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Research Article

The Effectiveness of Silver Nanoparticles for the Treatment of Abdominal Dropsy in *Labeo rohita*

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ABSTRACT

Despite using antibiotics, in aquaculture of freshwater sp. facing severe challenges of bacterial strain resistance occur. In order to combat disease-causing bacterial species, the present study was designed to assess the value of green synthesized silver nanoparticles (Ag NP´s) from the extract of plant leaves for abdominal dropsy treatment in *Labeo rohita*. For this purpose, an extract of lemon leaves extracts in powder form was prepared followed by a dry method and a mixture of ethanol and distilled water (4:6) was added. Prepared nano-solution was then subjected to further characterization of particles where color changes, UV-VIS spectroscopy, particle analysis, and FTIR were done. Statistical results showed maximum (SGR%) value (2.65 ± 0.010) in T1 nano solutions treated group, had a significant difference ($P<0.05$) as respect to other treatments and control groups. Water physicochemical properties showed a significant result ($P<0.05$). At the end of the trial, total protein, albumin, globulin, glucose, ALT, and AST were analyzed from serum samples of both (diseased & healthy fish) and showed significant results ($p<0.05$) compared to other treatment groups. By histopathology detection, no hepatocyte damage, vacuolization and, regular shape nuclei were detected in healthy fish, also fibrosis and coagulative necrosis were absent, while in diseased fish, damaged hepatocytes were detected with irregular shaped nuclei and vacuolization. Thus, our study suggests that the lemon plant leaves extract solution could be very effective for the treatment of bacterial disease in *Labeo rohita*.s.

Keywords: Aquaculture; Nanoparticles; Plant extract; Medicinal treatment; Abdominal dropsy; *Labeo rohita*

INTRODUCTION

In aquaculture production, disease has developed into one of the major limiting factors, exclusively with the recent expansion of aquaculture process [1]. Among the causative agents of bacterial fish diseases, (*Pseudomonas* and *Aeromonas* sp.) is one of the most important fish pathogens [2]. Gill rot, septicemia disease, dropsy, tail fin rot, and ulcers including epizootic ulcerative syndrome disease are common diseases of freshwater fishes, caused by these bacteria [3]. Virulence of different Aeromonads and *Pseudomonads* bacterial isolates have been studied elsewhere in diseased fish in cultured and capture fisheries [4]. In a natural environment like soil, fresh, brackish water, sewage, and wastewater generally, *Aeromonas* are present [5]. Furthermore, members of the genus *Aeromonas* are generally spread in different foods like vegetables, chicken, fish, and meat [6]. Disease occurrence is significant violence to the development of aquaculture. For instance, severe complications have been affected by the appearance of furunculosis, sea lice, infectious salmon anemia and pancreatic necrosis in Scottish Atlantic-salmon farms [7-9].To control bacterial diseases, a spectrum of antibiotics is registered for use despite concerning to health matters of human being, few states sometimes not consent the registration of antibiotics because of non-medicinal reasons like use of growth promotant [10]. While some researcher [11] did not promote the antibiotics used in aquaculture. According to them antibiotics practice in aquaculture vary extensively one state to another state. In established kingdoms, here is an inadequate number of yields, administrative regulator is active, and usage of antibiotics is downturn because of advancements in administration and improvement of useful preparations [12,13].

Yet, *Aeromonas hydrophila* evolved protection to various antibacterial such as ampicillin, rifampicin, amoxicillin, lincomycin, novobiocin, penicillin, tetracycline, and oxacillin [14]. It has been reported that shrimps, shrimp pods, shellfish, eels and aquaculture environments have developed resistance against antibiotics [15-22] reported that

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resistance evolves in a period of not many centuries of handling contamination with drugs that kill bacteria. So, this is a factor, which restricts their importance in the restriction of diseases that are caused by bacteria in fish [23], aside since some public health concerns. So, research aimed to find antibiotics alternatives are earning momentum [24]. Approaches have been evolved to recognize and control material by nanoscale magnitudes, ranging from one to one hundred nanometer using nanotechnology. Such molecules that have this size range have molecules, which paves unique uses [25].

Because of their unique physical chemistry, optoelectronic properties and dimensions that are administered through their magnitude, form, and size dispersal, nanoparticles gained much attention [26,27]. According to previous studies, synthesis of nanoparticles through biological entities from different methods like microorganisms also plants are non-toxic, cheap, and an eco-friendly [28,29].

Considering these remarkable properties of plants, a major eco-friendly way for manufacturing metal nanoparticles via biological entities for detoxification applications have been recognized [30,31]. Different researchers focused on the synthesis of silver nanoparticles from plants [32-36]. These have a few benefits to their ability to reduce metal ions stability [37]. They ranged 15 to 65 nm and a regular size of 34 nm in size and they are mostly rectangular and cuboidal. Many researchers found the effect of silver nanoparticles against *Klebsiella pneumonia*, *Pseudomonas fluorescence*, *Escherichia coli, Pseudomonas aeruginosa*, *Shigella flexaneri, Proteus mirabilis* and *Shigella sonnei* [38-40]. Most of the plants that are used for the synthesis of Ag nanoparticles include *Anacardium occidentale* (cashew nut), *Azadirachta indica* (neem), *Swietenia mahagony* (West Indies mahogany), cruciferous vegetable extracts, *Citrullus colocynthis*, *Eucalyptus macrocarpa*, *Mangifera indica*, *Camelia sinensis*, *Rhododendron dauricum*, *Citrullus colocynthis* and Aloe vera [41-51]. Ag nanoparticles have been acquired together with antibacterial and anti-inflammatory properties that can encourage quicker wound healing. Considering their valuable properties, Ag nanoparticles have been involved in pharmaceuticals, medical transplant coatings and wound dressings [52,53]. There is no related study yet for the treatment of abdominal dropsy in *Labeo rohita,* Thus, I have firstly aimed present study to explore the antibacterial properties of naturally occurring plant leaves nanoparticles solution in *Labeo rohita* and to find the effect of NP's on blood chemistry and histo-pathological parameters.

