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FUCOIDAN NANOENCAPSULATION FROM BROWN ALGAE (*Sargassum polycystum*) AS A POTENTIAL MARINE IMMUNOMODULATORY AGENT

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Abstract

The quest for better and more effective treatments has encouraged the search for therapies derived from natural sources to obtain effective immune therapy, considering that several pandemics have arisen caused by viruses. Developing fucoidan from brown algae in drug encapsulation as an immunomodulator could be more promising. This study aimed to produce nanoencapsulation loaded with fucoidan-purified extract from brown algae and to evaluate the influence of nanoencapsulation formulation on the immunomodulatory activity of fucoidan. Fucoidan was obtained from brown algae and extracted by hot aqueous, followed by ethanol purification. Nanoencapsulation of fucoidan purified extract was prepared using the ionic gelation method. The carbon clearance method was carried out for the immunomodulatory activity test of the nanoencapsulation of fucoidan purified extract. Nanoencapsulation of fucoidan purified extract with the optimum composition of maltodextrin 9.9% and S-TPP 0.1% (1:5) resulted in particle size of 715.4 nm, zeta potential -0.1 mV, pH 7.54, transmittance 97.54%+0.08, and entrapment efficiency 89.94%+0.17. The carbon clearance test showed that the nanoencapsulation of fucoidan was a strong immunostimulant with a phagocytosis index of 1.65. The development of nanoencapsulation could increase the phagocytosis index of fucoidan purified extracts from brown algae. Further molecular studies are needed to demonstrate the molecular activity of this preparation as an immunomodulator.

Keywords: fucoidan, brown_algae, nanoencapsulation, immunomodulator

Introduction

The global pandemic due to SARS-CoV-2 and other viral diseases, as well as the search for better and more effective treatments, has encouraged research regarding the search for therapies derived from natural sources based on the regulation of the immune response. The immune system is an organism's defense mechanism that prevents and eliminates potentially dangerous pathogens.[1] In addition, the immune response is enhanced by certain compounds called immunomodulators that influence the cellular and humoral immune systems by increasing or inhibiting function. Immune stimulants have been widely used to fight cancer, bacterial and viral infections, and immunodeficiency diseases. At the same time, immunosuppressants are used to treat autoimmune diseases and organ transplantation.[2] Another study indicates that the ingestion of fucoidan notably elevated the activity of natural killer cells, a fundamental component of the innate immune system involved in defending against tumor cells, among male cancer survivors. The immunostimulatory effect of fucoidan on macrophage differentiation has been shown to contribute to its anti-tumor efficacy in laboratory experiments and living organisms.[3]

The potential of marine sulfated polysaccharides such as brown algae-derived fucoidan as immunomodulators in preventing and treating viruses, such as COVID-19, has received considerable interest.[4-6] The main problem that limits its application in therapy is its high molecular weight, and the absorption rate of fucoidan in the intestine was only 0.6%.[7] A study is needed to formulate fucoidan in a delivery system such as a nanoparticle. Therefore, stomach acid does not damage fucoidan and can be absorbed in the intestine with a higher absorption capacity. Nanoparticles can be made using various methods, including the ionic gelation method. A polycationic polymer is mixed with a polyanion in the ionic gelation technique. Nanoparticles are made with positively charged maltodextrin, with an anionic phospholipid sodium tripolyphosphate (S-TPP).[8]

Drug formulation development is increasingly focused on drug delivery systems. The nanoparticle matrix of the encapsulated drug consisting of polymers can be derived from natural or synthetic polymers.[9] Multiple drug analogs or target drugs encapsulated for a particular tissue may theoretically be more promising. It can reduce the dose of the drug, minimize side effects, and improve patient compliance and therapeutic effect.[10] Various biocompatible and degradable natural polymers can form nanoparticles through self-assembly methods. Several studies have successfully formed nanoparticles as encapsulated drugs by the ionic gelation method, including combining chitosan with maltodextrin chitosan with sodium tripolyphosphate (S-TPP).[11,12] Therefore, research about encapsulating a marine drug such as fucoidan from brown algae (Sargassum polycystum) with a cross-linking agent such as chitosan, maltodextrin, and or S-TPP needs to be carried out. This study aimed to produce nanoencapsulation loaded with fucoidan-purified extract from brown algae in the optimum combination of cross-linkers and to evaluate the influence of nanoencapsulation formulation in the immunomodulatory activity of fucoidan.

