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Investigation of gold nanoparticles for Ganoderma fungi treatment in oil palm trees

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ABSTRACT

In recent years, oil palm has been one of the industries that contribute substantially to the gross domestic product of producing countries. As per Plantation Industries and Commodities, palm oil accounted for roughly 66.1% of the total export earnings, reaching RM 44.63 billion in 2022. However, several threats to palm trees, such as Ganoderma basal stem rot disease (BSR), pose risks to our country's gross domestic product. BSR, caused by the fungus *Ganoderma boninense*, leads to the breakdown and degradation of the lignin trunk of palm trees. Therefore, gold nanoparticles have been fabricated in this project because of their ability to treat fungi. Gold nanoparticles were synthesized in two forms: gold nanorods (GNRs) and gold nanobipyramids (GNBPs) utilizing a wet chemical technique called the Seed-Mediated Growth Method (SMGM). Two plasmon peaks were obtained for synthesized nanoparticles corresponding to transverse surface plasmon resonance (t-SPR) and longitudinal surface plasmon resonance (l-SPR). GNRs have a t-SPR peak at 536 nm and an l-SPR peak at 713 nm, while for GNBPs, a t-SPR peak was obtained at 563 nm and 809 nm for l-SPR peak. For structural properties, the intensity peak at the plane (111) for GNRs and GNBPs occurs at 38.19° and the plane (200) occurs at 44.39°. The surface density of GNRs is 65.26±3.44% and for GNBPs is 69.44±2.94%. For the Ganoderma treatment process, the observation was made for three samples; no control, GNRs and GNBPs. It was found that GNBPs have a high ability to inhibit fungal growth GNRs with no fungal found in their area. In the GNRs area, 20% of fungal growth was found, and there was 60% of fungal growth around no control. In conclusion, GNBPs successfully inhibited fungal growth due to their curvature and sharp edge tips that will interact with microbial cell membranes, resulting in membrane damage and leakage of cellular contents of fungi.

Keywords: *Gold nanorods, Gold nanobipyramids, Fungi treatment, Ganoderma boninense*

1. INTRODUCTION

Malaysia diversified its export of palm oil and oil palm products to over 200 global markets, encompassing regions such as the Indian subcontinent, West Asia, Africa, and Asia. The Malaysian oil palm sector has emerged as a significant driver of Malaysia's Gross Domestic Product (GDP), foreign exchange revenue, and job creation. Nonetheless, numerous threats persist, with Ganoderma basal stem rot (BSR) disease standing out as a significant risk to oil palm plantations [1]. In the family Ganodermataceae, the genus Ganoderma contains over 80 species of polypore fungi, many of which are native to tropical areas. Ganoderma has double-walled basidiospores and may be distinguished from other polypores. They are also known as bracket fungi or shelf mushrooms, and they have large, perennial, and woody brackets known as “conks” that are characterized by basidiocarps [2].

The Ganoderma BSR disease is attributed to a particular fungus called *Ganoderma boninense*, which can deteriorate or break down the lignin trunk of palm trees [3]. All species of wood, including gymnosperms, woody dicots, and palms, are home to the wood-decaying fungi in the genus Ganoderma. In Florida, there are numerous species of this fungus, but only one of them is a disease of palm trees; it’s called *Ganoderma boninense*, and there is no disease caused by this fungus in any other plant family [4]. This fungus is a white rot fungus that develops a variety of enzymes that enable it to break down lignin and then cellulose. Ganoderma BSR cannot be identified in a palm until the basidiocarp (conk) grows on the trunk or until the interior colour of the trunk is seen after the palm has been cut down [5]. The spores that are created and released from the basidiocarp spread the fungus (conk). As soon as the conks start to appear on the trunk, a palm needs to be taken off. When the palm is gone, a little of the stump and root system needs to be taken or otherwise crush the stump. Besides, planting another palm in the same spot is not advised without taking particular care since the fungus persists in the soil. Figure 1 shows the affected oil palm trees at a plantation in Kota Tinggi.

