

## Synthesis of biopesticides using nutmeg essential oil (*Myristica fragrans* Houtt) and its application as a lichen growth inhibitor

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### ABSTRACT

Essential oils are environment-friendly natural ingredients that can be used as biopesticides. Biopesticide formulation using nutmeg seed oil (*Myristica fragrans* Houtt) and its application as an inhibitor of lichen growth on rocks. This study aimed to synthesize biopesticides and determine their inhibitory activity on lichen growth on rocks. The biopesticide was synthesized by adding AgNO<sub>3</sub> powder directly to the nutmeg seed oil, Tween 80 surfactant, and PEG 400 co-surfactant. The synthesized biopesticide had a dark brown color, indicating that the reduction process was successful, as shown by the formation of absorption at a wavelength of 437 nm. The results of the PSA and SEM-EDX analyses showed that the biopesticide had a particle size of 454.8 nm and was spherical in shape with Ag, C and O contents of 59.07%, 28.94% and 11.99% respectively. The addition of Tween 80 and PEG 400 surfactants resulted in particle sizes of 235 nm and 406.2 nm respectively, and a round and non-uniform shape. Tween 80 affects the synthesis of nutmeg oil biopesticide, that is, it is more stable. The synthetic biopesticide is effective as an anti-lichen on stone surfaces.

**Keywords:** Nutmeg oil, Silver nanoparticles, Biopesticide, Anti-lichen activity

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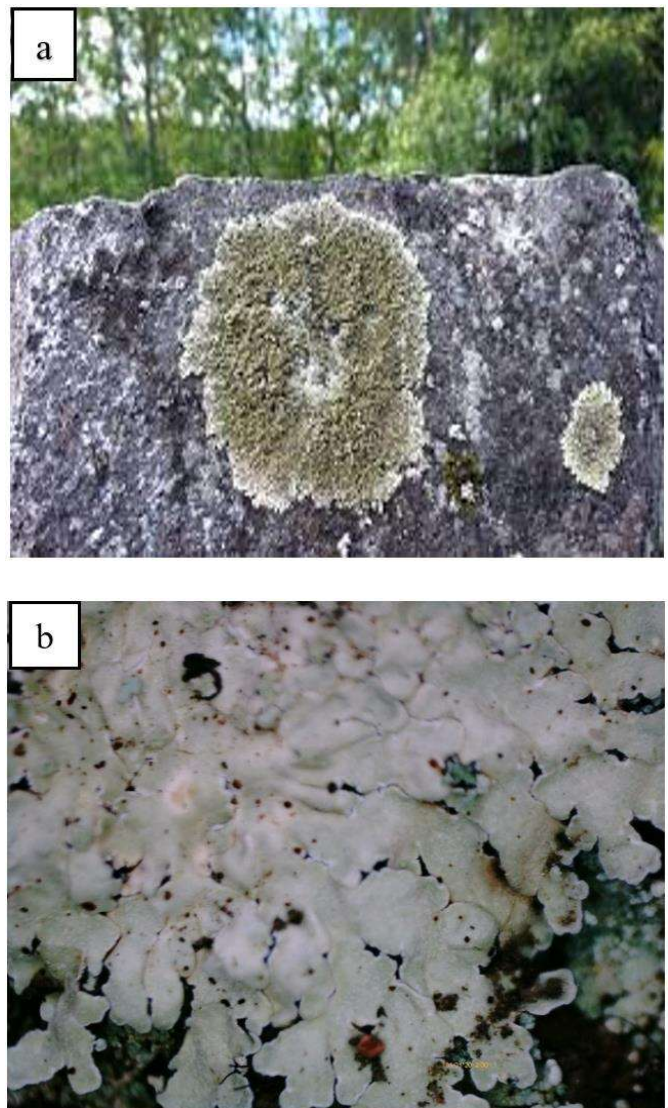
### INTRODUCTION

Cultural heritage must be protected from extinction and damage due to natural processes such as acid rain and attacks by insects, fungi, lichens, and microbes. These microbes grow easily on sandstone, granite, limestone, and gypsum (Burford *et al.*, 2003). Bio-deterioration agents include fungi, algae, lichens, and bacteria. Lichens can attach to man-made property files, such as concrete and scrap metal from abandoned cars and park benches. Lichen comes from two different organisms that are symbiotic with each other. These organisms are fungi and photosynthetic organisms, namely algae, so morphologically and physiologically, it is a unit where the fungus obtains food from algae through the process of

