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Green synthesis of silver nanoparticles using algaes *(Fucus vesiculosus, Euchema spinosus* and *Gracilaria verrucosa)* as antioxidants and anticollagenase

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Green Synthesis of Silver Nanoparticles using Algaes *(Fucus vesiculosus, Euchema spinosus* and *Gracilaria verrucosa)* as Antioxidants and Anticollagenase

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Abstract. The green synthesis of silver nanoparticles is an environmentally friendly method to obtain compounds that are useful as anti-aging candidates. The algae extract used contains secondary metabolites such as terpenoids and flavonoids, hence it acts as a bio-reductant agent for the production of silver nanoparticles. This research aims to synthesize silver nanoparticle using algae, antioxidant activity test and anticollagenase enzyme activities of silver nanoparticle algaes are *F.vesiculosus, E. spinosus,* and *G. verrucosa*. The silver nanoparticle character test was performed using a Particle Size Analyzer (PSA), and Scanning Electron Microscope (SEM). Furthermore, the antioxidant test was conducted using the DPPH method while the anticollagenase activity test was performed using spectrophotometry with standard collagenase. The silver nanoparticles produced from *Fucus vesiculosus* extract had a particle distribution with a size of 74.06 nm. Also, the particle size distribution from the extract of *Euchema spinosum* ranges in sizes from 53.70 nm to 100 nm. Meanwhile, the particle size distribution from the extract of *Gracilaria* spp has a size of 123.4 nm. The SEM imaging results showed that the obtained ZnO was generally spherical in shape, but the gap between the particles was not visible. Therefore, the antioxidant and anticollagenase enzyme activity test results showed that *F. vesiculosus* > *E. spinosum* > *G. verrucosa*, respectively.

INTRODUCTION

Nanoparticles are particles with sizes between 1 and 100 nanometers that function as a unit to their properties and transport. Scientific study on nanoparticles is very intensive because it is useful for applications in medicine, physics, optics, and electronics [1]. Silver nanoparticles (NPAg) are silver metal particles with a size of less than 100 nm. Furthermore, the synthesis is performed using both physical and chemical methods which has negative impacts, such as the use of toxic solvents, high energy consumption, and the release of hazardous waste [2]. The development of chemical or physical methods has led to environmental pollution because the chemical procedures involved in the synthesis of nanomaterials produce a large number of harmful by-products [3]. Therefore, it is necessary to develop an environmentally friendly method, such as using natural ingredients with plant extracts. In this process, nanoparticles are synthesized using organic compounds contained in plants to reduce metal ions.

Some plant species that contain certain chemical compounds, act as reducing agents. Currently, many studies are being conducted on the synthesis of silver nanoparticles with silver ions and plant extracts which contain secondary metabolites such as terpenoids and flavonoids which have antioxidant activity hence it acts as a bio-reductant to produce silver nanoparticles [3]. Green synthesis is a relatively inexpensive method of converting nanoparticles which presents a low risk of environmental pollution. Furthermore, it produces safer and more environmentally friendly products and is used in various fields, including health and biomedicine [4]. Additionally, it is a synthesis method that forms metallic nanoparticles using natural materials derived from terrestrial and marine organisms (plants and microorganisms). Silver nanoparticles are those that are synthesized by the green synthesis method [5].

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Green synthesis systems use biological microorganisms such as bacteria, fungi (yeast), and plant extracts [6]. Meanwhile, Chemical and physical manufacturing methods are more effective in producing pure nanoparticles but are quite expensive and potentially harmful to the environment. In the utilization of biomass such as microorganisms, a plant extract is an option for the green production of nanoparticles [7]. The main requirement for green synthesis of AgNPs is a solution of silver metal ions and reducing agents of biological compounds. In the vast majority of cases, reducing agents or other constituents in the cell act as stabilizers and caps, hence there is no need to add external capping and stabilizing agents.

Currently, the synthesis of nanoparticles using seaweed has been widely developed. Based on the analysis results, there are certain lipids, minerals and vitamins, polysaccharides, proteins, and polyphenols that are potentially anti-cancer, antioxidant, anti-inflammatory, antiallergic, thrombosis, lipidemia, hypertension, and other degenerative diseases. Phytochemical compounds such as hydroxyl, carboxyl, and amino functional groups, serve as effective metal reducing agents and as covering agents to provide a strong coating on metal nanoparticles [8]. Algae is widely used as a basic ingredient in the cosmetics industry, and this species is one of the marine resources considered to be safe and has little cytotoxicity effect on humans. Furthermore, it is rich in bioactive substances that have a beneficial effect on the skin, especially in the treatment of pigmentation, aging, and cancer. Algae is applied as a skin whitening, anti-aging, anticancer, antioxidant, anti-inflammatory, and antimicrobial agent [9].