MATERIALS AND METHODS

Experimental design and sampling

Preparation of plant extract: Fresh leaves of Lemon (*Citrus limon)* were collected from the botanical garden of University of Veterinary and Animal Sciences, Lahore. Extract of the plant was prepared by using dry method. Leave were washed, dried in shade, made fine and kept in plastic bags. Mix 3 g powder in 100 ml of solution (40 ml ethanol + 60 ml water) in a beaker and covered the beaker with aluminum foil placed the mixture for 2 days with 1 hour stirring daily. After 2 days filter the solution and stored in the refrigerator [54] (Figure 1).

Phytochemicals screening: Phytochemicals of plant extract were determined, and the following contents were tested.

Detection of carbohydrates:

Benedict's test: In 1 ml of plant extract, 2 ml of Benedict's reagent added in the test tube and heated moderately on the water bath. Formation of orange-red precipitates confirms the presence of reducing sugars.

Detection of proteins:

Xanthoproteic test: 2 ml of plant extract was treated with a few drops of conc. HNO_3. Due to the reaction between both reagents yellow color formed which assures the presence of proteins.

Detection of flavonoids

Alkaline reagent test: Extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on the addition of dilute acid, indicates the presence of flavonoids.

Detection of phenols

Ferric chloride test: Extract was treated with 3-4 drops of ferric chloride solution. Due to reaction formation of bluish.

Detection of tannins

Copper acetate test: Extract was dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green color indicates the presence of diterpenes.

Test for steroids and terpenoids

Salkowski's test: 2 ml of extract and 2 ml chloroform was taken in a test tube and treated them with few drops of conc. Sulphuric acid, shaken well and allowed to stand. The red color in the lower layer confirms the presence of steroids while, the formation of reddish-brown color in the middle area after addition of conc. Sulphuric acid to the side indicated the presence of terpenoids.

Detection of saponins

Foam test: 0.5 ml of extract was mixed and shaken with 2 ml of water. Formation of foam and continues for ten minutes it confirms the presence of saponins.

Test for oil: Between two filter papers, a small quantity of the extract was pressed. Oil stain on the filter papers ensures the presence of oil.

Test for phlobatannin: 2 ml of 1% hydrochloric acid added to 1 ml of plant extract. Red precipitates formed when boiled the solution.

Detection of glycosides

Extract was hydrolyzed with dil. HCl, and then subjected to test for glycosides.

Keller Killiani test: Extract was treated with 2 ml of glacial acetic acid with a few drops of $FeCl_3$. 1 ml of H_2SO_4 was added along the sides of the test tube carefully. A brown ring at the interface

indicated deoxy sugar of cardenolides, violet ring beneath the brown ring and greenish ring appear.

Detection of alkaloids

Extract was dissolved individually in dilute Hydrochloric acid and filtered.

Hager's test: Filtrate was treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow-colored precipitate.

Synthesis of nanoparticles

5 ml of extract was added to 45 ml of 1 mM aqueous solution of silver nitrate and the reaction mixtures were placed at hot stirrer (60°C) temperature till a change of color of the mixture to yellow brown was observed as a confirmation of nanoparticle formation.

Characterization of silver nanoparticles

The Litesizer™ 500 is an important instrument for illustrating the size of nanoparticles in solutions. Through measuring DLS (dynamic light scattering), it detects the particle size also measures the sample's refractive index. This instrument has combined expertise with modern software creativity to create intuitive particle analyzers. The reduction of pure Ag^+ ions was monitored by measuring the UV–visible spectrum of the reaction medium at 5 h after diluting a small aliquot of the sample into distilled water. UV–visible spectral analysis was done by using UV-vis spectrophotometer (Perkin Elmer, Lambda 35). FT-IR (Fourier transform infrared) analysis was carried out on Tensor-27 (Bruker) in the diffuse reflectance mode operated at a resolution of 4 cm^{-1} in the range of 400 to 4,000 cm^{-1} to evaluate the functional groups that might be involved in nanoparticle formation.

Growth trial

Individuals of healthy fish were collected from fish hatchery, University of Veterinary & Animal Sciences, Lahore (Ravi Campus, Pattoki) whereas, diseased fish was collected from nearby fish farms. The healthy and diseased fish were divided into two groups. Healthy fish was designated as control and Treatment #1 whereas diseased fish was designated as Treatment #2 and Treatment #3. Ten individuals of fish were selected for each group (healthy and diseased fish) and kept in aquaria of 60-liter water capacity, separately, with each had three replicates. Each aquarium was filled with dechlorinated tap water at 30°C. The standard method recommended by the Animal welfare committee was followed. (Control group did not receive any chemical and fed with commercially available fish feed. Treatment #1 and Treatment #2 was fed with feed also bath of synthesized silver nanoparticles daily (2 ppm) while treatment #3 was treated with antibiotics (Oxytetracycline, 75 mg/kg) and artificial feed) (Table 1). Artificial feed with 30% crude protein (CP) level was prepared from Oryza Organics and fed to experimental fish twice a day for 6 days a week up to satiation (Table 1).

Water physicochemical properties

Physicochemical parameters viz. temperature (°C), pH, dissolved oxygen (mgL^{-1}), salinity (mgL^{-1}), total dissolved solvents (mgL^{-1}) and electrical conductivity ($mScm^{-1}$) were recorded on daily basis using digital meters and HANNA Nitrate Test Kit HI3874.

Feeding

The artificial feed with 30% crude protein (CP) level was prepared

Table 1: Experimental design for growth trial.