Methods

Tools

The tools used in this research were digital scales (O'Hauss), analytical scales, measuring cups, filter paper, magnetic stirrer, stopwatch, centrifuge (PLC series),

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sonicator (Branson 1800), UV-Vis 1280 spectrophotometer (Shimadzu), pH meter, FTIR spectrophotometer, and Particle Size Analyzer (Malvern).

Materials

The materials used for the manufacture of nano encapsulations and determination of fucoidan were brown algae (Sargassum polycystum) from Pailus Beach, Jepara-Indonesia, fucoidan standard (Sigma Aldrich, catalog no F1890), aquadest (MKR Chemicals Ltd, Indonesia), ethanol (Mallinckrodt Chemicals), chitosan (CV. Biochitosan Indonesia), maltodextrin (Qinhuangdao Strach, co. ltd, China), sodium tripolyphosphate (Arrow Fine Chemical, India). The materials used for the immunomodulator test were Swiss albino male mice, heparin (Inviclot®, Indonesia), gelatin (MKR Chemicals Ltd, Indonesia), sodium carboxy methyl cellulose (MKR Chemicals Ltd, Indonesia), carbon ink (Yamura®, Indonesia), formalin (MKR Chemicals Ltd, Indonesia), Sancoidan® (Sanbe Farma, Indonesia).

Preparation of Fucoidan Extraction and Purification from Brown Algae

The fucoidan extraction procedure was performed as described by Junaidi[13] with slight modification. Five hundred grams of dried brown algae were extracted with hot distilled water (1:10) in four hours. It would be air-dried and milled into powder before extraction by water solution to produce fucoidan crude extract. The fucoidan crude extract was then centrifuged at a speed of 3000 rpm for 10 minutes. Sediment was separated, and the liquid part (filtrate) was added with 70% ethanol for purification with the same amount of the liquid part until a precipitate was formed. Centrifugation will then be performed once again to separate sediment from the liquid. The final precipitate was separated and dried; these were the final stages of fucoidan purification. Fucoidan purification extract was analyzed for fucoidan content with FT-IR.

Preparation of Nanoencapsulation Containing Fucoidan Purification Extract

The fucoidan purification extract solution was made at 1 mg/mL concentration. Preliminary tests were carried out to select the right combination of cross-linkers. In the preliminary test, several cross-linkers were tried to be combined. FP I is the combination of chitosan and S-TPP, FP II is the combination of chitosan and maltodextrin, and FP III is the combination of maltodextrin and S-TPP.

Fucoidan was weighed as much as 100 mg, then dissolved with distilled water at 100oC in a beaker glass and stirred until homogeneous. The solution was put into a measuring flask and stirred 100 mL with distilled water to obtain a fucoidan solution with a concentration of 1 mg/mL. The chitosan solution was made by dissolving powdered chitosan within an acetate buffer solution of pH 4 and stirring it with a magnetic stirrer until it was dissolved. The solution of maltodextrin and S-TPP as a cross-link was made in an aqueous solvent.

The cross-linker combination that produced the highest transmittance in the preliminary test would be optimized to obtain nanoencapsulation preparations with the highest fucoidan adsorption efficiency. The optimization of the cross-linker combination was carried out with the help of software Design Expert 10. Cross-linkers were dissolved with distilled water and stirred with a magnetic stirrer until completely dissolved. The first cross-linker solution was put into a vial. Then, it was added to a fucoidan solution in a 1 mg/mL concentration and stirred with a magnetic stirrer at 1500 rpm for 30 minutes. The second cross-linker solution was taken using a volume pipette and then added to the vial. Stir again using a magnetic stirrer for 60 minutes. The mix solution formed was sonicated for 60 minutes. The manufacturing of nanoencapsulation was carried out for three cycles with a stirrer for 60 minutes and sonication for 60 minutes. Evaluation of the nanoencapsulation solution test as an optimization response was:

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pH test

The pH test was carried out using a pH meter (Trans Instruments Walklab Series®). Before measuring the pH of the preparation, the pH meter was calibrated with standard buffer solutions of pH 4 and pH 7.

