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Research paper



Synthesis of ethanol nanoparticle preparations of white oyster mushroom (pleurotus ostreatus) extract using chitosan and the ionic gelation method

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Abstract

Indonesia is a country rich in natural resources, many of which have the potential to be used as traditional medicinal ingredients—one example is the White Oyster Mushroom (Pleurotus ostreatus). The White Oyster Mushroom is known for its high nutritional value, popularity among the public, and ease of cultivation. It contains various active compounds with potential antioxidant and anti-inflammatory properties, including vitamin C, ergothioneine, flavonoids, alkaloids, tannins, saponins, and other phenolic compounds. Since ancient times, the use of mushrooms as medicine in the community has been widely reported, and more research is being conducted to see their potential to become standardized herbal medicine or phytopharmacology. The study aims to describe the synthesis of ethanol-based nanoparticle preparations of White Oyster Mushroom extract using chitosan as a stabilizing agent and the ionic gelation method. Based on the results of the Particle Size Analyzer, Potential Zeta, and Transmission Electron Microscope (TEM) tests, the oyster mushroom extract nanoemulsion showed that the preparation successfully qualified for stable status as a nanoparticle with a size of less than 1000 nm and a potential zeta of more than -30 mV.

Keywords: *Chitosan; Ionic Gelation Method; Nanoparticles; Phenolic; White Oyster Mushroom Ethanol Extract.*

1. Introduction

In recent years, there has been a significant rise in the global use of herbal medicine. The WHO reports that almost 80% of the population in the world uses herbal medicine for primary health care. In Indonesia, as one of the developed countries, the use of herbal medicine reaches 75 - 80% of the population, around 2 to 3 times the consumption compared to conventional treatment. The widespread use of herbal medicine is evident not only in Asia and Europe but also in various other countries, such as Australia 48%), France 49%), Canada 70%), and 158 million adult people in the US. The World Health Organization defines herbal medicine as a packaged product made from plant parts with active ingredients. The plant parts commonly used in herbal medicines are derived from medicinal plants(Indonesia's Potential Herbal Products Gain in the European Market, 2021).

One of the herbal products showing significant growth in recent years is the White Oyster Mushroom (*Pleurotus ostreatus* Jacq: Fr Kumm). This mushroom has potential medicinal properties and serves as a food source rich in essential nutrients (primary metabolites) required by the body, including protein, unsaturated fats, glucans, and various vitamins and minerals. The utilization of P. ostreatus in the culinary realm extends beyond its role as a fundamental ingredient, encompassing its evolution as a spice and flavoring that has garnered significant popularity within the community [2]. A comprehensive series of trials has revealed the advantageous effects of P. ostreatus consumption on glucose metabolism, characterized by a reduction in fasting and/or 2-hour postprandial glucose levels, along with a decline in lipids, evident by a decrease in total cholesterol, LDL cholesterol, and/or triglycerides. Furthermore, certain trials have documented a reduction in blood pressure. Conversely, there was no alteration in body weight. The assessment did not encompass the subject of appetite sensations(Dicks & Ellinger, 2020; Thomas et al., 2014).

Previous studies have reported that mushrooms are a beneficial food in the prevention of several diseases, including hypertension, hypercholesterolemia, and carcinoma. White oyster mushrooms, in particular, have demonstrated proven therapeutic potential, such as antioxidant and anti-inflammatory properties. Numerous studies have confirmed that white oyster mushrooms possess strong antioxidant activity both in vivo and in vitro (Bhekti Rahimah, Yuwono Soeroto, et al., 2021a; Rahimah, Kharisma, et al., 2019)(Bhekti Rahimah, Yuwono Soeroto, et al., 2021b; *Jurnal Sperma-Rokok, Santun-UII*, n.d.; Rahimah, Djunaedi, et al., 2019). The extract of White oyster mushroom has a hepatoprotective effect on hepatotoxicity induced by carbon tetrachloride (CCl_4), and the effect has also been observed in other organs, such as the kidneys and brain. Previous research demonstrated that the ethanolic extract of white oyster mushrooms exhibits antioxidant effects, helping to prevent an increase in malondialdehyde (MDA) levels and a decrease in lung surface density (S/V) (Jayakumar et al., 2011). The potent antioxidant activity of white oyster mushrooms has also been observed in rats exposed to cigarette smoke, as