photosynthesis, and the fungus provides the exchange of water, gas and minerals. Lichen is a component of microflora that can occur in all types of rocks in symbiosis with cyanobacteria, chemolithotrophic and chemoorganotrophic fungi, and algae, which are part of the epilytic and endolithic microbial communities. Epilytic fungi are usually located on rock surfaces where symbiosis occurs with algae or cyanobacteria, such as lichens. In addition, fungi can also occupy the sub-surface of rocks as endoliths in pre-existing cracks and fissures, or as cryptoendoliths in mineral pores and voids (Burford *et al.*, 2003). The use of natural ingredients was developed to prevent side effects and to reduce the use of synthetic materials. Natural plant ingredients have

the potential to control microorganisms because they contain active substances (Ibrahim, 2020). Essential oils are among the natural ingredients that contain active substances. Essential oils are volatile compounds with a characteristic odor (Bakkali *et al.*, 2008). Most of these contain natural antioxidants and antimicrobials (Mehra *et al.*, 2015). The registration of pesticides made from essential oils has passed the Environmental Protection Agency (EPA) and is declared safe by GRAS so that it is environmentally friendly and safe for humans. One type of essential oil is nutmeg. The contents of various active compounds in nutmeg essential oil include carotenoids, phenols, vitamins, and flavonoids and have major components such as sabinene, myristicin, terpineol,  $\alpha$ -pinene,  $\beta$ -pinene, methyl eugenol, and limonene (Shekarforoush *et al.*, 2007). These compounds can also interfere with fungal growth. Nutmeg oil also has the potential to reduce metal ions, scavenge radicals, and be antimicrobial, antibacterial, antifungal, and anti-inflammatory (Mousavi *et al.*, 2019). It possesses insecticidal, antioxidant, and antifungal properties (Valente *et al.*, 2014). Nanomaterial-sized biopesticides have a distinctive size compared with micrometer-sized materials because of their larger surface area (Iglesias-Silva *et al.*, 2007). Metal nanoparticles (NPs) are a developing nanomaterial. Metal nanoparticles have a high surface area and specific surface atoms because of their extraordinary physicochemical characteristics, including optical, catalytic, electronic, magnetic, and antimicrobial properties (Verma & Mehata, 2016). Silver nanoparticles (AgNPs) are widely researched because of their wide range of applications, one of which tends to be highly developed in the antimicrobial field (Kim *et al.*, 2007). Silver is highly toxic to microorganisms because its attachment to the cell membrane surface disrupts respiration and permeability of microbial cells. This paper reports the results of biopesticide synthesis using nutmeg (*Myristica Fragrans* Houtt) seed oil and its application as a lichen growth inhibitor on rocks. The synthesis of biopesticides by adding  $\text{AgNO}_3$  directly into

nutmeg essential oil, Tween 80, and PEG 400 were investigated in this study. Nutmeg essential oil is effective as a biopesticide, but it evaporates easily. The effectiveness was increased by adding  $\text{AgNO}_3$  to form nanoscale biopesticides. The stability of the biopesticide was improved using Tween 80 and PEG 400. The performance of the biopesticides was observed in their inhibition of lichen growth. An example of lichen on a rock surface is shown in Figure 1.



**Figure 1.** (a) Lichen observed visually and (b) lichen observed using a handy microscope

## MATERIALS AND METHODS

### Materials and instrumentation

The required materials include nutmeg oil, silver nitrate ( $\text{AgNO}_3$ ), anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ), polysorbate 80 (Tween 80), polyethylene glycol 400 (PEG-400), fresh samples of lichens, Potato Dextrose Agar (PDA), and 70% ethanol. The instruments used were Gas Chromatography Mass Spectrometry (GC-MS), Ultraviolet-Visible Spectrophotometer (UV-VIS), Infrared Fourier Transform Spectroscopy (FTIR), particle size analysis (PSA), and Scanning Electron Microscopy-Energy Dispersive X-Ray (SEM) -EDX).

### Formulation of nutmeg oil biopesticide

The formulation of the biopesticide was carried out by mixing 0.017 g of solid  $\text{AgNO}_3$  directly in 10 mL of nutmeg oil in a 25 mL beaker. The mixture was stirred and placed in a 10 mL bottle until homogeneous and stored for 24 hrs at room temperature. The stability of the biopesticide was observed after 1, 3, 5, 7 and 9 days. The biopesticides formed were analyzed using a UV-Vis spectrophotometer. Biopesticide synthesis was also carried out with  $\text{AgNO}_3$  concentrations of 1.0, 3.5, 5.0, 7.5 and 10 mM.

### The effect of Tween 80 addition on the stability of biopesticides

Biopesticide synthesis was performed by varying the weight of silver nitrate ( $\text{AgNO}_3$ ) 0.2, 0.4, 0.6, 0.8 and 1.0 mM.  $\text{AgNO}_3$  was mixed directly in nutmeg oil in a 25 mL beaker. Then, 1 mL of Tween-80 and PEG 400 were added and stirred until homogeneous. The homogeneous solution was then stored for 1 hrs at room temperature.