Transmittance Test

An amount of approximately 5 mL of a nanoencapsulation solution was put into a cuvette. Then, the transmittance was measured at a wavelength of 650 nm. The blank used when measuring transmittance was aquadest.[14]

Entrapment Efficiency

A good nanoparticle system should have a high drug-loading capacity to reduce the material's use as a matrix. Drug loading can be done by two methods, namely, the combining method and the absorption method. Drug loading and adsorption efficiency are highly dependent on the solubility of the drug in the matrix or polymer, the drug's molecular weight, and the polymer's interaction in the presence of ester or carboxyl functional groups.[15] Fucoidan was not absorbed in the encapsulation (free fucoidan in solution) and was quantitatively determined by FT-IR. Entrapment efficiency[16] was calculated by using the following equations :

$$\textit{\% Entrapment Efficiency} = \frac{(total\ amount\ of\ fucoidan\ in\ solution - free\ fucoidan\ in\ solution)}{total\ amount\ of\ fucoidan\ in\ solution} \textit{x 100}$$

Evaluation Characteristics of Nanoencapsulation Containing Fucoidan

After obtaining the optimum formula from the nanoencapsulation solution, the particle size and potential zeta were analyzed with Particle Size Analyzer (PSA-Horiba®). The solution was dried using a freeze dryer, and its characteristics were evaluated. The morphological structure of nanoencapsulation was evaluated using Scanning Electron Microscopy (SEM-Horiba®). The samples were prepared by dropping solutions into copper grids coated with carbon using the auto carbon coated for 5 minutes before the samples dried. The samples stayed in the copper network for 2–3 min. The samples were then immersed in 2% phosphotungstic acid stain and stained for 2–3 min. After natural drying, the samples were placed under SEM for observation.[16]

Immunomodulatory Effect

Carbon Ink Suspension

1.6 ml of carbon ink (Yamura®) was taken and put into a mortar, and 0.5% sodium carboxy methyl cellulose (S-CMC) suspension was dissolved in a 10 ml volumetric flask.

Macrophage Phagocytosis by Carbon Clearance Method

Tests for non-specific immune responses were based on phagocytic activity.[17] Swiss albino male mice (25-30 g) were used. The animals were fed with a standard pellet diet and water *ad libitum* and maintained under standard environmental conditions (22 ± 5°C with 12 h of light/dark cycle). All experimental protocols were approved by the Health Research Ethics Commission (305/AHW-SW/KEPK/STIFAR /EC/XII/2021), Sekolah Tinggi Ilmu Farmasi Yayasan Farmasi Semarang, Indonesia.

Mice were divided into five groups of five mice each. Drugs were administered in various groups, i.e., Group I served as normal control (NC) and received distilled water, Group II served as negative control and received S-CMC 0.5% suspension, Group III received standard drug fucoidan as an aqueous suspension at a dose of 580 mg/kg, Group IV received nanoencapsulation containing fucoidan purification extract at a dose

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580 mg/kg, and Group V received Sancoidan® at a dose of 580 mg/kg. Every Group was given each drug orally at once a day for seven days. On the eighth day, all the groups were given carbon ink suspension at 10 ml/kg through the tail vein. Blood was collected from the retro-orbital plexuses of individual animals at 0, 5, 10, 15, and 20 min immediately after the injection of carbon suspension. Blood (25 μl) was lysed with heparin 0.25 ml of heparin solution and 4 ml of 1% acetic acid, and the absorbance was measured spectrophotometrically at 640,5 nm to determine optical densities. Phagocytosis activity was determined by comparing the linear regression line slope between transmittance concerning time in the test and normal groups.

Lymphoid Organ Index Testing

The mice were euthanized using ether for several minutes until the mice lost consciousness. The mice were dissected, and their lymphoid organs (liver, spleen, and lymph nodes) were removed Imus). The lymphoid organs were weighed and compared with the control group and the organ index (%).