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evidenced by the suppression of 4-hydroxynonenal (HNE) level elevation and the preservation of glutathione concentrations (*Jurnal Sperma-Rokok, Santun-UII*, n.d.)(Bhekti Rahimah, Yuwono Soeroto, et al., 2021b).
White oyster mushrooms contain secondary metabolites with pharmacological effects, including vitamin C, beta-carotene, selenium, ergothioneine, alkaloids, saponins, tannins, and phenolic components. Phenolic components are the main responsible components for antioxidant activity. Several other metabolites also act as anti-inflammatory agents, including phenolic compounds, flavonoids, terpenoids, polysaccharides, lectins, steroids, glycoproteins, certain lipid components, and ergothioneine (ET). One type of carbohydrate found in mushrooms with notable anti-inflammatory activity is β-glucan (beta-glucan). It is isolated from fungi and has various biological activities, including antitumor, immunomodulatory, and anti-inflammatory [14].
The activity of phenolic antioxidants is mainly due to their ability as a hydrogen donor reducing agent and *singlet oxygen quencher*, in addition to which this component also has *a potential metal chelation* effect. Phenolic antioxidants also affect transcription factors in the GSH synthesis process, which increases the outcome. Oyster mushrooms, including white oyster mushrooms, have higher antioxidant activity, reducing power, scavenging abilities, and total phenol content compared with the winter and shiitake mushrooms. In *vitro* tests for the antioxidant power of white oyster mushrooms expose good *hydroxyl and superoxide radical scavenging* activity and the potential to inhibit lipid peroxidase activity with an *inhibitory concentration* value of 50 (IC50), almost the same as vitamin C, which is 6–8 mg/mL.
Research on the advancement of medicinal herbs derived from white oyster mushrooms has been conducted between 2010 and 2019. These studies included phytochemical analysis, identification of marker compounds, and evaluation of the antioxidant and anti-inflammatory activities of the ethanol extract of white oyster mushrooms. Preclinical tests using rat models were also carried out to assess the efficacy and toxicity of the ethanol extract. There is a need to promote the advancement of herbal preparations of white oyster mushrooms in nanoparticle form. Nanoscience offers the ability to manipulate medicinal compounds at the molecular and macromolecular levels, where their properties differ significantly from those at larger scales. This manipulation can enhance the solubility of the extract, improve absorption, and ultimately amplify its therapeutic effects. Advancing nanoparticle formulations through nanotechnology can improve solubility, control the release rate of active substances, and enhance bioavailability in the body.(Bhekti Rahimah, Firmansyah, et al., 2021; Bhekti Rahimah, Yuwono Soeroto, et al., 2021a; Fitri et al., 2020; Jayakumar et al., 2011; Mr Sharad Kamble et al., 2022; Ningsih et al., 2017; PATIL et al., 2021; Rahimah, Djunaedi, et al., 2019; Rahimah et al., 2010; Rahimah, Kharisma, et al., 2019).
This study aims to analyze the synthesis process of white oyster mushroom ethanol extract nanoparticles utilizing chitosan and ionic gelatin methodologies. Future research will aim to develop these nanoparticles into standardized herbal formulations and phytopharmaceutical products.

2. Method

This study is an in vitro study that uses white oyster mushroom simplicia.

