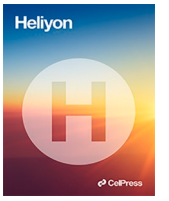
Heliyon 9 (2023) e21663

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Polychaeta-mediated synthesis of gold nanoparticles: A potential antibacterial agent against Acute Hepatopancreatic Necrosis Disease (AHPND)–causing bacteria, *Vibrio parahaemolyticus*.

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ARTICLE INFO

Keywords:
Gold nanoparticles
Green synthesis
Marphysa moribidii
AHPND
Antibacterial agent
Vibrio parahaemolyticus

ABSTRACT

Gold nanoparticles (AuNPs) have emerged as a promising application in aquaculture. Their nano-sized dimensions, comparable to pathogens offer potential solutions for combating antibiotic resistance. In this study, AuNPs were synthesized by using polychaetes, *Marphysa moribidii* as the bio-reducing agent. Modifications were made to reduce agglomeration in green-synthesized AuNPs through ultrasonication. The antibacterial activities of AuNPs against *V. parahaemolyticus* were evaluated. The physicochemical characteristics of the green synthesized AuNPs were comprehensively investigated. The successful formation of AuNPs was confirmed by the appearance of a red ruby colour and the presence of surface Plasmon resonance (SPR) absorption peaks at 530 nm as observed from UV–vis spectroscopy. Scanning electron microscopy (SEM) revealed spherical-shaped AuNPs with some agglomerations. Transmission electron microscopy (TEM) showed particle size of AuNPs ranging from 10 nm to 60 nm, meanwhile dynamic light scattering (DLS) analysis indicated an average particle size of 24.36 nm. X-ray diffraction (XRD) analysis confirmed the high crystallinity of AuNPs, and no AuNPs were detected in the polychaetes extracts prior to synthesis. A brief ultrasonication significantly reduced the tendencies for AuNPs to coalesce. The green-synthesized AuNPs demonstrated a remarkable antibacterial efficacy against *V. parahaemolyticus*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests revealed that a concentration of 0.3 g/ml of AuNPs effectively inhibited *V. parahaemolyticus*. These findings highlighted the potential of green-synthesized AuNPs as antibacterial agents for the prevention and management of AHPND in aquaculture.

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https://doi.org/10.1016/j.heliyon.2023.e21663
Received 21 June 2023; Received in revised form 8 October 2023; Accepted 25 October 2023
Available online 28 October 2023

1. Introduction

A specific virulent strains of *Vibrio parahaemolyticus* is the aetiology of Acute Hepatopancreatic Necrosis Disease (AHPND). This pathogen initially observed to colonize the stomach of infected shrimps [1–3], progressively reached the hepatopancreas (HP) causing HP degeneration and eventually lead to high mortality [4,5]. The shrimp production in AHPND outbreak regions has dropped to 60 % and resulted to a collective loss of approximately USD 43 billion across Asian region [6–8]. In addition, *V. parahaemolyticus* has attracted a great public attention owing to a frequent seafood rejection exported to EU countries considering this bacterium contributed to 25 % of seafood-borne diseases [9–11]. This has further exacerbated the impact on the aquaculture industry.

V. *parahaemolyticus* has been reported to acquire resistance to penicillin, ampicillin, apramycin, cephalothin, gentamycin and trimethoprim [12,13]. This multiple antibiotic resistance has raised a serious concern to public health and economy, indicating that a new strategy for AHPND prevention and management is urgently needed. World Organization of Animal Health (OIE) highlighted a well-established general husbandry practice is imperative to reduce the impact of shrimp disease, including AHPND [14]. However, this exclusion strategy is not practical for intensive farming systems because it has a tendency that leads to negligence especially due to intensification in the number of stocking density to meet market demands. Therefore, a future vision of sustainable production of shrimps calls for a combination of basic and new innovative and efficient technology for a better management of aquaculture diseases.

Nanotechnology is a growing interdisciplinary field with many potential applications in disease diagnosis, disease control, removal of contaminants, gene delivery, etc [15]. Numerous reported biological potentialities of metal nanoparticles (MNPs) include their application as antibacterial, antioxidants, anti-diabetic, catalytic degradation of organic dyes, anti-inflammatory and anti-biodeterioration agents [16–19]. These diverse biological properties open door to various potential applications concerning aquaculture challenges. Nevertheless, the conventional synthesis method to produce MNPs is expensive, energy demanding, and involves the use of toxic ingredients, leading to the production of pernicious byproducts [20,21]. Underscoring the aforementioned concerns, it greatly limits their relevance in aquaculture applications. Therefore, safer, facile, cost-effective, and eco-friendly manufacturing methods are actively explored with fabrication towards utilizing biogenic domains such as plants, animals, bacteria, fungi, etc. This biogenic fabrication of MNPs offers an eco-benign synthesis process where it is not harmful to the animal health and ecosystem.

The engineering of the MNPs nano-size (1 nm–100 nm) contributes to their efficiency as an antibacterial agent, whereby the size is small enough to attach to pathogens without changing its function [22]. These unique properties offer a valuable tool as a new mechanism to kill bacteria. The utilization of conventional synthesized MNPs has been proven to be effective in enhancing the immune system of shrimps against AHPND, as evidenced by increased expression of immune-related genes (mRNA), higher haemocytes count, lowering hepatopancreas damages, and improved survival rates [23–25]. However, the utilization of conventional synthesized MNPs has been reported to cause various toxic effects in shrimps, including suppression of antioxidant enzymes, reduced total antioxidant capacity, apoptotic cells formation, and DNA damage [26,27]. Therefore, the utilization of bio-inspired fabrication of MNPs offers massive advantages to reduce the associated toxicity effects along with easier manufacturing protocol, less generation of harmful waste products, and a straightforward approach.

Amongst various MNPs, gold nanoparticles (AuNPs) are considered the most suitable candidate for therapeutic vector due to its unique and tunable physico-chemical properties for instance size, shape, plasmon resonance, and surface area to volume ratio [19,28]. AuNPs have demonstrated a significant potential in drug delivery, cancer therapy, tumor diagnosis, and pathogens detection [29–31]. Nevertheless, the aspect of sustainability and impact on the environment must be considered for a safer application of AuNPs in aquaculture. Considering this, a straightforward and economical biosynthesis of AuNPs stabilized by bio-inspired domain using aqueous extract of marine invertebrates (*Marphysa moribidii)* underlying the principle of green chemistry was developed. This adoption of biological-mediated synthesis, also termed as green nanotechnology provides a crucial route towards reducing the destructive and toxicity effects associated with conventional laboratory procedures used to synthesize AuNPs [32,33].