Figure 1. Affected oil palm trees in a plantation at Kota Tinggi, Malaysia. a) basal rooting, b) Basidiocarp at oil palm base, c) Ganoderma, d) Extraction of Ganoderma fungus

Therefore, the need to eliminate this kind of fungus is crucial, as Ganoderma fungus will live as a parasite on the oil palm tree and cause damage to the tree. It will destroy the structure of the oil palm tree and spread the disease to other oil palm trees until the tree is dead. This factor will have a huge impact and cause a loss in our country's GDP. There are a few methods that have been invented to treat the disease in oil palm plantations: physical, chemical, and biological control. These methods have been used for a long time to treat oil palm plantations. Two examples of methods of physical control that have been used to prevent the development of Ganoderma fungus are windrowingto minimize the spread of the fungi and surgery and mounding treatment. However, these methods are very expensive and require a lot of physical energy to proceed with them. For windrowing, this method is not very efficient in stopping the survival of fungi that infect the palm, so we will use open burning to reduce the wound on the palm tree. Although open burning is used, this method has been banned by the Environmental Quality Act in Malaysia to avoid fire incidents [6]. The surgical and mounding interventions on palm trees led to a reduced incidence of BSR on oil palms during the initial 9 years post-planting, with the BSR rate consistently kept below 5% [1], [7]. This treatment involves removing the affected part of the outer stem, and the open surfaces will be treated with a protectant chemical, coal tar and Trichoderma, to stop the decay caused by Ganoderma fungi. Meanwhile, the soil mounding and soil compacting processes can delay the development of BSR and prolong the infected palm tree's lifespan for 2 to 3 years [8].

An alternative method employed is chemical intervention. Fungicides, a longstanding chemical treatment, have been utilized to address *Ganoderma boninense* infections in oil palm trees. For instance, organomercury formulations and potassium hydroxyquinoline sulphate are administered by injecting fungicides into the affected palms [9], [10]. However, this method is very challenging and has its limitations to success because it will be difficult to measure the size of the trunk wound due to the large mass of numerous wounds. Furthermore, the progression of BSR can be impeded by administering systemic fungicides like fusilazol and hexaconazole. Arifin and Idris conducted a study indicating that injecting a fumigant fungicide, dazomet, into the stem can halt the spread of BSR by inhibiting the growth of fruiting bodies [7], [11]. Although fungicides have a good and efficient result in preventing BSR disease, this method can cause environmental damage to our ecosystem and the evolution of fungicide resistance. Another alternative is biological control. In Malaysia and Indonesia, a method developed to combat BSR disease involves utilizing bacteria and fungi as biocontrol agents. This approach has demonstrated promising outcomes in inhibiting the proliferation of *Ganoderma boninse* in plantations and their natural habitats, employing Trichoderma species like *T. harzianum*, *T. reesei*, and *T. asperellum* [11], [12]. Nevertheless, this Trichoderma species demonstrates effectiveness solely against pathogens in the initial stages of infection. Other examples of biocontrol agents that have reduced the severity of BSR by suppressing the growth of *Ganoderma boninense* are Burkholderia and Streptomyces spp. In Malaysia, two commercially available products contain biocontrol agents that are efficient against *G. boninense.* One of the products is Hendersonia GanoEF3, which uses inorganic and organic fertilizers as a carrier and contains the endophytic fungus Hendersonia toruloidea. This mixture can promote soil fertility and vegetative development and prevent pathogen infection. Another product that can reduce the infection and inhibit the growth of *Ganoderma boninense* is EMBIOTM ActinoPLUS [12].

GNPs exhibit inherent antifungal activity, which can be leveraged to target and inhibit *Ganoderma* directly. Their small size and high surface area enable better interaction with fungal cells, potentially leading to improved antifungal effects. Besides, using GNPs can potentially reduce the likelihood of resistance development compared to conventional antifungal chemicals. Their unique mechanism of action might decrease the chances of *Ganoderma* developing resistance. Hence, in this study, chemical control using GNPs has been proposed for the *Ganoderma* fungi treatment. The ability of GNPs to prevent the *Ganoderma* basal stem rot disease from the beginning is more effective in eliminating the *Ganoderma* fungi and, at the same time, will not damage our ecosystem. GNPs have been synthesized and characterized into rod and bipyramid shapes, and the effectiveness of these GNPs towards *Ganoderma* fungi has been studied and analyzed.

