

Green synthesis of chitosan nanoparticles using *Camellia sinensis* plant extract and its effect on lymphocytes in *Metapenaeus affinis* shrimp

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Abstract

The results of the study showed the possibility of manufacturing chitosan in the laboratory from uneconomic shrimp waste, in addition to the importance of this product in preparing nano-chitosan, which showed the results of the UV, AFM, SEM and TEM tests, the production of chitosan nanoparticles using the green method and by relying on green tea extract. When these nanomaterial were applied to shrimp juveniles of *Metapenaeus affinis*, the results showed strengthening the immune system through a clear increase in the number of lymphocytes. In the wild, the total number was $38 \times 10^6 \pm 6 \times 10^4$, from these $34 \times 10^6 \pm 5 \times 10^4$ small cytes and $10 \times 10^6 \pm 2 \times 10^4$ large cytes. In the laboratory with chitosan nanoparticles and control treatments, the total lymphocytes were $45 \times 10^6 \pm 7 \times 10^4$ and $42 \times 10^6 \pm 5 \times 10^4$ respectively, small cytes formed $38 \times 10^6 \pm 5 \times 10^4$ and large cytes $7 \times 10^6 \pm 3 \times 10^4$ in chitosan nanoparticles compared with $31 \times 10^6 \pm 3 \times 10^4$ and $11 \times 10^6 \pm 1 \times 10^4$ in control respectively.

Keyword: *Green synthesis. Chitosan nanoparticles, lymphocytes, shrimp*

I. Introduction

Chitosan is found in the exoskeleton of marine organisms such as crustaceans, and is considered an important material in many industrial applications such as food, medicine, cosmetics, and environmental purification materials (Syamdidi et al., 2020 and Sabry et al., 2021). Shrimp is one of the main sources of chitin, as its shells contain high levels of chitin that can be converted into chitosan, these shells are usually collected through industrial processes that process shrimp and chitosan is extracted through several stages that include converting chitin into chitosan using chemical and biological processes or green synthesis (Islam et al., 2016). Lymphocytes in shrimp *Metapenaeus affinis* and other are play an important role in the cellular immune response, including clotting, non-self-recognition, phagocytosis, melanization, encapsulation, cytotoxicity, and cell-to-cell communication (Soderhall, 1999 and Le Moullac and Haffner, 2000).

The word nanometer means one millionth of a millimeter (range size between 1 to 100 nm (Agnihotri and Mukherji, 2014). Nanotechnology means complete and precise control in the production of materials through control the interaction of the molecules involved in the reaction and the direction of these molecules through the production of a specific substance (Muruyan and shanmugasndaram, 2014 ; Manjunatha, 2016 ; Quinones et al., 2022). There are three ways to synthesize nanomaterial, including the physical, chemical, and biological methods, one of the disadvantages of the physical and chemical methods is that they are expensive to manufacture, and the chemical methods use some toxic materials (Hulkoti and Taranath, 2014). While biological methods are characterized by being less expensive and less toxic, they also produce nanomaterial that are extremely small and more homogeneous in shape (Ribeiro et al., 2020). Chitosan is one of the natural organic polymers that has received great attention because



of its biological properties, Recently, chitosan has been widely used in pharmaceuticals and paint as it enhances cell assimilation because of the attraction of charge (Mohammadi et al., 2016). Using green methods, the environmental impact can be reduced and sustainability can be enhanced (Widatalla et al., 2022). Green tea *Camellia sinensis* extract contains biologically active compounds such as polyphenols, which have antioxidant and antibacterial properties, these compounds are of particular importance in the manufacture of nanomaterial, due to their unique properties, in addition, using tea as a source of nano composites is an environmentally friendly option and less impactful compared to traditional chemical techniques that use toxic substances (Nigam et al., 2022 and Wang and Li, 2011).

The current study aims to manufacture chitosan in the laboratory from uneconomic shrimp and then use it in manufacturing chitosan nanoparticles based on green tea extract and to know the effect of these nanoparticles on the immune system in Juveniles shrimp *M. affinis* through the number of lymphocytes.

