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Detection of green synthesized silver nanoparticles and their antagonistic effect on fish larvae pathogenic bacteria

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ABSTRACT

The effect of green synthesized nanoparticles on fish larvae pathogenic bacteria has so far been rarely investigated. In the present study, silver nanoparticles "AgNPs" were synthesized biochemically from the reduction of $AgNO_3$ by leaves extract of mangrove (*Avicennia marina*) plants, and their antibacterial activity against fish larvae pathogens was assayed. To define the characterization of the green synthesized AgNPs, UV-Visible spectra, IR (infra-red), X-ray powder diffraction (XRD) and photoelectron spectroscopy (XPS), high resolution transmitted electron microscope (HRTEM), and selected area electron diffraction (SAED), were utilized. Results determined the formation of nano-size AgNPs with ranges 15 to 25 nm. The isolated bacteria, from fish larvae (sardines, pipefishes, cardinalfishes, dragonets, silversides, and wormfishes), were identified by morphological characterization and biochemical tests. Thus, the isolated bacteria was suggested to belong to *Vibrio* spp. (*Vibrio alginolyticus*, *Vibrio vulnifacus*, *Vibrio parahaemolyticus*, *Vibrio harveyi*, and *Vibrio cholera*). Except for *A. marina* leaves extract, the green synthesized AgNPs, with an inhibition zone radius of ~13-20 mm, showed an effective impact on all those pathogenic bacteria.

INTRODUCTON

Nanoparticles (NPs) have unrivalled features due to their minute size (1-100 nm dimensions), distribution, and morphology **(Keat *et al.*, 2015; Sarkheil *et al.*, 2016; Abdi *et al.*, 2017, 2018, 2019**). Due to their antibacterial features against many species of pathogenic bacteria, NPs could be used to control bacterial diseases as substitutional to antibiotics (**Wang *et al.*, 2017**). Silver nanoparticles, for instance, are highly toxic to

several pathogenic organisms, hence, play a vital role in treating many diseases. Consequently, it has long been recognized for owing a potent inhibitory effect towards many bacterial strains and microorganisms (**Sarkheil *et al.*, 2017**).

Nowadays, different chemical and physical methods are available for synthesis of metal nanoparticles (**Abdi *et al.*, 2018**). However, the cost of these methods and their negative impact on ecosystems are high. Biological methods, such as utilization of plant extracts (leaves, root, fruits … etc.) are suitable alternatives to synthesize nanometer scale particles. This green synthesis of nanomaterials is easy, ecofriendly, and, moreover, consumes low energy during industrialization processes (**Ahmed *et al*., 2016; Singh *et al*., 2018**).

Bacterial diseases are tremendous causes of mortality in fish and hatcheries (**Grisez & Ollevier, 1995**). As a result, mortality during the early stages leads to natural variations in the size and recruitment strength of marine fish populations. Larval mortality has frequently been related to adverse environmental conditions and intrinsic factors affecting feeding ability, vulnerability to predators, and bacterial infection (**Garrido *et al.*, 2015**).

Mangroves are woody plants, shrubs or trees that grow at the costal transition zone, are able to perform well in extreme environmental conditions. They are adapted to cope with high temperature, salinity, flooding, and strong winds stresses s well (**Basheer *et al*., 2018**). Mangroves may play a special role as nursery habitat for juveniles of fish whose adults occupy other habitats (e.g., coral reefs and sea grass beds). The majority of them have medicinal and commercial importance (**Biswas & Biswas, 2019**). They form a rich source of the wide range of bioactive compounds and natural products, which could be effective factors in reduction and stabilization of metal ions during the manufacture of nanoparticles. Those plant ingredients include sugars, amino acids, proteins, aldehydes, phenolic acids, flavonoids, alkaloids, vitamins, ketones, terpinoids, and glycosides such as saponin **(Thakkar *et al.*, 2010; Ahmed *et al.*,2016)**.