2. MATERIALS AND METHODOLOGY

All the materials and processes in this study will be explained. The process involved the synthesizing and characterization of GNRs and GNBPs, followed by the disc diffusion method used in Ganoderma fungus treatment.

2.1. Materials

To synthesize GNRs and GNBPs, the following materials were utilized: hydrogen tetrachloroaurate ($HAuCl_4.3H_2O$, ≥99.9%), chloroplatinic acid hydrate ($H_2PtCl_4.H_2O$,

99.99%), cetyltrimethylammonium bromide (CTAB, ≥98%), sodium borohydride (NaBH4, ≥98.0%), ascorbic acid (C6H8O6), and agar medium sourced from Sigma Aldrich. Hydrochloric acid (HCl, 37%) was procured from RCI Labscan. Silver nitrate (AgNO3, ≥99.0%) was acquired from Honeywell Fluka and QreC. All solutions of these chemicals were prepared using deionized water (DIW) with a resistivity of 18.2 MΩcm obtained from a Millipore RiOs water purification system. Prior to preparation, all glassware underwent washing with decon and water, followed by sonication in DIW for 15 minutes. This cleaning process was repeated for acetone and 2-propanol, respectively, before drying in an oven at 50°C for 1 hour.

2.2. Synthesis of Gold Nanorods (GNRs) and Gold Nanobipyramids (GNBPs)

The synthesis procedures were conducted utilizing the Seed-Mediated Growth Method (SMGM), comprising two primary stages: the seeding process for planting nanoseeds and the growth process for nurturing the nanoseeds to the desired growth duration. The methodologies employed were adapted from our prior publication on GNRs [13] and GNBPs [14], [15].

In the seeding process for GNRs, three distinct chemicals were employed: gold chloride, CTAB, and sodium borohydride. Initially, 15 mL of 0.5 mM was combined with 15 mL of 0.2 M CTAB solution and stirred for 15 minutes. The interaction between surfactant agents (CTAB) and precursors led to the quantitative displacement of ions by ions in CTAB micelles. Subsequently, 1.8 mL of ice-cold 0.01 M $NaBH_4$ was introduced to the CTAB mixture, facilitating the reduction of the borohydride anion and the formation of quasi-nanoparticles stabilized with CTAB bilayers during the reduction process. Consequently, the seeding solution of GNRs gradually transitioned in colour from yellow to light brown throughout the process. This colour alteration is attributed to the conversion of gold ions into gold particles in the presence of water, with the seeding process completed after two hours at room temperature. For the growth process of GNRs, four different types of chemicals were used, which are $AgNO_3$, CTAB, gold chloride, and ascorbic acid. First, a newly prepared 0.6 mL of 4 mM $AgNO_3$ was added to 15 mL of 0.2 M CTAB solution. Next, 15 mL of 1 mM was added to the mixture solution. Quantitative displacement of ions by CTAB micelles was generated because of a reaction between surfactant agents (CTAB) and precursors. Then, 210 uL of 0.1 M ascorbic acid was added to the mixture solution. Subsequently, the solution colour transitioned from yellow to colourless as the bound CTAB micelles were reduced to Au(I) by L-ascorbic acid. Following this, 30 uL of the seed solution was introduced into the growth solution containing stable ions and stirred for 1 minute to homogenize the mixture. The colour of the GNRs' growth solution gradually shifted from colourless to purple throughout the process. The growth phase was allowed to proceed undisturbed for 20 hours at room temperature. Subsequently, the GNRs' growth solution underwent centrifugation for 30 minutes at 5000 rpm to separate the precipitate from the supernatant. The precipitate was then dispersed in 1 mL of deionized (DI) water, collected, and deposited onto the substrate surface.