Data Analysis

Test data for fucoidan purified extract nanoparticles from brown seaweed (*Sargassum polycystum*) includes pH, transmittance, and entrapment efficiency tests. Optimal formula parameters include pH test, transmittance, and adsorption efficiency. Determination of the optimal formula is calculated using Design Expert software using the Simplex Lattice Design method. The selected formula was then created again with the same test and tested for validity using the T-test. Meanwhile, the phagocytosis index data in the immunomodulatory activity test will be analyzed statistically using one-way ANOVA using SPSS software.

Result

Brown algae (Figure 1) was taken from the shores of Pailus Beach, Jepara, Central Java. The sample was washed with seawater and dried in a cabinet for approximately 24 hours. Before drying, the sample was first cut into pieces to speed up the drying process. The brown algae used in the study was determined in the laboratory of the Department of Biology, Faculty of Math and Science, State University of Semarang, Indonesia. The determination aimed to get a clear identity of the raw materials under study and avoid errors in collecting the main research materials.

A: Fucoidan purified extract
B: Fucoidan standard (Sigma Aldrich, catalog no: F1890)

Figure 1. a) Brown algae (*Sargassum polycystum*) from Pailus Beach, Jepara, Indonesia; b) FT-IR profile of fucoidan purified extract of brown algae

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Table 2. Physical Characteristic of Optimization Formulas of Fucoidan Purified Extract Nano-encapsulation

Formula	Proportion		Response		
	Maltodextrin 9,9%	S-TPP 0,1%	Transmittance	pH	%EE
1	1	5	96.4	7.59	90.5
2	4	2	93.9	5.74	77.28
3	5	1	91.7	5.05	74.35
4	3	3	95.5	6.94	83.9
5	5	1	91.3	4.95	74.28
6	1	5	98.7	7.61	89.27
7	2	4	96.2	7.03	84.75
8	3	3	95.1	5.63	82.9

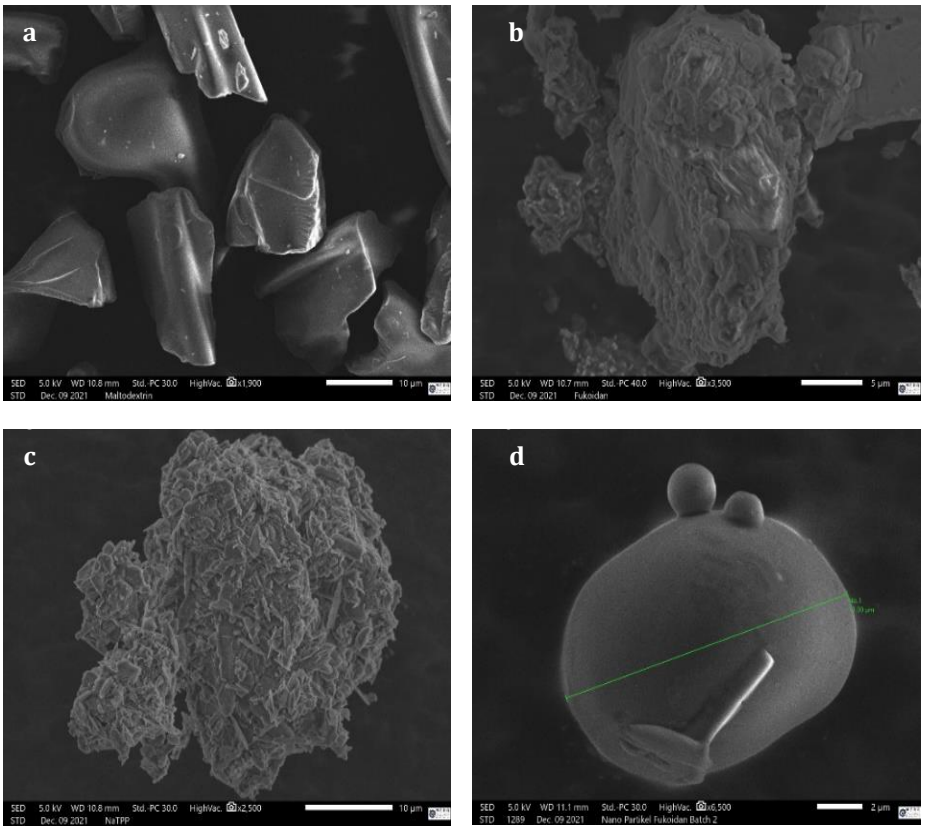

Figure 3. Scanning Electron Microscopy (SEM) result a) maltodextrin b) fucoidan purified extract c). S-TPP d) fucoidan purified extract encapsulation

