FORMULATION AND CHARACTERIZATION OF SERUM COLLAGEN OF SEA CUCUMBER EXTRACT STICHOPUS HORRENS AS AN ANTIOXIDANT

SOFI NURMAY STIANI1*, TARSO RUDIANA2, YUSUB SETIAWAN3, ETI SETYOWATI3, SOFIAN ANSORI4

1Sekolah Tinggi Ilmu Kesehatan Salsabil, Serang Banten, Jalan Raya Serang-Pandeglang KM 06, Curug Serang Banten 42211, Indonesia,
2Chemistry Study Program Faculty of Science, Pharmacy and Health, Universitas Mathla’ul Anwar Banten, Jalan Raya Labuan KM 23, Saketi Pandeglang Banten 24273, Indonesia, 3Pharmacy Study Program Faculty of Science, Pharmacy and health Universitas Mathla’ul Anwar Banten, Jalan Raya Labuan KM 23, Saketi Pandeglang Banten 24273, Indonesia, 4Fish Residues and Medicine Laboratory Fish Disease and Environment Examination Center (LP2IL) Serang, Banten 42167 Indonesia
Email: sofia240586@gmail.com, arso.rudiana@gmail.com

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ABSTRACT

Objective: The study was aimed to extract, formulate and characterize collagen extract of Stichopus horrens into serum preparations and decide antioxidant activity in powders and serum preparations.

Methods: The sea cucumber meat was extracted collagen in three stages, namely the pretreatment stage using 0.3 M NaOH solution 1:10 (w/v) for 48 h, the hydrolysis step in the 0.3 M 1:10 acetic acid solution (w/v) for 48 h, and the extraction stage in distilled water 1:2 (w/v) for 2 h at 45 °C. The collagen extract was freeze-dried to obtain collagen powder. Collagen powder was characterized by HPLC and its antioxidant activity was determined using the DPPH method. Collagen powder formulated with extract variation of 0, 0.5 and 1%.

Results: The results showed that collagen powder had a % yield of 0.24%, which consisted of the amino acids glycine, proline, alanine, and glutamic acid as the dominant amino acids. The % Free radical inhibition of collagen powder at concentration of 5000 ppm was 63.23%. IC50 values were obtained at 4045.37 ppm. The stability test resulted in stable serum preparations without significant changes at 4 °C and 27 °C±2 °C storage temperatures.

Conclusion: The measurement of DPPH Radical reduction activity in the highest serum preparation was 1% extract with a value of 2.4%.

Keywords: Serum, Collagen, Stichopus horrens, Antioxidant

INTRODUCTION

Currently, the use of medicinal ingredients is not only derived from plants but marine life is also being developed because it has great potential as a source of medicinal ingredients. Sea cucumbers are one of the most potential marine resources. Approximately 53 species of sea cucumbers are found in Indonesia, there are 22 species that can be consumed, and 8 of them have a high market value. The 8 species are sand sea cucumbers (Holothuria scabra), milk sea cucumbers or koro (Holothuria nobilis and Holothuria fuscogilva), rock sea cucumbers (Actinopyga echinites), biliao sea cucumbers (Actinopyga lecanora), lotong sea cucumbers (Actinopyga miliaris), cat’s eye sea cucumber (Bohadschia argus) and pineapple sea cucumber (Thelenota anaana) [1].

Sea cucumbers have complete nutritional content due to being referred as seabed ginger and are used as supplements. The results of laboratory analysis showed that the dried sea cucumber extract contained up to 86.8% protein, 80% collagen, minerals, mucopolysaccharides, glucosaminoglycans (GAG), natural anti-septics, chondroitin, omega 3, omega 6, and omega 9, as well as various amino acids [2].

The active ingredients in various types of sea cucumbers have been reported in various publications, including the antibacterial activity of the sea cucumber Cucumaria frondosa was detected in extracts mainly coelomocytes and eggs from Cucumaria frondosa and potential for discovery novel antibiotics [3], isolate from sea cucumber Psolus patogencus are glycocide and patagonicocide A have the antifungal activity against fungus Cladosporium cucumerinum [4], isolation of arginine kinase enzyme in sea cucumber Stichopus japonicus was success cloned the gene for sea cucumber into an E. coli expression vector purified functional enzyme [5], the activity of serum amyloid A in sea cucumber Holothuria glaberrina can activation of the immune system [6], glycoside structure in sea cucumber Stichopus mollisto classify S. mollis in the new genus Australostichopus levin [7], and isolation of fucan sulfite in sea cucumber Stichopus japonicus as an inhibitor of osteoclastogenesis [8].