For the seeding process of GNBPs, four different types of chemicals will be used: gold chloride, CTAB, chloroplatinic acid, and sodium borohydride. First, 0.15 mL of 0.01 M is mixed with 0.1 mL of chloroplatinic acid 0.01 M in 9.75 mL of CTAB 0.1 M solution. The colour of the mixed solution will be yellow because of the quantitative displacement of ions by ions in CTAB micelles. Next, the mixture solution was added to 0.9 mL of ice-cold water and continued with gentle shaking. Then, the colour of the seeding solution for GNBPs will change from yellow to brown during the process. The colour changes because of the borohydride anion will reduce to and produce quasi-nanoparticles stabilized with a CTAB bilayer in the reduction process, and the seeding process will complete after two hours at a temperature of 27 °C. After developing for two hours, the seed solution was ready to use in the growth process. For the growth process of GNBPs, six different types of chemicals will be used, which are gold chloride, CTAB, chloroplatinic acid, hydrochloric acid, and ascorbic acid.

Firstly, 20 mL of 0.1 M CTAB is added to 0.875 mL of 0.01 M, 0.025 mL of 0.01 M chloroplatinic acid, 0.2 mL of 0.01 M, and 0.4 mL of 1.0 M hydrochloric acid. The mixture solution will form a yellow colour due to the quantitative displacement of ions by ions in CTAB micelles, and we continued the process by adding 0.16 mL of 0.1 M ascorbic acid. The colour of the mixture solution will immediately change from yellow to colourless after mixing with the ascorbic acid. This is because bound CTAB micelles are reduced to Au(I) by L-ascorbic acid. Then, the growth procedure proceeded by adding 50 L of the GNBPs seed solution into the growth solution and leaving it undisturbed for 2 hours at 25 °C. The GNBP growth solution was centrifuged for 15 minutes at 5000 rpm to separate the precipitate and supernatant. This centrifuge process was repeated three times, and the precipitate was dispersed in 1 mL of DI water and then collected and dropped onto the substrate surface.

2.3. Characterization

The structural characteristics of both GNRs and GNBPs were assessed utilizing the Bruker D8 Advance XRD system from Germany. X-ray diffraction was conducted with CuKα radiation at a wavelength of 1.5406 Å, covering diffraction angles from 20° to 60°. The measurement parameters included a step size of 0.03°, scanning speed of 0.5°/minute, and operating current and voltage set at 40 mA and 40 kV, respectively. Optical properties were examined using a UV-Vis spectrometer, specifically the UV-1800 Spectrophotometer by Shimadzu from Japan, with wavelengths ranging from 300 nm to 800 nm. The sample morphology was analyzed using a Joel JSM-7600F Schottky FESEM from the USA, operating at an accelerating voltage of 5 kV.

2.4. Disc Diffusion Method

The disc diffusion technique was executed on Mueller-

Hinton Agar (MHA), commencing with the preparation of the agar medium. The agar medium was prepared by using agar powder to help the growth of the fungus to provide nutrients and a place to grow. Since the agar is indigestible to the fungus, they cannot eat or destroy it. Agar powder was weighed at 2.5 g per 50 ml of DI water and mixed using a magnetic stirrer at 100 °C for around 40 minutes to get a good form of agar medium. After the agar powder is fully dissolved in the solution, the pH of the solution needs to be checked using a pH meter before pouring it into the petri dish. Figure 2 shows the process flow for preparing the agar medium.

For the fungi sample, Ganoderma was obtained fresh from a palm oil plantation located in Johor. After obtaining the Ganoderma, the plant needs to be reseeded to keep it fresh. After that, a fungi sample can be obtained by scraping the Ganoderma directly with a spatula and placing it into a petri dish for the next treatment process. Figure 3 shows the process used to get the fungus sample.

For the disc diffusion method setup, the antibiotic discs used have a 6 mm diameter and are carefully stored at 2 to 8 °C in a desiccant-filled container that is tightly sealed before being used. Firstly, the antibiotic disc was placed on the surface of the inoculated agar medium using sterile forceps. Then, the disc was slowly pushed using forceps to ensure complete contact between the disc and the agar surface. The disc should not be moved after insertion because some diffusion can occur instantaneously. In this study, three samples have been tested, which are no control, gold nanorods, and gold nanobipyramids samples. The setup is shown in Figure 4.