### Characterization of biopesticide

Biopesticides were characterized using a Hitachi UH 5300 UV-Vis Spectrophotometer and Fourier-Transform Infrared Spectroscopy (FT-IR) Perkin Elmer Spectrum Version 10.5.1 to determine functional groups. Analysis of the particle size distribution and polydisperse index was performed using a Horiba SZ-100 Pore Size Analyzer (PSA), Japan and the surface morphology and elemental content of the materials were analyzed using

Scanning Electron Microscope-Energy Dispersive X-Ray (SEM-EDX) (Phenom World).

### Application of biopesticide to inhibit lichen.

The tools to be used were sterilized by spraying with 70% ethanol, wrapped in paper, and placed in an oven at 180 °C for 1 hour. Potato Dextrose Agar (PDA) was used as medium. PDA of 19.5 g media was dissolved in distilled water in a 500 mL Erlenmeyer flask and heated. After the agar had dissolved, it was sterilized using an autoclave at 121°C for 1.5-2 hrs. The sterile medium was transferred to a petri dish and stored at room temperature until the medium solidified. The lichen was scraped from the rock and placed in sterile microtubes to avoid contamination. Next, the sample was washed using a 0.9% salt solution ( $\text{NaCl}$ ) and centrifuged for 10 min at 1000 rpm to separate the lichen from its impurities. Lichen isolation was performed using sterile medium. The tube wire was burned, and lichen samples were collected using a tube and planted in sterile agar media. After planting, the Petri dish was covered with plastic wrap and incubated for 3-5 days. After incubation, the fungus was selected as lichen and separated using the shear method to separate the fungal cells. The obtained lichens were planted in new media and propagated to the test stock. The lichen obtained from the isolation process was then tested for the effectiveness of its inhibitory power by providing a small filter paper soaked in the test solution and placed on the surface of the media where the lichen was planted. After incubation at 27 °C for 48 hrs, inhibitory power was measured using a scan 500 tool, namely an automatic colony counter, and the diameter of the inhibition zone in the lichen colony was obtained.

## RESULT AND DISCUSSION

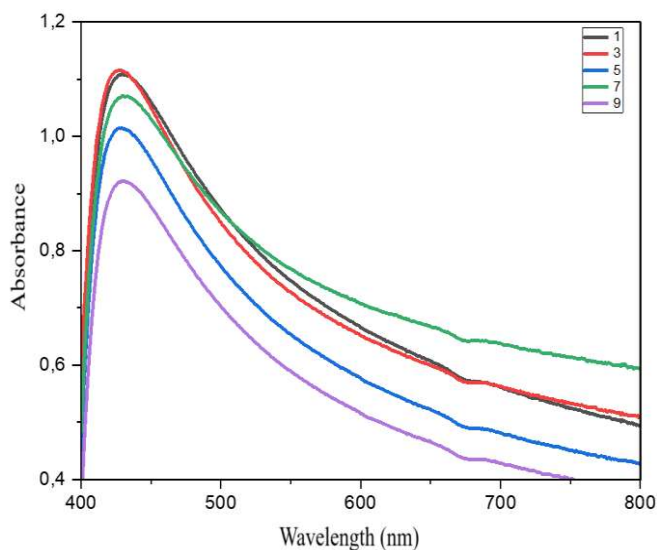
### Variation of storage time on the stability of biopesticide

Variations in storage time were observed every 24 hrs for nine days. Visually observing the effect of storage time on biopesticides, the change in color to dark brown is shown in Figure 2. The color changed to brown on day 1, dark brown on day 3, and dark brown on days 5, 7 and 9, with a



**Figure 2.** Visual observation of stored biopesticide (a) nutmeg seed oil (b) 1 (c) 3 (d) 5 (e) 7 and (f) 9 days

distinctive silver shiny coating attached to the surface of the bottle. The distinctive color of the silver nanoparticle solution was caused by plasmon resonance on the silver surface and the color produced in each sample indicated the influence of the reducing agent used.



**Figure 3.** UV-Visible spectra of biopesticides with varying storage times

The stability of the colloidal AgNP solution can be observed from the changes in the absorption peaks. The nutmeg oil biopesticide with silver has a maximum wavelength ( $\lambda_{max}$ ) in the range of 400-500 nm (Almasoud *et al.*, 2021). In this study,

measurements were carried out in the wavelength range–380-800 nm. UV-visible spectra showed that from day to 1-9 (Figure 3), silver nanoparticles were formed with the appearance of a characteristic peak, and reduction of  $Ag^+$  to  $Ag^0$  occurred in the liquid phase.