II. Materials and methodology

Preparation of chitosan

Individual of *Macrobrachium nipponese* shrimp were collected and dried at 80 °C until the weight was constant. Firstly, 65 grams of dried shrimp and grind them well with mortar, place the powder in a 500 ml glass beaker and add 2 % sodium hydroxide (NaOH) for two hours at 60 °C. Wash well with ionic water and dry in an incubator at 60 °C for 24 hours, then add 7 % of HCl for four hours at room temperature. Wash well with ionic water and dry for 24 hours in an incubator at 60 °C and this chitin. Secondly, take the chitin and add 50 % NaOH to it for 3 hours at a temperature of 24 °C (room temperature), then wash it well with ionic water and dry it at a temperature of 60 °C inside the incubator and keep it dry, away from moisture in the laboratory (Duraismy et al., 2022).

Preparation of plant extract from green tea extract

One gram of dry powder of green tea was boiled in 100 ml of distilled water. After boiling, the mixture was covered and left to cool in a dark place under room temperature and pressure conditions for 24 hours. Whatman paper no. 1 was used to filter the mixture in order to obtain an aqueous solution of green tea extract. The filtrate obtained was kept at 4 °C for 14 hours overnight (Nigam et al., 2022).

Synthesis of chitosan nanoparticles

One gram of chitosan was dissolved in 1% glacial acetic acid with stirring at speed 5000 rpm for 15 min until a clear solution was obtained. Then 20 ml leaf extract was added drop-wise to 80 ml of chitosan. The addition of leaf extract to the chitosan solution was done under constant stirring at 100 rpm for 30 minutes. The nanoparticle-containing solution obtained was subjected to centrifugation at 13000 rpm for 20 minutes to remove any unreacted chitosan impurities (Mohamed et al., 2021).

Lymphocytes in juvenile shrimp *M. affinis*

The lymphocytes of juvenile shrimp of *M. affinis* under study were counted by taking 25 individuals and withdrawing a quantity of lymph and placing it in tubes containing a known volume of sodium esters as an anticoagulant, based on Stolen et al., (1995) and Kondo (2003). A Hemocytometer counting slide was used to estimate the total number of cells and their shape according to the following equation:

Number of lymphocytes (THC) = Average number of cells in four squares x Dilution factor x 10⁴

The dilution factor was estimated in the previous equation through:

Dilution factor = Volume of extracted lymph + Volume of anticoagulant / Volume of extracted lymph





Picture 1: Laboratory preparation of chitosan: A= drying of *Macrobrachium nipponese* for get on waste; B= drying after digestion; C = add NaOH to get chitosan ; D= add HCl ; E = filtration and F= Chitosan produced.

III. Results and discussion

UV-Visible Spectrophotometer

The UV-Visible spectrum of chitosan nanoparticles was measured after their formation in the solution, where the solution turned brown. The absorption spectrum was recorded, sthe appearance of an absorption peak at a wavelength of 271 nm as a result of the formation of chitosan nanoparticles (Figure 1).The study showed that the UV-Vis absorption spectrum of the extract prepared using chitosan and green tea was within the wavelength of 200-800 nm. Two peaks were identified, the first at 205.5 nm with an absorption of 3.98, indicating strong absorption in the UV range, which is probably related to the aromatic compounds present in the green tea extract, and the second peak at 271 nm with an absorption of 1.186, which is likely related to the phenolic compounds commonly present in green tea. This peak is characteristic of flavonoids, catechins or other polyphenols, which are known to absorb in this region. This is in agreement with Farcas et al., (2019) who discussed the antioxidant properties of chitosan and green tea extract, and used UV-Vis spectroscopy to determine the optical properties of these compounds, and reported similar absorption related to polyphenols.

It is clear that the nanoparticles are in the form of spheres or irregular and somewhat clumped structures, with a surface structure in the form of tangled fibers. The size of the particles ranges between 50-200 nm (Figure 2 A).

It was observed from the AFM examination that there is a height estimated at about 43.33 nm, indicating the surface roughness resulting from nanofabrication. It can also be observed that the distribution of nanoparticles on the surface of chitosan may have effects on the properties of the material such as porosity and interaction with other materials (Figure 2 B).

The analysis shows a prominent peak, indicating the presence of a type of crystalline or semi-crystalline structure in the particles. Chitosan usually appears semi-crystalline in this range and this pattern indicates that the addition of green tea extract did not significantly affect the crystalline nature of the structure (Figure 2 C).

When TEM examination of chitosan nanoparticles is performed, it is evident that the particles have an irregular spherical shape with sizes ranging from nm. The particles appear to be slightly clustered with the internal crystalline structures of the particles being observed, which confirms that these particles are of crystalline nature (Figure 2 D).

