for 24 hours. The carbomer pH was set to 7 by the addition of triethanolamine. The neutral carbomer was transferred into a Beaker glass, added a small amount of water, and homogenized to form a gel base. The propylene glycol was added and mixed until homogeneous. The potassium sorbate and sodium metabisulfite in purified water were dissolved at 30 rpm for 10 minutes and added to the gel base. Each extract or the extract-loaded gelatin nanoparticles, as the actives, was added to the gel bases of F1 or F2. The actives had been dissolved previously in propylene glycol. The green tea oil was added as flavor and homogenized at 150 rpm for 15 minutes. Finally, serum gel formulations were evaluated, including organoleptic, pH, homogeneity, spreadability, viscosity, flow

properties, stability, and anti-elastase activity [15,16,17].

2.2.4 Anti-elastase assays of the extracts, nanoparticles, and serum gels

The anti-elastase assays are in several steps [18,19].

a. Chemical preparation:

There are two, namely first, 0.1 M Tris HCl buffer solution of pH 8.0 and second, N-Suc-(Ala)3-substrate solution.

b. Sample solution preparation (The extract, the extract-loaded gelatin nanoparticles, and serum gels)
c. Preparations of five working-solution concentrations use sample solutions of 1000, 500, 250, 125, and 62.5 µg/mL, and final concentrations: 66.67; 33.33; 16.67; 8.33; 4.17
d. Preparations of control (Buffer + Enzyme), test solution (Buffer + Sample + Enzyme), and blank (Buffer + Sample)
e. Measurement of visible absorption at λ 410 nm with a microplate reader (96-well microtiters) to calculate %-inhibition and look for a linear regression equation
f. IC50 value of the extract, nanoparticles, and serum gels.

3. RESULTS AND DISCUSSION

3.1 Extract Preparation and Characterization

Identification of the plant was at the Herbarium Depokensis (UIDEP) of the Department of Biology, Faculty of Mathematics and Natural Sciences at the Universitas Indonesia, and received a letter of No. 087/UN2.F3.11/PDP.02.00/2022 describing that the plant leaves belong to the Vaccinium varingiaefolium (Blume) Miq. from the Ericaceae family. Plant identification remains the primary method in identifying plants worldwide. While chemotaxonomy and DNA methods have provided great strides in changing the classifications of traditional taxonomy, however, there is no technology has supplanted botany as the primary method for this purpose [20].

The fineness degree of simplicial powder was that 100% passed through the sieve of #4, and 24.35% passed through the sieve of #18. The fineness degree might affect the compound number of the extract. [9] The extract preparation using the above method, 548.48 grams of dry simplicial powder with 5 liters of 70% ethanol, resulted in 65.75 grams thick extract (11.99% and a DER-native of 8.34). The DER-native value was the starting material (simplicia) amount used to make a unit of extract [21].

Table 1 provided the characteristics of the plant extract, namely organoleptic (color, odor, and appearance), pH, solubility in various solvents, moisture content, extract contents in water and ethanol, ash contents, phytochemistry, and heavy metal contents. The parameters are parts of specific and non-specific and extract standardization [9].

The organoleptic examination showed the dry extract was reddish brown with a specific Vaccinium varingiaefolium odor. The pH of the extract was 3.29, and the flavonoid content probably caused this low pH extracted by the 70% ethanol solvent. The anthocyanins were stable at a pH of around 3 [22,23,24].

The solubility results in solvents showed that the dry extract was soluble in 70% ethanol, 96% ethanol, propylene glycol, DMSO, and Tris HCl buffer and was slightly dissolved in acetone and difficult to dissolve in purified water. Based on this solubility data, a mix or co-solvent was required to make serum gel formulations. The extract solubilities in some solvents were stored and used in the next steps of this study [25].

The water content assay of the extract resulted in 7.06%, which was within the water content requirements of <10%. The water content of the extract should be limited because water is an appropriate medium for microbial growth [9].

Heavy metals like lead (Pb) and cadmium (Cd) were toxic. If they contaminate an extract, there would be a high risk of chronic poisoning and weakness. The analysis results provided no heavy metal content or less than the limit of detection (LOD) [9].

The anti-elastase activity of the extract was 49.37 ppm, meaning it has a potent category [26]. This result was acceptable for further processes of nanoparticles and formulations because, during the last step (formulation), the active ingredients would have concentration dilution.

