The correlation between total protein content and antioxidant activity of collagen isolated from a marine sponge *Stylissa flabelliformis* collected from North Lombok Indonesia coast

The correlation between total protein content and antioxidant activity of collagen isolated from a marine sponge *Stylissa flabelliformis* collected from North Lombok Indonesia coast

A L Sunarwidhi[1]*, A Rosyantari[1], E S Prasedya[2], N Ardiana[2], B T K Ilhami[2], A S Abidin[2], Y Ambana[2], I A P Kirana[2], D G Wirasisya[1], W Anugrah[1], R D Fersiyana[1], N M A R Dewi[1]

[1]Department of Pharmacy, Faculty of Medicine, University of Mataram, Indonesia
[2]Bioscience and Biotechnology Research Centre, Faculty of Mathematics and Natural Sciences, University of Mataram, Indonesia

*Corresponding author: anggit.sunarwidhi@unram.ac.id

Abstract. Collagen is a fibrous protein that has recently gained high attention from the pharmaceutical industry due to its benefits on the skin. Collagen can be isolated from various resources including marine sponges. Marine sponges are found in a large amount in Indonesia and has not been widely explored for its pharmacology benefits. Here we isolate collagen from a marine sponge *Stylissa flabelliformis* found in North Lombok Indonesia coast. The isolation of collagen was performed followed by total protein content analysis using modified Bradford protein assay and antioxidant activity measurement using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The total yield of the collagen isolate obtained was 3.5% and it had a total of 0.755mg/ml protein. DPPH assay has shown that the collagen isolate had antioxidant activity with an IC_{50} of 61.5±2.132 ppm. Based on Spearman correlation assay, the antioxidant activity was found to be correlated with the protein content of the isolate (r value=0.8). These results show the potency of using the collagen isolated from marine sponge *Stylissa flabelliformis* for further antioxidant benefits.

Keywords: Marine collagen, Marine sponge, Total protein, Antioxidant

1. Introduction

Collagen represents the most abundant part of the skin's extracellular matrix, which determines the skin physiology by maintainence of the skin structure [1, 2]. As the skin ages, collagen production decreases, especially collagen type I and III, which may be caused by the aging of cellular fibroblast and also the defect of mechanical stimulation [3]. Collagen degradation is also increased in aged and damaged skin caused by the increased expression of the degradation enzymes [4]. To address issues related to skin aging, the use of additional collagen supplementation has now gained a wide interest due to its benefits on the skin, including improvement of skin hydration, skin elasticity, skin roughness, skin density, and reduction of skin wrinkling [5, 6]. Collagen intake has also shown benefits in increasing the expression of skin collagen and also suppression of matrix metalloproteinase 2 activity which is responsible for collagen degradation [7]. Up until now, collagen has been collected from various sources including bovine and porcine origins [8, 9]. Unfortunately, collagen collected from bovine or porcine comes with several disadvantages including the risk of transmissible diseases such as bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE) [10, 11]. Moreover, it might also not be suitable for certain population with vegetable-based diet or non-porcine-based diet due to religious reasons [12]. Due to these issues, marine collagen has become a more preferable choice. Marine collagen are available in a high content in various sources of marine organisms, including fish, jellyfish, sharks, starfish, and sponges [13-18].

Indonesia is a country with high biodiversity of the marine ecosystem. One marine resource that is found abundantly in Indonesia especially Lombok coast is marine sponges. Among the marine organisms, marine sponges has gained high attention due to its various medical properties [19]. It is also a rich source of collagen [14] which brings a lot of benefit to health as mentioned above.
Stylissa flabelliformis is a marine sponge species that could be easily found in North Lombok Indonesia coast, but it is still underutilized. To address this issue, this study was conducted as a preliminary study for the development of *Stylissa flabelliformis* as an alternative source of collagen for cosmetic and pharmaceutical products. In order to understand the characteristic of collagen from *Stylissa flabelliformis* collected from North Lombok coast Indonesia, we observed the total protein content and antioxidant activity, and in additional, we also analysed the correlation between them.

2. Materials and Methods

2.1 Sponge collection and characterization

Marine sponge *Stylissa flabelliformis* was collected from North Lombok District, West Nusa Tenggara, Indonesia (8°27'47.3"S 116°02'08.1"E), and was rinsed with seawater to clean any debris from the sample. The clean marine sponge sample was then preserved in 50% ethanol before further analysis. The sponge was also observed under microscope to evaluate the type of the sponge spicule.