Characterization of nanoparticles nanomaterial manufacturing involves the production of nanosized materials with dimensions ranging from 1-100 nanometers, there are different production methods, but in general there are two main methods: the first is from top-down, in which the size of the larger material is reduced to the nano dimension through mechanical or physical processes such as grinding or physical precipitation, and the second method is from bottom-up, Bottom-Up, in which atoms or molecules are collected to produce nanomaterials (Mourya and Inamdar, 2009 and Zhang et al., 2016). First method requires high costs in addition to the danger of the chemicals used, but the green method that uses living organisms or biological processes such as bacteria, fungi and plants to produce nanoparticles is environmentally friendly, less expensive and less harmful to the environment (Riberiro et al., 2020). Therefore, the choice fell on adopting green methods in preparing nanomaterial for the current study, as we are dealing with a living organism that can be consumed by humans, and it is necessary to use materials that are less harmful to health (Attia and Elsheery, 2020).

The use of aquatic plants in the manufacturing processes of nanoparticles is characterized by being a clean, sustainable and inexpensive technology, with wide horizons in industrial, medical and agricultural applications (Singh et al., 2018; Zhang et al., 2020 and Widatalla et al., 2022). Despite the availability of different methods for synthesizing nanoparticles, their synthesis by biological methods using plant products has attracted great interest because they are environmentally friendly and economically feasible products (Widatalla et al., 2022). The choice of the green tea plant *C. sinensis* is based on the fact that it is rich in polyphenolic compounds that act as reducing agents and have a role as capping agents for the resulting Nano products, so it is widely used (Camacho-Jiménez et al., 2020). Kumari et al., (2017) in their study of green tea components showed polyphenols, that these compounds have absorption peaks at 270 nm, which is consistent with the results obtained in the present study. Riaz et al., (2018) also discussed the interaction of chitosan with green tea extract and showed different absorption spectra of phenolic compounds present in the extract of this plant. Sivaroban et al., (2016) discussed the antioxidant properties of natural extracts present in chitosan and green tea and used spectroscopic analysis to study the absorption and indicated that the use of UV-visible spectroscopy to analyze the polyphenols present in green tea has an absorption peak at the range of 270-280 nm, which supports that this spectrum is consistent with the presence of polyphenols and flavonoids, and that green tea extract is rich in polyphenolic compounds such as catechins, which have strong absorption of UV rays, especially in the range of 200-300 nm.

One of these plants is green tea, which is a rich source of phenolic compounds, that have antioxidant properties, these compounds play an important role in many applications, including the manufacture of nanomaterial, as its phenolic compounds have reducing properties, allowing metals and other materials to be reduced to a nano state without the need to use harmful chemicals (Attia and Elsheery, 2020). This contributes to providing safer and more environmentally friendly methods for manufacturing nanomaterial (Khan et al., 2020). Green tea is also a readily



available and affordable material compared to the chemicals used in traditional nanomaterial manufacturing techniques, which contributes to reducing the cost of operations (Iravani, 2011). Therefore, the use of green tea in the manufacture of nanomaterial represents an innovative and sustainable approach that opens new doors for scientific and technological applications (Zhang et al., 2020).

Chitosan extracted from shrimp shells is used in many environmental applications such as water purification and removing heavy metals and pollutants from water, it is used in pest control as a natural treatment for fungi and is used in the manufacture of biofilms or natural cosmetic powders (Khan et al., 2019). Reduce environmental pollution by reducing harmful chemicals and minimizing waste through the use of solvents and processes that do not leave harmful waste (Singh et al., 2018). Green tea contains biologically active compounds such as polyphenols, which have antioxidant and antibacterial properties (Wang and Li, 2011). These compounds act as powerful antioxidants, which helps protect nanomaterials from reacting with oxygen and deteriorating, and have properties that contribute to improving the chemical stability of nanomaterial, in addition, using tea as a source of nanocomposites is an environmentally friendly option and less impactful compared to traditional chemical techniques that use toxic substances (Iravani, 2011 and Nigam et al., 2022). On the other hand, this plant contains substances capable of reacting with metals and minerals, which helps in preparing nanomaterial through a process called bio reduction, this method is considered a sustainable and safer alternative to traditional chemical methods (Widatalla et al., 2022).