Polychaetes or marine worms are a desirable food source for aquaculture sector due to their high protein and polyunsaturated fatty acids (PUFAs) content [34]. Therefore, they are used as feed for shrimp brooders, widely practiced in shrimp hatcheries. Polychaetes are reported to have potential as a host for bacteria [35], which has grown concern over their use as food source for shrimps that could lead to a spread of disease. *M. moribidii* are soft bodied marine worms, widely distributed in the mangrove forest across the west coast of peninsular Malaysia [36]. *M. moribidii* extracts are reported to possess valuable bioactive compounds that have potential as reducing agents for metal nanoparticles synthesis as well as in wound healing treatment [37]. AuNPs previously have been successfully synthesized from various invertebrates such as brittle star, soft corals, snail body, earthworms, and jellyfish [18,19,38–40]. However, the exploitation of invertebrates as a reducing agent for MNPs synthesis is relatively limited as compared to plants species. Therefore, this study aimed to explore the utilization of marine invertebrate of polychaetes, *M. moribidii* extract as a bio-reducing agent in the green synthesis of AuNPs which offers a safer, cost-effective, and rapid synthesis approach. Considering the reported acquisition of antibiotic resistant in *V. parahaemolyticus* against multiple antibiotics [41–43], these biosynthesized AuNPs hold potential as a novel antibacterial agent against *V. parahaemolyticus* that causes AHPND in shrimp and offer a new alternative to antibiotics. There is currently no report on the antibacterial assessment of AuNPs synthesized using *M. moribidii* against *V. parahaemolyticus.* Therefore, the finding of this study provided a new avenue to explore the antibacterial efficacy of AuNPs derived from marine resources in the control of AHPND disease.

2. Material and methods

2.1. Sampling and preparation of aqueous M. moribidii crude extract

Polychaetes, *M. moribidii* was collected from the west coast of peninsular Malaysia, Morib mangrove forest. The specimens were kept in the aquaria that mimic their natural habitat. Polychaetes with complete morphology of body width ~6 mm–8 mm were selected throughout the synthesis. Polychaetes crude extract was prepared according to Refs. [44,45]. The sediments and other adherent materials were completely removed from the polychaetes by using ddH_2O. The polychaetes samples were then cut and finely pulverized by using mortar and pestle, prior to segregation by weight of 15 g in a separate beaker and mixed with 100 ml of ddH_2O. The mixture was incubated for 1 h before filtration with filter paper (Whatman, 110 mm). Finally, the filtrated aqueous polychaetes crude extract was freshly used for AuNPs biosynthesis.

2.2. AuNPs biosynthesis

The biosynthesis of AuNPs was prepared as described from the previous method [45]. The triplicate of the filtered polychaetes crude extracts solution (5 ml) were mixed with 20 ml of gold (III) chloride trihydrate ($HAuCl_4.3H_2O$) (1 mM) solution. The reaction solutions were incubated at room temperature with 150 rpm agitation in the dark for 24 h. The observation of the colour changes to red-ruby colour of the triplicate samples were periodically checked by naked eyes. In order to prevent particle aggregation and obtain a stable solution of AuNPs, the AuNPs solution was ultrasonicated for 60 s after 24 h of incubation, utilizing Powersonic 405 ultrasonicator at frequency of 40 kHz and power of 350 W.

2.3. UV–visible spectrophotometry

The bio-reduction of gold metal ions (Au^{3+}) into AuNPs (Au^{+} to Au^{o} ions) were monitored through the measurement of surface Plasmon resonance (SPR) spectrum subjected to 300 nm–600 nm wavelength range by using the UV–Vis spectrophotometer (UV 1800, Shimadzu, Japan) [45]. Approximately, 3 ml of biosynthesized AuNPs were filled in the glass cuvette and the UV spectra was obtained from the automated software.

2.4. Dynamic light scattering (DLS)

Particle size and particle size distribution of the biosynthesized AuNPs were characterized according to Ref. [46] with some modifications by using DLS (Malvern Instruments Zetasizer Nano ZS, Malvern Analytical USA) at 25 °C with a scattering angle of 90°. The sample measurements were performed in triplicate.

2.5. Scanning electron microscope (SEM)

The morphology of the biosynthesized AuNPs was observed according to the method conducted by Ref. [47] using a JEOL SEM JSM-6390 LA (USA) operating at an accelerating voltage of 15 kV–20 kV. To prepare the samples, a drop of AuNPs was deposited onto a dry poly-L-lysine and left to dry overnight. The samples were then coated with gold by using an Auto Fina Coater-JEOL and examined under the microscope.

2.6. Transmission light microscope (TEM)

To visualize the average particle size and shape of biosynthesized AuNPs, a TEM Tecnai G2 Spirit Biotwin (FEI Company, USA) operating at a voltage of 100 kV–200 kV was used according to the previous report [44]. For TEM imaging, the biosynthesized AuNPs were diluted and drop-casted onto a copper grid coated with a thin layer of carbon, followed by drying at room temperature and examination under the microscope. The size distribution of biosynthesized AuNPs was analyzed utilizing ImageJ software.

2.7. X-ray diffraction (XRD) analysis

The polychaete crude extract and dried biosynthesized AuNPs were prepared according to Ref. [45] and structurally identified by using a MiniFlesx II diffractometer (Rigaku, Japan) equipped with X'celerator. Cu Kα radiation was used in a range of 0 and 110° (2θ) and operated at 30 kV voltage and 30 mA current.

2.8. Identification of V. parahaemolyticus strain

The *V. parahaemolyticus* bacteria used in this study was obtained from the Institute of Marine Biotechnology (IMB), Universiti Malaysia Terengganu. It was isolated from the shrimp tissue samples that showed signs of AHPND, and its strain was confirmed by using PCR through nanopore sequencing. The full-length microbial 16S rRNA sequence was amplified by using 27F (TTTCTGTTGGTGCTGATATTGCAGRGTTYGATYMTGGCTCAG) and ITS-R(ACTTGCCTGTCGCTCTATCTTCTTTTCRYCTTTCCCTCA CGG) primers with Nanopore partial adapter on the primer 5' end [48,49]. The PCR was conducted utilizing WizBio HotStart 2×

Mastermix (WizBio, Korea) with the PCR condition of 95 °C for 3 min followed by 30 cycles of 95 °C for 20 s, 50 °C for 20 s and 72 °C for 180 s. The PCR products ~2500 bp were visualized on gel and purified by using SPRI Bead [50], and the PCR indexed using EXP-PBC001 kit (Oxford Nanopore, UK). The barcoded libraries were pooled based on band intensity and size-selected using 0.8X vol of SPRI magnetic bead to remove fragments smaller than 500 bp. The pooled barcoded amplicons were quantified by using Denovix high sensitivity, and an appropriate amount (~150 fmol) of the amplicons were used as the input for LSK110 library preparation (Oxford Nanopore, UK). Finally, sequencing was performed on a Nanopore Flongle Flowcell for 24 h.