Group	Fish	Treatment
Control	Healthy	No treatment
T1	Healthy	NP's bath
T2	Diseased	NP's bath
T3	Diseased	Antibiotics

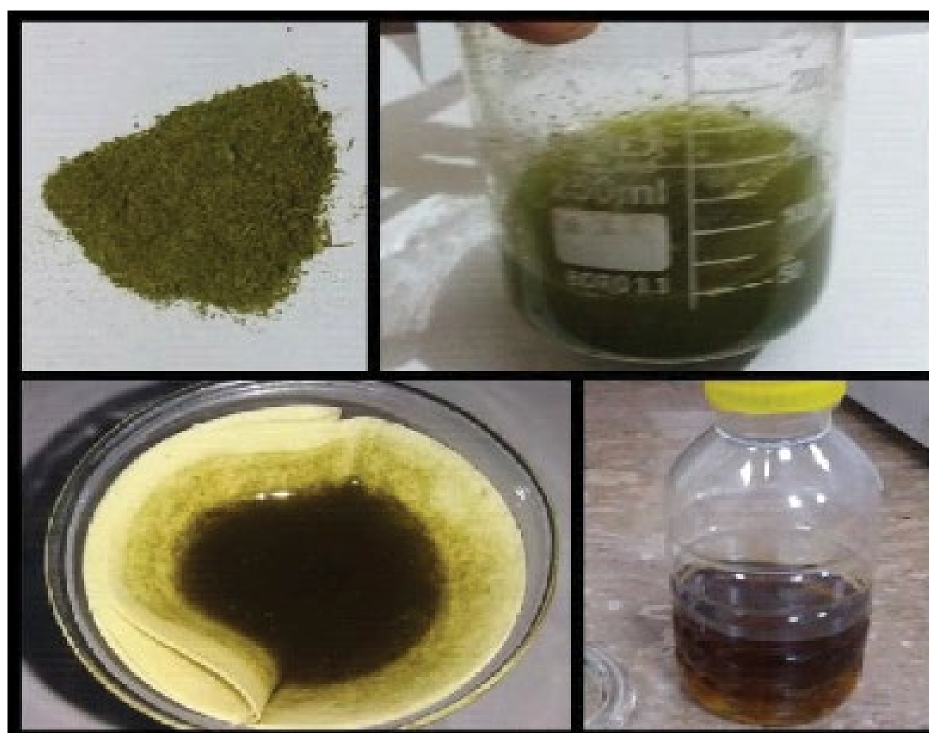

Figure 1: Preparation of lemon (*Citrus limon*) extract.

from Oryza Organics and fed to experimental fish twice a day for 6 days a week up to satiation.

Histopathological examination

Healthy and diseased fish indiscriminately selected from each treatment. Fish samples were dissected for the collection of liver and gills. The liver and gills were carefully removed a weighted. The samples were washed with normal saline and preserved in 10% neutral buffered formalin (NBF). For light microscopy technique, tissues were processed by paraffin embedding the technique [55]. To analyze the samples under Light microscopy thin tissue sections (5-6 μm) were required that obtained with paraffin embedding technique.

Serological analysis

Blood from both specimens, diseased and healthy fish were collected in Eppendorf tube with a red cap, which was not contained EDTA, allowed the tubes to stand in tilted position at room temperature to separate out the serum for following tests.

Total serum protein: Total serum protein (albumins and globulins). For the checking of total protein level in serum, 20 μL serums along with 1000 μl reagent added in 2 ml Eppendorf tube. A purple complex was formed due to the reaction of protein with cupric ions in alkaline medium. The absorbance was checked at 520-580 nm wavelengths which was proportional to protein concentration in the sample, against reagent blank that was only one per series required.

Albumin: To determine the albumin level in serum, 1000 μl reagent and 10 μL serum was mixed and gently sharked. Bromo cresol green forms a complex with albumin in citrate buffer and observed the absorbance at 578 nm wavelength, against reagent blank.

Globulin level: From the serum, globulin level was recorded, albumin values were subtracted from the total serum protein.

Glucose: For the checking of glucose level in serum, 1000 μL biuret reagent and 20 μL serum added in Eppendorf. The absorbance was checked at 520-580 nm wavelength which was proportional to glucose concentration in the sample.

Table 2: Phytochemical analysis of Lemon plant extract.

Phytochemical constituents	Chemical tests	Lemon extract
Carbohydrates	Benedict's test	-
Proteins	Xanthoproteic test	-
Flavonoids	Alkaline reagent test	+
Phenolic compounds	Lead acetate test	+
Tannins	Copper acetate test	+
Steroids	Salkowski's test	+
Terpenoids	Salkowski's test	+
Saponins	Froth test	+
Oils	Filter paper	-
Glycosides	Keller-killiani test	+
Phlobatannin	Hcl Test	+
Alkaloids	Hager's test	-

Key: + =Present, - =Absent

ALT: Alanine transaminase test (ALT) is also known as serum glutamate pyruvate transaminase (SGPT). For the determination of SGPT level in the serum sample, 200 μL substrate and 800 μL reagent was preheated at 37°C for 5 minutes. Mildly blunt 100 μL serum sample. At 340 nm wavelength activity was checked within few seconds.

AST: Aspartate transaminase test is also known as serum glutamate oxaloacetate transaminase (SGOT). For the checking of SGOT level in serum, 750 μL of reagent pipetted with 250 μL of substrate and incubate it for 5 minutes at 37°C. After incubation, 100 μL of serum sample was added in it. The absorbance was checked at 340 nm within few minutes of sample preparation.

Statistical analysis

Statistical analysis was performed using the analysis of variance (ANOVA) and Duncan multiple range test. Multiple Range Test was carried to determine the differences between treatments (mean at a significance level of $p < 0.05$). All analysis was run on the computer using the SAS program [56].