2.1. Simplicity and determination of natural materials

The simplicia used for extract preparation was derived from fresh white oyster mushrooms cultivated in Solo by CV Masusi. The selection of these natural materials was based on the following characteristics. White oyster mushrooms have been determined to be natural materials at the School of Life Sciences and Technology (SITH), Bandung Institute of Technology (ITB). The results of the determination of natural materials are as follows:
Class name/ class: Basidiomycota
Name of the nation/ Ordo: Agaricales
Tribal name/ family: Tricholomataceae
Type Name/species: Pleurotus ostreatus (Jacq. Ex Fr.) Quel.
Common name: Oyster Mushrooms (Indonesia), Oyster Mushroom (United Kingdom)

2.2. Preparation of ethanolic extract of white oyster mushroom

The primary raw material for the ethanol extract of white oyster mushrooms was 11.5 kg of fresh white oyster mushroom simplicia. The initial stage involved washing the mushrooms with reverse osmosis (RO) water, followed by separating the caps and stems to reduce their size. Extraction was carried out using the maceration method with 15 liters of 70% ethanol solution. The resulting macerate was collected in a vessel and subjected to evaporation using an apparatus equipped with a double-jacket heating system, maintained at a temperature of approximately 60–80 °C. Upon completion of the evaporation process, the concentrated extract was removed from the apparatus. In the subsequent step, amylum (starch) was added to the extract and mixed until a homogeneous macerate mixture was formed. This mixture was then oven-dried at 60 °C. The final dried product was ground to obtain a powdered extract(Khan & Rehman, 2005; Mujianto et al., 2024; Ningsih et al., 2017).
The preparation of the ethanolic extract of white oyster mushroom is carried out in the production laboratory of PT Phytomed Neofarma, which is one of the herbal medicine industries and makes extracts according to the standard of good traditional medicine manufacturing methods (CPOTB). This procedure is a government provision to maintain the quality of herbal medicines in Indonesia(BADAN PENGAWAS OBAT DAN MAKANAN REPUBLIK INDONESIA, n.d.).



Fig. 1: Preparation Of White Oyster Mushroom Ethanol Extract. A) White Oyster Mushroom, B), Maceration of White Oyster Mushroom, C) Heating of Macerate, D). Addition of Amylum

2.3. Preparation of nanoparticles ethanolic extract of white oyster mushroom

The ingredients used are Oyster Mushroom Extract (0120724-C045), Technical Ethanol (PA), 100% Glacial Acetic Acid (RLC2.0057.0500), ddH2O, Chitosan (Phy Edumedia), Propylene Glycol (PT Dwilab Mandiri Scientific), Ethanol 70%, DMSO 10% (Supelco - Sigma Aldrich, 1,029,521,000), and Sodium Tripolyphosphate (Na-TPP) 0.4%.
The instruments used are Stirring Hotplates (Fisher Scientific, 1110217SH), Nanoparticle Analyzer (Horiba, SZ100), Transmissions Electron Microscope (Hitachi, HT770), Analytical Balance (AXIS, AGN220C), Measuring Cup (pyrex), Droppipette (Onemed), Erlenmeyer Flask (pyrex), Funnel (pyrex) and Filter Paper.
The nanoparticle preparation procedure begins with the mixing of a liquid extract by dissolving 1 g of white oyster mushroom extract in 30 mL of pro analysis (PA) ethanol, followed by filtration using filter paper. The filtrate is then taken to make nanoparticles. The preparations of nanoparticles is carried out in the following way: 1 gram of chitosan is dissolved in 100 mL of 1% glacial acetic acid using a magnetic stirrer so that a concentration of 1% chitosan is obtained. A total of 15 mL of white oyster mushroom extract is added to 60 mL of mixed solvent (20 mL of propylenglycol, 20 mL of ethanol 70%: 20 mL of DMSO 10%) and 100 mL of aquadest. Then, a 1% chitosan solution of 40 mL was added so that the chitosan concentration was 0.2%. The mixture is stirred using a magnetic stirrer for 10 minutes. It is then dripped with 20 mL of 0.4% Na-TPP at a rate of 1 drop/3 sec with a burette and in a magnetic stirrer at rpm 300 until nanoparticles are formed, characterized by homogeneous turbidity. Then it remains on the magnetic stirrer for 15 minutes so that a solution of oyster fungus nanoparticles, turbidity, and precipitation is obtained.(Lidia et al., n.d.; Mr Sharad Kamble et al., 2022; Murdock et al., 2008; Utami et al., 2023).