2.2 Collagen isolation

Collagen was isolated by using the previous method in the literature [20] with modification. 50g of ethanol-conserved sponge was washed three times under running tap water, then it was cut into small pieces and homogenized. The homogenized sponge was then soaked in 250ml of 100mM Tris–HCl buffer (pH 9.5; 10mM EDTA; 8M urea; 100mM 2-mercaptoethanol). Sodium hydroxide (Sigma Aldrich, UK) was added to this solution to increase the pH of the solution to 9. This solution was then stirred for 24h continuously at room temperature. After 24h, the viscous extract was centrifuged again (5000xg; 5mins; 2°C). The obtained pellet was then discarded followed by collagen precipitation from the supernatant. This was achieved by adjusting the pH to 4 with acetic acid (Sigma Aldrich, UK). Collagen was then collected by centrifugation (20,000xg; 30mins; 2°C). The pellet from centrifugation was then resuspended in distilled water, centrifuged (20,000xg; 30mins; 2°C) again and lyophilized for preservation. Isolated collagen was then diluted in different solvents, including water, acetic acid, sulfuric acid, acetone, hydrogen peroxide, dimethyl sulfoxide (DMSO), and methane sulfonic acid, to observe its solubility. All solvents used in the solubility assay were from Sigma Aldrich, UK.

2.3 Collagen total protein measurement

The total protein of the collagen was measured by using modified Bradford assay. A serial dilution of bovine serum albumin (BSA) with water and 0.0035% of SDS was prepared for the standard curve. 20µL of isolated collagen was mixed with 1mL of solution B (dH_2O and Bradford reagent), and then the absorbance was read at 595nm using a multiplate reader (Multiscan Go Thermoscientific, UK).

2.4 Collagen antioxidant activity assessment

The antioxidant activity of the collagen was assessed by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay. DPPH Assay was performed based on [21] method with modification. The collagen isolate was diluted into a serial dilution (10µL, 20µL, 30µL, 40µL, 50µL, 100µL, 250µL, 300µL and 400µL). 100µL of the sample and blank (ethanol) was added to 100µL of 200µM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (Sigma Aldrich, UK).
This was done in triplicates and the antioxidant activity was calculated using formula 1.

$$\%Inhibition = \frac{(Acontrol - Asample)}{Acontrol}\ x\ 100 \quad (1)$$

2.5 *Data analysis*

All experiments were performed in triplicates. The correlation between the total protein of collagen and the antioxidant activity of the isolated collagen was analysed by Spearman correlation assay.

3. Results and Discussion

Due to its moisturizing and anti-ageing ability, collagen has been used in cosmetic creams and used as supplementation in the pharmaceutical industry [22-24]. Collagen used in pharmaceutical industry has

been sourced from bovine or porcine, which has several limitations [8-12]. These limitations increased the demand of alternative sources of collagen. Here we study an alternative source of collagen from a marine sponge named *Stylissa flabelliformis* collected from North Lombok Coast. This study would be a pilot study to initiate the development of the marine collagen for skin health purpose.

In North Lombok Indonesia coast, various marine sponge species was present in the area, however the marine sponge *Stylissa flabelliformis* was chosen for this study due to the abundance of this marine sponge in this area, which makes it a convenient and suitable choice for further pharmaceutical and cosmetic developments. Moreover, *Stylissa flabelliformis* belongs to the class of Demospongiae, which is known to have high amount of collagen [25].

3.1 Collection of Stylissa flabelliformis and collagen isolation

Marine sponge *Stylissa flabelliformis* was collected from North Lombok Indonesia coast followed by preservation in 50% ethanol, before further analysis. The collected marine sponge was then partially characterized by light microscopy observation. Literature has shown that marine sponges, especially the phylum of Porifera, can be classified by the morphology of the spicules, a siliceous skeletal elements ranging in size, which are the result of enzymatically polymerized silica that are deposited to sponge cells [26]. Based on the microscopy observation of the collected marine sponges, it showed that the shape of spicules where classified as a monoaxon shape (Figure 1). This spicule observation also confirmed that this sponge was classified in the class of Demospongiae and a Stylissa spp.