Macroalgae are classified into three major classes, namely *Phaeophyceae* (brown algae), *Rhodophyceae* (red algae), and *Chlorophyceae* (green algae). Furthermore, it is a rich source of catechins and flavonoids, and macroalgae also contain a diversity of compounds 10 times greater than terrestrial plants and have a different flavonoid composition than vegetables and fruits [10]. Phlorotannin algae compounds have a unique structure, which is not found in land plants and these compounds make up to 25% of the dry weight of brown algae. Furthermore, a variety of primary metabolites, such as unsaturated fatty acids, polysaccharides, vitamins, and essential amino acids are all produced from algae. However, results have shown that secondary metabolites derived such as fucoidan, fucoxanthin, sulfated polysaccharides, polyphenols, and fucosterol which are derived from algae were discovered to have anti-inflammatory, antioxidant, anticancer, antibacterial, and anti-aging effects [9].

Algae is a natural resource with potential for cosmetic development. In ancient times, this species was used as a medicine to treat diseases related to the skin, such as atopic dermatitis and skin aging. The demand for algae bioactive compounds for cosmetics is increasing rapidly as they contain natural extracts which are considered safe and have fewer side effects in humans [11]. Antioxidants are substances which in small concentrations are significantly able to inhibit or prevent oxidation of the substrate caused by free radicals which are molecules of high reactivity because of the unpaired electrons in their outer orbitals hence they react with body cell molecules by binding to the molecular electrons. Free radicals, which are constantly produced during normal metabolic processes, are considered to cause damage to the functioning of body cells, which eventually triggers the onset of degenerative diseases [12].

Nature provides an effective and relatively safe source of antioxidants such as flavonoids, vitamin C, beta carotene, and others. Flavonoids are a group of phenolic compounds that function as antioxidants [13]. Also, it plays a role in preventing damage to cells and their cellular components by reactive free radicals [14]. The antioxidant role of flavonoids occurs through the donation of hydrogen atoms or through their ability to chelate metals. These compounds exist in the form of glucosides containing chains, or in the free form, called aglycones. Aging is a complex phenomenon in the form of changes in the structure and function of the human body. Furthermore, free radicals are one of the factors that play a role in aging. The process is caused by the activity of the enzymes elastase and collagenase, whereby inhibition is performed using natural ingredients that act as antiaging which have antioxidant, antielastase, and anticollagenase activities. Aging can be caused by factors originating from the inside (intrinsic) and outside the body (extrinsic). Meanwhile, the intrinsic factor is the activity of certain enzymes, such as elastase, hyaluronidase, collagenase, and tyrosinase in the skin aging process. Collagen is the main component of the skin with a percentage of 70-80% of the total weight of the skin, and it has been widely known as an enzyme that plays a role in the breakdown of collagen [15]. Some studies show that compounds in plants have the ability to act as antioxidants that inhibit enzymes such as elastase, hyaluronidase, collagenase, and tyrosinase. Phytochemical compounds such as polyphenols contained in plants counteract as free radicals that have the antiaging potential [16].

MATERIALS AND METHOD

The Material and Tools

The materials used are the algae species of *F.vesiculosus, E. spinosum,* and *G. verrucosa*, a dry preparation with a moisture content of 15%, aquadest, and $AgNO_3$. The tools used are centrifuge, Scanning Electron Microscopy (SEM) Brand FEI, Type: Inspect-S50, Apodization, Shimadzu Happ-Genzel, X-ray Diffraction (XRD), and Malvern Zetasizer Nano Particle Analyzer.

The working procedure of nanoparticle compounds using the green synthesis method Green Procedure for Synthesis of Silver Nanoparticles with Modification

Sample Preparation

The algae samples were dried in an oven at 80 °C for 10 minutes (until the moisture content was approximately 10%) and were blended until smooth, afterward, it was filtered using a sieve with a size of 100 mesh [17].

Sample Extraction

The fine sample was dissolved into aquadest in a ratio of 1:10 and was homogenized using a stirrer on a hotplate at room temperature. The solution was set into a 15 mL tube, then centrifuged at 4000 rpm for 15 minutes at 4 °C, after which the supernatant was extracted.