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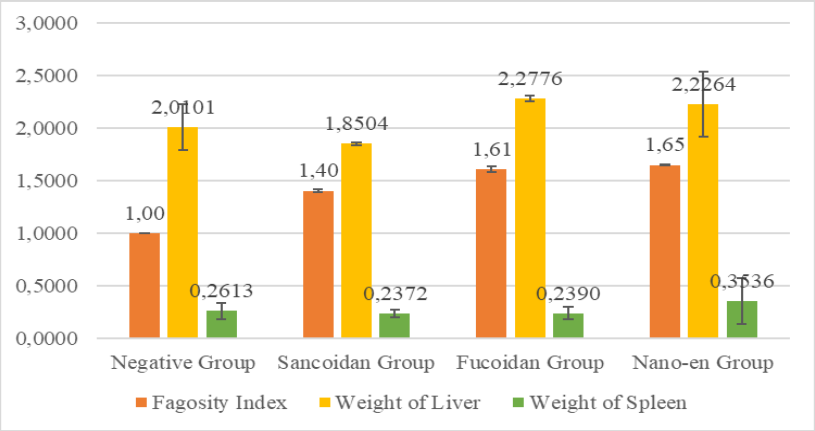

Figure 4. Phagocytosis index, weight of liver, and weight of spleen value of animal group on immunomodulatory activity test

Discussion

In the process of fucoidan extraction, the dried brown algae was blended to reduce its size. The size reduction could cause the brown algae cell wall and membrane to rupture. The rupture of cell walls causes the compounds contained in brown algae to be easier to extract.[18] When the particle size was smaller, the surface area in contact with the filter solution became larger, so the filter solution diffused more easily into the cell. This caused the extraction process to be maximized, and the yield was greater.

The extraction process was carried out for 4 hours. The 4-hour extraction time was determined based on Junaidi's research,[13] of which produced the largest yield. The extracted filtrate was separated by centrifugation at 3000 rpm for 10 minutes, then added with 70% ethanol in a ratio of 1:1. The purpose of adding ethanol was to attract, purify, and precipitate fucoidan compounds. The residue obtained was separated by centrifugation for 10 minutes at 3000 rpm. The residue was dried in an oven at 40^{o}C for approximately 24 hours. The fucoidan purified extract obtained was dark brown and odorless, in the form of thin flakes with a yield of 2.14%.

Identification of fucoidan with FT-IR spectrophotometer was carried out for qualitative and quantitative analysis based on its functional Group. FT-IR spectrophotometry can be used to analyze the characteristics of the functional groups of a compound.[19] In this study, analysis was carried out at wave numbers 4000-400 nm^{-1}. The FT-IR profile result can be seen in Figure 3.

According to Maciel[20], the Functional Group of fucoidan was C-O-S with a wave number range of 820-840 cm^{-1}. Compared with this study's results, it was known that the standard fucoidan obtained the C-O-S group at a wave number of 839 cm^{-1}, and the purified extract obtained a wave number of 816 cm^{-1}. The analysis results also found several other functional groups, namely S=O, indicating sulfated polysaccharides. In addition, C=C, C=O, and CH2-S groups were also found on the standard, and the two samples with wave numbers are listed in the wave number table above. These results showed that the two samples have similar functional groups with standard fucoidan. This indicates that both samples contain fucoidan compounds. The results of this experiment will also be used as a reference for determining fucoidan levels by IR spectrophotometry.