RESULTS

The nanoparticles were synthesized in Organic chemistry lab of Chemistry department of UET and the experimental trail was conducted in the fish hatchery of Department of Fisheries and Aquaculture, UVAS, Ravi Campus, Pattoki. Silver nanoparticles (AgNPs) were prepared from leaf extract of lemon.

Phytochemical analysis

During the present study, the phytochemical analysis of plant extract (lemon) was done after extract preparation. Phytochemical components viz. carbohydrates, proteins, flavonoids, phenolic compounds, tannins, steroids, terpenoids, saponins, oils, glycosides, phlobatannins and alkaloids were determined in extract (Table 2). In determined results, carbohydrates, proteins, oils and alkaloids were absent while flavonoids, phenolic compounds, tannins, steroids, saponins, glycosides, and phlobatanins were found in the lemon leaf extract.

Visual observations

The mixture of the metal solution and plant extract were mixed, and gradually visible color changes were observed. Because of the reaction of the metal solution with phytochemical contents of each extract, which convert Ag^+ to Ag. In other words, silver ions that are less beneficial than silver nanoparticles converted to nano-sized particles ranged from 0-100nm. In present studies, Color changes of silver nanoparticles (Ag NP's) from the extract of lemon (yellow to brown) were observed and showed (Figure 2).

UV-visible spectroscopy studies

UV-visible spectroscopy is one of the important methods to check the stability and formation of nanoparticles from the reaction mixture of plant extract and metal solution. Due to the reduction of silver ions. In this study, absorbance plotted on Y-axis and wavelength in nanometers were plotted on X-axis. Figure 3 showed the formation of nanoparticles from the lemon. A visible broad

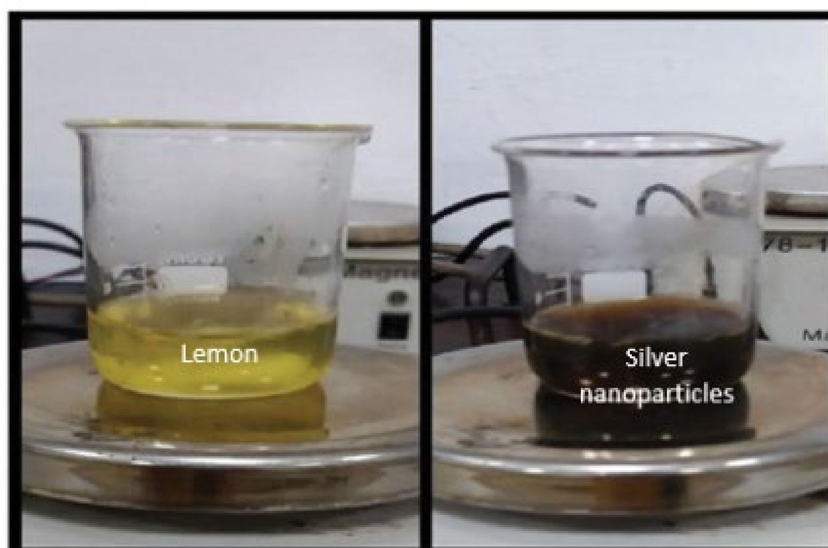

Figure 2: Silver nanoparticles from lemon (*Citrus limon*).

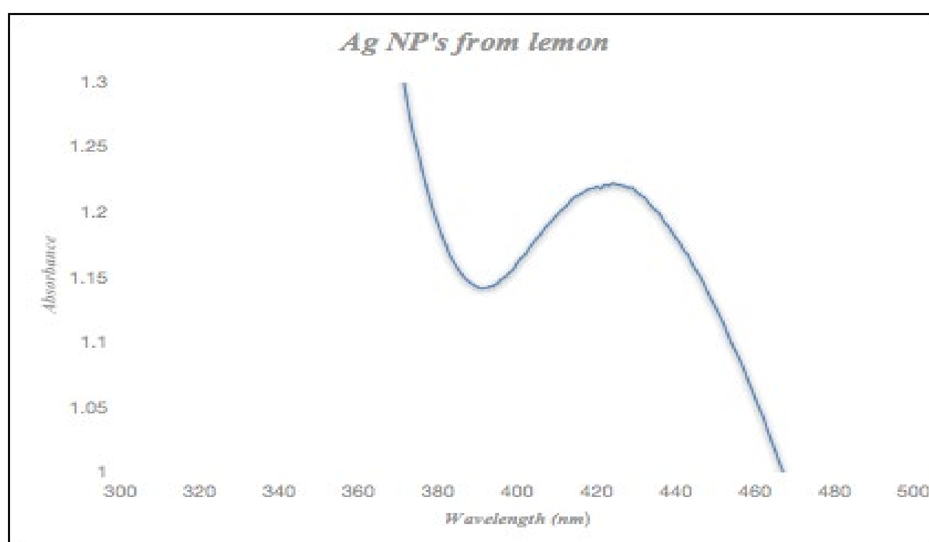

Figure 3: Formation of Ag NP's from lemon (*Citrus limon)* extract.

peak was observed and displayed its strong peak at around 410-435nm.

Particles size analysis

The particle size of the sample was detected from particle analyzer and graphs were plotted between relative frequency (%) and particle diameter (nm). When Ag NP's from lemon extract were prepared their hydrodynamic diameter was 54.13958 nm and the polydispersity index was 26.90228%. In Figure 4, two peaks were observed, peak 1 was 61.810126 nm and peak intensity 2 was 4.4635463 nm in diameter.

FT-IR studies

The FT-IR (Fourier transform infrared spectroscopy) was used to detect the functional groups that might involve in the formation of nanoparticles from plant extract and metal solution. Ag nanoparticles that were prepared from both plant extracts were subjected to FT-IR and different stretches and bending peaks were observed, showing different absorption bands. In Figure 5, four peaks were observed at 3387.437, 2199.437, 1629.536 and 1398.441 cm^{-1} from the solution of Ag NP's. Broad peak on 3387.437 indicates the O-H group (alcohols) and stretches of N-H group (amines) while, peak on 2195.437 showed variable stretches of alkyne group (-C-E-). Peaks on 1629.538 and 1398.441 were indicated the variable stretches of alkenes (-C=C-) and strong stretches of alkyl halides (C-F) (Figure 5).