Table 1. Characteristics of the plant extract

Test parameters		Results
Organoleptic:	Color	Reddish brown
	Odor	Specific
	Appearance	Thick
pH		3.29±0.11
Solubility:	in 70% ethanol	Soluble (10-30)
	in 96% ethanol	Soluble (10-30)
	in DMSO	Very soluble (<1)
	In acetone	Sparingly soluble (30-100)
	in propylene glycol	Soluble (10-30)
	in purified water	Slightly soluble (30-100)
	in Tris HCl buffer	Soluble (10-30)
Moisture content (%)		7.06+1.56
Extract content (%)	Water solub.	20.52+0.01
	Ethanol sol.	21.80+0.01
Ash content (%)	Total	6.28+0.01
	Acid insoluble	0.03+0.01
Phytochemistry:		Alkaloids: - Saponins: + Flavonoids: + Steroids: + Quinones: - Tannins: +
Heavy metal content:	Pb	Undetectable
Cd		Undetectable
Anti-elastase activity (IC_{50}, ppm)		49.37±0.94 ppm (The extract) 12.45±0.99 ppm (Elastanial, control)

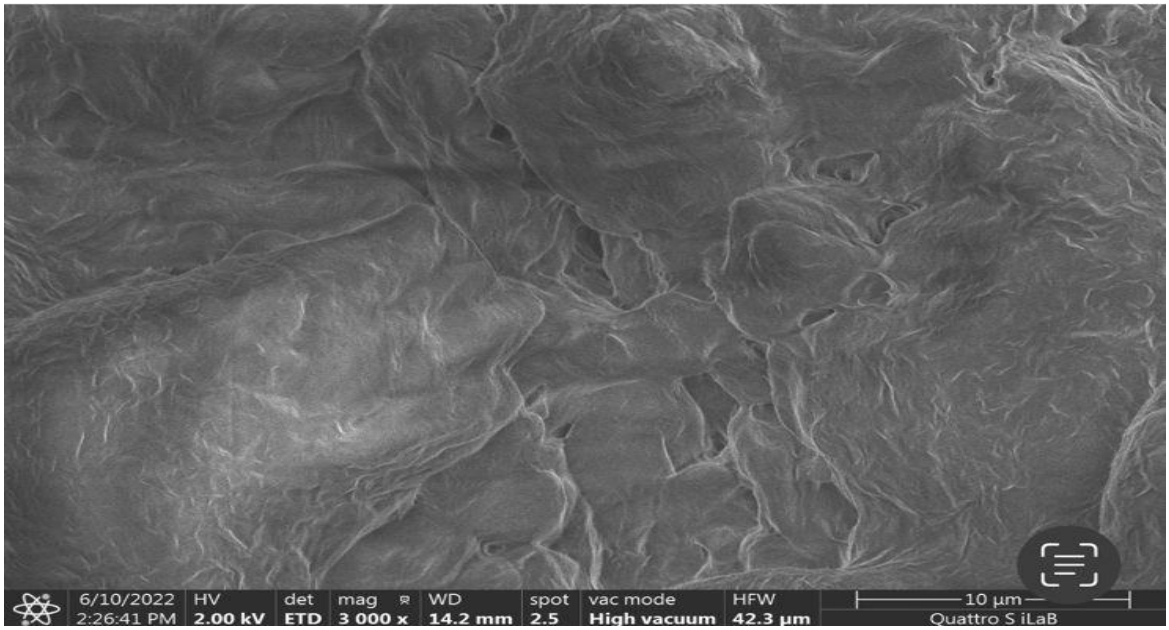

Fig. 1. Shape (morphology) of the extract-loaded gelatin nanoparticles as the lump nanoparticles using SEM

The quality parameters of the extract, such as specific and non-specific, required a standardization that could be used as a reference to determine whether the extract met the specified requirements. The Vaccinium varingiaefolium leaf extract so far had no official standardization published by the Ministry of Health or other sources, so as a reference for this study, "the general extract requirements" were used as the non-specific parameters [9].

3.2 Synthesis and Characterization of Gelatin Nanoparticles

Fig. 1 shows the shape (morphology) of the extract-loaded gelatin nanoparticles as the lump nanoparticles using SEM. However, the nanoparticle data was presented clearly in Table 2. In general, the nanoparticles form fulfill the specifications, except for entrapment efficiency and zeta potential, which are slightly low. Entrapment efficiency gave an idea about the drug percentage successfully entrapped (adsorbed) into nanoparticles. Nanoparticle characterizations included organoleptic (color, odor, shape), particle size, polydispersity index, zeta potential, moisture content, entrapment efficiency, drug loading, and FTIR [7,12].