2.9. Culture of *V. parahaemolyticus*

The *V. parahaemolyticus* strain used in this study was retrieved from 15% glycerol stock and cultured in Marine broth (MB) at 30 °C for 24 h [51]. After centrifugation at 10 000 rpm for 15 min, the resulting cell pellets were washed and resuspended in PBS buffer. The concentration of the bacterial suspension was determined by measuring the absorbance at $\lambda = 600$ nm and adjusted to OD_{600}.

2.10. Well diffusion method to determine the zone of inhibition (ZOI)

To evaluate the antibacterial properties of the biosynthesized AuNPs, the Agar well diffusion method was employed according to Ref. [52] with some modifications. A sterile cork borer (0.8 mm in diameter) was used to prepare wells on Mueller Hinton Agar (MHA) plates, onto which the inoculum *V. parahaemolyticus* was spread uniformly. AuNPs solutions of 60 μL at concentrations of 100%, 70%, 40%, and 10% were added to the respective wells after serial dilution. The agar plates were chilled at 4 °C–8 °C for 1 h to allow the AuNPs solution to diffuse into the agar, after which they were incubated at 30 °C for 24 h. Tetracycline was used as a positive control, respectively.

2.11. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) was conducted by using the micro dilution technique [53]. Approximately 10^8 CFU/ml (0.5 McFarland's standard) of the target bacteria was used to the MIC of biosynthesized AuNPs against *V. parahaemolyticus* followed by the standard broth dilution method according to CLSI M07-A8. Serial two-fold dilution of AuNPs with concentration ranging from 30% to 0.06% in marine agar (MA), with two assigned controls, a positive control (10^8 CFU/ml, 0.5 McFarland's standard) and a negative

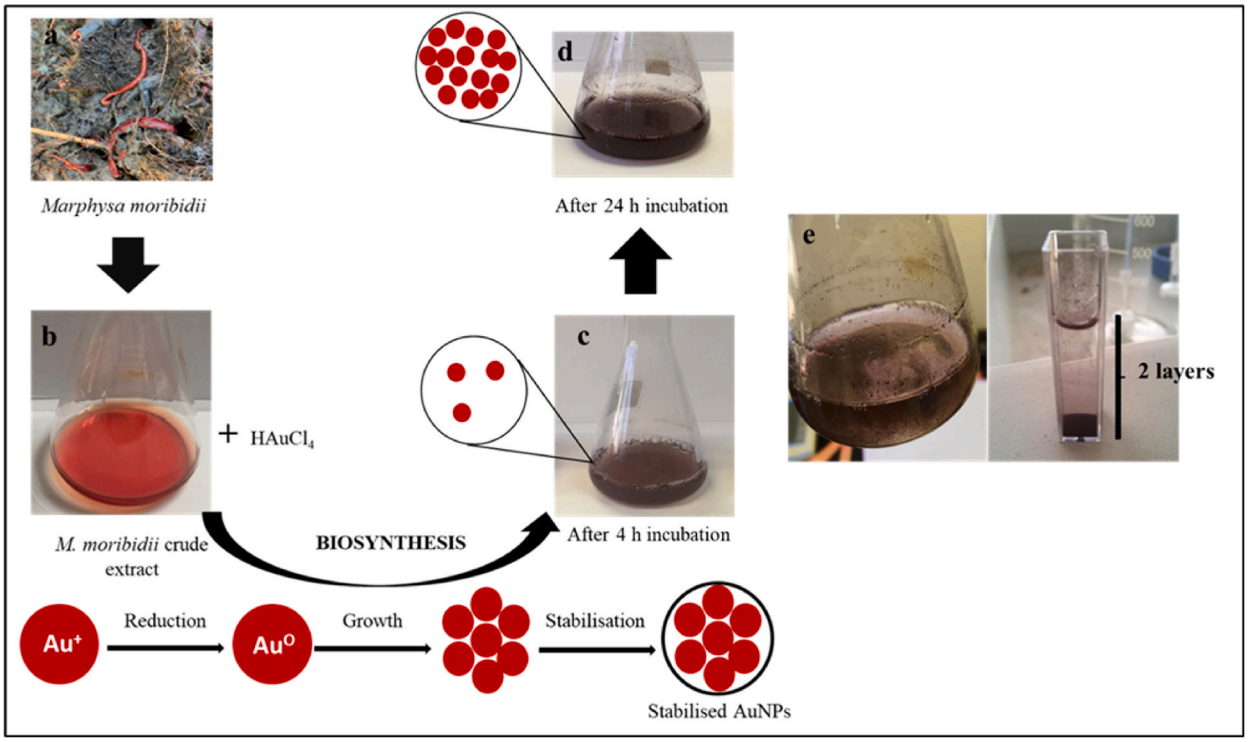

Fig. 1. Biosynthesis of AuNPs utilizing *M. moribidii* as biogenic reducing agents: (a) *M. moribidii* (polychaetes) collected from its habitat, (b) The colour of polychaetes crude extract, (c) Reaction solution of polychaetes crude extract with gold precursor, $HAuCl_4.3H_2O$ after 4 h incubation, (d) The formation of AuNPs after 24 h incubation and (e) AuNPs after 24 h incubation with high sedimentation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

control (MA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye was used to evaluate the visible growth of *V. parahaemolyticus*, whereby a clear yellow colour indicated visible inhibition of bacteria, while turbid (dark blue colour) indicated visible growth of bacteria. This test was conducted by using 96 well plate and incubated for 24 h at 37 °C. The MIC endpoint is the lowest concentration of AuNPs whereby no visible bacterial growth occurs in the well plates. The visual turbidity of the well plates was evaluated after 24 h of incubation period to confirm the MIC value.

The minimum bactericidal concentration (MBC) test was conducted according to Ref. [54] after determining the MIC value of AuNPs. 10 μL from each well plate that showed no visible bacterial growth was seeded on marine agar (MA) plates and incubated them for 24 h at 37 °C. Observation for the presence and absence of bacterial growth was made after 24 h of incubation and MBC value was determined when 99.9% of the bacterial population from the lowest concentration were killed.

2.12. Statistical analysis

Statistical analysis Bonferroni's test was performed on the antibacterial assessment of biosynthesized AuNPs against *V. parahaemolyticus* measuring zone of inhibition values (ZOI) by using GraphPad Prism 5.01, and a p value < 0.05 was considered statistically significant to determine the differences. The ZOI data were expressed as mean ± S.D.