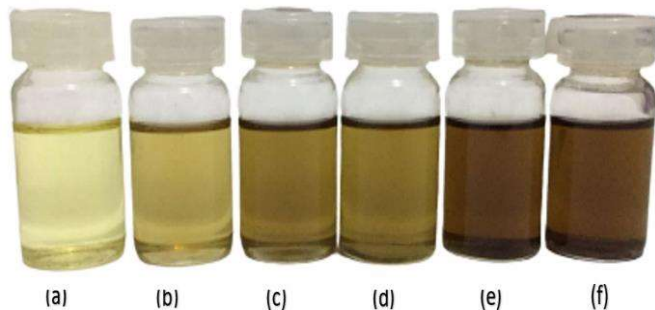
If there is a shift in the absorption peak to a longer wavelength, the stability of colloidal AgNPs is still low (Wahyudi *et al.*, 2011). The increased wavelength shift is due to changes in the plasmon resonance.

#### **Effect of variations in $AgNO_3$ concentration**

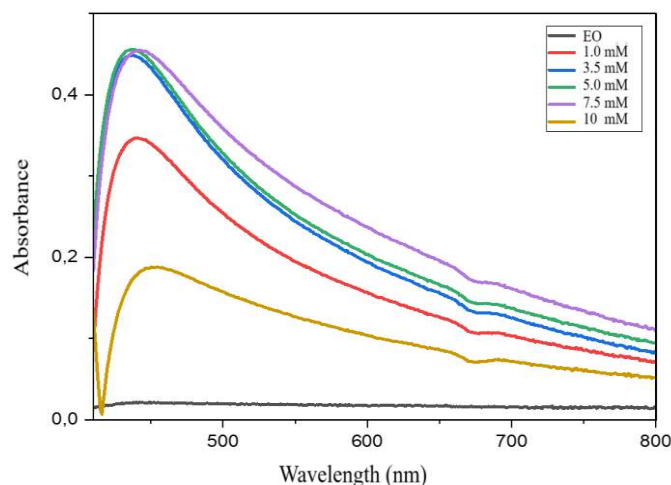
Synthesis of AgNPs was carried out with  $AgNO_3$  concentrations of 1.0, 3.5, 5.0, 7.5 and 10 mM. Visually observing the color change in the formation of silver nanoparticles is shown in Figure 4, where the nutmeg oil is pale cat colored. The addition of  $AgNO_3$  at a concentration of 1.0 mM the solution turned clear brown and dark brown color. The higher the precursor concentration, the darker the color of the resulting solution because the reduction process is faster.

Figure 5 shows the visible UV-visible spectra of the effects of variations in the addition of  $AgNO_3$ . The absorbance intensity increased with an increase in the  $AgNO_3$  concentration.  $AgNO_3$  concentration influences the formation of silver nanoparticles. The increase in concentration causes a broadening and shift of the peak at





**Figure 4.** Visual observation of variations in  $\text{AgNO}_3$  concentration, (a) EO-100%, (b) 1, (c) 3.5, (d) 5 (e) 7.5 and (f) 10 mM.

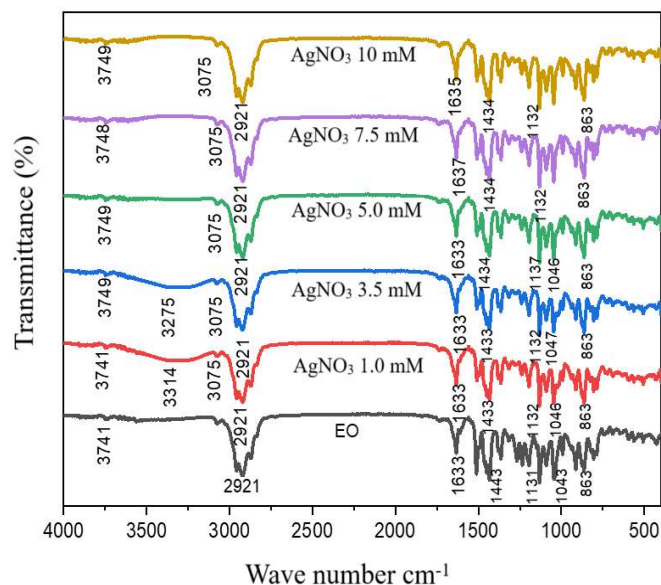


**Figure 5.** UV-Visible spectra of the effect of  $\text{AgNO}_3$  concentrations

$\text{AgNO}_3$  concentrations of 7.5 and 10 mM. Qualitatively, the higher the absorbance value, the more AgNPs will be formed in the solution. The shift of the absorption peak to a longer wavelength indicates that the stability of AgNPs is still low due to agglomeration events (Wahyudi *et al.*, 2011).