Avicennia marina (Forssk.) Vierh. is a mangrove tree belonging to the plant family Acanthaceae (formerly in the Verbenaceae or Avicenniaceae). Extracts from mangroves and mangrove-dependent species have proven active against human, animal, and plant pathogens (**Kathiresan & Bingham, 2001; Patra & Mohanta, 2014**). Mangrove extracts are suitably scaled up for large scale biosynthesis of AgNPs in a controlled manner according to their size, shape, and sensitivity (**Balakrishnan *et al.*, 2016**). The antibacterial activity of green synthesized AgNPs was investigated by some authors using the diffusion disk method, where their antagonistic effects on various pathogens were realized **(Roy *et al.*, 2019).** However, the antibacterial effect of these nanoparticles on fish larvae pathogens was not previously evaluated. Thus, in the present study, the biosenthesis of AgNPs, using *A. marina* leaves extract, were characterized, and their antibacterial efficacy against isolated bacteria from fish larvae was assessed. Furthermore, morphological, and biochemical aspects of the pathogenic bacteria were identified.

MATERIALS AND MATERIALS

1.1.Collection of plant samples

Fresh leaves of *A. marina* were collected from mangrove tiny area at El-Gouna resort on the northern Red Sea coast of Egypt. It is located about 20 kilometers north of Hurghada at 27º 21′ 25.77″ N and 33º 41′ 5.47″ E. The leaves were thoroughly washed in distilled water, and then, shade-dried for 15 days. Finally, leaves were crushed to attain fine powder, and then, kept at room temperature until usage.

1.2. Preparation of plant extract

A known mass (10 g) of plant leaves powder was mixed with 100 ml distilled water and placed in a shaker for 24 hours for extraction. The extract solution was filtered through Whatman No.1 filter paper (pore size 25 μm). Then, samples were collected and used for synthesis of silver nanoparticles.

1.3. Synthesis of silver nanoparticles (AgNPs)

The leaves extract of *A. marina* (0.25 ml) was added to 99.75 ml of aqueous solution (0.5 mM) of $AgNO_3$ (Cica) in a conical flask. Drops of 0.1 M NaOH (Cica) solution were used to adjust the pH of the solution in the slightly alkaline medium (pH 7.5). The reaction mixture was kept in a dark place (to prevent photo-activation of silver nitrate) for 72 hours at room temperature. An observed color change from pale yellow to brown confirmed the reduction of Ag^+ to Ag of the extract. The solution was taken in centrifuge tube to be centrifuged for 20 min at 10000 rpm. The supernatant was discarded, and the residue was isolated, oven-dried at 30°C and kept for further use.

1.4.Characterization of AgNPs

UV-visible

The observation of a resonance plasmon band in the UV-visible spectra is characteristic of biochemical reduction of Ag^+ and formation of AgNPs. This phenomenon was observed in the absorption spectra of $AgNO_3$-extract mixture that recorded on Shimadzu UV visible spectrophotometer (UV-1650PC, Japan), which is a double beam scanning spectrophotometer. The sample was scanned from 200 to 800 nm in 1 cm quartz corvette (**Singh *et al.*, 2014**).

Fourier-transform infrared (FT-IR) spectra

FTIR analysis was done on a Bruker FT-IR spectrophotometer as KBr discs in the wave number with ranges 4000-400 cm^{-1}. FT-IR is helpful in identifying the organic groups that exist in the extract and bind to Ag. The spectra were achieved at the Center of Biotechnology and Mycology, El-Azhar University, Cairo, and compared with a reference chart (**Deepa *et al.*, 2013**).

1.5. X-Ray powder diffraction (XRD)

XRD patterns were undertaken on a Philips XPERT-PRO with nickel filtered Cu Kα (λ=1.5405 Å) radiation at National Research Center, Cairo, Egypt .

1.6.Transmission Electron Microscopy (TEM)

(HR-TEM) images were taken using a transmission electron microscope (TEM) (JEOL JEM-2100, HR TEM, 200 Kev) at National Research Center.

1.7.X-ray photoelectron spectroscopy (XPS)

XPS measurements were performed on a PHI 5000 Versa Probe XPS spectrometer (ULVAC Physical Electronics, USA) based on a classic X-ray optic scheme in the binding energy range of 0 to 1500 eV. It was carried out at National Research Center.