3. Results and discussions

3.1. AuNPs biosynthesis by using M. moribidii (polychaetes) extract as biogenic reducing agents

The unique ability of *M. moribidii* to self-regenerate suggests that this species composes of various bioactive compounds [55], which have potential for various applications. The main functional group in polychaetes extracts such as phenols, esters, sterol, and fatty acid are reported to have probable chemical reactions that contribute to the success of NPs stabilization and synthesis process [45,56]. The successful synthesis of AuNPs produced from polychaetes (marine worms), *M. moribidii* as biogenic reducing agent (Fig. 1a and b) was first demonstrated through the colour changes from pale yellow solution to pale red-ruby solution after 4 h incubation (Fig. 1c). This phenomenon is due to the reduction of gold metal ions to the AuNPs whereby the reducing agent could interact directly by changing the Cl^- ion in the gold precursor, $HAuCl_4$ by hydroxyl (–OH) functional group from polychaetes extract resulting the hydroxyl group was deprotonated to reduce Au^{3+} to become Au^0 [57]. The changes of colour to a more intense red-ruby colour were progressively observed after 24 h (Fig. 1d). However, AuNPs solution tended to coalesce into large clusters or formed as agglomeration due to robust van der Waals interactions which increased the sedimentation and further deteriorating the thermo-physical properties [58,59] which was observed subsequent to 24 h incubation period as indicated in Fig. 1e. Therefore, ultrasonication assisted method was performed to break down large clusters of nanoparticles into individual or smaller clusters of nanoparticles.

Fig. 2 presents the absorption spectra of biosynthesized AuNPs before (Fig. 2a) and after (Fig. 2b) ultrasonication treatment demonstrating an improved stability of AuNPs solution. The absorption peak profile of AuNPs (530 nm) was consistent after ultrasonication treatment, as shown by the triplicate measurements. Fig. 2 illustrates the significant decrease in the sedimentation of biosynthesized AuNPs following ultrasonication treatment. This is evident in the observation of a darker and more intense shade of red ruby colour of the biosynthesized solution.

This observation suggested that the ultrasonication treatment enhanced the dispersion of nanoparticles, and further increase the stability of nanoparticle in the solution, as indicated by the increase of the absorbance peak profile of biosynthesized AuNPs after the ultrasonication treatment [41]. This treatment aligned the principles of green synthesis as it eliminates the need for additional chemical reagents. In contrast, various physical and chemical methods employed to synthesize MNPs utilized toxic chemicals, expensive, labour-intensive processes, and resulted in various biological risks and hazardous byproducts [60,61]. The present study offered a simple, cost-effective, and eco-friendly approach that rapidly produced AuNPs within 24 h period. This biological-mediated

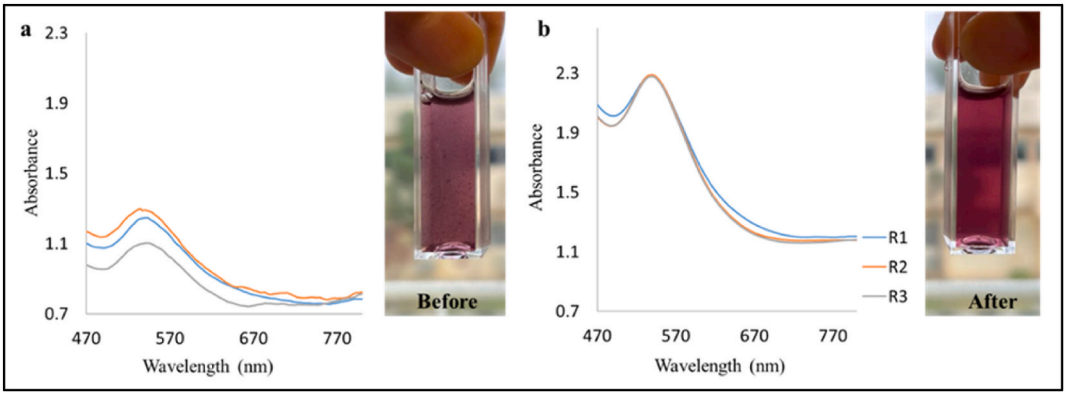

Fig. 2. UV Vis absorption spectra of biosynthesized AuNPs: (a) before and (b) after treatment with ultrasonication method.

approach overcame the challenges associated with the conventional methods, including high cost and threat to health and the environment [62,63] making it more suitable and safer option for future applications.

3.2. Characterization of biosynthesized AuNPs

3.2.1. UV–visible spectroscopy

The formation of AuNPs involves the reduction of gold metal ions (Au^{3+}) to AuNPs (Au^{+} to Au^{o} ions) owing to high reduction potential of Au^{3+}. Polychaetes extract functions as a bio-reducing agent that is responsible to reduce Au^{3+} to AuNPs. The formation of AuNPs was confirmed by the surface Plasmon resonance (SPR) bands presence that are generally exhibited in the range of 520 nm–560 nm [64–66]. Biosynthesized AuNPs showed a prominent SPR peak at 530 nm which was relatively close in comparison to the SPR peak of positive control, citrate stabilized AuNPs at 520 nm (Fig. 3). This observation indicated that the green synthesis method by using polychaetes extract as biogenic reducing agent can reduce gold metal ions (Au^{3+}) with almost relatively similar of SPR peak without reliance to the addition of chemicals that were used for the positive control synthesis.

Additionally, this finding also in consonance with the previous reported work [45], where almost similar range of SPR peak (530 nm–540 nm) was observed for AuNPs produced by using *M. moribidii* as bio-reducing agent. Almost similar range of SPR peak also observed in *S. koreensis* DC4-AuNPs [67] and *T. diffusa*-AuNPDam [68]. However, a blue shift in the SPR peak was observed in the present study, which can be attributed to the fact that the SPR peak wavelength of metal NPs is dependent on several factors, including their size, shape, composition, and dielectric environment [69,70]. Therefore, it is possible that the observed shift was due to the ultrasonication method, which is known to break down large clusters of particles into smaller particles.

3.2.2. Particle size and morphology of biosynthesized AuNPs

The particle size, morphology, and shape of biosynthesized AuNPs were investigated by using DLS, SEM and TEM. DLS was used to predict the particles size and particle size distribution of molecules in solution which exploits the Brownian motion phenomenon. The particle size is measured based on their hydrodynamic diameter (D_h) while polydispersity index (PDI) acts as an indication of the particles size distribution [71].The DLS results showed an average particle size of biosynthesized AuNPs was 24.36 nm with the PDI value of 0.2 (Fig. 4a), indicating a polydisperse distribution of the particles.