The results of the characterization of the biopesticides using FTIR are shown in Figure 6. Functional groups in essential oils include C=C, -CH<sub>2</sub>, -CH<sub>3</sub>, -OH, CH, C=O, COC, CH<sub>3</sub>-C-CH<sub>3</sub>, CO, and -CN. Functional groups in essential oils are used to reduce Ag<sup>+</sup> ions to Ag silver (Sana *et al.*, 2021). Figure 6 shows the characteristic peaks of absorption at 3749 and 3671 cm<sup>-1</sup>, detected as stretching vibrations of the OH group, in the flavonoid and phenolic compounds of nutmeg oil. This functional group was assumed to reduce the

Ag metal ions in the NPs. The characteristic absorption of biopesticide at 3275 cm<sup>-1</sup> shows OH stretching vibrations, while the peak at 3075 cm<sup>-1</sup> indicates the presence of =C-H vibrations and CH<sub>2</sub> stretching vibrations at 2921 cm<sup>-1</sup> absorption, while the absorption peak at 1633 cm<sup>-1</sup> is an aromatic C=C stretching vibration, and peaks at 1433 cm<sup>-1</sup> and 1437 cm<sup>-1</sup> are caused by the asymmetric bending vibration of C-H<sub>3</sub>. In absorption, the absorption is 1132 cm<sup>-1</sup> as the C-H bending vibration, and the C-O peak appears at 1043 cm<sup>-1</sup> and 1047 cm<sup>-1</sup> (asymmetric stretching vibration) C-O alcohol from the ester group.



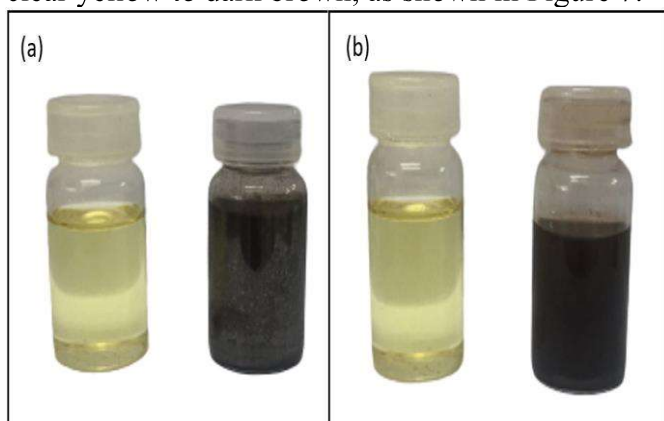
**Figure 6.** FTIR spectra of the influence of variations in  $\text{AgNO}_3$  concentration

#### Effect of Tween 80 and PEG 400

Surfactants and co-surfactants were added because the biopesticide particle size was still high. Biopesticide synthesis requires surfactants to coat its surface (Mout *et al.*, 2012). Nanoparticles tend to agglomerate; thus, stabilizers play an important role in the synthesis of nanoparticles (Kadam *et al.*, 2015). The choice of stabilizing agent for nanoparticles is very important because stabilizers often affect various properties of nanoparticles, including their size, shape, and interaction with solvents (Ajitha *et al.*, 2016). The use of Tween and PEG surfactants improves the solubility of solutes in the dispersion medium by increasing the

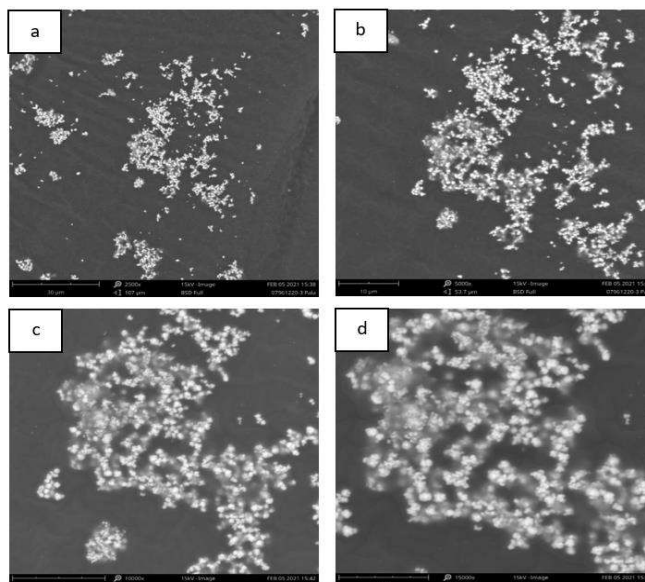
flexibility of the surface layer of the particles (Kadam *et al.*, 2015). The stabilizers or surfactants used in this study were Tween 80 and PEG 400 because they are non-toxic and function as emulsifying agents, solvents, and particle-size stabilizers. The non-toxic organic composition of the stabilizer can increase the rate of internalization and retention of the nanoparticles (Rodrigues *et al.*, 2013).