Synthesis of silver nanoparticles with natural ingredients/Green synthesis silver nanoparticles

The sample filtrate was dissolved in 1 mM $AgNO_3$ solution in a ratio of 1:9, the solution was left in a dark room for 1 hour, after which it was inserted into a 15 mL tube and centrifuged at 4000 rpm for 30 minutes at 20 °C, the supernatant was discarded and the pellet was then dried. in an oven at 45 °C for 24 hours, after which it was grounded into a powder.

Particle Size Analyzer (PSA) Characterization

Particle size testing was carried out using a digital microscope and PSA testing. Furthermore, samples were taken using a spatula, then dissolved in aquadest in a ratio of 1 mg in 10 mL of aquadest and then vortexed. The solution was then put into a tube at a maximum height of 15 mm, then the diameter distribution of the sample was measured using the Malvern Zetasizer Nano Particle Analyzer [18].

Characterization of Nanoparticle Compounds using a Scanning Electron Microscope (SEM)

The nanoparticles were placed on the stub using double-sided tape, and the powder was conditioned to be electrically conductive with a tuft of a thin platinum layer from the coating for 30 seconds at a pressure below 2 Pa and a current strength of 30 mA. The photo was taken at an electron voltage of 10 kV with the desired magnification [19].

Determination of antioxidant activity (IC_{50}) in red algae samples

The sample was weighed to 25 mg, then dissolved with aquadest and put into a 25 mL volumetric flask, and labeled (1000 ppm mother liquor). Standard solutions of 100, 200, 300, 400, and 500 ppm were made by pipetting 1, 2, 3, 4, and 5 mL of the mother liquor into a 10 mL volumetric flask and then marked with ethanol. Afterward, 3 mL of each standard solution was pipetted and 1 mL of 0.2 mM DPPH was added. The control solution was prepared by pipetting 3 mL of ethanol and adding 1 mL of 0.2 mM DPPH, after which the solution was incubated at 37 °C for 30 minutes and the absorbance value was measured using a UV-Vis spectrophotometer at the maximum wavelength of (516 nm) [20].

The absorbance data obtained from each concentration of the extract was calculated as the percent (%) inhibition value which is obtained from the following Equation 1.

$$\%\ Inhibition\ = \frac{Control\ absorbace - Sample\ absorbance}{Control\ absorbance} \times 100\% \quad (1)$$

After obtaining the % inhibition, the value of IC50 was then calculated by obtaining a regression equation using the "*Graph Pad prism8 software, Regression for analyzing dose-response data*" program which shows the relationship between the log extract concentration (*x*) and % inhibition (*y*). Therefore, the smaller the IC_{50} value, the stronger the sample's ability as an antioxidant.

Anticollagenase Activity Test

400 µL enzyme + 2 mL substrate + 2 mL Tris-HCl buffer of 0.05 M with pH 8 was incubated at 37 °C for 10 minutes, then 0.2 M TCA (Trichloroacetic acid) was added, after which it was incubated at 37 °C for 10 minutes. Furthermore, it was centrifuged at 2000 × g for 10 minutes, then the supernatant (if there is no supernatant use all and measure how many ml) was collected and place in a test tube containing 0.4 M Na_2CO_3. Also, Folin ciocalteau reagent (1:2) was added, afterward incubated at 37 °C for 10 minutes. The results were measured by spectrophotometer at 578 nm, and 1 unit of enzyme activity was defined as the amount that liberated 1 mol of tyrosine per minute at optimum temperature & pH [21].

UA = Asp-Abl/Ast-Abl × P × 1/T

Asp = Sample Absorbance Value

Abl = Blank Absorbance Value

Ast = Standard Absorbance Value

P = Dilution Factor
T = Incubation Time

The activity of the anticollagenase enzyme can be calculated by the following Equation 2.

$$\text{UA} = \frac{(Absorbance\ sample - absorbance\ blanko}{(Absorbance\ standart - absorbance\ blanko} \times \text{P} \times \frac{1}{T} \quad (2)$$

Note
UA/mL = the amount of tyrosine produced per enzyme per minute
P = dilution factor
T = incubation time (10 minutes)

Percentage of inhibition (Equation 3).

$$\frac{activity\ collagenase\ with\ inhibitor}{activity\ collagenase\ without\ inhibitor} \times 100\% \quad (3)$$

Data Analysis

Antioxidant and anticollagenase data were tested for homogeneity and normality and then analyzed by ANOVA using SPSS 16.0 application. if the ANOVA test results are significantly different then further tested using Tukey Test HSD with a level of 5%