3. RESULTS AND DISCUSSION

The properties of GNRs and GNBPs have been explained, and the inhibition ability of Ganoderma fungi has been analyzed for this study.

3.1. Properties of GNRs and GNBPs

The synthesis of GNRs and GNBPs was effectively achieved through the seed-mediated growth method. The properties of both gold nanoparticles have been measured and analyzed. The structural properties have been investigated, and it was found that two intensity peaks in planes (111) and (200) at diffraction angles 2Θ of 38.19° and 44.39° occurred for GNRs. The diffraction pattern is matched with previous research mentioned by Ahmad [16] and the dominant orientation is the (111) plane. The angle reading for GNBPs is the same as for GNRs. The peak positions align with the standard Inorganic Crystal Structure Database (ICSD) file No. 98-061-1625, indicating that the gold particles exhibit a f face-centered cubic (FCC) crystal structure. The structural characteristics of both samples are illustrated in Figure 5.

The optical characterization is obtained using a UV-Vis spectrometer to determine the localized surface plasmon resonance (LSPR) response for the synthesized GNRs and GNBPs. From the UV-Vis graph for GNRs, the longitudinal and transverse surface plasmonic resonances, or l-SPR and t-SPR, were determined using the results. As shown in Figure 6, the two peaks are produced in response to the dimensions of the nanorods and bipyramids, with the lower-energy peak of the t-SPR being caused by the length and the higher-energy peak of the l-SPR being caused by the diameter. Referring to the results, both samples show two absorption peaks that correspond to t-SPR in the wavelength range of 500 to 600 nm (536nm and 563, respectively) and l-SPR in the wavelength range of 700 to 900 nm (713nm and 809nm, respectively). This is consistent with the plasmon resonance peaks for GNRs and GNBPs as previously reported by Suratun [15].