Previous research regarding hydrolyzed collagen derived from golden sea cucumbers has an antioxidant effect (IC50 of 5.25±0.15 mg/ml) [9]. Other studies confirm that sea cucumbers are a source of collagen and need further research [10]. Collagen plays an important role in the food, cosmetic and pharmaceutical industries [11]. Collagen which is a connective tissue in bones and skin, can be used for skin beauty and can increase the regeneration of dead cells due to wounds so that it can accelerate healing. Therefore, sea cucumber extract can be used as a cosmetic ingredient and ointment to heal wounds [2].

Collagen is used in the cosmetic field as an active ingredient in skin care products with the function to increase skin moisture, prevent wrinkles, keep the skin from the bad effects of radiation and maintain elasticity. Collagen is a fibrillar protein and is suitable for connective tissues in the human body, both skin, joints, and bones. Due to its abundance in the body, its strength and relationship are directly proportional to skin aging. Collagen fibers are damaged over time, losing thickness and strength, which are closely related to the phenomenon of skin aging and aging [12]. The addition of collagen in cosmetic formulations is intended to replace damaged collagen due to environmental influences and age factors. The specialty of using collagen is related to the physicochemical characteristics of collagen, including non-toxicity, low antigenicity, biocompatible and biodegradable, making collagen the main source in medical applications [13]. Cosmetics have developed rapidly into various dosage forms that function to increase user comfort. One form of cosmetic dosage has been widely developed is topical serum. Serum is a gel with a lower viscosity. Serum has the advantage that it can provide a more comfortable effect and is easier to spread on the surface of the skin because its viscosity is not too high [14]. Based on this description, no research has been found regarding the serum from this sea cucumber using Stichopus horrens collagen as an antioxidant serum. This study aims to extract, formulate and
characterize Stichopus horrens collagen extract into serum preparations that meet SNI requirements and decide antioxidant activity in powder and serum dosage forms.

MATERIALS AND METHODS

The materials and tools used are knife, analytical balance, beaker, erlenmeyer, stirrer, volumetric pipette, measuring cup, stirring rod, filter cloth, freeze dryer, spatula, test tube, micropipette, conical tube, vial, vortex, oven, microtube, pH meter, rotary viscometer (NDJ 5S), UV-Vis Spectrophotometer (ShimadzuUVmini-1240 Japan), HPLC (Water Corporation USA), and IR spectrophotometry (Bruker Tensor 37 German). Stichopus horrens from Sangiang beach, aquadest, DPPH (1,1-diphenyl-2-picrylhydrazyl), 0.3 M NaOH, 0.3 M acetic acid, methanol, natrosol, glycerin, DMDM hydantoin, ethoxydiglycol.

Identification of sea cucumber Stichopus horrens

The identification of Stichopus horrens; sea cucumber was carried out at the School of Biological Science and Technology, ITB, Labtek V C Building Forestry Building, Jl. Let. Gen. retired. Dr. (Hc) Mashudi No.1, Sayang, Jatinangor, Sumedang Regency, west java.

Collagen extraction

Collagen extraction was carried out in three stages, namely the pretreatment stage using sodium hydroxide solution, the hydrolysis stage in acetic acid solution, and the extraction stage with distilled water. Sea cucumber meat of Stichopus horrens as much as 1000 g was soaked in 0.3M sodium hydroxide with a ratio of meat to solution 1:10 (w/v) for 48 h to remove non-collagen protein then filtered and the filtrate is neutralized with water to a neutral pH. The filtrate soaked in 0.3 M acetic acid with a ratio of meat to solution 1:10 (w/v) for 48 h, followed by the neutralization process again until the pH was neutral. Last step was extraction using distilled water for 2 h at 45 °C with a ratio of 1:2 (w/v) sample to solvent. The results of extraction are obtained in the form of liquid collagen which is then freeze-dried [15].