Growth trial

The healthy and diseased fish were divided into two equal groups. Healthy fish was designated as control and Treatment #1 whereas diseased fish was designated as Treatment #2 and Treatment #3. Ten individuals of fish were selected for each group (healthy and diseased fish) separately, with three replicates each. Each treatment specimens were acclimatized and then treatment protocol was followed as given in materials and methods. All the groups were given with an artificial feed of 30%. Fish in treatment #1 and #2 were given a bath of AgNP's (2ppm) on daily basis while treatment #3 was treated with antibiotic (Oxytetracycline). Their growth parameters were examined also the physical changes in diseased and healthy fish were observed for fifteen days. Fishes of treatment

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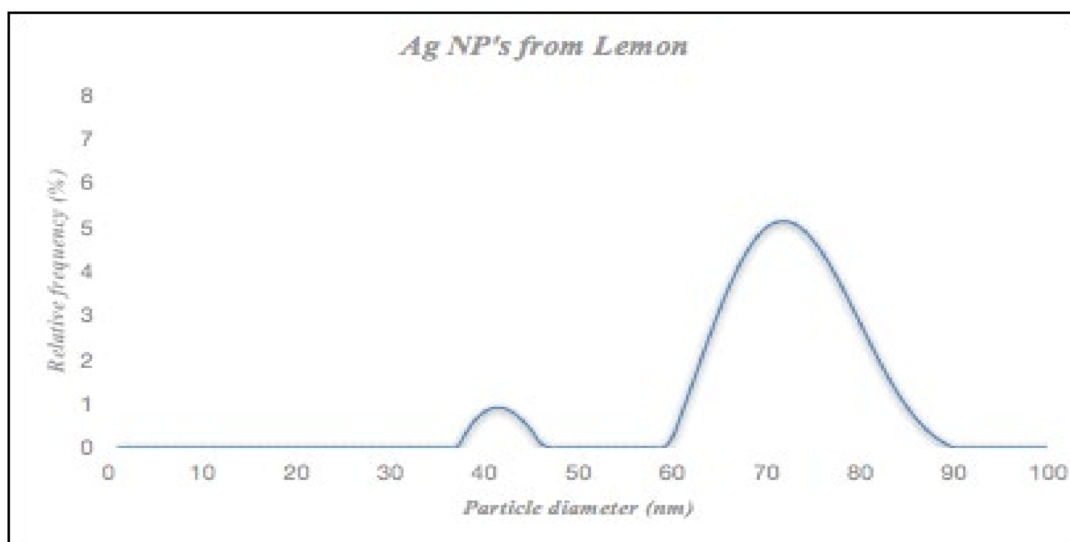

Figure 4: Particle analysis of Ag NP's from lemon (*Citrus Limon).*

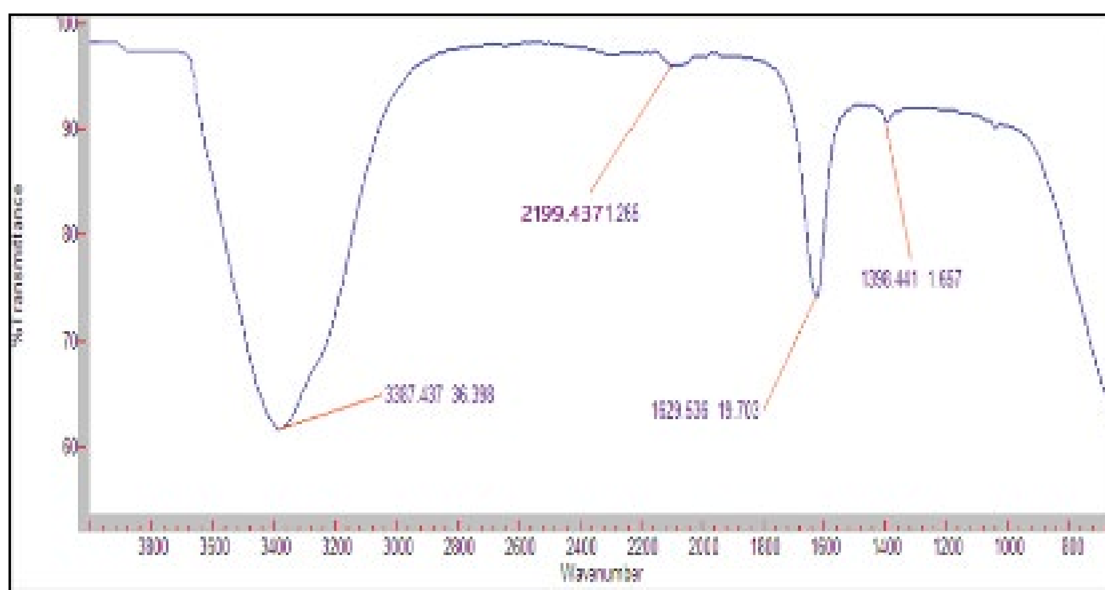

Figure 5: The FT-IR spectra of Ag NP's from lemon (*Citrus limon).*

#2 were cured within 3 days of the trial while treatment 3 which contained diseased fish and received antibiotics (oxytetracycline) cured after 4th to 5th day of experiment trial.

In Table 3, growth parameters of all treatments are given, showing the significant result (P <0.05). Maximum weight (g), length and fork length (mm) gain was recorded in T_1 viz. (6.41 ± 1.35, 27.36 ± 1.79 and 26.88 ± 1.25), which contained healthy fish and received both feed and nanoparticles. However, minimum weight (g), length and fork length (mm) gain was recorded in treatment 3 which contained diseased fish and received antibiotics, 26.88 ± 1.25, 9.86 ± 2.41 and 10.69 ± 2.5 respectively.