Table 2 shows the component materials of synthesis of the extract-loaded gelatin nanoparticles. Gelatin was the polymer dissolving in water, while extract was the active dissolving in a co-solvent of purified water, ethanol, and DMSO. Poloxamer 188 was the surfactant as a stabilizer of nanoparticles that form [14]. 0.1N HCl 0.1N HCl was used to adjust the pH of the solution to 3.00. Acetone was a desolvating agent that can promote nucleation and precipitation of the polymer molecules to aggregate. The last material was glutaraldehyde as a cross-linker to react carboxylate groups with amino groups of gelatin to make the aldimine groups at a wave number of 1545 cm-1 [26].

The anti-elastase activity of the extract-loaded gelatin nanoparticles was 72.92 ppm, which was lower than that of the extract. This phenomenon was because the extract needs more time to release from gelatin nanoparticles, while the extract in non-nanoparticles dissolved faster [26].

3.3 Serum Gel Formulations

Finally, serum gel formulations were evaluated, including organoleptic, pH, homogeneity, spreadability, viscosity, flow properties, stability, and anti-elastase activity. The F1 and F2 contained 100 times its IC50 as active agents as the extract or nanoparticles. Other excipients in weight percent were carbomer-940 as a gelling agent, propylene glycol as a solvent and moisturizer, triethanolamine as a pH adjuster, potassium sorbate as an anti-microbe, sodium metabisulfite as an antioxidant, green tea oil as flavor, and purified water as a solvent [15,16,17].

Table 2. Formulation and characteristics of the extract-loaded gelatin nanoparticles including FTIR

No.	Materials	Quantity	Parameters of nanoparticles	
1	Gelatin	200 mg	Organoleptic: Color	Light brown
2	Purified water	25 mL	Odor	Specific odor
3	Plant extract	100 mg	Shape	Dry powder
4	96% Ethanol	10 mL	Particle size (nm)	174.77±0.32
5	DMSO	7 mL	Index polydispersity	0.302 ± 0.01
6	Purified water	5 mL	Zeta potential (mV)	2.82±0.39
7	Poloxamer 188	300 mg	Entrapment efficiency (%)	38.60+0.03
8	0.1N HCl	q.s	Shapes of SEM	Lump nanoparticles
9	Acetone	37 mL	Moisture content (%)	7.18% ± 0,01
10	Glutaraldehyde	0,3 mL	Solubility: in purified water	Soluble
			in propylene glycol	Soluble
			in DMSO	Soluble
			Anti-elastase activity (ppm)	72.92+0.53

FTIR Profile

No.	Bond type	Wave number (cm^{-1})	Gelatin (cm^{-1})	Extract (cm^{-1})	Nanoparticles (cm^{-1})
1.	O-H	3150-3650	3280,29	3326,98	3397,92
2.	N−H	3000-3500	3074,91	-	-
3.	C−H stretching (aliphatic)	2700-3000	2960,82	2947,92	2881,99
4.	C=O	1400-1690	1625,88	1716,65	1640,66
5.	C=N	1650-1900	1449,03	1620,27	1545,33 (Aldimine)
6.	C-H	1300-1475	1334,93	1237,83	1342,07
7.	C=C	650-1000	918,48	916,09	954,29