3. Result

3.1. Particle size distribution

From the results of the particle size examination using the *Particle Size Analyzer* tool with three repetitions, the following results were obtained:

Table 1: Results of Particle Size of Oyster Mushroom Extract Nano Emulsion

Repetition Wed-	Average size (nm)	PI
1	799.1	0.651
2	791.1	0.502
3	699.4	0.619
Average	763.2	0.590

The oyster mushroom extract nano-emulsion has an average particle size of 763.2 nm with three iterations. This shows that the nanoemulsion from the oyster mushroom extract has met the requirements, where a particle is said to be a nanoparticle if it is in the range of 1-1000 nm.(Rahmat & Wirawan, 2020; Yusuf et al., 2023).

3.2. Potential zeta

From the results of the nano emulsion charge examination using the Zeta Potential Analyzer tool with three repetitions, the following results were obtained:

Table 2: Potential Zeta Test Results

Repeated to	Zeta Potential (mV)
1	-25.7
2	-25.7
3	-25.2
Average	-25.53
Standard deviation	0.28

The potential zeta value indicates the surface charge of a particle. The particle charge causes particles to experience a tendency to aggregate or rejection. According to Murdock 2008, the potential zeta value of a stable preparation is more than +30 mV or less than -30 mV. A good potential zeta value indicates that the strength of the particles to repel each other is getting stronger, resulting in a stable dispersion of the preparation. Meanwhile, the poor potential zeta value shows that the strength of the particles to reject is getting weaker, so that the particles experience a tendency to aggregate and cause unstable dispersion of preparations. Based on the results of the study, the zeta value of the oyster mushroom extract nanoemulsion has a value of less than -30 mV, which indicates that the nanoparticles are stable.

3.3. Transmission electron microscope (TEM)

From the results of the examination of the form of Nano emulsion of oyster mushroom extract using a tool *Transmission Electron Microscope* (TEM), with three repetitions obtained the following results:

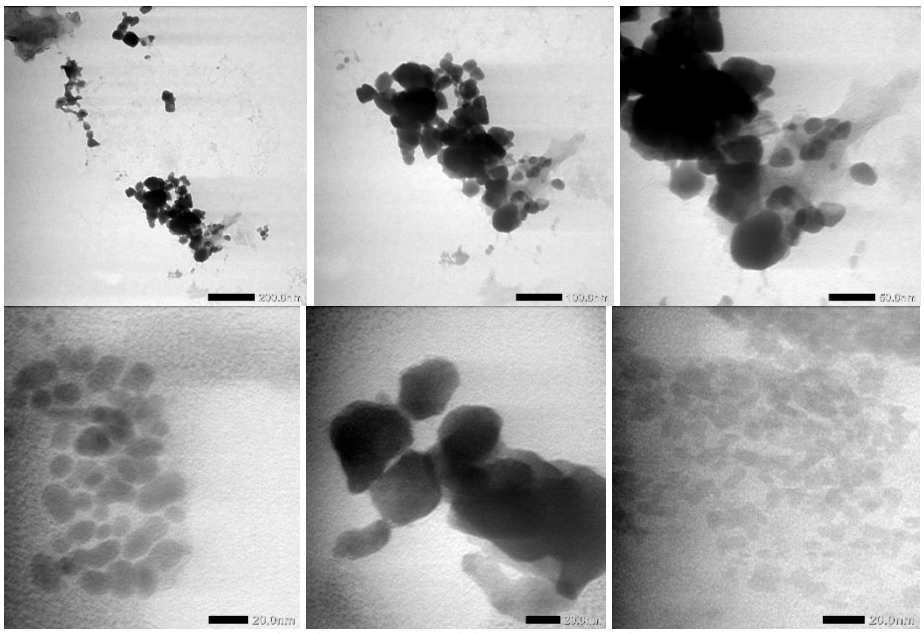


Fig. 2: Results of TEM Nanoemulsion of Oyster Mushroom Extract.