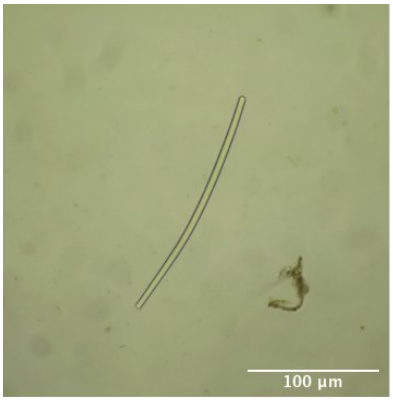

Figure 1. Spicule microscopic observation of *Stylissa flabelliformis*. Scale 100 μm.

Collagen was then isolated from the powdered air-dried marine sponge using modified [20] method. This isolation process resulted in the isolation yield of 3.5% collagen. This yield was very low but better compared to other methods used for collagen extraction from fish skin [27], which resulted into no yield. This may be due to the difference in the collagen types in both sources. However, further optimisation of the collagen isolation method may be worth exploring.

To understand the solubility of the isolated collagen, a solubility test was performed. The collagen isolate was dissolved in various types of solvents at room temperature and 4°C. Those solvents included water, acetic acid, sulfuric acid, acetone, hydrogen peroxide, dimethyl sulfoxide (DMSO), and methane sulfonic acid. The isolated collagen was not dissolved in any of these solvents at room temperature. Interestingly, it was completely dissolved in methane sulfonic acid after incubation for 4 days at 4°C.

3.2 Total protein content analysis

Protein content analysis by modified Bradford assay was performed to understand the total amount of collagen in the sample. This assay is a well-known method to quantify total protein concentration. The principle of this assay is the measurement of blue colour which represents the binding of Coomassie dye to the protein [28]. In this case, this assay was used to detect the amount of collagen that has been isolated. Due to the insensitivity of the standard Bradford assay to collagen, SDS was added to the

solution. A small amount of SDS has been shown to increase the response of this assay towards collagen [29]. This technique was chosen due to the simplicity and rapidness of the protocol. Based on the modified Bradford assay conducted, the isolated collagen contained 0.755mg/ml of total protein (Figure 2).

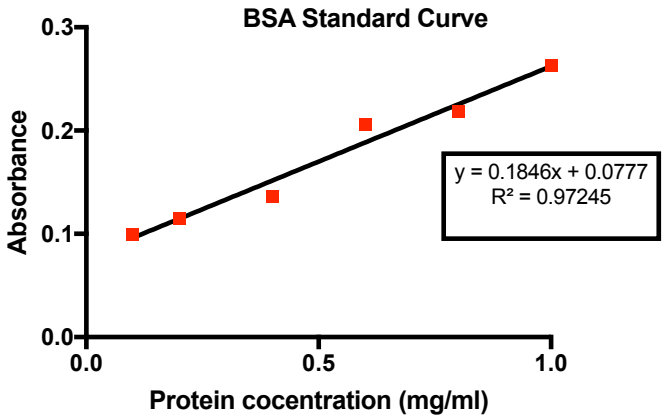

Figure 2. Standard curve of Bovine Serum Albumin used for Protein Content analysis. The equation used for total protein calculation was y=0.1846x + 0.0777.

3.3 Radical scavenging analysis

Several marine sponge species such as *Rhabdastrella globostellata* and *Spirastrella inconstans* has been reported to have antioxidant activity [30]. Collagen itself also has antioxidant activity, as reported in an antioxidant study conducted on collagen extracts from fish skin [31, 32]. This antioxidant activity is related to many pathological processes. Antioxidants also has benefit in supporting skin health, including prevention of skin photoaging and UV-induced cancer [33]. Hence antioxidants have now been included in pharmaceutical products. To understand further wheter the isolated collagen from *Stylissa flabelliformis* in this study had antioxidant activity or not, we proceeded with analysing the radical scavenging activity of the isolated sample. Radical scavenging analysis reveals the ability of the isolated collagen to reduce ROS production, which is responsible for causing a wide amount of skin diseases. The result in this study showed that the isolated compound has antioxidant activity with an IC_{50} value of 61.5±2.132 ppm (Figure 3). A small IC_{50} value indicates a high antioxidant activity [34], which shows that this isolate has the potency to be developed for ROS-related pharmacology treatments.