RESULTS AND DISCUSSION

Synthesis and Characterization of Red Algae Silver Nanoparticles

The results of the nanoparticle characterization of F. *vesiculosus* extract based on the size distribution histogram showed that the particle size had a dominant volume percentage of about 86.3% with a diameter of 113.3 nm and the smallest volume percentage of about 13.7% with a diameter of 400 nm (Figure 1a). This indicates that the F. vesiculosus algae extract has been successfully used in the synthesis of silver nanoparticles. Zeta potential serves to determine the surface charge of nanoparticles in a solution. The nanoparticle compound shows a zeta potential value of 0.2 mV, this indicates that the particle is in agglomeration state. The zeta potential value ranges from 0-5 mV

indicating that the particle agglomerates due to interactions between particles, including van der Wals bonds, hydrophobic interactions, and and hydrogen bonds. Zeta potential analysis is very important to do to determine the successful functionalization or surface modification of nanoparticles. MI (Mean Intensity Diameter) 155.1 nm, MN (Mean Number Diameter) 108.4 nm, MA (Mean Area Diameter) 125.2 nm, and PDI (Polydispersity Index) 0.0486. This value indicates that the tested particles are included in the monodispers category, which has a high level of homogenity.

The size of the nanoparticles synthesized using E. spinosum ranged from 36.10 - 687 nm (Figure 1b). The nanoparticle size of 296 nm has the highest percentage (96%) when compared to other sizes. The average nanoparticle size in this study was 296 nm. Zeta Potential +200 mV, MI (Mean Intensity Diameter) 284.9 nm; MN (Mean Number Diameter) 65.40 nm; MA (Mean Diameter) 201.6 nm, and PDI (Polydispersion Index) 0.171. The zeta potential value of silver nanoparticles *E. spinosum* is +200mV, this value is in the category of very good stability. Zeta potential shows the stability of a particle that is formed. The diameter of the average intensity (MI) produced is 284.9 nm, while the diameter of the average number (MN) which is the average diameter of the distribution results is 65.40 nm. The mean area diameter (MA) is the average particle distribution surface of 201.6 nm. The results of the Polydispersity index show the number 0.1710 with a narrow distribution category, which means that the silver nanoparticle sample is narrow and uniform.

The distribution of particle size of silver nanoparticle *G. verrucosa* extract shown in the Figure 1c with sizes ranging from 53.70 nm to 100 nm. The particle shape is spherical, zeta potential +200 mV, MV (Mean Volume Diameter) 22.72 nm, MN (Mean Number Diameter) 10,67 nm, MA (mean diameter) 11.54 nm, PDI (Polydispersion Index) 0.135. Zeta potential value show the stability of nanoparticles. The zeta potential value of silver nanoparticles *G.verrucosa* is +200 mV, this value is in the category of very good stability. Zeta potential is a technique that can determine the surface charge of nanoparticles. Categories with a value of 0 to ±5 mV have easy coagulation properties. Values between ±10 to ±30 mV indicate that the nanoparticles are unstable. Stability in the moderate category is indicated by the value of ±31 to ±40 mV. Then the value of ±41 to ±60 has good stability. Stability in the very good category is indicated by the zeta potential value greater than ±60 [22].