1.8. Bacteriological examination

Bacterial isolation and characterizatio:

Fish larvae samples, with average length 0.5±2 (cm), were captured from coral reef area near the National Institute of Oceanography and Fisheries (NIOF) in Hurghada (Egypt) using light traps and identified by relevant literature **(Abu El-Regal *et al*., 2008; Abu El-Regal, 2013; Abu El-Regal *et al*., 2019).** A flame-sterilized loop was used to sample and inoculate macerated tissues (external skin ulcers) of the fish larvae onto primary isolation plate's media of trypton soy agar (TSA) (Oxoid Ltd, England) supplemented with 1.5% (w/v) NaCl. The plates were incubated under aerobic conditions at 25.5°C for 24 hours (**Farmer & Hickman-Brenner, 1992**). The suspected colonies were then subcultured on thiosulfate citrate bile salts sucrose (TCBS) agar (Oxoid Ltd) plates and incubated at 25.5°C for 18-24 hours. The putative *Vibrio* spp. Colonies, showing yellow or greenish color, on TCBS agar media, were picked up and subjected to bacterial identification through Gram staining and biochemical tests as described by **Alsina and Blanch (1994)** and **Nicky (2004)**. Commercial API20E strips (BioMerieux, France) were further used for identification of the organisms according to the manufacturer's instructions.

Antibacterial assay

Antibacterial activity of the green synthesized silver nanoparticles was evaluated by well-cut diffusion technique. In this method, wells (5 mm) were punched in Muller Hinton agar (Oxoid Ltd) plates inoculated with the isolated pathogenic bacteria. Then, 50 µl of AgNPs were transferred into each well and the plates were incubated at 25ºC overnight. Afterwards, the radius of the inhibition zone around each well was measured in mm (**Priyaragini *et al.*, 2013**). This test was carried out in three replicates for each treatment.

RESULTS

AgNPs were biosynthesized by reduction of $AgNO_3$ using leaves extract of mangrove (*A. marina*). On mixing the extract to the solution of $AgNO_3$, the color changed from pale yellow to yellowish- brown after 72 hours of incubation as a result of reduction of Ag^+ to Ag^0 (Ag nanoparticles) (Fig.1). The obtained AgNPs were characterized by the following techniques:

1.9.UV-visible spectra

The spectrum of $AgNO_3$ and extract mixture (Fig. 2A) was measured in wavelength range from 200-800 nm. Abroad beak centered at 420 nm was observed. The position of this peak between 410-450 nm suggested the presence of AgNPs.

1.10. FT-IR spectra

FT-IR spectra were carried out to shed some lights on functional groups characteristics of some biomolecules that can reduce silver ions and/or stabilize the formed AgNPs. The spectrum (Fig. 2B) showed broad strong band centered at 3320 cm^{-1}, that may be attributed to the starching vibrations of amino groups of protein residue, phenolic, and enolic hydroxyl groups. The spectrum was sharp and strong at 1638 cm^{-1} ,with some shoulder bands in the region 1600-1500 cm^{-1} due to carboxyl and amide groups of proteins. A broad band was observed at 632 cm^{-1} due to the presence of carbon halide bond of alkyl halide molecules. The presence of those bands in the IR spectra suggests the presence of some capping agents coating and stabilizing gaps.

1.11. XRD and XPS

The formation of AgNPs and the crystallinity of the product were confirmed by X-ray diffraction (XRD). XRD pattern (Fig. 3A) of the formed product from the reduction of $AgNO_3$ with the extract exhibits strong diffraction peaks at 2θ = 27.4, 32.45, 46.35, 54.75, and 67.5 correspond to the planes (311), (111), (200), (300), (220), and (222) of Ag metal. Furthermore, there was a peak at 2θ = 56.82 that may be due to the presence of some crystallized biomolecules. The pattern was well indexed to cubic silver (JCPDS Card No.006-0 480).

The crystallite size was calculated from Debye-Scherrer equation, $D = K\lambda / \beta \cos\theta$. Where D is the crystallite size, λ is the wavelength of x-ray source (1.5406 Å), β is the width of the peaks at half intensity, θ is Bragg′s angle and K is Sherrer ′s constant (K =

0.89). The calculated crystallite size was 25 nm. The formation of metallic silver was further confirmed by XPS high-resolution of Ag3d spectra (Fig. 3B). It showed two characteristic doublet peaks of Ag3d (Ag 3d5/2) and (Ag 3d3/2) around 368 and 374eV, respectively. The spin energy separation of the two peaks was 6.0 eV confirming the existence of metallic Ag nanoparticles.