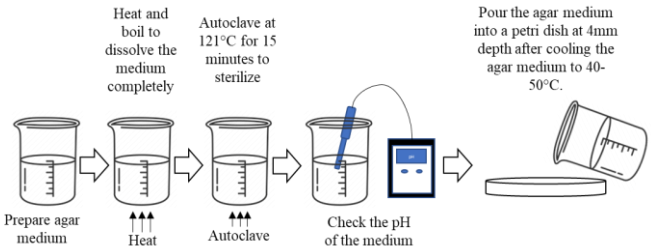


Figure 2. Process flow of the preparation of agar medium

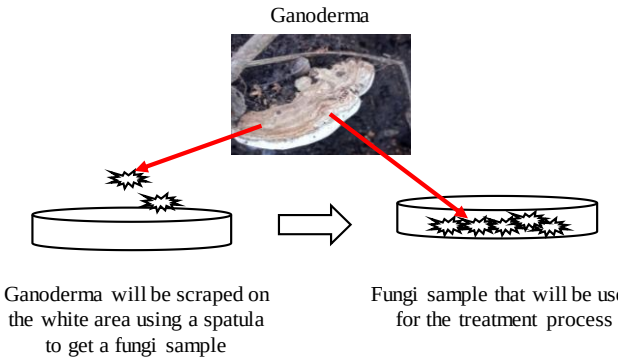


Figure 3. Process of Preparing the Fungus Sample

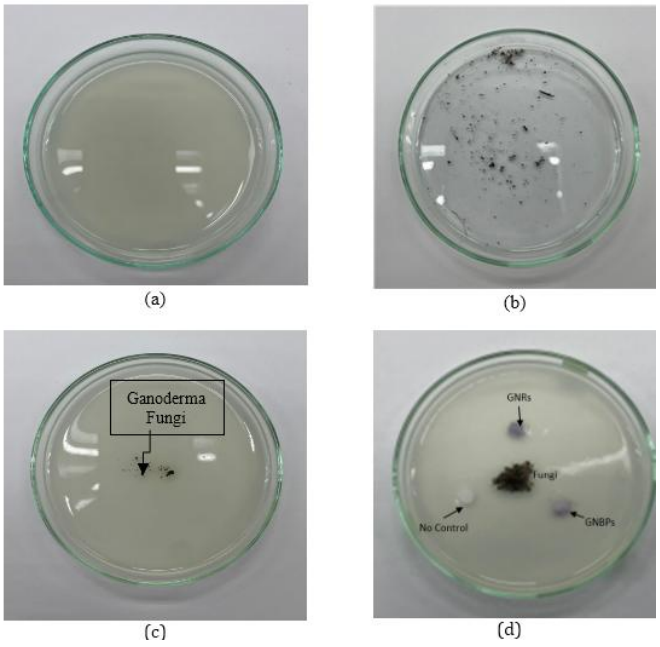
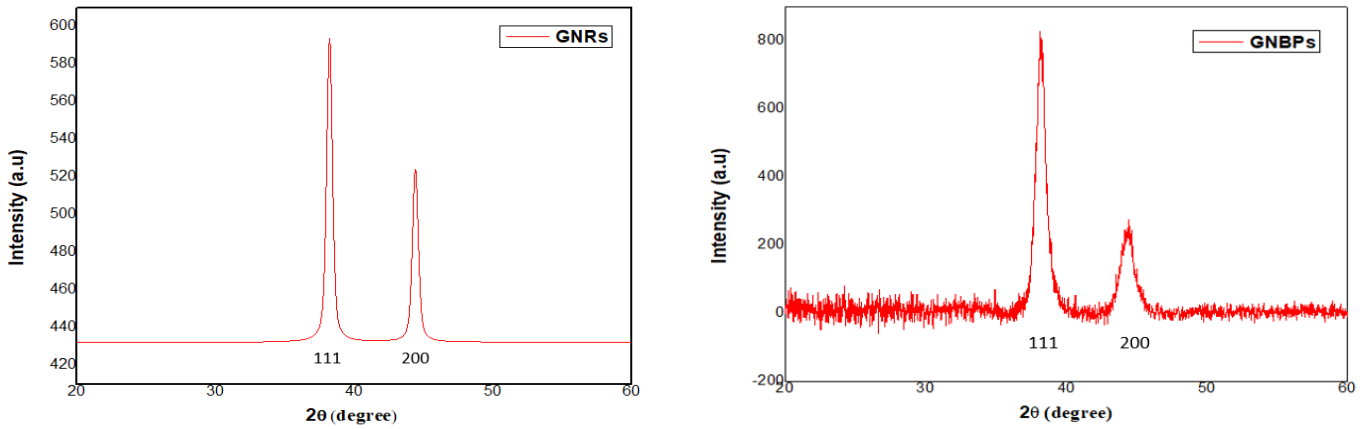
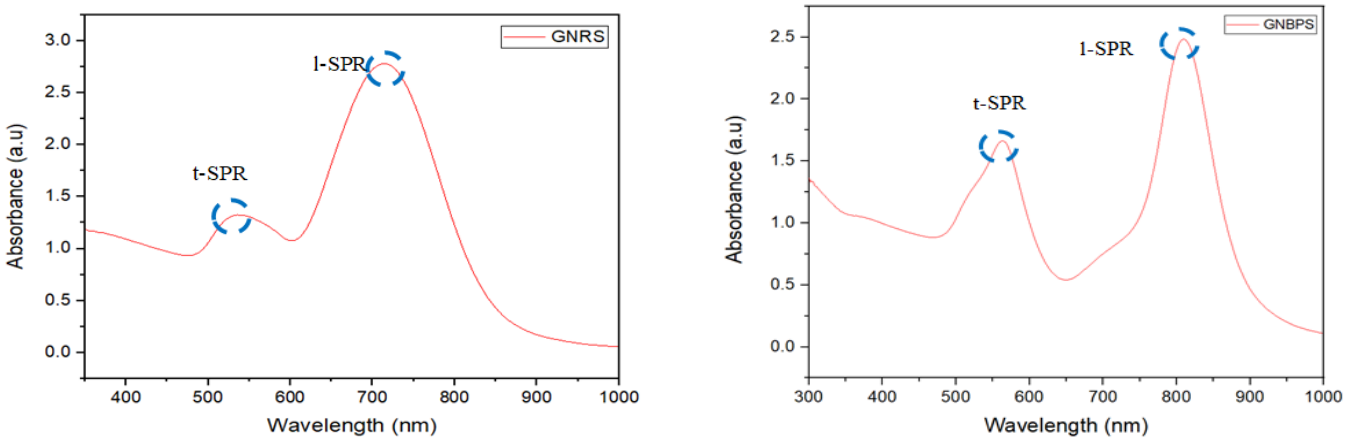