Characterization using HPLC and FTIR

The amino acid composition was determined by HPLC (AOAC 1995) and FTIR analysis. The HPLC instrument was rinsed with eluent to be used for 2-3 h. The syringe that will be used is rinsed with distilled water. Amino acid analysis using HPLC consisted of 4 stages, namely:

a. Making protein hydrolyzate

The sample was weighed as much as 0.1 grams and crushed, the crushed sample was added with 5-10 ml of 6 N HCl. The solution was heated in an oven at 100 °C for 24 h. This is done to remove gas or air present in the sample so as not to disturb the resulting chromatogram. After heating, the protein hydrolyzate was filtered using a millipore measuring 45 microns.

b. Drying

Drying The filter results were taken as much as 10 µl and added 30 µl of drying solution. Dryer solution made from a mixture of methanol, sodium acetate, and trimethylamine in a ratio of 2:2:1. After the sample is dried with a vacuum pump to speed up the process and prevent oxidation.

c. Derivatization

The derivatization solution was prepared from a mixture of methanol, picoiodothiocyanate, and trimethylamine solutions in a ratio of 3:3:4. The derivatization process is carried out so that the detector is easy to detect the compounds present in the sample. Further dilution was carried out by adding 10 ml of 60% acetonitrile and 1 M sodium acetate and then left for 20 min, filtered again using a millipore measuring 45 microns. 30 µl of derivatization solution was added to the drying product.

d. Injection

The filter results were taken as much as 20 µl to be injected into the HPLC FTIR analysis to determine the typical functional groups of collagen. 100 mg KBr and 2 mg of the test sample were ground until smooth and well mixed in a mortar and then molded into a pellet mold. Measurements were made with the FTIR tool at wavenumbers between 4000-500 cm-1.

Antioxidant activity test

Antioxidant activity is decided using the DPPH method. 10 mg of sample was dissolved in methanol p. a then made in series with concentrations 5000, 4000, 3000, 2000, and 1000 ppm. Each concentration series was inserted into the microplate as much as 160 µl added to the DPPH solution (0.3 mg/ml) each 40 µl. The DPPH solution was prepared by dissolving 3 mg of DPPH into 10 ml of methanol p. a. Sample blanks were made by inserting 160 µl of sample into the well and adding 40 µl of methanol p. a. Negative control was made by adding 160 µl of methanol p. a with 40 µl of DPPH and 200 µl of methanol p. a as a blank. Ascorbic acid was used as a positive control with a concentration series of 4.2; 1; and 0.5 ppm. The microplate was incubated at room temperature for 30 min and the absorbance was read using a UV-Vis spectrophotometer at a wavelength 517 nm.

Serum preparation formula and test the antioxidant activity

The serum was formulated by adding Stichopus horrens collagen extract with a concentration of 0.5% and 1% into the serum formula with a Natrosol concentration of 0.75%. Testing of antioxidant activity was carried out using the DPPH method. The DPPH solution was made by weighing 0.002 g of DPPH then dissolved in 100 ml of methanol p. a with 40 µl of DPPH and 200 µl of methanol p. a as a blank. Ascorbic acid was used as a positive control with a concentration series of 4.2; 1; and 0.5 ppm. The microplate was incubated at room temperature for 30 min and the absorbance was read using a UV-Vis spectrophotometer at a wavelength 517 nm.

Table 1: Serum dosage formulation

| Material                | Use                   | Composition (%) |
|-------------------------|-----------------------|-----------------|
| Collagen Extract        | Active substances     | F I             |
| Stichopus horrens       |                       | 0.5 g           |
| Natrosol                | Gelling agent         | 0.75 g          |
| Glycerine               | Humectants            | 0.75 g          |
| DMDM hydantoin          | Preservative           | 10 ml           |
| Ethoxydiglycol          | Emulsifier             | 10 ml           |
| Aquadest                | Solvent               | 2 ml            |

Table 2: Serum dosage formulation

| Material                | Use                   | Composition (%) |
|-------------------------|-----------------------|-----------------|
| Collagen Extract        | Active substances     | F I             |
| Stichopus horrens       |                       | 0.5 g           |
| Natrosol                | Gelling agent         | 0.75 g          |
| Glycerine               | Humectants            | 0.75 g          |
| DMDM hydantoin          | Preservative           | 10 ml           |
| Ethoxydiglycol          | Emulsifier             | 10 ml           |
| Aquadest                | Solvent               | 2 ml            |

Physical evaluation of Stichopus horrens collagen extract serum preparations

Physical evaluation and stability tests of serum preparations include organoleptic, homogeneity, pH, viscosity. Then the antioxidant activity test was carried out on serum preparations of Stichopus horrens collagen extract using the DPPH method (positive control of vitamin C). The stability of the preparation was evaluated at 4 °C and 27 °C for one month by observing organoleptic, homogeneity, pH, and viscosity measurements.