Based on the analysis results, the Transmission Electron Microscope (TEM) in Figure 1 shows that the nano-emulsion particles of oyster mushroom extract are spherical. The outer part of the formed nanoparticles is the result of ionic cross-linking of chitosan and Na-TPP When nanoparticles are formed, the extract or active compound is inside the nanoparticle circle (nucleus) This indicates that the particles have good stability because spherical particles tend to have lower surface energy compared to irregular shapes, Then it can be seen that most of the particles in the nano emulsion are spherical, indicating that the particle formation process takes place consistently and produces particles with uniform morphology. The round shape and uniform size can improve the efficiency of delivery of active ingredients to specific targets in the body or on the skin, which can be used in biomedical applications.(Murdock et al., 2008; Putri & Atun, n.d.; Utami et al., 2023).

4. Discussion

The preparation of nanoparticles can use several methods, including solvent evaporation, emulsification, and ionic gelation. In this study, the ionic gelation method is used because it is considered the common method. The ionic gelation method involves the process of cross-linking polyelectrolytes with their multivalent ion pairs. Ionic gelation is often followed by polyelectrolyte complexation with opposite polyelectrolytes. The formation of these cross-linked bonds will strengthen the mechanical strength of the particles formed(Ahmed et al., 2022; Mr Sharad Kamble et al., 2022).

A common strategy to enhance nanoemulsion characteristics is the use of chitosan, a biodegradable and mucoadhesive polymer. Chitosan contributes to improved emulsion stability and functional performance by forming a coating around nanoparticles, which can prevent

coalescence and enhance bioavailability [7]. Moreover, chitosan has been reported to facilitate the sustained release of active compounds while preserving particle integrity in biological environments [8]. When used in combination with natural extracts, chitosan-based nanoparticles often result in favorable physicochemical properties and bioactivities.(Chakraborty et al., 2018; Indira Muzib Sri Padmavati Mahila Visvavidyalayam et al., 2014; Yanat & Schroën, 2021)

The nanoemulsion formulation of Pleurotus ostreatus (oyster mushroom) extract produced in this study exhibited an average particle size of 763.2 nm. This particle size lies well within the range for nanoparticles, generally defined as 1–1000 nm [1]. The nanometer scale enhances the surface area-to-volume ratio, which is crucial for improving solubility, dissolution rate, and biological absorption of encapsulated bioactives [2]. In addition, particle size influences the physical stability and kinetic behavior of emulsions, as smaller droplets reduce the risk of gravitational separation and aggregation [3].(Ahmed et al., 2022; Mr Sharad Kamble et al., 2022; Vani et al., n.d.; Yanat & Schroën, 2021)