Similarly, among all the treatments better FCR was observed in treatment 1 (1.12 ± 0.25) and maximum in Treatment 3 (1.97 ± 0.11) while excellent condition factor was shown in treatment 3 that was 4.05 ± 0.31 and fair in T_1 that was 2.20 ± 0.03 (P <0.05). Specific growth rate (SGR) was recorded maximum in treatment 1 and minimum in treatment 3. The gradual decrease in SGR was as the following sequence: Treatment 1> Control> Treatment 2> Treatment 3 viz. 1.25 ± 0.25>1.03 ± 0.07>0.99 ± 0.14>0.73 ± 0.03 (Table 3).

Water physicochemical properties

Water physicochemical properties were recorded on daily basis and their means were showing Table 4, showing significance results (p <0.05). Maximum temperature (°C) was recorded in the control group and minimum in treatment 1, 29.74 ± 0.03 and 29.09 ± 0.85 respectively. Maximum DO (mg/l) was recorded in the control group (572 ± 0.04) and minimum in treatment 2 (5.12 ± 0.46). Higher pH and EC (mScm^{-1}) was observed in treatment 2 viz. 7.53 ± 0.01 and 2218.17 ± 226.87 while lower pH was found in treatment 1 (7.19 ± 0.28) and electrical conductivity in treatment 3 (2176.06 ± 201.76). Maximum TDS (mg/l) was recorded in treatment 1 and minimum in treatment 3, 1146.201 ± 126.90 and 1102.35 ± 183.16 respectively. However, salinity was remained constant in all the treatment throughout the experimental period.

Histopathology of fish

Liver and gills samples of both healthy and diseased fish were observed, and their tissues slides were prepared. In the liver,

round-shaped hepatocytes were observed in healthy fish, which were prominent with dark-colored nucleus. No hepatocyte damage and vacuolization were detected in healthy fish. However, fibrosis and coagulative necrosis were absent. Regular shaped nuclei were detected (Figure 6A). While in diseased fish, damaged hepatocytes were detected with irregular shaped nuclei and vacuolization. Fibrosis and coagulative necrosis were observed in focal areas. Blood clots and hemolysis were observed in blood vessels of the liver (Figure 6B).

Gills of fish have close contact with water and from the water any pesticide can easily enter the blood stream of fish through their gills. Gills of healthy fish contained filaments from which the primary and secondary lamellae arose. One to two thick layers of cells were detected in primary lamellar epithelium. At the base of secondary lamellar, chloride cells were visible (Figure 7A). No recognizable changes were detected in healthy fish from the normal one. However, in diseased fish excessive mucus secretions and upward position of epithelium observed which provide the direct route to bacteria into bloodstream (Figure 7B).

Serological analysis

Total protein, albumin, globulin, glucose, ALT and AST were analyzed from serum samples of both, diseased and healthy fish and showing statistically significant results (p <0.05) given in Table 5. Total protein was recorded 3.8 ± 0.3 mg/dl in healthy specimens of *Labeo rohita* while, 2.13 ± 0.25 mg/dl in diseased fish. Albumin level in healthy and diseased fish was recorded 1.31 ± 0.02 and 0.76 ± 0.05 mg/dl respectively. Level of globulin in diseased fish was 1.36 ± 0.20 mg/dl and 2.48 ± 0.28 mg/dl were recorded in healthy fish. Maximum glucose level was recorded in diseased fish, 123.69 ± 11.07 mg/dl while the minimum was in healthy fish, 86.43 ± 7.64 mg/dl. ALT and AST level were observed minimum

Table 3: Growth parameters of all treatments.

Growth Parameters	Treatments				
	Control	Treatment #1	Treatment #2	Treatment #3	Mean
Total Wt. Gain (g)	5.13 ± 0.40b	6.41 ± 1.35a	4.64 ± 0.49b	3.54 ± 0.20c	4.93 ± 1.18
Total L. gain (mm)	21.01 ± 1.97b	27.36 ± 1.79a	14.66 ± 2.84c	9.86 ± 2.41d	18.22 ± 7.61
Total FL. Gain (mm)	19.98 ± 0.98b	26.88 ± 1.25a	12.99 ± 0.27c	10.69 ± 2.5c	17.63 ± 7.32
FCR	1.36 ± 0.11c	1.12 ± 0.25bc	1.51 ± 0.16b	1.97 ± 0.11a	1.49 ± 0.35
CF	2.99 ± 0.27c	2.20 ± 0.03bc	3.01 ± 0.02b	4.05 ± 0.31a	3.06 ± 0.75
SGR	1.03 ± 0.07a	1.25 ± 0.25a	0.99 ± 0.14bc	0.73 ± 0.03c	1.00 ± 0.21

Means with same letters in a single column are statistically similar at P<0.05.
*Total wt. Gain: Total Weight Gain; Total L. Gain: Total Length Gain; Total FL. Gain: Total Fork Length Gain; FCR: Feed Conversion Ratio; CF: Condition Factor; SGR: Specific Growth Rate.

Table 4: Physico-chemical parameters of water recorded during experiment.