1) Organoleptic test

Organoleptic tests can be observed by determining changes in color, odor and texture that occur during 1 mo of storage [16].
2) Homogeneity

The serum preparation of *Stichopus horrens* collagen extract was placed between two slides and then observed for the presence of coarse particles or inhomogeneities under light [16].

3) pH measurement

pH measurement using a digital pH meter.

4) Viscosity

Viscosity measurements were carried out using a rotary viscometer with a rotational viscometer type (NDJ 5S). The preparation is put into a beaker and the appropriate spindle is lowered to the limit of the spindle immersed in the preparation, then the motor and spindle are turned on. The rotational speed is set at 60 rpm. The viscosity number shown on the display screen shows the viscosity value.

RESULTS

The sea cucumber samples used in this study were identified at the SITH ITB Zoological Museum. Based on the results of No. 1272/11. CO/2/11/2019 the analysis of the types of sea cucumbers in this sample is *Stichopus horrens*, family Stichopodidae.

The collagen extract obtained was in the form of a cloudy white collagen solution and a freeze dryer process was carried out so that the solid form was brownish in color with a cotton-like texture with a yield of 0.24%. The extraction process is carried out by soaking with NaOH solution, causing sea cucumber meat to expand (swelling) so that it can dissolve non-collagen proteins in sea cucumber meat [17]. The addition of acetic acid will break the cross-linked strands in the collagen so that it dissolves the non-cross-linked collagen and the collagen can dissolve completely [18].

(a)    (b)

Fig. 1: a. Liquid extraction of collagen b. Collagen extract after freeze dryer

![HPLC chromatogram of sea cucumber collagen extract Stichopus horrens](image)

Fig. 2: HPLC chromatogram of sea cucumber collagen extract *Stichopus horrens*

Qualitative and quantitative analysis of amino acids using HPLC instruments. The results of the amino acid analysis of *Stichopus horrens* sea cucumber extract obtained 15 amino acid peaks consisting of 9 types of essential amino acids and 6 types of non-essential amino acids. The essential amino acids found in *Stichopus horrens* sea cucumbers are isoleucine, leucine, lysine, methionine, phenylalanine, histidine, threonine, valine, and arginine. The non-essential amino acids analyzed were aspartate, serine, glutamate, glycine, alanine, and tyrosine. The basic molecule of collagen is formed from three polypeptide chains that are twisted together to form a triple helix structure with a typical amino acid arrangement, namely Gly-XY, at position X is proline and Y is hydroxyproline. Interpretation peak can be seen in table 2.

Based on table 2 shows that among the 15 amino acids obtained, three types of essential amino acids are dominated. There are arginine 3.76%, leucine 1.27% and threonine 1.51%, besides non-essential amino acids are dominated by glycine 8.29%, proline 3.04%, alanine 3.09% and glutamic acid 4.09%. This study is in accordance with the results of previous research [19] who reported that glycine is an amino acid that is dominant in collagen and all families. Collagen is characterized by the presence of repeats of the amino acid sequence Gly-X-Y. Amino acids in the form of proline and hydroxyproline are important for the structural integrity of collagen because they play an important role in the bond formation of intramolecular hydrogen [20].
### Table 2: Composition of collagen amino acid content of sea cucumber *Stichopus horrens*

| Amino acid | Retention time (Rt) | Concentration (%) |
|------------|---------------------|------------------|
| Aspartic acid | 6.799 | 2.660 |
| Glutamic acid | 7.377 | 4.890 |
| Glycine | 6.198 | 8.290 |
| Histidine | 4.400 | 0.300 |
| Arginine | 5.946 | 3.760 |
| Threonine | 7.798 | 1.510 |
| Alanine | 8.354 | 3.090 |
| Proline | 9.126 | 3.040 |
| Tyrosine | 10.370 | 0.750 |
| Valine | 10.896 | 1.070 |
| Methionine | 10.801 | 0.330 |
| Isoleucine | 11.759 | 0.870 |
| Leucine | 11.852 | 1.270 |
| Phenylalanine | 11.957 | 0.960 |
| Lysine | 10.370 | 0.470 |

FTIR absorption peaks showed *Stichopus horrens* collagen has a distribution of absorption peaks at several wavenumbers indicating certain functional groups. The results of the analysis can be seen in table 3.