1.12. High Resolution Transmission Electron Microscopy (HRTEM) and selected area electron diffraction (SAED)

The morphology Ag nanoparticles were described by HR-TEM. TEM images (Fig. 4A) showed the formation of irregular spherical particles with particle size in the range of 15-25 nm. SAED pattern of AgNPs (Fig. 4B) showed circular ring indicating the formation of crystalline Ag nanoparticles.

1.13. Bacterial identification and characterization

The colonies on TSA appeared as smooth, rounded, buff white-to-cream-colored, and 2-5 mm in diameter. After sub-culturing on TCBS media, all colonies grew, indicating they were related to *Vibrio* spp., whereas the colonies on TCBS were yellow-greenish, smooth, circular, and convex (Fig. 5). The isolates showed initial phenotypic properties typical of the genus *Vibrio*; being Gram-negative rods, motile, oxidase positive, and possessing requirements for sodium chlorides. The biochemical and physiological characteristics (Table 1) of all isolates were almost different and allowed the presumed identification of bacteria as *V. alginolyticus*, *V. vulnifacus*, *V. parahaemolyticus*, *V. harveyi*, *V. cholera* .

1.14. Antibacterial activity of silver nanoparticles

Generally, silver nanoparticles synthesized from *A. marina* leaves extract exhibited an excellent antibacterial activity against all tested pathogenic bacteria (*V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus*, *V. vulnifacus*, and *V. cholera*), whereas *A. marina* leaves extract failed to inhibit the growth of all tested pathogens (Fig. 6). The inhibition zones of silver nanoparticles, synthesized from *A. marina* leaves extract, were ranged between 13.5 to 20 mm (Table 2). It can be seen from this table that the green synthesized NPs exhibited strong action, whereas the highest antibacterial activity was against *V. alginolyticus* (20 mm), and the lower effect was against *V. cholera* (13.5 mm).

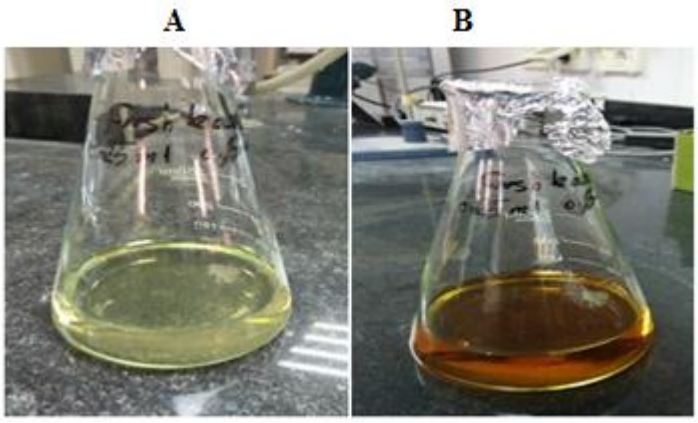

Fig.1. Change in color of leaf extract of *A marina* from yellow (A) to brown (B) after mixing)with 5m M $AgNO_3$