Parameters	Treatments				
	Control	Treatment #1	Treatment #2	Treatment #3	Treatment #4
Temperature (°C)	29.74 ± 0.03a	29.09 ± 0.85a	29.13 ± 0.95a	29.12 ± 0.75a	29.27 ± 0.31a
DO (mg/l)	5.72 ± 0.04a	5.38 ± 0.96a	5.12 ± 0.46a	5.34 ± 0.12a	5.39 ± 0.24a
Ph	7.36 ± 0.03a	7.19 ± 0.28a	7.53 ± 0.01a	7.48 ± 0.12a	7.39 ± 0.15a
EC (mScm-1)	2198.28 ± 148.37a	2214.84 ± 247.02a	2218.17 ± 226.87a	2176.06 ± 201.76a	2201 ± 19.07a
TDS (mg/l)	1126.50 ± 134.62a	1146.20 ± 126.90a	1118.72 ± 118.02a	1102.35 ± 183.16a	1123.44 ± 18.20a
Salinity(mg/l)	1.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a

Means with same letters in a single column are statistically similar at P <0.05.
*DO: Dissolved Oxygen; EC: Electrical Conductivity; TDS: Total Dissolved Solvents.

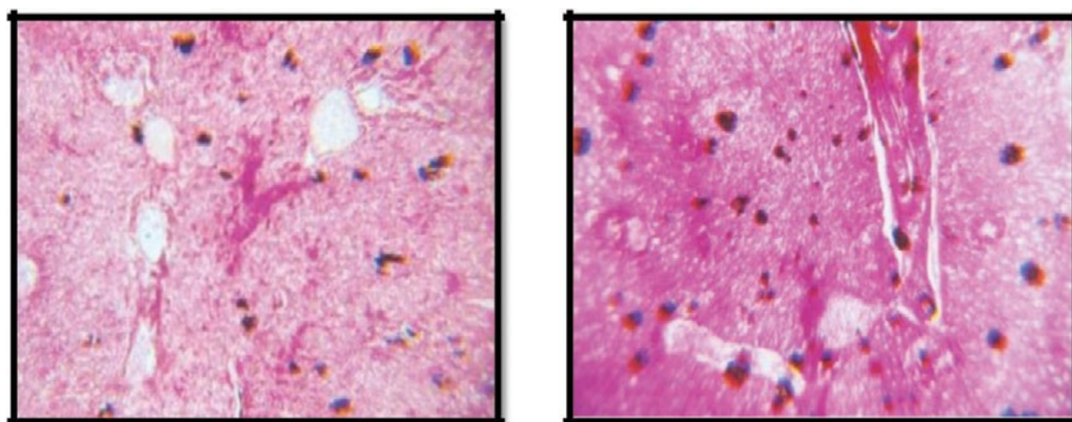

Figure 6: Histopathology of liver tissues of healthy fish (A) and diseased fish (B).

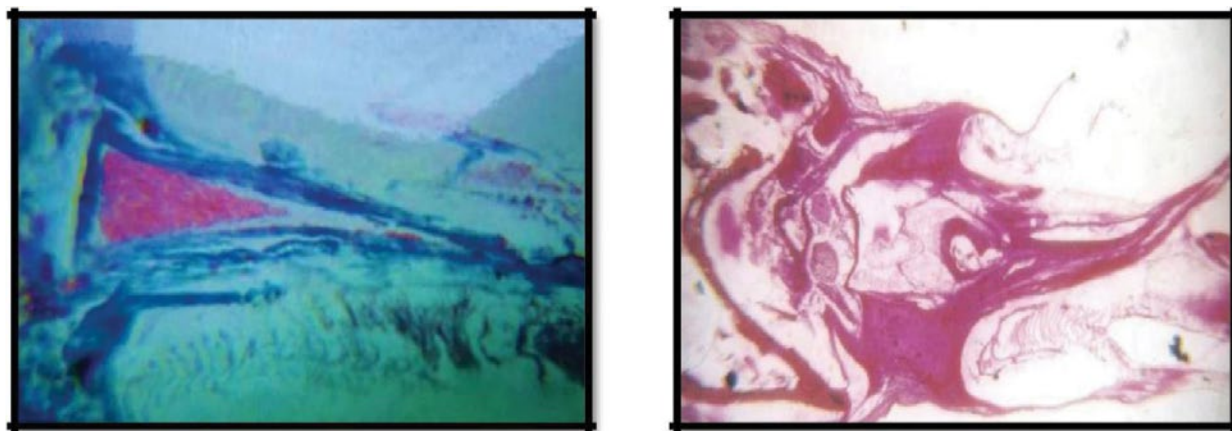

Figure 7: Histological section of healthy gill (C) and of the disease gill (D) (H &E, Gill arches).

Table 5: Serological analysis of both diseased and healthy fish showing statistically significant results.

Serum Parameters	Fish	
	Healthy	Diseased
Total Protein (mg/dl)	3.8 ± 0.3a	2.13 ± 0.25b
Albumin (mg/dl)	1.31 ± 0.02a	0.76 ± 0.05b
Globulin (mg/dl)	2.48 ± 0.28a	1.36 ± 0.20b
Glucose (mg/dl)	86.43 ± 7.64b	123.69 ± 11.07a
ALT (IU/L)	18.09 ± 1.67b	32.36 ± 2.11a
AST (IU/L)	36.17 ± 1.51b	79.39 ± 2.42a

Means with same letters in a single column are statistically similar at P<0.05

ALT: Alanine Transaminase and AST: Aspartate Transaminase

in healthy fish and maximum in diseased fish viz. 18.09 ± 1.67, 36.17 ± 1.51, 32.36 ± 2.11 and 79.39 ± 2.42 (IU/L).