After the fucoidan purified extract was obtained, the next process was the selection of the constituent components for fucoidan encapsulation. The results showed that the cross-linker of chitosan with Na-TPP resulted in the lowest transmittance. Low transmittance could indicate that the particles in it were quite large. The highest

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transmittance value resulted from the combination of maltodextrin and S-TPP, so the encapsulation formula was optimized with these two materials (Table 2).

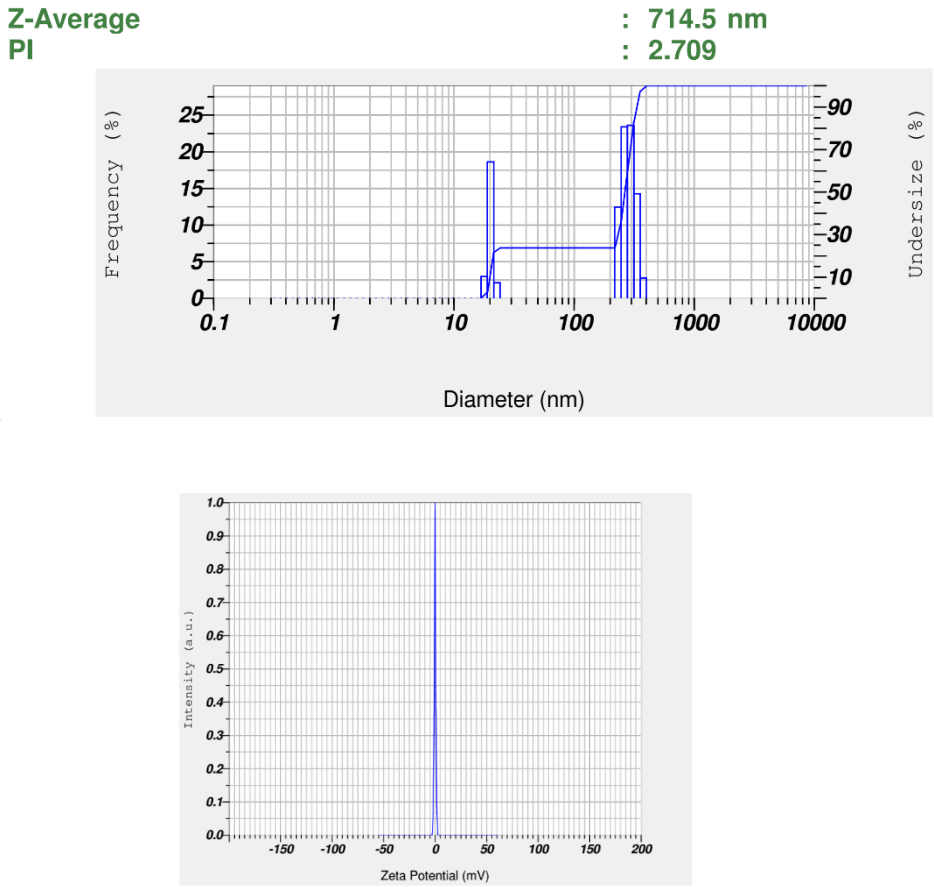

Figure 5. The measurements result of the optimum nanoparticles of fucoidan purification extract average size: Z-average of 714.5 nm; polydispersity index of 2.709, and zeta potential of -0.1mV

Based on Design Expert analysis, the optimum formula of nanoencapsulation of fucoidan purified extract consists of maltodextrin: S-TPP 1: 5 with desirability 0,921. The average encapsulation size of fucoidan purified extract was 714.5 nm. A polydispersity index value that is close to zero indicates a homogeneous or uniform distribution of particles. In contrast, a polydispersity index value that exceeds 0.5 indicates that the particles have a high level of heterogeneity. The polydispersity index value produced in this study was 2.709, still indicating high heterogeneity, so further research is needed to optimize the time and speed of stirring when making nanoparticles so that they can produce more homogeneous nanoparticles.[21]