Figure 4. (a) Agar in the petri dish (b) Ganoderma Fungus in Petri Dish (c) Ganoderma Fungi on the Agar Medium and (d) Disc Diffusion Setup for Ganoderma Fungi Treatment



(a) (b)

Figure 5. The structural properties of (a) GNRs and (b) GNBPs



(a) (b)

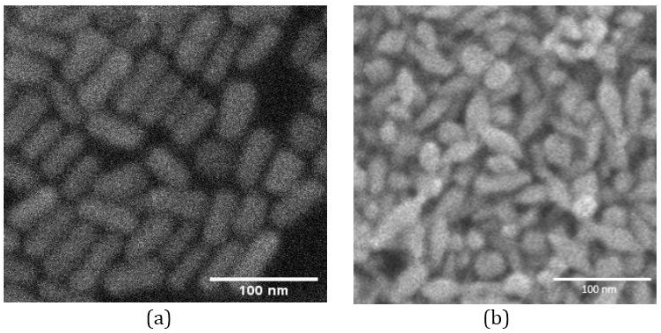
Figure 6. The optical properties of (a) GNRs and (b) GNBPs

Morphological characterization of the synthesized GNRs and GNBPs has been done by analyzing the results of the 50k magnification of FESEM images. This characterization is to determine the shape of both gold nanoparticles successfully synthesized into two shapes, which are rods and bypiramids. Three areas of FESEM images have been chosen to find the average length, width, aspect ratio, surface density, total surface density, and standard deviation of the synthesized GNRs and GNBPs by using ImageJ software. Fig. 7 shows the morphology of GNRs and GNBPs.

From Figure 7 (a), the parameters have been measured and calculated; the average length of the synthesized GNRs is 69.55±1.40 nm while the average width is 22.83±0.73 nm. The average aspect ratio is 3.04±0.036 and the total average surface density that has been obtained is 65.26±3.44 %. The data on surface density that has been obtained, which is more than 60%, indicates that all nanoparticle products were formed as GNRs. According to Akrajas et al., surface densities that exceed 60% will be considered high-yield growth for GNPs [17]. For GNBPs (Figure 7 (b)), the average length is 63.40±3.04 nm while the average width is 22.50±2.86 nm. The average aspect ratio is 2.81±0.00 and the total average surface density is 69.44±2.94 %. Both results show the high-density formation of gold nanoparticles.

3.2. Ganoderma Fungi Treatment using Disc Diffusion Method

Ganoderma fungi have been treated by using the disc diffusion method, which involves three controls. These controls have been set up in a petri dish that contains



(a) (b)

Figure 7. The morphology of (a) GNRs and (b) GNBPs using FESEM

Ganoderma fungi and an agar. The controls that have been used in the experiment are no control, which means no sample has been added to the disc, and two other controls are GNRs and GNBPs. The observation has been carried out for a week to verify whether these fungi can be treated by GNRs and GNBPs. The fungus in the Petri dish has been observed under the microscope to verify the presence of this Ganoderma fungus. Figure 8 shows the image of the fungus using 4x and 40x magnification. Figure 9 shows the image of fungi samples on the agar medium.

In the initial phase, the Ganoderma fungi are placed in the center of the petri dish with all the controls put at a distance of ~ 2 cm from the fungi. It can be seen that there are no fungi in the nearby area of the controls. After 7 days of incubation, the fungal growth was observed again in the control: no-control, GNRs, and GNBPs. The observation images on Day 1 and Day 7 are shown in Figure 10.

From the observation, the control area for each sample was divided into four areas, and it was found that there was 60% of fungal growth around no control, 20% for GNRs, and 0% for GNBPs. As stated in the previous study, GNPs have also been proven for their fungi treatment function since they can penetrate the cell walls of a wide range of microorganisms [18]. For GNBPs, the structure forming from its curvature and sharp edge tips will interact with the microbial cell membranes of fungi, resulting in membrane damage and leakage of the cellular contents. Therefore, it can be claimed that GNPs having been proven to be used for Ganoderma treatment with GNBPs have been successfully inhibiting fungal growth.