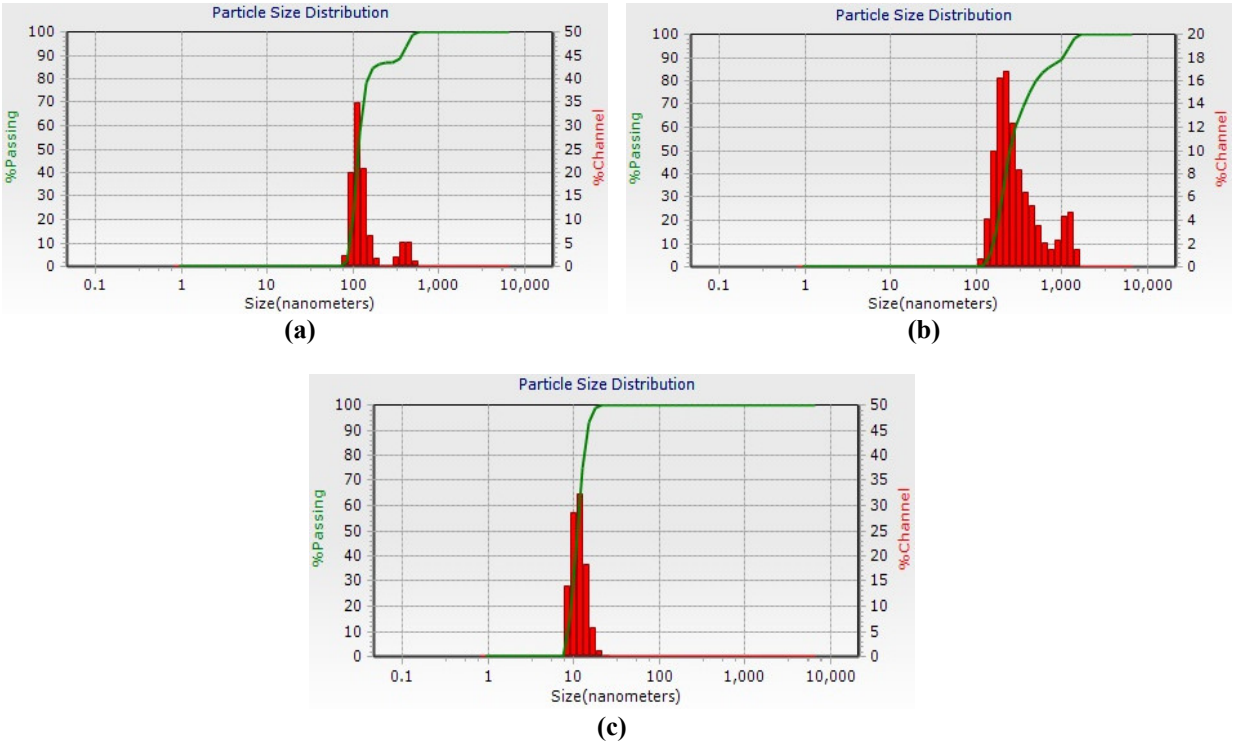

FIGURE 1. Particle size distribution of silver nanoparticles (a). *F.vesiculosus, (b). E. sspinosum (c). G. verrucosa*

The observation of silver nanoparticles have a spherical shape. The spherical shape of the nanoparticles has several advantages are increase cell absorption, speed and delivery of a drug. (Ankamwar (2012)). The spherical shape of

nanoparticles is a good option for drug delivery systems. Spherical nanoparticles have lower toxicity even in heterogeneous or homogeneous conditions and have a higher strength to interact with the cell surface [23]. Nanoparticles compounds that have been tested have an MV value of 27.22 nm. The MN value of the tested silver nanoparticles is 10.67 nm. The value is calculated from a volume distribution that shows small particles and is a type of average particle size in the population. The MA value is 11.54 nm. The MA value indicates the particle diameter and the particle surface [1]. The PDI (Polydispersion index) value for nanoparticle compounds was 0.135. This value indicates that the tested particles are included in the monodispers category, which has a high level of homogenity. This is in accordance with the PDI value of less than 0.5 indicating that the tested nanoparticles are monodisperse samples [24].

Nanoparticles are defined as the dispersion of particles with sizes between 10-1,000 nm. However, particles having a diameter of <1,000 nm are accepted as nano-sized carriers which are used in the pharmaceutical industry. Some of the advantages of using nanoparticle compounds are their particle size and surface characteristics which are easily manipulated to achieve passive and active drug targeting after parenteral administration.

Nanoparticle compounds control and maintain drug release during transport and localization, therefore altering subsequent distribution to achieve therapeutic enhancement of drug efficacy and reduction of side effects. The drug loading is relatively high such that it is fed into the system without chemical reactions, and this is an important factor to maintain drug activity. Specific site targeting is obtained by attaching the targeted ligand to the particle surface or through the use of magnetic guides. This system is used for various routes of administration including oral, nasal, parenteral, and intra-ocular [25].

The reducing agent used is algae extract which acts as a trap for silver precursors ($AgNO_3$). Furthermore, after the Ag^+ cation is reduced to a metal with zero charges, the algae extract compounds are localized around the surface of the nanoparticles formed. Particle size in the angstrom scale and the algae extract compound as a trapping agent is more dominant than the cation when the silver metal is positively charged therefore the resulting particle size follows that of the algae extract the compound. Reduction reactions become more frequent over time, therefore the particles combine and produce larger sizes. However, the tendency of particles to aggregate is caused by the effect of Brown motion or the continuous motion of particles that occurs in solution, thereby causes the particle diameter to be non-uniform. The aggregation of nanoparticles occurs through two stages. In the first stage, the particles approach each other and collide and in the second stage, the colliding particles stick together.