The biosynthesized AuNPs were observed to be nearly spherical in shape, as shown in the SEM image (Fig. 4b, indicated by red circle), which in consonance to the biological-mediated synthesis approach reported in both plant and animal species [19,45,72–74]. However, attributable to the poorly dispersed biosynthesized AuNPs, the agglomeration of particles was observed. Moreover, the limited resolution of SEM prevented viewing of the smaller size of biosynthesized AuNPs. Therefore, to confirm the morphology and structure of the biosynthesized AuNPs, further examination was performed utilizing TEM. The TEM image revealed the spherical shape of biosynthesized AuNPs (Fig. 4c), with particle sizes ranging from 10 nm to 60 nm. The average particle size distribution of

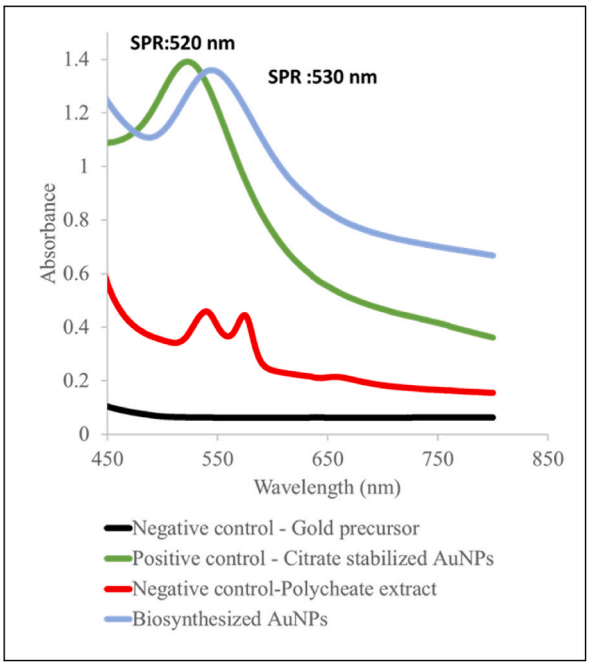

Fig. 3. Comparison of UV Vis absorption spectra of biosynthesized AuNPs, negative controls (gold precursor, $HAuCl_4$ and polychaetes crude extract) and positive control (citrate stabilized AuNPs). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

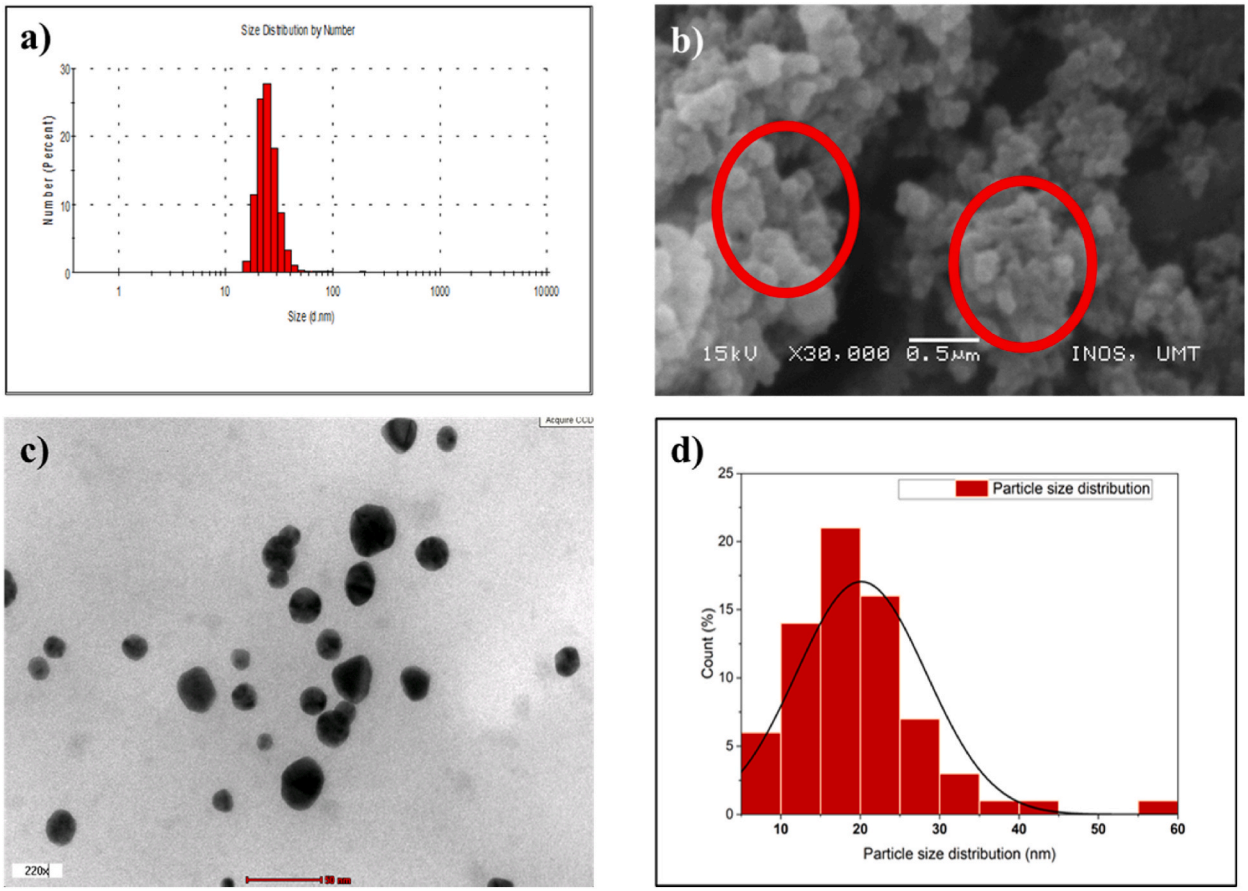

Fig. 4. Characterization of biosynthesized AuNPs using: (a) DLS analysis (b) SEM (c) TEM and (d) particle size distribution of biosynthesized AuNPs via TEM after treatment with ultrasonication.

biosynthesized AuNPs was also tabulated, with a preference for smaller particle size (~20 nm) (Fig. 4d).

The present study revealed a slightly smaller size of biosynthesized AuNPs as compared to the data reported by Ref. [45] using the same species of polychaetes. On account of high sedimentation and precipitation occurred during the biosynthesized of AuNPs in this study, ultrasonication was performed to improve the stability of AuNPs solution. The reduction in AuNPs size was further validated through DLS (Fig. 5a) and TEM analyses (Fig. 5b), revealing that the size of biosynthesized AuNPs was larger before treatment with ultrasonication as compared to after treatment.