The oyster mushroom is known for its rich content of polysaccharides, phenolic compounds, and antioxidants, all of which are sensitive to environmental degradation [4]. Encapsulating these bioactives in nanoemulsion systems helps to protect them from oxidative and thermal degradation and enables controlled release. The particle size of 763.2 nm found in this study is consistent with other research involving nanoencapsulation of plant and fungal extracts, which typically report stable systems within the 200–800 nm range [5,6]. The reproducibility of the particle size over three iterations in this study suggests robustness of the preparation method. This size also implies potential for cellular uptake and targeted delivery in biological systems, as particles below 1000 nm are more readily internalized by cells via endocytosis [9](Lidia et al., n.d.; PATIL et al., 2021). The incorporation of this nanoformulated mushroom extract into nutraceutical or therapeutic systems could enhance its efficacy, especially considering the growing demand for bioactive-rich functional foods and natural therapies.(Mansingh et al., 2023; Yusuf et al., 2023)The extract is dissolved in a solvent mixture of ethanol, propylene glycol, and DMSO, as the extract contains many components that must be well-mixed with the chitosan solution to enter the formed nanoparticles. The results of the optimization of the number and ratio of solvents used have not been able to dissolve the extract perfectly, so it is necessary to have a filtration process to remove the insoluble part, which is a nonpolar compound. The addition of tripolyphosphate to the mixture of extract solution and chitosan solution aims to form nanoparticles by ionic gelation method. Chitosan will cross-link with the help of tripolyphosphate because the positive charge of chitosan will interact with the negative charge of tripolyphosphate. Meanwhile, the extract components will be trapped in the cross-linked chitosan matrix. By adjusting the stirring speed and adding the tripolyphosphate solution, nanoparticles will be formed (Ahmed et al., 2022; Lidia et al., n.d.; PATIL et al., 2021; Putri & Atun, n.d.; Yusuf et al., 2023).

The characterization of nanoparticles is closely linked to their surface properties, where zeta potential plays a crucial role in determining the electrostatic interactions that influence the stability, aggregation behavior, and overall performance of the nanoparticle system. Zeta potential is a critical parameter in assessing the stability of colloidal dispersions, such as nanoemulsions. It reflects the magnitude of electrostatic repulsion between similarly charged particles in a suspension. A high absolute zeta potential value (either more positive than +30 mV or more negative than −30 mV) typically indicates strong repulsive forces, which can prevent particle aggregation and thus confer stability to the system.(Mansingh et al., 2023; Salvia-Trujillo et al., 2018)

In the present study, the oyster mushroom (Pleurotus ostreatus) extract nanoemulsion exhibited a zeta potential of less than −30 mV, suggesting a stable colloidal system. This negative surface charge likely results from the ionization of functional groups present in the mushroom extract and the emulsifiers used during formulation. Several recent studies have reported on the zeta potential values of nanoemulsions containing plant extracts. Cucumis melo var. agrestis Extract Nanoemulsion: A study developed a nanoemulsion gel loaded with Cucumis melo var. agrestis extract, reporting a zeta potential of -21.5 ± 0.12 mV. Despite being less negative than −30 mV, the formulation was considered stable, attributed to the use of nonionic surfactants like Tween 80, which provide steric stabilization rather than an electrostatic stabilization mechanism. (Brotons-Canto et al., 2021)Parijoto (Medinilla speciosa) Fruit Extract Nanoemulsion: Another study formulated a nanoemulsion using Parijoto fruit extract, reporting zeta potential values ranging from -22.2 ± 0.74 mV to -28.2 ± 1.6 mV. These values, while approaching −30 mV, still indicated a moderately stable system, with stability influenced by factors such as pH and the presence of acetic acid in the formulation. (Ananingsih et al., 2024) Woodfordia fruticosa Extract Nanoemulsion: A study developed a nanoemulsion using Woodfordia fruticosa extract, reporting zeta potential values ranging from −34.4 mV to −23.5 mV, depending on the concentration of the extract. The more negative zeta potential values were associated with higher concentrations of the extract, suggesting enhanced stability due to increased electrostatic repulsion.(Sridhar et al., n.d.) Compared to these studies, the oyster mushroom extract nanoemulsion's zeta potential of less than −30 mV falls within the range considered optimal for electrostatic stabilization, suggesting superior stability.