The formation of biopesticides was observed visually after 24 hrs. Visually, the observed results of the addition of Tween 80 surfactant showed a change in the color of the colloidal nanoparticles from clear yellow to dark brown, and the addition of the PEG 400 co-surfactant also changed from clear yellow to dark brown, as shown in Figure 7.



**Figure 7.** Visual observation of biopesticides using (a) PEG 400 and (b) Tween 80

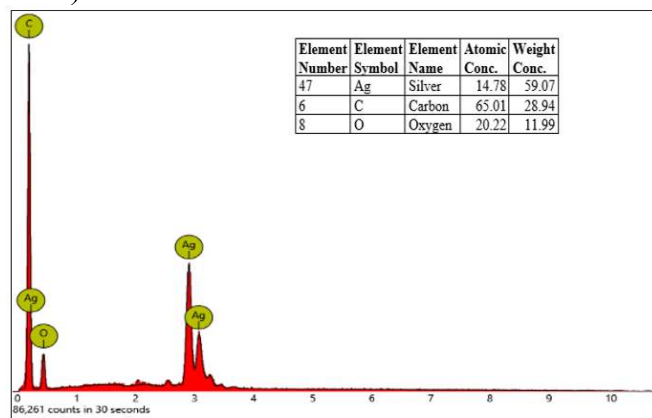
SEM analysis was used to determine the surface morphology of the biopesticides (Figure 8). Image magnification was performed at scales of 2500, 5000, 10,000 and 15000x scales. In this study, the visible morphology was round and non-uniform. The AgNPs were mostly spherical (Sharma *et al.*, 2014). SEM results at magnification of 2500x (Figure 8a) and 5000x (Figure 8b) show that most of the silver particles are scattered so that they are good enough to be observed, and it is estimated that the shape of the particles undergoes aggregation, causing the particle size to be non-uniform. Observations at 10,000 × magnification (Figure 8c) and 15,000 × magnification (Figure 8d) show a clearer morphology of the distribution of the silver particles.



**Figure 8.** Morphology of biopesticides at magnification a) 2500, b) 5000, c) 10000 and d) 15000x

The EDX spectrum is shown in Figure 9, with the elemental composition involved in the formation of Ag, C, and O silver nanoparticles. Tween 80 is believed to undergo a redox reaction in which free radicals are formed from  $\alpha$ -carbon oxygen ethers through hydrogen separation at the initial stage. Therefore, radicals can be further oxidized to esters or degraded to aldehydes (Pal *et al.*, 2012). Metal ions such as  $\text{Ag}^+$  can accelerate oxidation (Li *et al.*, 2012). PSA characterization (Figure 10) aims to determine the distribution of particle sizes in the sample. The homogeneity of a solution analyzed by PSA can also be determined from the polydispersity index (PI), which has a range from 0-1. A value close to 0 indicates homogeneous dispersion, while a value greater than 0.5 indicates heterogeneous dispersion (Avadi *et al.*, 2010). The polydispersity value indicates the spread of the particle-size distribution. The smaller the polydispersity index value, the smaller is the particle size distribution. Determining the characterization of the size distribution of nanoparticles is an important part to know because the size obtained determines the effectiveness of the test material and the ease with which the particles are absorbed by microbial cells. In general, size and shape are important factors that

determine the biological effects of nanoparticles, such as cellular uptake, cellular activation, and distribution between cells (Helmlinger *et al.*, 2016).



**Figure 9.** EDX spectrum of the formulated biopesticides

**Table 1.** Particle size distribution of biopesticides added Tween 80 surfactant.

AgNO <sub>3</sub> concentration (mM)	Particle size (nm)	Polydisperse index value (PI)
0.2	138.3	0.143
0.4	299.9	0.551
0.6	439.9	0.246
0.8	389.8	0.143
1.0	248.4	0.260

Table 1 shows the results of the particle size analysis with the addition of Tween 80 and various concentrations of AgNO<sub>3</sub>. The PI value represents the spread of the particle size distribution. The smaller the polydispersity index value, the more uniform is the particle size. PI has a range of 0-1, where a value close to 0 indicates homogeneous dispersion, while a value greater than 0.5 indicates heterogeneous dispersion, and the particle size tends to be non-uniform. The PI value at a concentration of 0.4 mM > 0.5 means that the solution was not dispersed homogeneously. The large particle size is influenced by several factors, such as temperature, amount of reducing agent and precursor used, and reaction holding time (Sileikaite *et al.*, 2006). In addition, it is possible