The synthesis of AgNP by biological matter is due to the presence of a large number of organic chemicals such as carbohydrates, fats, proteins, enzymes & coenzymes, phenols, flavonoids, terpenoids, alkaloids, and gums, etc which are capable of donating electrons for the reduction of Ag^+ ions to Ag^0. The active ingredient responsible for the reduction of Ag^+ ions varies depending on the type of organism and the extract used [6].

Scanning Electron Microscope (SEM)

The results of SEM imaging show that the obtained silver nanoparticles are generally spherical in shape but the gaps between the particles are not visible which is a result of agglomeration between them. Furthermore, the agglomeration between uniform and non-uniform particles occurs due to the influence of polarity, electrostatic power, and large energy on the sample surface which usually occurs during the synthesis process [26,27]. Also, it occurs presumably as a result of many chemical compounds contained in the algal extract which act as traps or templates for AgNO precursors. The size of the resulting ZnO is highly dependent on the size of the template surrounding the surface of the nanoparticle [28].

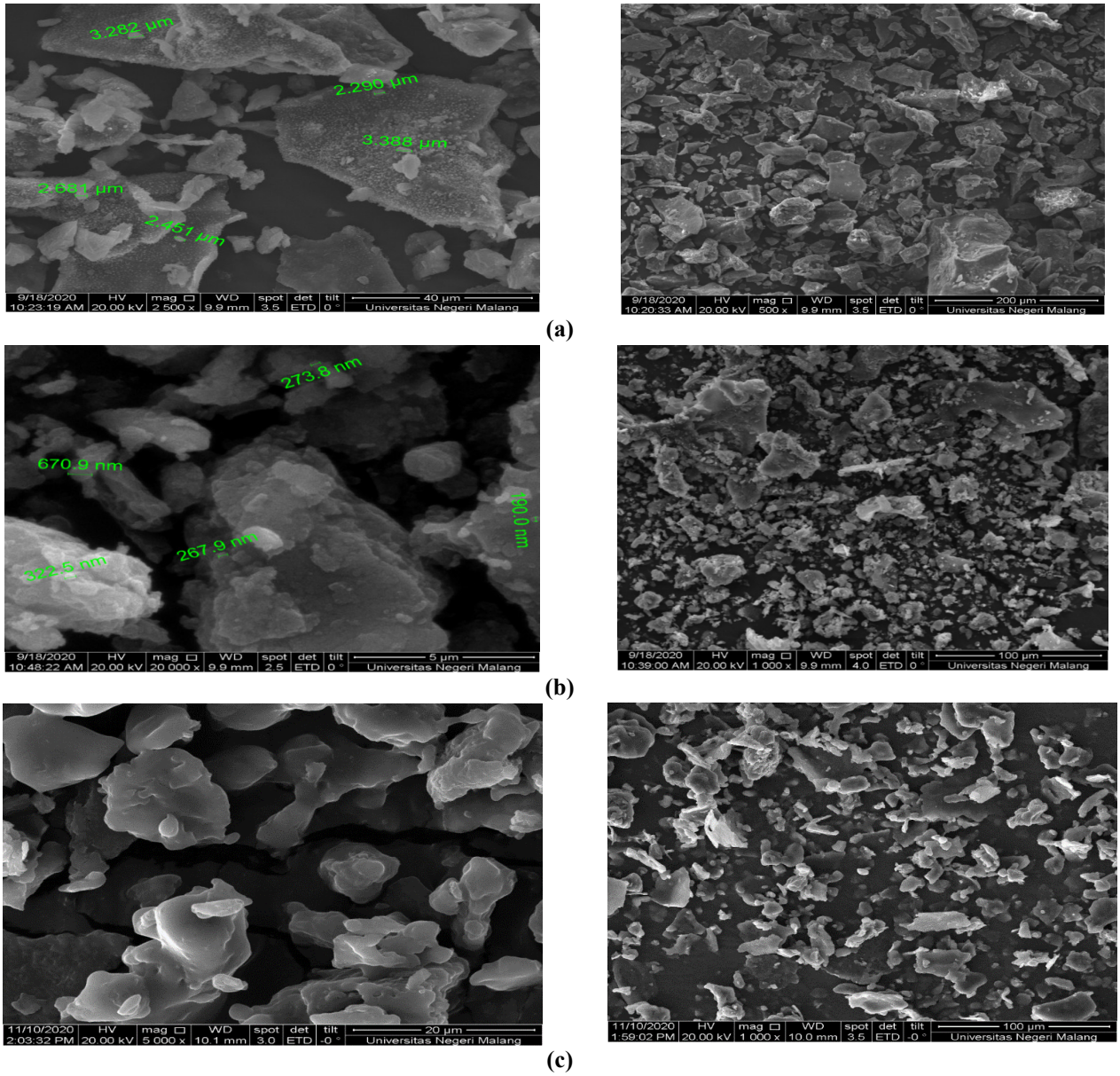

FIGURE 2. Imaging Scanning Electron Microscope (SEM) Silver Nanoparticles (a). *F.vesiculosus, (b). E. spinosum, (c). G. verrucosa*

Antioxidant Activity