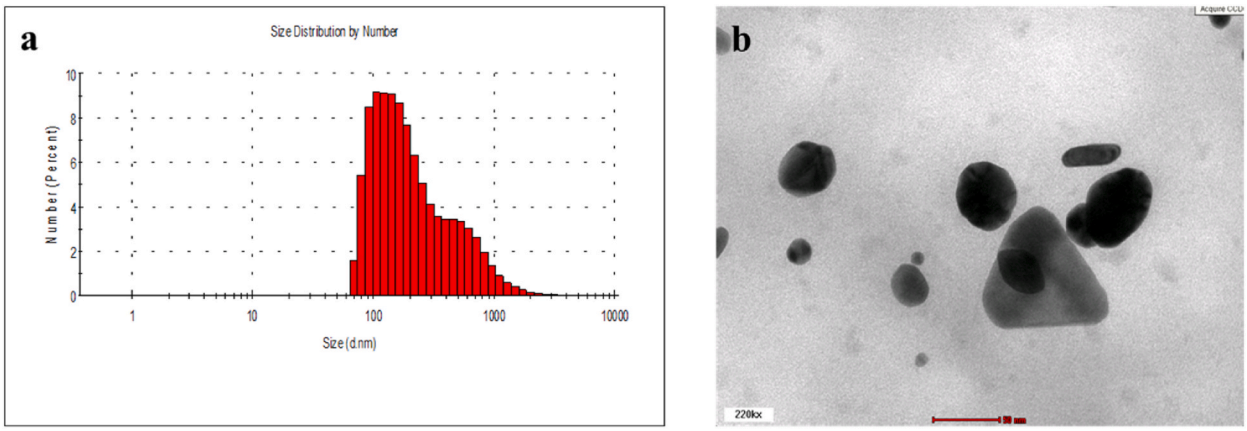

Fig. 5. Characterization of biosynthesized AuNPs using: (a) DLS and (b) TEM analysis before treatment with ultrasonication.

Previous studies also have reported that ultrasonication can lead to a satisfactory dispersion and reduction in particles size. For instance Ref. [75], investigated the influence of ultrasonication on chitosan nanoparticles and reported a decrease in particles size, while [76] observed a reduction in the size of nanoclusters of Pb doped ZnO nanocomposites after an ultrasonication period. In the present study, the ultrasonication was performed, reducing the size of AuNPs particles as compared to the particle size of AuNPs reported by Ref. [45]. The results also demonstrated that a short ultrasonication duration of only 60 s was sufficient to achieve a homogenous and dispersed AuNPs solution, considering that the sonication time required is unique to each solution depending on its response [77]. This suggested that a stable AuNPs solution can be obtained from the green synthesis method with only a brief ultrasonication treatment.

3.2.3. X-ray diffraction (XRD) analysis

X-Ray diffraction analysis was performed on the crude extract of *M. moribidii* (polychaetes) to confirm that the synthesized AuNPs were not originally presented in the polychaete's body. Instead, they were successfully produced through the reduction of Au precursors by using the polychaete extract (Fig. 6a). *M. moribidii* crude extract did not exhibit any possible diffraction peaks of AuNPs confirming the presence of a broad peak with low intensity that belongs to the organic compounds in the polychaetes sample [45]. On the contrary, the XRD spectrum (Fig. 6b) of the biosynthesized AuNPs exhibited distinct primary diffraction peaks at 2θ values of approximately 38°,44°,64° and 77°. These peaks were indexed as the (111), (200), (220), and (311) Bragg's reflections, respectively, corresponding to the cubic structure of metallic gold [78].

3.3. Antibacterial efficacy of biosynthesized AuNPs against V. parahaemolyticus

Metal NPs are known to have different mechanism to kill the bacteria as presented in Fig. 7. The unique non-specific bacterial toxicity mechanisms in metal-based NPs have made it difficult for bacteria to acquire resistance [79]. The antibacterial compounds induced its toxicological effect through a direct contact with the bacteria cell surface [80]. Both Gram-positive and Gram-negative bacteria have a negatively charged surface [81] such as of *V. parahaemolyticus.* Through electrostatic interactions, positively charged nanoparticles will be attracted to the negatively charged bacteria cell wall surface, resulting in disruption of cell wall (Fig. 7a) [79]. Furthermore, NPs will release the metal ions from the extracellular space, entering the cell and consequently causing disruption to biological processes of the bacteria [82]. Once the NPs and metal ions enter the cell, they will induce the production of reactive oxygen species (ROS), leading to oxidative stress due to the oxidation of glutathione, thus suppressing the antioxidant defence

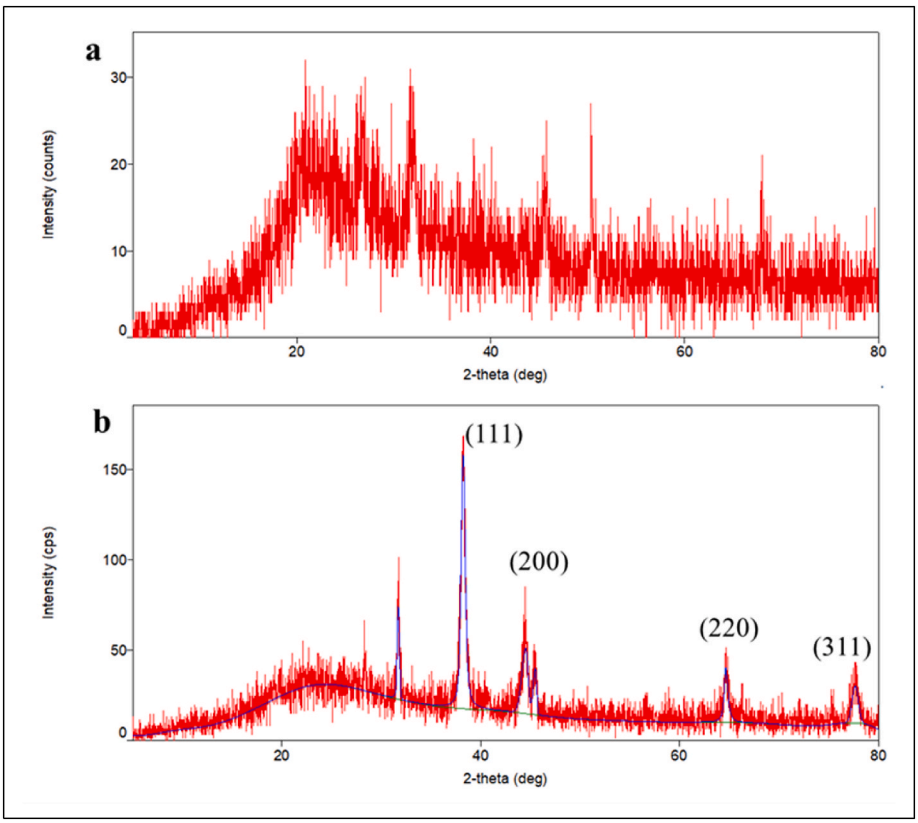

Fig. 6. X-ray diffraction spectrum of (a) *M. moribidii* crude extract and (b) biosynthesized AuNPs.