### Table 3: IR absorption of *Stichopus horrens* sea cucumber collagen extract

| Wavenumber (cm⁻¹) | Absorption region (cm) | Group | Type of compound |
|------------------|------------------------|-------|------------------|
| 3305.23          | 3300-3500              | OH    | Alcohol          |
| 2919.11          | 2850-3000              | C-H   | Alkanes          |
| 1640.91          | 1640-1680              | C=C   | Alkene           |
| 1540.42          | 1500-1600              | C=C   | Aromatic         |
| 1228.39          | 1180-1360              | N-H   |                  |
| 1163.28          | 1080-1300              | C=O   | Amine, aldehydes, esters, carboxylic acids and ketones |

The functional group can be known by FTIR spectroscopy analysis of collagen extract of *Stichopus horrens* sea cucumbers in table 3, showing the absorption peaks of amide A, amide B, amide I, amide II and amide III (fig. 3). Amide I has an absorption region in the range of 1600-1690 cm⁻¹ which shows the stretching vibration of C=O. The frequency of the amide I wavenumber is related to the secondary structure of the protein. Amide I consists of four components of the secondary structure of proteins there are-helix,-sheet,-turn, and random coils that overlap each other [21].

Percentage inhibition of free radical activity of the collagen extract of *Stichopus horrens* sea cucumber was carried out at a concentration series of 5000, 4000, 3000, 2000, 1000, 500 ppm with DPPH solution as a radical source. The test results can be seen in table 4.

### Table 4: Reduction activity of DPPH radical

| Sample           | Concentration (ppm) | Absorbance (ppm) | % Inhibition |
|------------------|---------------------|------------------|-------------|
| Extract          | 5000                | 0.076            | 69.230      |
| Collagen         | 4000                | 0.132            | 46.560      |
| Sea cucumber     | 3000                | 0.167            | 32.390      |
| *Stichopus horrens* | 2000              | 0.217            | 12.150      |
| Vitamin C        | 1000                | 0.254            | 86.046      |
| 4                | 0.012               |                  | 57.747      |
| 2                | 0.086               |                  | 20.175      |
| 1                | 0.182               |                  |             |
| 0.5              | 0.228               |                  |             |
The results of the serum preparation of *Stichopus horrens* collagen suppress oxidative reactions [23]. Hydrophobic amino acids (THAA) have high antioxidant activity to by the amino acid content. Peptides containing total high antioxidant activity. The difference in IC50 value is influenced by the amino acid content. Peptides containing total high antioxidant activity. The difference in IC50 value is influenced by the amino acid content. Peptides containing total high antioxidant activity. The difference in IC50 value is influenced by the amino acid content. Peptides containing total high antioxidant activity. The difference in IC50 value is influenced by the amino acid content.

The results of the serum preparation of *Stichopus horrens* collagen extract, formula A produces a transparent color because, without extract, formula B is light brown with 0.5% collagen extract and formula C produces a dark brown serum with a concentration of 1% collagen extract (fig. 4).

Stability testing was carried out by storing the serum preparation of *Stichopus horrens* collagen extract at a low temperature of 4 °C and 27 °C at room temperature for 1 mo. Observation of the stability test included organoleptic examination, homogeneity, pH, and viscosity.

![Fig. 4: Results of serum preparations of *Stichopus horrens* collagen extract](image)

**Table 5: Formulation of *Stichopus horrens* collagen extract**

| Time   | Temperature (°C) | Formula 0%          | Formula 0.5%         | Formula 1%          |
|--------|------------------|----------------------|----------------------|----------------------|
|        |                  | Color | Smell texture | Fragrant | Color | Smell texture | Fragrant | Color | Smell texture | Fragrant |
| Week I | 4                | Transparent | More viscous, Smooth | Slightly thick, Slightly brown, Smelled | Light | Thicker, Smoother | Typical | Dark | Thicker, Brown | Typical of the sea |
|        | 27               | Transparent | Smooth | Slightly thick, Brown | Smelled | Light | Thicker, Smoother | Typical | Dark | Smoother | Typical of the sea |
| Week II| 4                | Transparent | More viscous, Smooth | Slightly thick, Slightly brown, Smelled | Light | Thicker, Smoother | Typical | Dark | Thicker, Brown | Typical of the sea |
|        | 27               | Transparent | Smooth | Slightly thick, Brown | Smelled | Light | Thicker, Smoother | Typical | Dark | Smoother | Typical of the sea |
| Week III| 4               | Transparent | More viscous, Smooth | Slightly thick, Slightly brown, Smelled | Light | Thicker, Smoother | Typical | Dark | Thicker, Brown | Typical of the sea |
|        | 27              | Transparent | Smooth | Slightly thick, Brown | Smelled | Light | Thicker, Smoother | Typical | Dark | Smoother | Typical of the sea |
| Week IV| 4                | Transparent | More viscous, Smooth | Slightly thick, Slightly brown, Smelled | Light | Thicker, Smoother | Typical | Dark | Thicker, Brown | Typical of the sea |
|        | 27              | Transparent | Smooth | Slightly thick, Brown | Smelled | Light | Thicker, Smoother | Typical | Dark | Smoother | Typical of the sea |

Homogeneity test is an important factor for uniformity of content and patient comfort in using the preparation. Based on the results of these observations do not appear coarse grains (table 6-9).