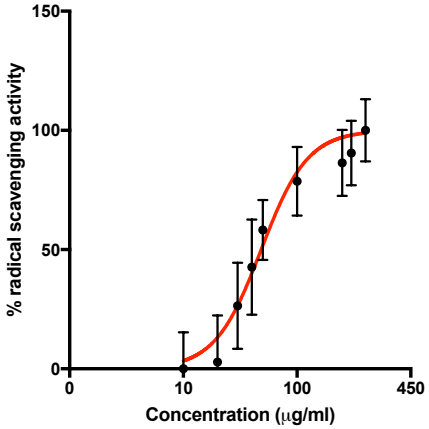

Figure 3. Percentage of radical scavenging activity of collagen isolated from *Stylissa flabelliformis* by DPPH Assay. Data obtained from three replicates, and presented as mean ± SD (n=3).

3.4 Spearman correlation analysis

The crude collagen isolate may contain non-protein compounds. Therefore to understand the contribution of the collagen to the whole antioxidant activity, a Spearman correlation analysis was performed. This analysis observed the correlation between the total protein content and antioxidant activity of the isolated collagen, and the results has shown that these two variables are positively correlated (r value=0.8) (Figure 4).

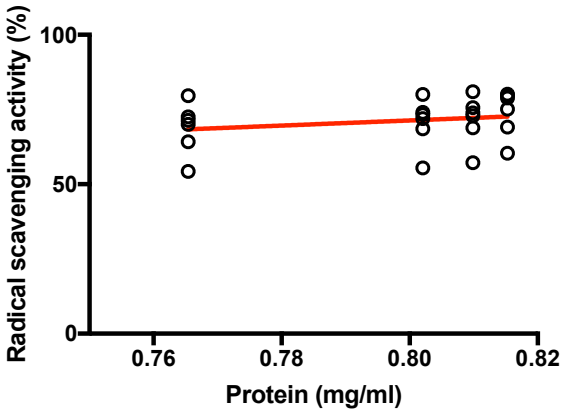

Figure 4. Correlation between radical scavenging activity and total protein content of collagen isolated from *Stylissa flabelliformis*, analysed with Spearman correlation analysis (r value = 0.8).

Proteins are known to have antioxidant activity through various mechanisms including inhibition of lipid oxidation. The pathways involved in the lipid oxidation process are inactivation of reactive oxygen species, scavenging free radicals, and reduction of hydroperoxides. Proteins antioxidant activity could also occur through other non-specific mechanisms [35]. The results in this study have shown that the protein components in the *Stylissa flabelliformis* collagen isolate highly contributes to the antioxidant activity. It could also be said that, in order to achieve a more potential antioxidant activity, the total protein concentration should be increased. More comprehensive study on this matter is encouraged to gain more information regarding the potential pharmacology activity of marine sponge collagen.

4. Conclusion

It could be concluded that the collagen isolated from the marine sponge *Stylissa flabelliformis* has antioxidant activity with the IC_{50} value of 61.5±2.132 ppm, and that the antioxidant activity also correlated with the protein content of the isolate (r value = 0.8). These results shows the isolated collagen from marine sponge *Stylissa flabelliformis* collected from North Lombok Indonesia coast is a promising antioxidant agent which would be beneficial for skin care development. This data could be used for further investigation on skin health benefits of this collagen isolate and the development of its pharmaceutical and cosmetic formulations.

Acknowledgement

We would like to thank Universitas Mataram for funding this research and also all parties that has contributed to this research.

References

[1] Cole, M.A., T. Quan, J.J. Voorhees, and G.J. Fisher, Extracellular matrix regulation of fibroblast function: redefining our perspective on skin aging. 2018, *Journal of Cell Communication and Signaling*. ***19***(5).