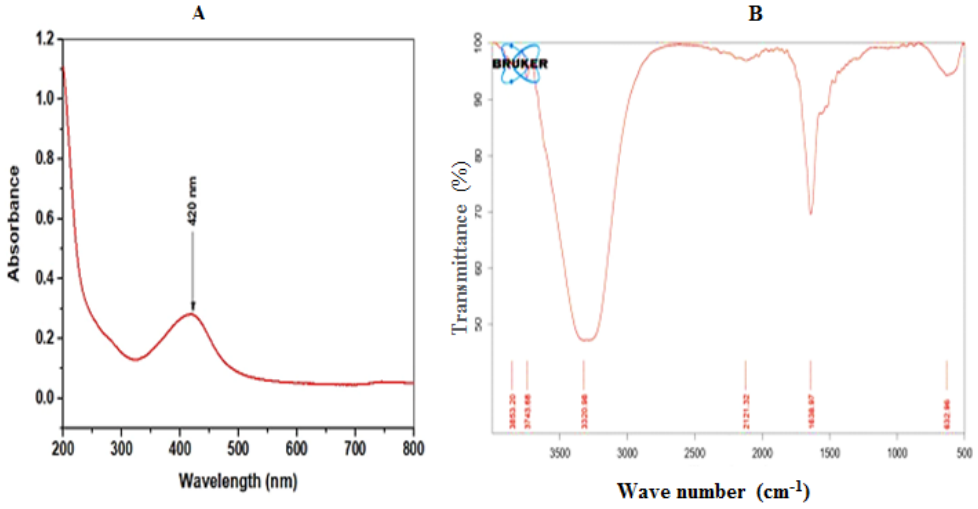

Fig.2. Changes in ultraviolet visible spectrum
A) Infrared spectrum B) of AgNPs

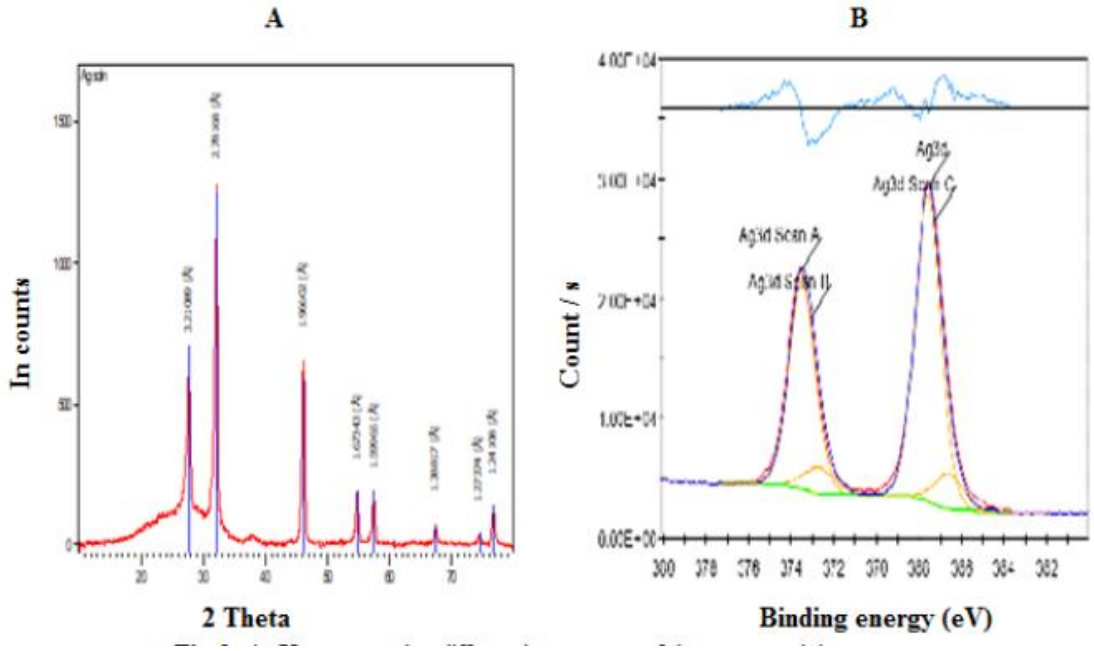

Fig.3. A. X-Ray powder diffraction pattern of Ag nanoparticles
B) X-Ray photoelectron spectroscopy of Ag nanoparticles

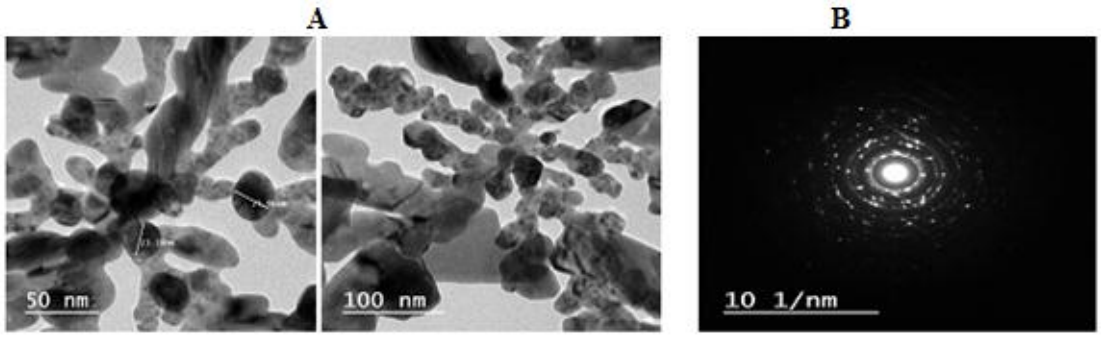

Fig. 4. A) High resolution transmission electron microscopy of Ag nanoparticles at different magnifications
B) selected area electron diffraction of Ag nanoparticles