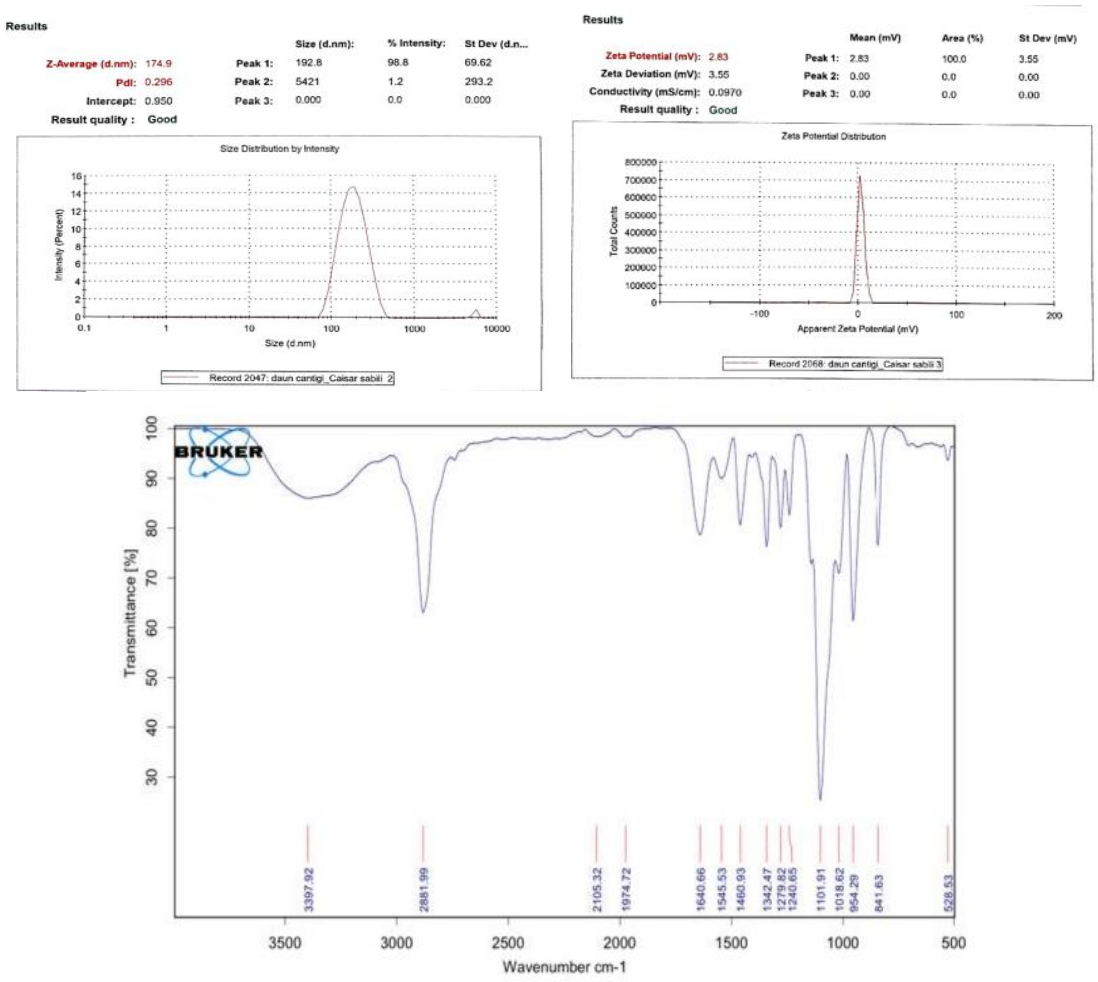

Fig. 2. Results of particle size, polydispersity index, potential zeta, and FTIR of the extract-loaded nanoparticles

The serum gel evaluation was according to their organoleptic, pH, homogeneity, viscosity, spreadability, viscosity, and flow properties. The serum gels have a semisolid form with a transparent blank color, while F1 containing the extract has a clear brownish color, and F2 with the active substance of nanoparticles has a clear yellowish color. The flavor of serum gels is a green tea oil that smells enough. The pH in the range of 5.35-5.60 meets the requirements for the skin pH range and is homogeneous. The serum gels can spread evenly to provide a better dose and effectiveness with low viscosity. The viscosity of serum gels from the highest to the lowest is the blank > F1 > F2. The viscosity of F1 and F2 decreased when compared to the blank because of the addition of active substances having low pH. The lower the pH of the active substance, the viscosity of the serum gel will decrease because it can affect the physico-chemical properties of the gelling agent used, namely carbomer forming a gel at pH 6-8. However, the viscosity of serum gels meets the specification of 500-5000 cPs. The flow property evaluation aims to determine the that of serum gels using various levels of force values, and in this study, used 4 rpm up and 3 rpm down. The flow properties are obtained by graphing the relationship between the force and speed (rpm) of the data. The results show that the blank serum gel, F1, and F2 have the plastic thixotropic flow properties. Their curves have an ascending curve on the right and a descending curve on the left, and they do not pass the (0,0) point and have yield value (Fig. 3). The serum gel spreadabilities are in the range of 3876-5109 mm2. Additions of active substances, namely the extract or the nanoparticles, increase their spreadabilities. The lower the viscosity of the serum gel, the higher the spreadability, and vice versa [15,16,17].

Table 3. The characteristics of the F0, F1, and F2 serum gel formulations at 40°C for one month