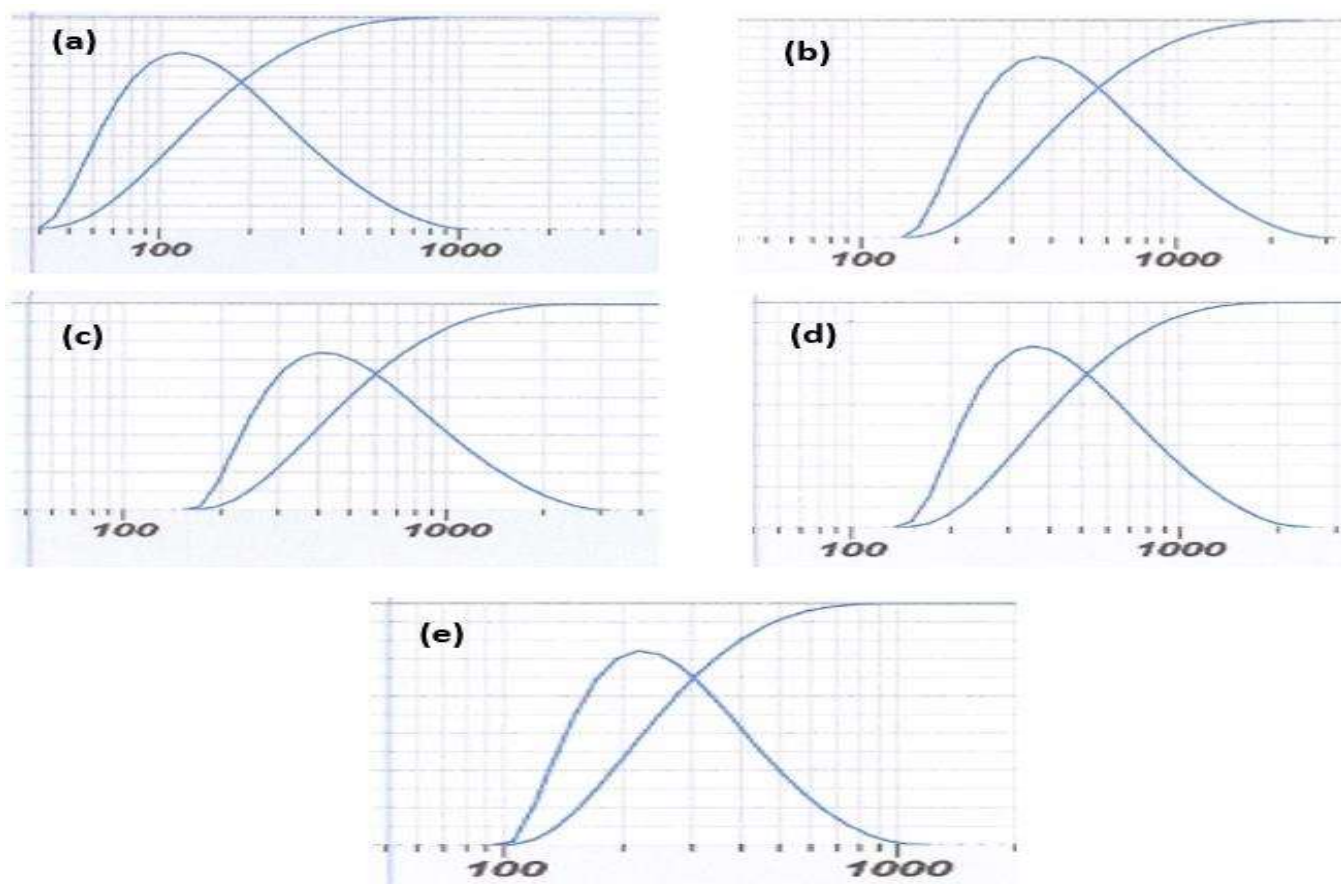
that the stabilizing agent used was not evenly dispersed in the AgNP solution.

Biopesticide testing was performed using the paper dish diffusion method (Figure 11). Biopesticides show inhibitory activity against lichen by forming clear zones on EO; 0.2mM; 0.4mM; 0.6 mM, 0.8 mM and 1.0 mM and the width of the inhibition zone diameter respectively 16.4 mm, 22.8 mm, 11.7 mm 13.5 mm, 16 mm, 21 mm and control 0. The size of the formed clear zone indicates the strength of the drag force. The wider the clear zone formed, the stronger was the active compound that inhibited lichen proliferation. Inhibition of microbial growth is influenced by active compounds in nutmeg seed oil (Rumopa *et al.*, 2016). Ag metal is toxic to cell membranes, affects respiration, and causes cell death. In addition, AgNPs also exhibit antimicrobial activity (Sharma *et al.*, 2014).