**Table 6: Results of homogeneity of pH at 4 °C and 27 °C**

| Formulation | Temperature (°C) | Week I | Week II | Week III | Week IV |
|-------------|------------------|--------|---------|----------|---------|
| Formula 0% (Base) | 4 | homogeneous | homogeneous | homogeneous | homogeneous |
|              | 27 | homogeneous | homogeneous | homogeneous | homogeneous |
| Formula 0.5% | 4 | homogeneous | homogeneous | homogeneous | homogeneous |
|              | 27 | homogeneous | homogeneous | homogeneous | homogeneous |
| Formula 1%  | 4 | homogeneous | homogeneous | homogeneous | homogeneous |
|              | 27 | homogeneous | homogeneous | homogeneous | homogeneous |

**Table 7: Results of pH measurements at 4 °C and 27 °C**

| Formulation | Temperature (°C) | Week I pH±SD | Week II pH±SD | Week III pH±SD | Week IV pH±SD |
|-------------|-----------------|-------------|-------------|-------------|-------------|
| 0%          | 4 | 7.3±0.01 | 7.3±0.01 | 7.3±0.01 | 7.4±0.02 |
|              | 27 | 6.8±0.02 | 6.4±0.01 | 6.1±0.01 | 6.0±0.02 |
| 0.5%        | 4 | 6.9±0.01 | 6.9±0.01 | 6.8±0.02 | 6.7±0.02 |
|              | 27 | 6.4±0.01 | 6.2±0.01 | 5.6±0.02 | 5.5±0.01 |
| 1%          | 4 | 6.8±0.01 | 6.7±0.01 | 6.6±0.02 | 6.4±0.01 |
|              | 27 | 6.3±0.01 | 6.1±0.01 | 5.9±0.02 | 5.7±0.01 |

Data represented as mean±SD (n=2) *SD= standard deviation
DISCUSSION

The sea cucumber samples were identified with the aim of proving the correctness of the materials used in the study. Fresh sea cucumber meat *Stichopus horrens* was cut into small pieces to facilitate the extraction process. The smaller the sample size, the solvent will be easily penetrated into the cells due to the target compound being extracted and dissolved in the solvent. The deproteinization process uses 0.3 N NaOH, which is to remove non-collagen proteins, fats, minerals, and pigments present in sea cucumber meat. During immersion in NaOH allows water to enter and causes non-collagenous proteins trapped in the collagen matrix to be more easily released. After pretreatment, the samples were washed with distilled water until the pH was close to neutral. The purpose of heating in the final stage is to hydrolyze collagen before formulation so that it has the opportunity to have good antioxidant activity.

serum. Based on the results of pH measurements, it can be observed that the longer storage, the pH decreases. The decrease in pH can be caused by due to the presence of ionic contamination from the materials used in the formulations, either ion positive or negative ions that can affect acidity or basicity preparation. The serum pH in this experiment all formulas met the quality standard of SNI 16-4399-1996 for serum or sunscreen preparations was 4.5-8. The decrease or increase in the value of the viscosity can be caused by the influence of temperature, which causes a change in the polymer structure of the dosage base becomes more tenuous or denser so that more serum preparation is thick from the initial preparation. The effect of adding collagen extract also increases viscosity. The viscosity required by SNI is 2000-5000 cPs, in this experiment, the formula with the addition of 1% active substance meets the viscosity requirements.

CONCLUSION

Collagen powder had a % yield of 0.24%, which consisted of the amino acids glycine, proline, alanine, and glutamic acid as the dominant amino acids. Collagen extracts can be used as serum preparations that meet SNI requirements. Collagen powder has a higher activity % Free radical inhibition at a concentration of 5000 ppm at 63.2% with IC50 value is 4045.37 ppm when compared to serum preparations with an extract concentration of 1% at 2.4%.

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