The zeta potential of nanoencapsulation was measured to determine the properties of surface charge related to the electrostatic interactions of the nanoparticles. The zeta potential was influenced by the composition of the particles and the medium in which the particles were dispersed. The zeta potential value describes the optimization between repulsion or attraction between particles. A good zeta potential value for nanoemulsion preparations was more than ±30mV. However, the zeta potential measurements of nanoemulsion preparations showed a value of -0.1 mV. From Albert 2016,[22,] the zeta-capability of maltodextrin became toward -30 mV when the concentration of maltodextrin was low, which characterize that excessive dilution has a high-quality effect on the

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balance of maltodextrin within the solution. The low zeta potential permits the particle to drag collectively, and flocculation happens due to the enchantment among Van Der Waal particles. A very high zeta potential will motivate a more repulsive force, while repulsion among debris with an identical electric charge will prevent particle aggregation.[23]

Figure 3 shows the SEM result of fucoidan purified extract encapsulation after the drying process with a freeze dryer. There was a physical interaction of the maltodextrin and S-TPP as cross-linkers. Figures 3a and 3C showed particle images of maltodextrin and S-TPP, and in Figure 3d, it can be seen that there are no intact particles from each cross-linker. This indicated that the two interact to form encapsulations with the active substance. The figure also showed that the fucoidan-purified extract could be encapsulated. In contrast, Figure 3b shows a rough surface of the fucoidan purified extract, which is not seen in the 3d encapsulation results.

Fucoidan purified extract and its nano-encapsulation proved immunostimulant abilities through non-specific immunomodulatory activity tests. Even fucoidan-purified extract in nanoencapsulation preparations was proven to have a stronger phagocytic index than fucoidan-purified extract and commercial preparations of Sancoidan®. The results of the non-specific immunomodulatory activity are summarized in the bar chart in Figure 4.

The immunomodulatory activity of fucoidan purified extract and its nanoencapsulation had been evaluated towards non-specific immune responses, which can be innate immune systems. This means that responses to foreign materials can arise even though the body has not been exposed to those materials before. The non-specific immune function features an early reaction to pathogens that enter the body. Phagocytic activity was determined by comparing the linear regression line slope between 100% transmittance and time in the test and negative Groups. The value of the slope of the linear regression line (K) for the negative Group, Sancoidan® group, fucoidan, and the nanoencapsulation of fucoidan purified extract can be seen in Figure 4. The slope value of the linear regression line (K) indicates the magnitude of the phagocytic index. Based on the one-way ANOVA test, the phagocytosis index value between groups had a significant difference ($p<0.05$). The average value of the phagocytic index indicates the phagocytic activity of phagocytic cells against carbon particles as antigens due to the effect of administering fucoidan purified extract and in nanoencapsulation dosage forms. If the average value of the phagocytosis index is greater than one, it means that the test substance has immunostimulant abilities.[24] The immunomodulatory activity of the test material is classified based on its phagocytic index. According to Wagner, the phagocytic index between 1 and 1.5 shows a moderate immunostimulation, and the phagocytic index >1.5 shows a strong immunostimulation effect.[25]

Then, the lymphoid organ index was analyzed. Mice were dissected, and the lymphoid organs (liver and spleen) were taken. The lymphoid organs were weighed and compared with the control group, and the organ index was calculated. As a secondary lymphoid organ, the spleen contains B lymphocytes and T lymphocytes, which play a role in specific immune responses. In addition, the spleen also contains dendritic cells and macrophages that act as APCs (Antigen Presenting Cells), which present antigens to lymphoid cells. The increase in these immune cells correlated with the weight of the spleen.[26] Although the results of statistical tests using one-way ANOVA showed no difference in the weight of the liver and spleen in all groups, it was seen that the increase in relative spleen weight indicates the effect of nanoencapsulation of fucoidan purified extract on the stimulant effect.

Nanoparticles can enhance the efficacy of drug therapy, particularly in the context of immune responses, due to their unique physical and chemical properties. One key reason is their size, as nanoparticles typically fall within the nanometer scale. This size increases surface area, facilitating better interactions with biological entities such as cells and tissues.[27,28]

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Conclusion

Based on the non-specific immune response assay results, it can be concluded that the development of nanoencapsulation may increase the phagocytosis index of fucoidan-purified brown algae extract. Further molecular studies were needed to demonstrate the molecular activity of this formulation as an immunomodulator.

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