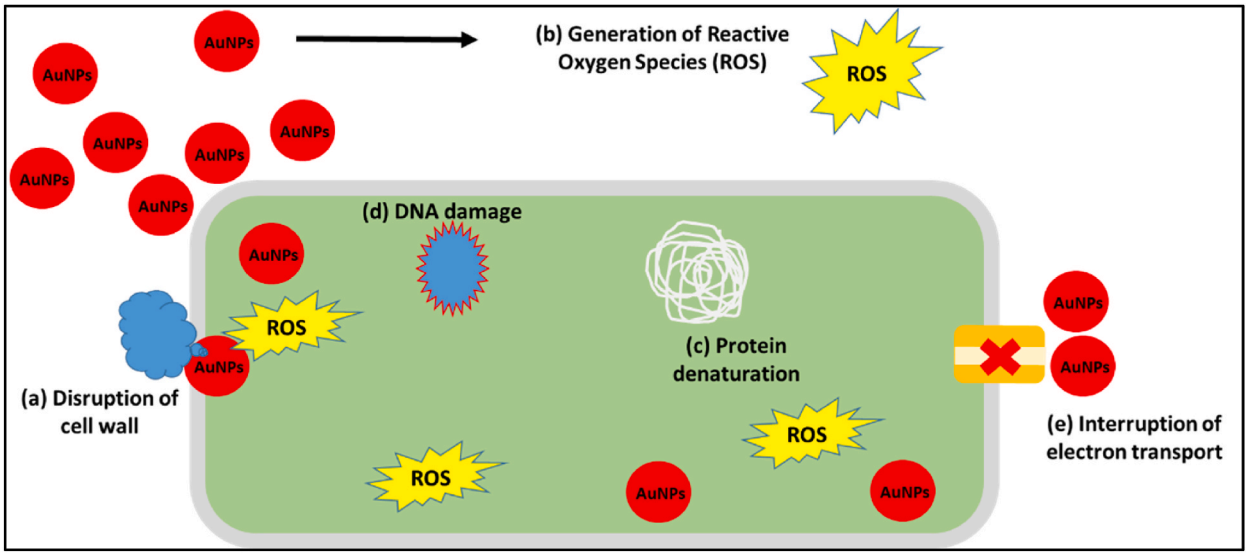

Fig. 7. Mechanism of AuNPs toxicity on bacteria. Interaction of AuNPs with bacteria can cause: (a) disruption of cell wall, (b) generation of ROS, (c) protein denaturation, (d) DNA damage and (e) interruption of electron transport.

mechanism of bacteria against ROS (Fig. 7b). The metal ions also are free to interact with cellular structures of the bacteria and consequently disrupting its cell functions (Fig. 7c–e) [82–84].

The antibacterial properties of biosynthesized AuNPs against *V. parahaemolyticus,* the causative bacteria of AHPND were assessed by using the agar well diffusion method. This method measured the zone of inhibition (ZOI) values obtained by testing different concentrations of the biosynthesized AuNPs against *V. parahaemolyticus,* as depicted in Table 1. This study has a limitation concerning the collection of biosynthesized AuNPs, primarily due to the possibility of product oxidation and the low yield of solid biosynthesized AuNPs obtained. As a result, it was challenging to express the concentrations in g/ml or μg/ml. Therefore, to address this limitation, the concentrations of the aqueous solution of biosynthesized AuNPs were expressed as a percentage (v/v) [85,86].

The antibacterial activity was observed at the highest concentration of 100% biosynthesized AuNPs, showing the largest inhibitory zone with ZOI of 17.10 ± 0.17 mm. This was followed by concentrations of 70% (ZOI: 15.83 ± 0.35 mm), 40% (ZOI: 15.67 ± 0.58 mm) and 30% (ZOI: 14.67 ± 0.58 mm) biosynthesized AuNPs. However, no inhibition was observed at the lowest concentration of 10% biosynthesized AuNPs. Statistically significant difference in means were observed amongst the AuNPs concentration groups ($p < 0.05$). The Bonferroni test was conducted for multiple means comparison. The results indicated that the 10% concentration did not exhibit a significant inhibitory effect on *V. parahaemolyticus,* suggesting that this concentration was insufficient to inhibit the growth of *V. parahaemolyticus*. However, higher concentrations (40%–100%) showed a dose-dependent inhibitory, which was statistically significant. The antibacterial activities observed in this study were comparable to previous reports that utilized polychaetes as biogenic reducing agent (Table 2) [44,45,47,56].

The *M. moribidii* crude extract has been reported to exhibit non-detectable MBC to MIC ratio against pathogenic bacteria, suggesting that the crude extract alone is not efficient as an antibacterial agent [55]. This lack of antibacterial activity (*M. moribidii* crude extract) against *V. parahaemolyticus* is confirmed by the results shown in Fig. 8a. Previous studies have reported higher extraction yields of antibacterial compounds when using less polar solvents and organic solvents with positive antibacterial effect [87–89]. Therefore, it can be inferred that the use of water as a solvent for *M. moribidii* extract was ineffective in extracting a sufficient amount of antibacterial compounds. Nevertheless, future studies may explore the utilization of different solvents such as methanol or ethanol to enhance the extraction of antibacterial compounds from *M. moribidii*.

Gold inert properties lead to active antimicrobial properties that induce antibacterial effect [90,91]. This was observed when crystalline gold ($HAuCl_4.3H_2O$, NC2) in the results exhibited ZOI value of 12.03 ± 0.14 mm as shown in Fig. 8b. A synergistic effect was observed when biosynthesized AuNPs (100%, 70%, 40%, and 30%) were tested against *V. parahaemolyticus,* resulting in a significantly enhanced inhibition effect ($p < 0.05$) against the bacteria as shown in Fig. 8c. This can be attributed to the nano sized AuNPs with increased surface area for exposure of microbial cells to gold ions.

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the biosynthesized AuNPs were both determined to be 0.3 g/ml which corresponded to 30% AuNPs concentration. This concentration was selected as the lowest concentration to be tested for MIC as this concentration showed the least value of inhibition effect against *V. parahaemolyticus* with significantly different in inhibition values ($p < 0.05$) as compared to 100% and 70% AuNPs. AuNPs showed no significant inhibition against *V. parahaemolyticus* as the MIC test demonstrated no visible turbidity at a concentration of 0.3 g/ml AuNPs, while apparent turbidity was observed at all other dilutions (0.15 g/ml, 0.08 g/ml, 0.04 g/ml, 0.02 g/ml, 0.009 g/ml, 0.005 g/ml, 0.002 g/ml, 0.001 g/ml, 0.0006 g/ml). The MBC was determined by using a plate counting method, which revealed no bacterial population after a 24-h incubation period. This finding suggested that a concentration of 0.3 g/ml AuNPs effectively inhibits the growth of *V. parahaemolyticus*.