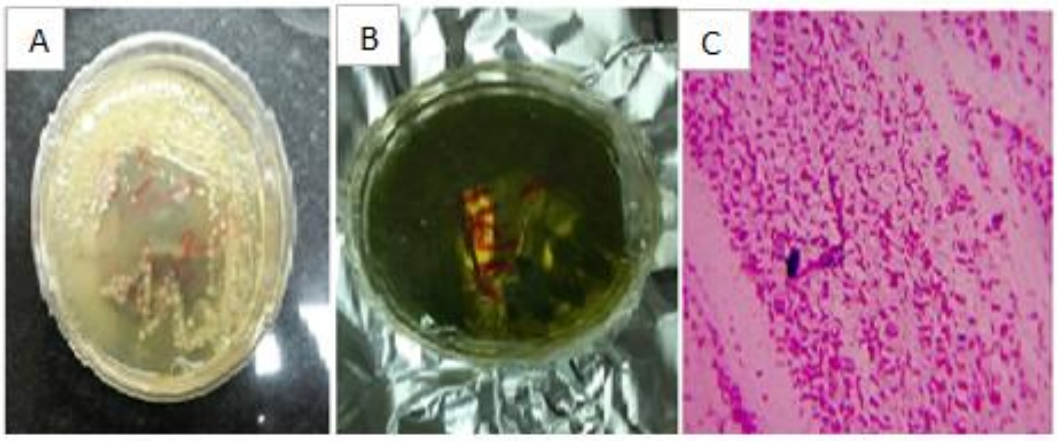

Fig.5. colony morphology, culture characteristics of the retrieved *Vibrio* spp.

A.Bacterial growth (colonies) on TSA after 24 h at 25C
B. *Vibrio* growth (colonies) on TCBs after 24 h at 25C
C. Gram negative rods

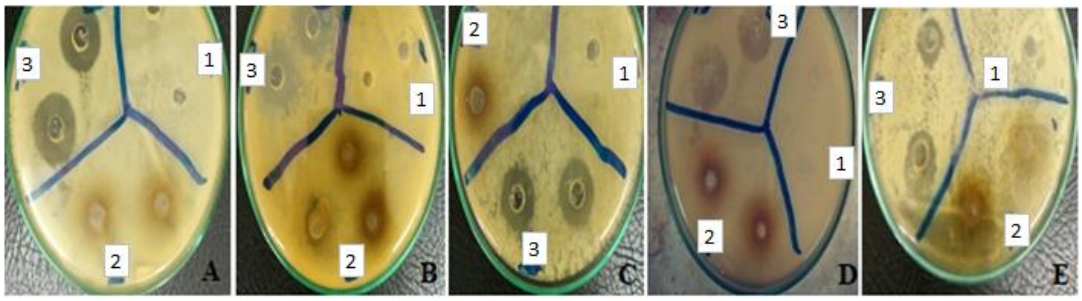

Fig.6. antibacterial effect of green synthesized AgNPs and leaves ectract of *A. marina* against:

A) V. parahaemolyticus
B) V. harveyi
C) V. alginolyticus
D) V. vulnificus
E) V. cholera

Table (1) Colony morphology, cultural and biochemical characteristics of the retrieved *Vibrio* spp. from diseased fish larvae **(Mohammed *et al*., 2021).**

1a. Colony morphology and cultural characteristics

Test		*V. alginolyticus.*	*V. parahemolatycum*	*V. vulnifacus*	*V. cholera*	*V. harveyi*
Colony shape		Round	Round	Round	Round	Round
Colony colour		White creamy	White	White	Small white	White creamy
Motility		+	+	Motile	Motile	-
Gram stain		-Ve rods	-Ve rods	-Ve rods	-Ve rods	-Ve rods
Growth in	0% NaCl	-	-	-	-	-
	1.5%NaCl	+	+	+	+	+
	3% NaCl	+	+	+	+	+
	8 % NaCl	+	+	-	V	+

Abbreviations: *V. Vibrio;* V, Variable strains; -Ve, Gram negative; + , Positive result; - Negative result.