Several factors can influence the zeta potential of nanoemulsions: Type of Surfactant: Ionic surfactants contribute to electrostatic stabilization, while nonionic surfactants like Tween 80 provide steric stabilization. The choice of surfactant affects the surface charge and, consequently, the zeta potential pH of the Medium: The ionization of functional groups on the particle surface is pH-dependent, influencing the zeta potential. Ionic Strength: The presence of electrolytes can compress the electrical double layer around particles, affecting the zeta potential. Composition of the Extract: The presence of charged molecules in the extract, such as proteins or polysaccharides, can contribute to the overall surface charge. (Mr Sharad Kamble et al., 2022; Salvia-Trujillo et al., 2018)

The high absolute zeta potential value observed in the oyster mushroom extract nanoemulsion indicates strong electrostatic repulsion between particles, reducing the likelihood of aggregation and enhancing stability. This stability is crucial for applications in pharmaceuticals, nutraceuticals, and cosmetics, where consistent delivery and shelf life are essential. Moreover, the stable nanoemulsion system can improve the bioavailability of the bioactive compounds present in oyster mushrooms, potentially enhancing their therapeutic efficacy.(Ananingsih et al., 2024; Brotons-Canto et al., 2021; Indira Muzib Sri Padmavati Mahila Visvavidyalayam et al., 2014)

The physical characterization of the oyster mushroom (Pleurotus ostreatus) extract nanoemulsion demonstrated promising stability and morphology, indicating its potential for biomedical applications. One of the key indicators of nanoparticle stability is zeta potential, which reflects the surface charge and electrostatic repulsion between particles. In this study, the nanoemulsion showed a zeta potential of less than −30 mV, which, according to Murdock (2008), qualifies the formulation as electrostatically stable. A zeta potential value beyond ±30 mV is often associated with a reduced tendency for particle aggregation due to strong repulsive forces, thus promoting a more uniform dispersion in the system.

This finding is supported by recent work by Sultana et al. (2023), who formulated a nanoemulsion from Woodfordia fruticosa extract with a zeta potential of −34.4 mV, resulting in enhanced dispersion stability and sustained bioactivity over time. Similarly, Kurniawati et al. (2022) reported zeta potential values of −28.2 mV in a Medinilla speciosa (Parijoto) nanoemulsion, which, while close to the stability threshold, still produced stable dispersions over a prolonged period.

Complementing the zeta potential data, Transmission Electron Microscopy (TEM) analysis revealed that the majority of the nanoparticles were spherical and uniformly distributed. Spherical particles are known to exhibit lower surface energy and better colloidal stability compared to irregularly shaped ones (Yadav et al., 2025) The spherical morphology observed suggests consistent particle formation and

indicates successful encapsulation of the mushroom extract within the nanoparticle matrix. The outer layer formation is attributed to ionic crosslinking between chitosan and sodium tripolyphosphate (Na-TPP), while the bioactive compounds are entrapped within the nanoparticle core.(Yadav et al., 2025)

Uniform and spherical nanoparticles not only offer better stability but also enhance targeted delivery and bioavailability of active compounds in biomedical applications. According to Jain et al. (2020), spherical nanoparticles demonstrate enhanced cellular uptake due to their ability to mimic natural biological carriers such as vesicles or viruses. This is particularly advantageous in drug delivery systems targeting epithelial tissues or transdermal pathways. In summary, the integration of high absolute zeta potential and consistent spherical morphology indicates a stable and effective delivery system. These characteristics are critical for ensuring long-term storage stability, controlled release, and targeted bioactivity of the oyster mushroom extract in pharmaceutical or cosmeceutical applications.(Antil et al., 2023)

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5. Conclusion

Test results Particle Size Analyzer, potential zeta, and Transmission Electron Microscope (TEM) white oyster mushroom extract nano emulsion showed that the preparation successfully qualified for stability as a nanoparticle with a size of less than 1000 nm and a zeta potential of more than -30 30 mV. In the future, this herbal preparation—already developed according to good traditional medicine standards—will need to undergo preclinical testing, both in vitro and in vivo, to confirm its effectiveness and safety. In Indonesia, traditional medicine is categorized into three levels: jamu, standardized herbal medicine, and phytopharmaca. Based on this classification, our goal is to advance this preparation to the phytopharmaca stage, so it can be more widely used by individuals and patients. To reach that point, it must first successfully pass both preclinical and clinical trials.

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