[2] Arseni, L., A. Lombardi, and D. Orioli, From Structure to Phenotype: Impact of Collagen Alterations on Human Health. 2018, *International Journal of Molecular Sciences*. ***19***(5).
[3] Varani, J., M.K. Dame, L. Rittie, S.E. Fligiel, S. Kang, G.J. Fisher, and J.J. Voorhees, Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. . 2006, *The American journal of pathology*. ***168***(6): p. 1861-1868.
[4] Varani J., Warner R.L., M. Gharaee-Kermani, S.H. Phan, S. Kang, J.H. Chung, Z.Q. Wang, S.C. Datta, G.J. Fisher, and J.J. Voorhees, Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. 2000, *Journal of Investigative Dermatology*. ***114***(3): p. 480-486.
[5] Bolke, L., G. Schlippe, J. Gerß, and W. Voss, A collagen supplement improves skin hydration, elasticity, roughness, and density: Results of a randomized, placebo-controlled, blind study. 2019, *Nutrients*. ***11***(10): p. 2494.
[6] Kim, D.U., H.C. Chung, J. Choi, Y. Sakai, and B.Y. Lee, Oral intake of low-molecular-weight collagen peptide improves hydration, elasticity, and wrinkling in human skin: a randomized, double-blind, placebo-controlled study. 2018, *Nutrients*. ***10***(7): p. 826.
[7] Zague, V., V. de Freitas, M.D.C. Rosa, G.A. de Castro, R.G. Jaeger, and G.M. Machado-Santelli, Collagen hydrolysate intake increases skin collagen expression and suppresses matrix metalloproteinase 2 activity. . 2011, *Journal of medicinal food*. ***14***(6): p. 618-624.
[8] Francis, G. and J. Thomas, Isolation and chemical characterization of collagen in bovine pulmonary tissues. . 1975, *Biochemical Journal*. ***145***(2): p. 287-297.
[9] Quereshi, S., A. Mhaske, D. Raut, R. Singh, A. Mani, and P. J, Extraction and partial characterization of collagen from different animal skins. 2010, *The Recent Research in Science and Technology*. ***2***(9): p. 28-31.
[10] Coppola, D., M. Oliviero, G.A. Vitale, C. Lauritano, I. D'Ambra, S. Iannace, and D. de Pascale, Marine collagen from alternative and sustainable sources: Extraction, processing and applications. 2020, *Marine collagen from alternative and sustainable sources: Extraction, processing and applications*. ***18***(4): p. 214.
[11] Pallela, R., H. Ehrlich, and I. Bhatnagar, *Biomedical applications of marine sponge collagens.* , in *Marine Sponges: Chemicobiological and Biomedical Applications* 2016, Springer: New Delhi.
[12] Hackett, C., B. Grim, M. Stonawski, V. Skirbekk, M. Potančoková, and A. G., The Global Religious Landscape. Pew Research Center. 2012, *Washington, DC, USA*.
[13] Avila Rodríguez, M.I., L.G. Rodríguez Barroso, and M.L. Sánchez, Collagen: A review on its sources and potential cosmetic applications. 2018, *Journal of Cosmetic Dermatology*. ***17***(1): p. 20-26.
[14] Felician, F.F., C. Xia, W. Qi, and H. Xu, Collagen from Marine Biological Sources and Medical Applications. 2018, *Chemistry & Biodiversity*. ***15***(5): p. e1700557.
[15] Langasco, R., B. Cadeddu, M. Formato, A.J. Lepedda, M. Cossu, P. Giunchedi, R. Pronzato, G. Rassu, R. Manconi, and E. Gavini, Natural collagenic skeleton of marine sponges in pharmaceutics: Innovative biomaterial for topical drug delivery. 2017, *Materials Science and Engineering: C* ***70***(Pt1): p. 710-720.
[16] Lim, Y.S., Y.J. Ok, S.Y. Hwang, J.Y. Kwak, and S. Yoon, Marine Collagen as A Promising Biomaterial for Biomedical Applications. 2019, *Marine Drugs*. ***17***(8).
[17] Rahman, M.A., Collagen of Extracellular Matrix from Marine Invertebrates and Its Medical Applications. 2019, *Mar Drugs*. ***17***(2).
[18] Parisi, J.R., K.R. Fernandes, I.R. Avanzi, B.P. Dorileo, A.F. Santana, A.L. Andrade, P.R. Gabbai-Armelin, C.A. Fortulan, E.S. Trichês, R.N. Granito, and A.C.M. Renno, Incorporation of Collagen from Marine Sponges (Spongin) into Hydroxyapatite Samples: Characterization and In Vitro Biological Evaluation. 2019, *Mar Biotechnol (NY)*. ***21***(1): p. 30-37.