Table 1b. Biochemical characteristics

Test	*V. alginolyticus.*	*V. parahemolatycum*	*V. vulnifacus*	*V. cholera*	*V. harveyi*
Catalase	+	+	+	+	+
Cytochrome oxidase	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-
Ornithine decarboxylase	+	+	V	+	+
Lysine decarboylase	+	+	V	+	+
Arginine dihydrolase	+	-	-	-	-
Hydrogen sulphide production	-	-	-	-	-
Citrate	+	+	+	+	+
Indole production	+	+	-	+	+
TDA	-	-	+	-	+
Urea hydrolysis	-	-	-	-	+
O- nitrophenyl-β-galactopyranoside	-	-	+	+	-

GEL	+	+	+	+	V
Xylose	-	-	-	-	-
Raffinose	-	+	-	+	+
Glucose	+	+	+	+	+
Manitol	+	+	-	+	+
Inositol	-	-	-	-	-
Sorbitol	+	-	-	-	+
Rhaminose	-	-	-	-	-
Sucrose	+	-	-	+	+
Malonate	-	+	+	-	-
Adonitol	-	-	-	-	-
Arabinose	-	+	-	-	-
Lactose	+	-	+	-	-

Abbreviations: *V., Vibrio*; V, Variable strains; + Positive result; - Negative result

Table 2 Inhibition zone (mm) of silver nanoparticles and *A. marina* leaves extract against some pathogenic bacteria isolated from fish larvae.

Pathogenic bacteria	**AgNPs inhibition zone (mm)**	***A. marina* leaves extract inhibition zone (mm)**
V. paraheamolyticus	15± 2.4	0
V. harveyi	16 ± 4.7	0
V. alginolyticus	19.5 ± 1.7	0
V. vulnificus	17 ± 1.4	0
V. cholera	13.5 ± 3.9	0

Values are presented as mean ± standard deviation.

DISCUSSION

Green synthesis of AgNPs is a promising and ecofriendly technique. In the current work, extract of mangrove (*A. marina*) leaves was utilized to mediate the green synthesis of silver nanoparticles. With respect to the visual observation of colour change on mixing the mangrove extract with silver nitrate solution, the formation of AgNPs was suggested. The change in color, resulting from the reduction of Ag^+ by the extract of *A.*

marina to AgNP, UV-visible spectrum was used to confirm the formation of AgNPs as indicated from the position of the absorption band of the reaction mixture. The color was changed (from yellowish to brown) due to surface plasmon resonance of AgNPs (**Abdi *et al.*, 2018; Bharathi *et al.*, 2018; Gopinath *et al.*, 2012; Sinha *et al.*, 2015)**. It is known that when nanoparticles are formed from their salts, characteristic absorption bands appear in their spectra. According to the previous studies, AgNPs have characteristic absorption band in the visible region, with ranges, 400 to 500 nm. In the present study, based on the obtained absorption band (420 nm), the formation of AgNPs by aqueous extract of *A. marina* is supported. The band around 420 nm has been been observed by other studies for biosynthesized AgNPs (**Jyoti *et al.*, 2016; Abdi *et al.*, 2019**) .

The observed FTIR spectra, in the current study, were used to assign the main functional groups in different chemical categories such as; flavonoids, polyphenols, and terpenoids. The positions of the observed characteristic bands in the spectrum of AgNPs indicated the presence of characteristics groups of phenols, aldehydes, flavenoides, and terpenoides that coordinated to AgNPs and stabilized their formations **(Prakash *et al.*, 2013; Abdi *et al.*, 2018)**. The observed peaks shown in XRD patterns confirmed the presence of Ag nanoparticles in crystalline form. Furthermore, the formation of metallic silver is confirmed by XPS high-resolution of Ag3d. The peaks in XPS pattern are characteristic to metallic silver, as indicated by **Wang *et al.* (2005, 2012)**. According to HRTEM images, the formed AgNPs have spherical shape with 15-25 nm size range, and likewise; SAED pattern of AgNPs showed circular ring indicating the formation of crystalline silver nanoparticles **(Abdi *et al.*, 2018).**

Vibrios are important pathogens, causing potential devastation to marine aquaculture **(Austin, 2009).** These pathogens are responsible for mortalities of fish larvae **(Olafsen, 2001; Rodrigo *et al.*, 2016; Mahmoud *et al.*, 2017)**. In this investigation, the pure bacterial isolates from naturally infected early stages of fish were identified as *V. alginolyticus*, *V. vulnifacus*, *V. parahaemolyticus*, *V. harveyi*, and *V. cholera* using the colony, morphological characters, and biochemical reactions including the API20E tests. This was also confirmed by the molecular characterization of these pathogenic isolates (unpublished data of the authors).