Nano chitosan particles are considered to be of great importance in biological applications, especially in improving the immune health of marine organisms such as shrimp, these particles consist of chitin, which is a natural substance extracted from the shells of some marine organisms such as shrimp itself, and it has an importance in the number of lymphocytes in shrimp, and works to enhance the immune system, as research indicates that nano chitosan can stimulate the immune system of shrimp, including increasing the number of lymphocytes, as these cells participate in immune defense against microbes and viruses, so increasing their number can enhance the ability of shrimp to resist diseases (Islam et al., 2016 ; Khan, et al., 2020 and Syamdi et al., 2020 and Sabry et al., 2021).

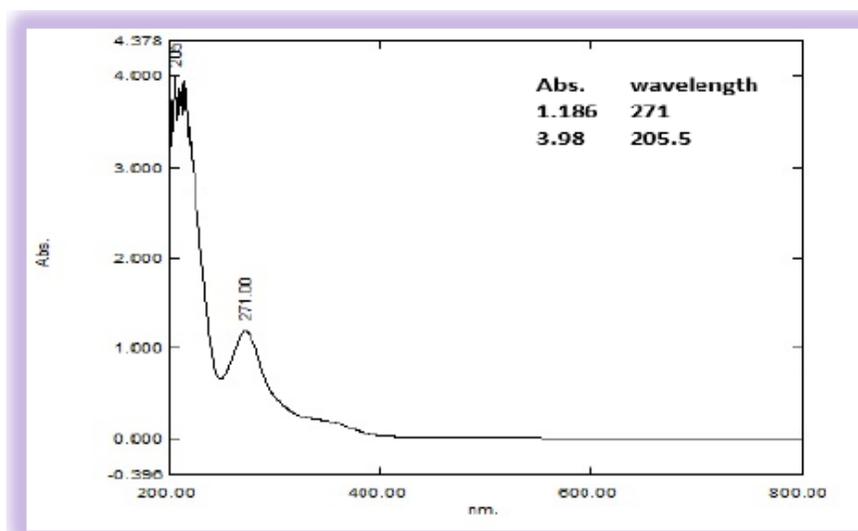


Figure 1: UV spectrum examination of a sample of nano-chitosan prepared using *Camellia sinensis* green tea extract.