Table 1
Antibacterial zone of inhibition (ZOI) of the biosynthesized AuNPs tested against *V. parahemolyticus*.

Concentration (v/v%)	Zone of Inhibition (mm)
100	17.10 ± 0.17
70	15.83 ± 0.35
40	15.67 ± 0.58
30	14.67 ± 0.58
10	No inhibition
PC (Tetracycline)	20.5 ± 0.71
NC 1 (*M. moribidii* extract)	No inhibition
NC 2 ($HAuCl_4.3H_2O$)	12.03 ± 0.14

Table 2
Comparison of antibacterial properties of biosynthesized AuNPs using polychaetes as bioreducing agents.

Metal NPs	Species of polychaetes	Tested Bacteria	Range of Zone of Inhibition (ZOI, mm)	References
AuNPs	*M. moribidii*	*V. parahaemolyticus*	14 ± 17	Present study
AuNPs	*M. moribidii*	*E. coli, S. epidermidis, S. typhi and S. aureus*	17 ± 18	[45]
AgNPs	*M. moribidii*	*E. coli, S. epidermidis, S. typhimurium, S. aureus, K. pnemoniae, Serratia* sp, *S. sonnei, P. aeruginosa*	6 ± 9	[44]
AuNPs	*Diopatra claparedii*	*E. coli, S. epidermidis, S. typhi and S. aureus*	14 ± 18	[47]
AgNPs	Unspecified species	*S. aureus, E. coli, P. aeruginosa, V. parahaemolyticus, S. typhi*	8 ± 13	[56]

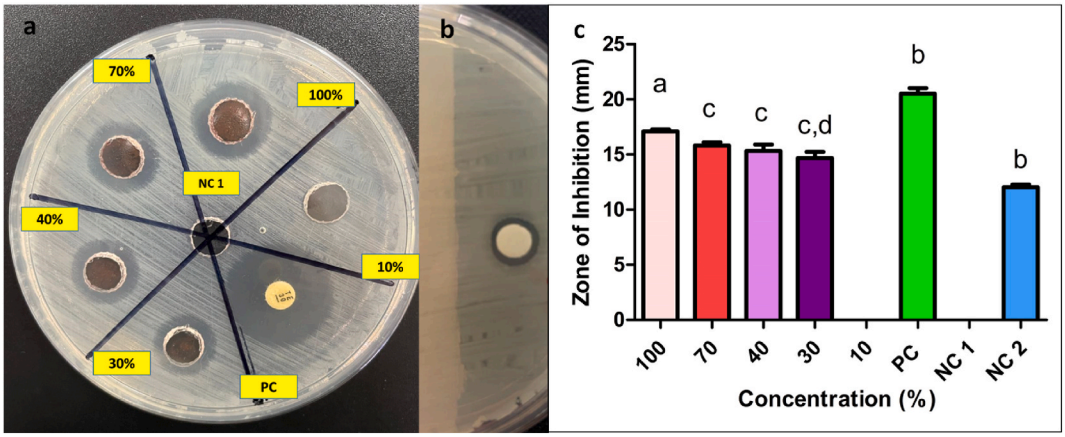

Fig. 8. Antibacterial assessment of biosynthesized AuNPs against *V. parahaemolyticus* using well diffusion method on Mueller Hinton Agar (MHA) tested by using: (a) different concentration (%v/v) of biosynthesized AuNPs (100%, 70%, 40%, 30% and 10%), tetracycline (positive control, PC) and *M. moribidii* extract (negative control, NC1), (b) gold precursor ($HAuCl_4.3H_2O$) (NC2) and (c) zone of inhibition values. Different letters indicate statistically differences between the means ($p < 0.05$) as tested with Bonferroni's test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Relevant potential applications concerning AHPND issue, for instance AuNPs-supplemented diets, water treatment utilizing AuNPs, and a *V. parahaemolyticus* detection technique involving AuNPs have been highlighted. Recent studies have demonstrated enhanced immune response and increased survival rates in shrimps fed diets supplemented with MNPs [92,93], inhibition of *Vibrio* sp. growth in water [94,95], and the development of a novel and rapid *V. parahaemolyticus* assay [96,97]. However, the deployment of a biologically mediated approach for the safer biosynthesis of AuNPs opens the door to safer adoption of these pertinent applications within the aquaculture sector.

4. Conclusion

In summary, green-synthesized AuNPs were successfully produced utilizing *M. moribidii* extract as a biogenic reducing agent. Through the modification of the synthesis method involving ultrasonication, enhanced stability and reduced agglomeration were achieved, resulting in a visually captivating red ruby-coloured solution of AuNPs. In addition, DLS and TEM analysis confirmed the presence of smaller particle sizes of the biosynthesized AuNPs. Significantly, the biosynthesized AuNPs displayed a remarkable

antibacterial efficacy against *V. parahaemolyticus*, the causative bacteria of AHPND. The highest concentration of biosynthesized AuNPs exhibited the largest ZOI, indicating the strongest antibacterial effect. Both the MIC and MBC of the biosynthesized AuNPs were determined to be 0.3 g/ml, in which effectively inhibited the growth of *V. parahaemolyticus*. The optimization of the extraction solvent is recommended to enhance the yield of bioactive compounds in *M. moribidii* extract for the discovery of more potent antibacterial agents in the future. Overall, this present study highlighted the promising application of biosynthesized AuNPs produced by using *M. moribidii* as highly effective antibacterial agents that offers an alternative approach to the conventional antibiotics for combating antibiotic-resistant diseases, particularly AHPND in aquaculture.

Funding statement

Ministry of Higher Education, Malaysia, under the Higher Institution Centre of Excellence (HICoE), Institute of Tropical Aquaculture and Fisheries (AKUATROP) (Vot No: 63933 & 56055 – UMT/PPPI/2-2/5 Jld. 2 (29)).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Mohamad Sofi Abu Hassan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Nurul Ashikin Elias:** Investigation, Methodology. **Marina Hassan:** Formal analysis, Validation, Visualization. **Sharifah Rahmah:** Syed Muhammad, Validation, Visualization. **Wan Iryani Wan Ismail:** Validation, Visualization. **Noor Aniza Harun:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The author acknowledged the support of the Ministry of Higher Education Malaysia, Malaysia under the Higher Institution Centre of Excellence (HICoE), Institute of Tropical Aquaculture and Fisheries (AKUATROP), UMT, Malaysia (Vot No: 63933 & 56055 – UMT/PPPI/2-2/5 Jld. 2 (29)).

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