AgNPs have wide applications in different biological fields; the most important of which is their toxicity against pathogenic bacteria. The antibacterial activity of AgNPs is attributed to their high surface to volume ratio, which is an interactive property of the nanoparticles (**Rai *et al.*, 2009; Sarkheil *et al.*, 2017**). In the present study, the green synthesized AgNPs exhibited an enormous antibacterial activity against all tested pathogenic bacteria (*V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus*, *V. vulnifacus*, and *V. cholera*), whereas *A. marina* leaves extract failed to inhibit the growth of all tested pathogens (**Balakrishnan *et al.*, 2016**). This finding can be supported by **Sarkheil *et al*. (2017)** who reported that silver nanoparticles are highly toxic to several pathogenic organisms and hence play a vital role in treatment of many diseases. Similarly, **Kamel *et***

al. **(2016)** confirmed the reactive antibacterial activity of AgNPs. During green biosynthesis of AgNPs, nanoparticles capping or coating occurs which leads to many advantages such as blocking agglomeration, toxicity reduction, and antibacterial action enhansement (**Roy *et al.*, 2019**). Therefore, it is expected that, the green synthesized AgNPs would be highly effective against various disease-causing pathogens.

CONCLUSION

Biosynthesis of Ag nanoparticles by *A. marina* leaves extract was simple, ecofriendly, and could be easily produced for large-scale syntheses. AgNPs, with particle size in the range of 15-25 nm, were obtained and characterized by different methods. The isolated pathogenic bacteria from fish larvae were mainly *Vibrio* spp. The green synthesized AgNPs showed an enormous effect against these bacteria, whereas the leaves extract alone did not show any effect. This may be the first report on the usage of green synthesized AgNPs against fish larvae pathogens. Thus, this study opens a door for a new range of antibacterial agents, which could reduce mortality in the early life stages of fish.

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الملخص العربى

الكشف عن جسيمات الفضة النانوية المخلقة خضريا وتأثيرها المضاد للبكتيريا المسببة للأمراض فى يرقات الأسماك

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لم يتم دراسة تأثير الجسيمات النانوية الخضراء المخلقة على البكتيريا المسببة للأمراض فى يرقات الأسماك بشكل كاف حتى الآن. فى هذا البحث تم تصنيع جسيمات الفضة النانوية "AgNPs" كيميائيًا حيويًا من خلال اختزال $AgNO_3$ عن طريق مستخلص أوراق نباتات المانجروف (*Avicennia marina*) وتم تقييم نشاطها المضاد للبكتيريا مسببات الأمراض فى يرقات الأسماك. تم استخدام الأطياف المرئية للأشعة فوق البنفسجية، الأشعة تحت الحمراء، حيود الأشعة السينية (XRD) والتحليل الطيفي الكهروضوئي (XPS)، والمجهر الإلكتروني عالي الدقة (HRTEM)، وحيود الإلكترون المحددة (SAED) في توصيف AgNPs المخلقة خضريا . أشارت النتائج إلى تكوين جسيمات فضة نانوية (AgNPs)بحجم نانو يتراوح من 15 إلى 25 نانومتر. وايضا تم التعرف على البكتيريا المعزولة من يرقات أسماك السردين من خلال التوصيف المورفولوجي والاختبارات الكيموحيوية واتضح أنها تنتمي إلى *Vibrio* spp. وهى (*Vibrio alginoly*ticus و *Vibrio vulnifacus* و *Vibrio parahaemolyticus* و *Vibrio harveyi* و *Vibrio cholera*).

وتبين ان AgNPs المخلقة خضريا فعالة ضد كل هذه البكتيريا المسببة للأمراض فى اليرقات المستخدمه بنصف قطر منطقة تثبيط ~ 13-20 مم. أما خلاصة أوراق االمانجروف لوحدها فلم تؤثر فعليًا على هذه البكتيريا المسببة للأمراض.