In the analysis of red algae powder *Fucus vesiculosus*, the results of the calculation of the IC_{50} value using the program "Graph Pad prism8 software, Regression for analyzing dose-response data" was 1135 ppm, while that of the analysis of red algae nanoparticles *Fucus vesiculosus* obtained an IC_{50} value of 177.6 ppm. Regression for analyzing dose-response data" program were 6160 ppm, and 739.9 ppm for *Euchema spinosum*. In the analysis of red algae powder *Gracillaria spp*, the results of the IC_{50} value calculated using the "Graph Pad prism8 software. Regression for analyzing dose-response data" program were 34777 ppm, and 1349 ppm. The antioxidant test results showed that *Fucus vesiculosus* > *Gracillaria verrucosa* > *Euchema spinosum*, respectively (Figure 3).

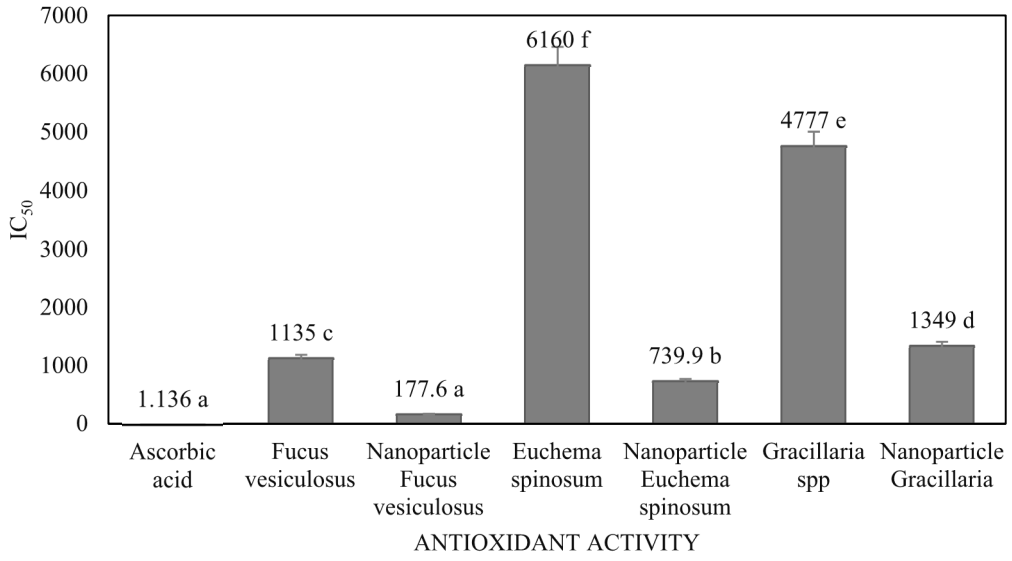

FIGURE 3. IC $_{50}$ extract algae and nanoparticle algae *F. vesiculosus, E. spinosum,* and *G. verrucosa*

The IC_{50} value of nanoparticles was greater than that of *F.vesiculosus* extract, which means that the synthesis of F.vesiculosus AgNPs was successful in increasing antioxidant activity. Polyphenolic compounds as constituents of AgNPs to produce stable nanoparticles and increase antioxidant activity [29].

Flavonoid compounds can inhibit free radicals through the HAT (Hydrogen-Atom Transfer) mechanism. The O-H bond of the hydroxyl group of the flavonoid will separate and the H atom will be transferred to the free radical. In addition, the size of the nanoparticles has a larger surface area when compared to the extract, thus, the potential of nanoparticles to bind to the active site of the enzyme is greater [30]. The large surface area of nanoparticles has a great capability in its activity [31].