	Accelerated temperature (40°C)								
Parameters	**F0**			**F1**			**F2**		
	Week 1	**Week 3**	**Week 6**	**Week 1**	**Week 3**	**Week 6**	**Week 1**	**Week 3**	**Week 6**
Color	C	C	C	B	B	B	Y	Y	Y
Odor	Gt	Gt	Gt	Gt	Gt	Gt	Gt	Gt	Gt
Shape	Thin gel	Thin gel	Thin gel	Thin gel	Thin gel	Thin gel	Thin gel	Thin gel	Thin gel
Homogeneity	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Rheology	Plastic thixo.	Plastic thixo.	Plastic thixo.	Plastic thixo.	Plastic thixo.	Plastic thixo.	Plastic thixo.	Plastic thixo.	Plastic thixo.
Viscosity (cP)	4800 +2679	4136 +2099	3936 +1970	4236 +2485	3707 +2008	3471 +1819	4600 +2708	3939 +2282	3739 +2094
Spreadability (mm^2)	3876 +36	4390 +32	4021 +28	4602 +28	4850 +42	4622 +9	4789 +20	5109 +29	4572 +43
pH	5,60± 0,01	5,52± 0,02	5,50± 0,01	5,35± 0,02	5,25± 0,02	5,22± 0,01	5,48± 0,02	5.40± 0,01	5.37± 0,01

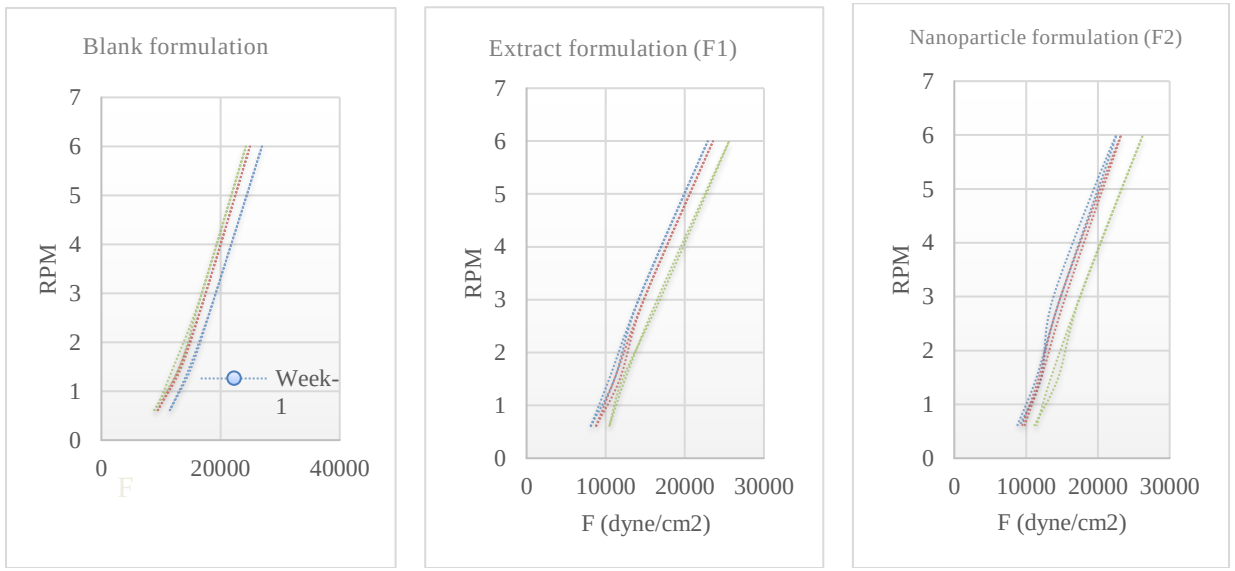

Fig. 3. The characteristics of the F0, F1, and F2 serum gel formulations at 40°C for one month

3.4 Anti-elastase Assays of the Extracts, Nanoparticles, and Serum Gels

Table 4 shows the results of anti-elastase assays of extracts, nanoparticles, and serum gels.The anti-elastase activity of the extract was 49.37±0.94 ppm, while the nanoparticles were 72.92±0.53 ppm, respectively. In addition, the serum gels of F1 were 117.92±0.63 ppm, and F2 were 142.92±0.21 ppm. F1 and F2 were stable during storage. It is still possible to enhance the activity of the serum gels, such as by increasing the addition of actives. This result is consistent with the previous study in a spray gel formulation [27,28].

Table 4. Results of anti-elastase assays of extracts, nanoparticles, and serum gels

Anti-elastase activity (IC_{50}, ppm)	
Control (Elastanial)	12.45±0.99
Extract of *Vaccinium varingiaefolium*	49.37±0.94
Gelatin nanoparticles	72.92±0.53
Serum gel F1	117.92±0.63
Serum gel F2	142.92±0.21

4. CONCLUSION

In conclusion, the extract, extract-loaded gelatin nanoparticles, and serum gels of F1 and F2 have anti-elastase activity in the potent category for the extract and nanoparticles, while moderate category for F1 and F2. The serum gel formulations are physically and chemically stable during storage. Increasing the activity of the serum gels is possible by adding the extract concentration.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been u0sed during writing or editing of manuscripts.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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