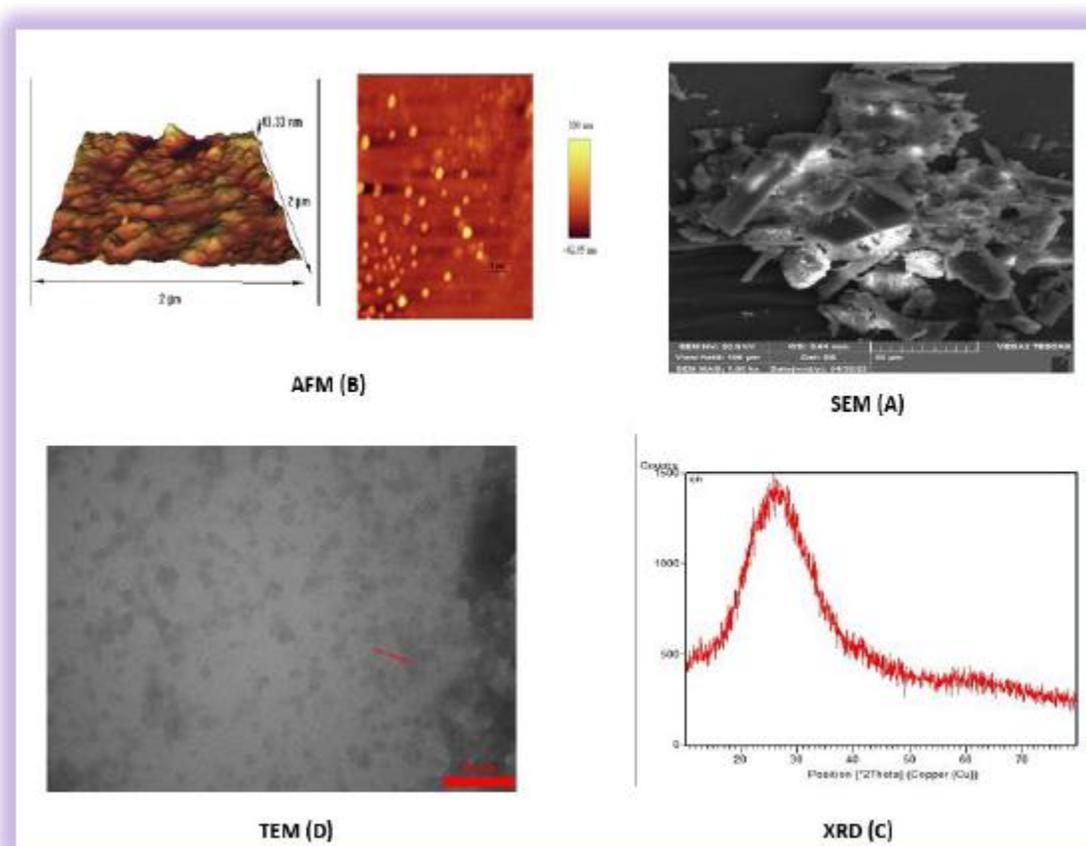


Figure (2): Characterization of chitosan nanoparticles prepared using *Camellia sinensis* green tea extract using scanning electron microscope (SEM) (A), atomic force microscope (AMF) (B), XRD (C), and transmission electron microscope (TEM).

Lymphocytes count

Table 1 shows the number of small, large lymphocytes and the total number in shrimp living in the natural environment (wild). The number of small cells was $28 \times 10^6 + 4 \times 10^4$ cell/ ml, while large cells $10 \times 10^6 \pm 2 \times 10^4$ cell/ ml, and the total number was $38 \times 10^6 \pm 6 \times 10^4$. The number of small, large lymphocytes and the total count of laboratory reared Juveniles shrimp treated with nano- chitosan, the number of small cells was $45 \times 10^6 \pm 7 \times 10^4$ cell/ml in the nano treatment compared with $31 \times 10^6 \pm 3 \times 10^4$ cell/ ml in the control treatment. Furthermore, large cells were $7 \times 10^6 \pm 3 \times 10^4$ cell/ ml compared with $11 \times 10^6 \pm 1 \times 10^4$ in the nano and control treatments respectively,. While the total count were $45 \times 10^6 \pm 7 \times 10^4 \pm 7 \times 10^4$ cell/ ml in the chitosan treatment, and in the control were $42 \times 10^6 \pm 5 \times 10^4$ cell/ ml.

Nano chitosan particles can also bind to immune receptors in the shrimp body, which leads to stimulating the immune response and increasing the production of lymphocytes, thus helping shrimp adapt to environmental challenges by strengthening the immune system, which leads to increasing its resistance to stress and increasing the

number of lymphocytes, and shrimp becomes more able to resist infections that may affect it from bacteria or viruses, and this contributes to improving the health and quality of shrimp in marine farms and enhances growth and reproduction (Soderhall, 1999 ; Mohammadi et al., 2016 ; Le Moullac and Haffner, 2000 and Quinones et al., 2022).

There are two types of lymphocytes in shrimp according to size, small and large, both of which play an important role in the immune response of this marine organism, and the coordination between small and large lymphocytes works together to stimulate an integrated immune response, as their roles complement each other in recognizing and attacking foreign bodies (Kakoolaki et al., 2010). Small lymphocytes primarily work to recognize foreign bodies and antigens and help coordinate the immune response, although shrimp do not have a complex immune system like vertebrates, small lymphocytes contribute to recognizing microbes and stimulating the appropriate immune response and contribute to monitoring the surrounding environment and interacting with foreign materials such as bacteria and viruses, which enhances the shrimp's ability to survive in environments full of biological hazards (Bachere et al., 1995). As for large lymphocytes, they are considered more effective in defending against infection. They are known as immune cells that show the ability to destroy cells infected with viruses or bacteria and engulf foreign bodies, these cells have a higher ability to engulf and destroy foreign bodies, and they are part of the inflammatory response that occurs when a microbe attacks the living organism (Barracco and Amirante, 1992 and Johansson et al., 2000).

Table 1: Shows the number of lymphocytes in the wild and laboratory- reared Juveniles of *M. affinis* treated with nano chitosan particles

Type of lymphocytes	Number of lymphocytes (cells/ ml)		
	Wild	Nano chitosan	Control
Small cytes	$28 \times 10^6 \pm 4 \times 10^4$	$38 \times 10^6 \pm 5 \times 10^4$	$31 \times 10^6 \pm 3 \times 10^4$
Large cytes	$10 \times 10^6 \pm 2 \times 10^4$	$7 \times 10^6 \pm 3 \times 10^4$	$11 \times 10^6 \pm 1 \times 10^4$
Total cytes	$38 \times 10^6 \pm 6 \times 10^4$	$45 \times 10^6 \pm 7 \times 10^4$	$42 \times 10^6 \pm 5 \times 10^4$

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