Anticollagenase enzyme activity test

Among the three types of algae tested, *F. vesiculosus* species had the highest anticollagenase activity compared to Gracillaria and Euchema spinosum, following the order *F. vesiculosus* > *E. spinosum* > *G. verrucosa*). (Figure 4). *Fucus vesiculosus* species are candidates for anti-aging ingredients or products, while *Euchema spinosum* and *Gracillaria verrucosa* are local Indonesian species. *F. vesiculosus* comprises the *Fucaceae* family with active ingredients such as Alginic acid, alginates, polysaccharides, and iodine [32]. Also, it contains bioactive compound/extract Fucoidan, which has beneficial activity as Anti-aging, and mechanism of action that stimulates collagen production, as an Anti-melanogenic, a mechanism of action which Inhibits tyrosinase and melanin, using an experimental method on B16 murine melanoma cells [11], and as anticancer, a mechanism of action that decreases melanoma growth, using the Mice experimental methods [33]. Furthermore, it also has beneficial activity as antioxidant, mechanism of action which prevent oxidation formation, using an experimental method in vitro, RAW 264.7 macrophage, and Mouse (ex vivo) [10].

The test results represented that *F.vesiculosus* algae AgNPs were able to increase the inhibitory activity of the collagenase enzyme because of its very strong antioxidant content. The natural antioxidant content in F. *vesiculosus* acts as an inhibitor of the collagenase enzyme. Natural antioxidant compounds such as terpenoids and flavonoids have a synergistic effect in inhibiting the collagenase enzyme which can cause wrinkles on the skin [32]. Nanoparticle compounds have better inhibitory activity than extracts, this is evidenced by the smaller IC50 value of nanoparticles compared to extracts [24].

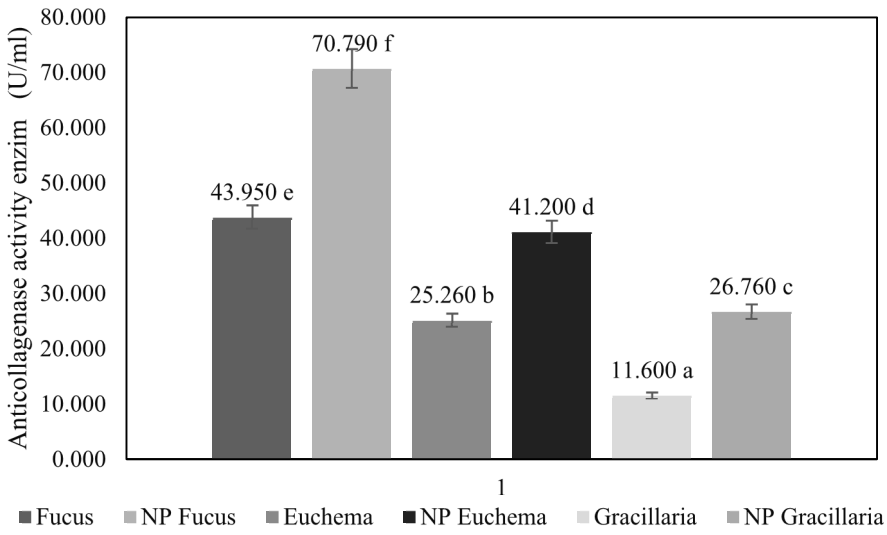

FIGURE 4. Anticollagenase activity enzim (U/ml) extract algae and nanoparticle algae *F. vesiculosus, E. spinosum,* and *G. verrucosa*

The activity of the collagenase enzyme in samples of *F.vesiculosus* in the form of silver nanoparticles can increase the inhibitory activity of the collagenase enzyme. Phytochemical compounds attached to silver can act as inhibitors for the collagenase enzyme. A comparison of the collagenase enzyme inhibitory activity between the extract and AgNPs showed that the AgNPs collagenase enzyme inhibitory activity was greater than that of the extract. The formation of silver nanoparticles had better collagenase enzyme inhibitory activity than the extract indicated by the IC50 value which was lower than the IC50 of the extract. The smaller the IC50 value, the greater the inhibitory activity [34].

CONCLUSION

The results of this study indicate the findings that silver nanoparticle *F. vesiculosus* compounds increase antioxidant activity and anticollagenase enzymes compared to non-nanoparticle compounds. The algae of the *F. vesiculosus* has the highest activity of both antioxidant and anticollagenase compared to the G. *verrucosa* and E. *spinosum*.

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