[19] Esposito, R., N. Ruocco, T. Viel, S. Federico, V. Zupo, and M. Costantini, Sponges and Their Symbionts as a Source of Valuable Compounds in Cosmeceutical Field. 2021, *Marine Drugs*. ***19***(8): p. 444.
[20] Diehl-Seifert, B., B. Kurelec, R.K. Zahn, A. Dorn, B. Jericevic, G. Uhlenbruck, and W. Muller, Attachment of sponge cells to collagen substrata: effect of a collagen assembly factor. 1985, *J. Cell Sci.* ***79***(1): p. 271-285.
[21] Zhang, W., H. Chen, Z. Wang, G. Lan, and L. Zhang, Comparative studies on antioxidant activities of extracts and fractions from the leaves and stem of Epimedium koreanum Nakai. 2013, *J. Food Sci. Technol.* ***50***(6): p. 1122-1129.
[22] Asserin, J., E. Lati, T. Shioya, and J. Prawitt, The effect of oral collagen peptide supplementation on skin moisture and the dermal collagen network: evidence from an ex vivo model and randomized, placebo - controlled clinical trials. . 2015, *Journal of cosmetic dermatology*. ***14***: p. 291-301.
[23] Borumand, M. and S. Sibilla, Effects of a nutritional supplement containing collagen peptides on skin elasticity, hydration and wrinkles. 2015, *Journal of Medical Nutrition and Nutraceuticals*. ***4***(1): p. 47.
[24] Elsner, P., E. , Berardesca, and H. Maibach, , Bioengineering of the Skin: Water and the Stratum Corneum. 1994, *CRC Press LLC*.
[25] Pallela, R., S. Bojja, and V.R. Janapala, Biochemical and biophysical characterization of collagens of marine sponge, Ircinia fusca (Porifera: Demospongiae: Irciniidae). . 2011, *International journal of biological macromolecules*. ***49***(1): p. 85-92.
[26] Müller, W.E., X. Wang, F.Z. Cui, K.P. Jochum, W. Tremel, J. Bill, H.C. Schroder, F. Natalio, U. Schlobmacher., and M. Wiens, Sponge spicules as blueprints for the biofabrication of inorganic–organic composites and biomaterials. 2009, *Applied microbiology and biotechnology*. ***83***(3): p. 397-413.
[27] Arumugam, G.K.S., D. Sharma, R.M. Balakrishnan, and J.B.P. Ettiyappan, Extraction, optimization and characterization of collagen from sole fish skin. . 2018, *Sustainable Chemistry and Pharmacy*. ***9***(19-26).
[28] Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. . 1976, *Analytical Biochemistry*. ***72***: p. 248-254.
[29] Duhamel, R.C., E. Meezan, and K. Brendel, The addition of SDS to the Bradford dye-binding protein assay, a modification with increased sensitivity to collagen. . 1981, *Journal of biochemical and biophysical methods*. ***5***(2): p. 67-74.
[30] Chairman, K., A.R. Singh, and G. Alagumuthu, Cytotoxic and antioxidant activity of selected marine sponges. . 2012, *Asian Pacific Journal of Tropical Disease*. ***2***(3): p. 234-238.
[31] Medina-Medrano, J.R., T.A. Quiñones-Muñoz, A. Arce-Ortíz, J.G. Torruco-Uco, R. Hernández-Martínez, M.A. Lizardi-Jiménez, and E. Varela-Santos, Antioxidant Activity of Collagen Extracts Obtained from the Skin and Gills of Oreochromis sp. 2019, *Journal of medicinal food*. ***22***(7): p. 722-728.
[32] Nurilmala, M., H.H. Hizbullah, E. Karnia, E. Kusumaningtyas, and Y. Ochiai, Characterization and antioxidant activity of collagen, gelatin, and the derived peptides from yellowfin tuna (Thunnus albacares) skin. 2020, *Marine drugs*. ***18***(2): p. 98.
[33] Briganti, S. and M. Picardo, Antioxidant activity, lipid peroxidation and skin diseases. What's new. . 2003, *Journal of the European Academy of Dermatology and Venereology*. ***17***(6): p. 663-669.
[34] Molyneux, P., The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. 2004, *Songklanakarin Journal of Science and Technology (SJST)*. ***26***(2): p. 211-219.
[35] Elias, R.J., S.S. Kellerby, and E.A. Decker, Antioxidant activity of proteins and peptides. . 2008, *Critical reviews in food science and nutrition*. ***48***(5): p. 430-441.