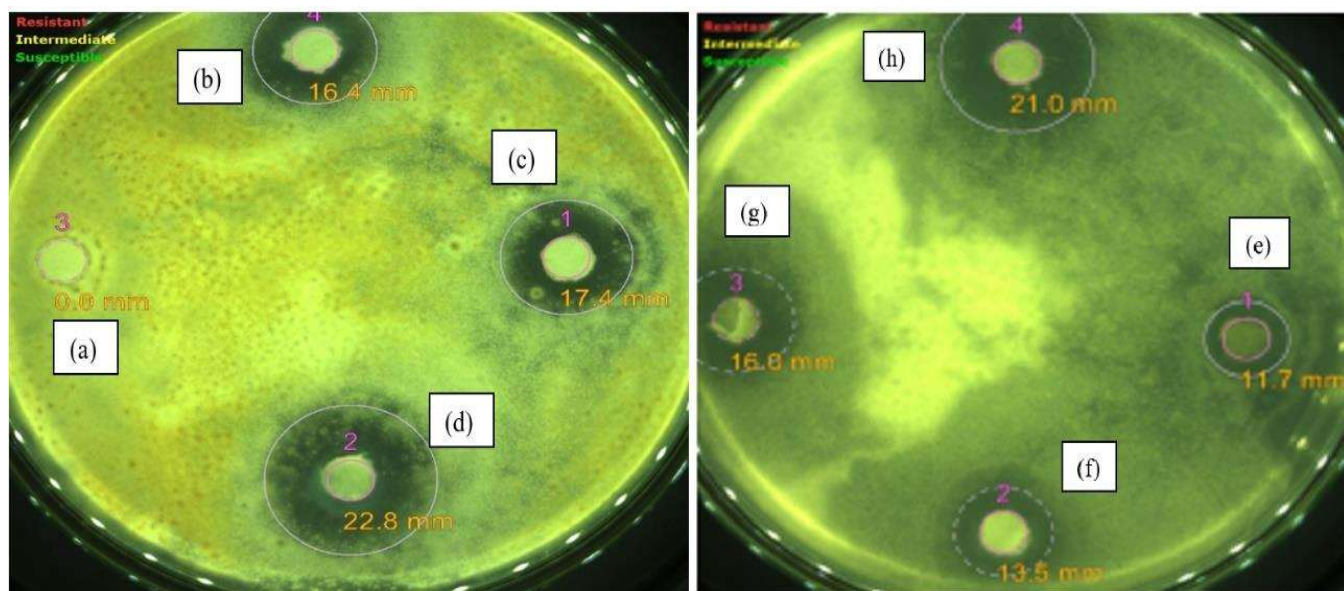
The mechanism for inhibiting lichen activity is due to the compounds present in nutmeg seed oil. Nutmeg seeds exhibit antimicrobial activity against bacteria and fungi (Gupta and Rajpurohit, 2011). The compounds  $\alpha$ -pinene and  $\beta$ -pinene have been reported to have antimicrobial activity (Dorman & Deans, 2000) and are supported by Ag metal, which has an antifungal effect. The AgNP solution diffuses and interacts with the sulfur and phosphorus contained in the DNA of microbes, causing damage to microbial cells. This interaction causes DNA to lose its replication ability, thereby preventing cell division and growth. Therefore, microbial cells died.

Biopesticide synthesis can be carried out directly using nutmeg seed oil by mixing solid AgNO<sub>3</sub>, which is characterized by a color change from brown to dark brown. Variations in the AgNO<sub>3</sub> concentration show changes in the color of the resulting solution, which becomes darker and tends to be stable with higher  $\lambda_{max}$  and absorbance values. Tween 80 produced a more stable biopesticide than PEG 400 did. The smaller the AgNO<sub>3</sub> concentration, the greater the inhibitory power against lichens. The biopesticide activity test using the inhibitory power test against





**Figure 10.** Particle size analysis where the effect of adding  $\text{AgNO}_3$  (a) 0.2 (b) 0.4 (c) 0.6 (d) 0.8 and (e) 1.0 mM



**Figure 11.** Anti-lichen activity test with concentrations of (a) control, (b) EO (c) 0.2 (d) 0.4 (d) 0.6 (e) 0.8 (f) 1.0 mM  $\text{AgNO}_3$



ichens was proven to be more effective than using essential oils alone.

#### ACKNOWLEDGEMENT

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