4. CONCLUSION

In conclusion, the gold nanoparticles have been successfully synthesized into two shapes, which are rods and bipyramids, by using the Seed Mediated Growth Method (SMGM). The high density of GNRs (65.26±3.44 %) and GNBPs (69.44±2.94 %) are potentially used to treat Ganoderma fungi. The findings show that GNPs have the potential to be used for Ganoderma fungi treatment. GNRs cannot fully inhibit fungal growth, while GNBPs can fully inhibit fungal growth. The structure of GNBPs consists of curvature and sharp edge tips that interact with microbial cell membranes and result in membrane damage and leakage of the cellular contents of fungi, in this case Ganoderma fungi.

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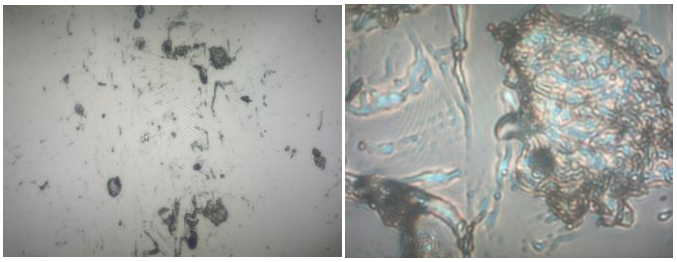

(a) (b)

Figure 8. Image of fungus sample under (a) 4x magnification and (b) 40x magnification

(a) (b)

Figure 9. Image of fungi samples on the agar medium under 40x magnification

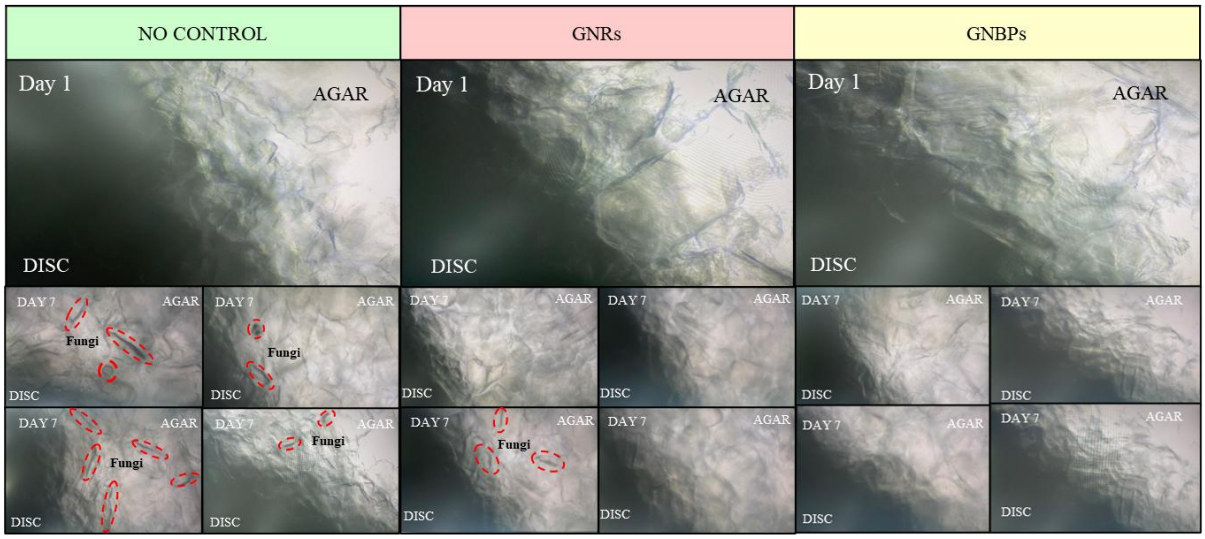

Figure 10. The observation in three areas area of the disc control; no-control, GNRs and GNBPs on Day 1 and Day 7

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