DISCUSSION

The synthesis of silver nanoparticles (AgNP's) from lemon plant extract is linked to the green synthesis of nanoparticles. The aim of the present research work was to synthesize nanoparticles from natural components that were extracted from lemon plant leaves in the form of their extract and check their efficacy against fish disease management. Phytochemical components are the type of components, which includes carbohydrates, phenolic compounds, proteins, flavonoids, phyobatonnins, alkaloids, tannins, glycosides, oils, saponins, steroids, and terpenoids [57,58]. Phytochemical analysis conducted on prepared extract and different components were observed in both. The extract contained flavonoids, steroids, terpenoids, and saponins. Singh et al. [59] found flavonoids, glycosides, oils, proteins and steroids in *H. coronarium,* and *H. rubrum* plants. Edeoga et al. [60] also found flavonoids and saponins in Nigerian plants. Synthesized nanoparticles were examined for change in color from yellow to brown in the present study. Light yellow or green to brown color was also observed in silver nano synthesis from *phlomis* leaves extract, eucalyptus, mango, papaya, banana, aloe vera and *C. spinose* [61-64]. Peaks under UV-vis spectroscopy were observed in the range of 400-450 nm [65,66]. From this analysis broad peak was observed at around 410-435. Benakashani et al. and Vigneshwaram et al. observed peak at 420 nm, while Allafchian et al. observed on 440 nm and Medda et al. observed at 400 nm [67-70]. The FT-IR studies during the present research trail showing many peaks on different absorption bands. Allafchian et al. recorded 9 peaks in *Phlomis* plant extract showing the involvement of nine functional groups in its synthesis [71]. Growth parameters were recorded during the experiment and the results of the present study and significant results were recorded. Maximum weight (g), length and fork length (mm) were recorded in Treatment 1 viz. 30.79 ± 1.10, 50 ± 1.00 and 46 ± 1.52. Yin et al. recoded maximum wet weight in group 1 which received 0.5% guava in their feed while Giri et al. recorded the average weight gain of *Labeo rohita* in group 2 which contained 1% guava leaves in feed. Yildirim et al. observed better growth results in the groups which received enzyme in addition to feeding than the groups that only received the feed. The results of Khalil et al. also correlates with them. The feed conversion ratio was better recorded in treatment 1 and maximum in treatment 3 viz. 1 0.97 ± 0.50 and 1.44 ± 0.10. Khalil et al. recorded 1.21 ± 0.01 FCR in their research trail. FCR values reported by Azimuddin et al., 1.73-2.04 and Ahmed et al. for three months of the experiment. SGR was ranged from 2.09 ± 0.015 (treatment 3) to 2.65 ± 0.010 (treatment 1). Yildirim et al. recorded maximum SGR in the treatment which received more enzymes, so as compare to others, better FCR and SGR recorded in this trail. All the water quality parameters or physico-chemical parameters such as temperature, dissolved oxygen (DO), pH and electrical conductivity were continuously measured on daily basis throughout the study period. The results of our study are in agreement with Wahab et al., Kohinoor et al. and Dewan et al. where they reported the temperature range from 24°C to 34°C for optimum growth. Azad et al. reported DO variation from 4.1 to 8.9 mg/L which is larger than variation found in our study. It was also reported that pH values between 6.5 and 9.0 is optimum for polyculture pond [72,73] and our values of pH are within that optimum range. For histopathology, liver and gills samples of both healthy and diseased fish were observed and their tissues slides were prepared. In healthy liver, round-shaped hepatocytes were observed, which were prominent with dark colored nucleus. No hepatocyte damage and vacuolization were detected in healthy fish. Regular shaped nuclei were detected. While in diseased fish, damaged hepatocytes were detected with irregular shaped nuclei and vacuolization. Fibrosis and coagulative necrosis were observed in focal areas. Blood clots and hemolysis were observed in blood vessels of the liver. Similar results of histopathology were recorded by Kumar et al. in liver, kidney and intestine of *Labeo rohita.* In

the present study, gills of healthy fish contained filaments from which primary and secondary lamellae arose. One to two thick layers of cells were detected in primary lamellar epithelium. At the base of secondary lamellar, chloride cells were visible. However, in diseased fish, excessive mucus secretions and upward position of epithelium observed which provide the direct route to bacteria into blood stream. Patnaik et al. studied the organs of *Cyprinus carpio* and observed vacuolization and fusion in gills of fish due to the accumulation of lead [74]. Different tests of serum were done to check the difference between normal or healthy fish from diseased fish. Total protein level was observed maximum in healthy fish (3.8 ± 0.3 mg/dl) and minimum in diseased fish (2.13 ± 0.25 mg/dl) as they indicated that due to disease protein contents automatically became low. Same as albumin and globulin level was also high in healthy fish and low in diseased fish. Kumar et al. recorded maximum total protein in treatment 3, which received non-gelatinized starch, crude protein and amylase enzyme in the specific ratio. However, they recorded albumin and globulin in treatment 1 viz. 1.13 ± 0.13 and 1.76 ± 0.42 mg/dl. Tiwari et al. recorded 2.73 g/dl total protein in healthy fish and 2.30 g/dl in fish after disease [75]. They also observed low albumin and globulin level in diseased fish. Maximum glucose level was recorded in diseased fish, 123.69 ± 11.07 mg/dl while the minimum was in healthy fish, 86.43 ± 7.64 mg/dl with the mean value of 105.06 ± 26.34 mg/dl and the results correlated with the results of Tiwari and Pandey. ALT (Alanine aminotransferase) level was recorded maximum in diseased fish (32.36 ± 2.11 IU/L) as we knew that ALT present in liver and entered to blood due to liver damage or any injury so its level raised in blood from the normal range. AST (Aspartate transaminase) level was recorded maximum in diseased fish (79.39 ± 2.42 IU/L) and its low level was recorded in healthy fish. Tiwari et al. recorded maximum AST in healthy fish and minimum in diseased fish as in the recent research their results matched. But they recorded maximum AST (150.4 IU/L) in healthy fish and minimum in diseased fish (96.6 IU/L).

CONCLUSION

This study deployed the use of green synthesis of silver nanoparticles from lemon leave extract, which have potent biomedical applications to cure diseased fish (*Labeo rohita*) from abdominal dropsy. Our study concluded that green synthesized silver nanoparticles helped to cure bacterial disease (abdominal dropsy) immediately as compared to antibiotics. Silver nanoparticles resulted to better hematological and growth rate of the fish during this experiment. Moreover, it has been revealed that the use of nanoparticles from lemon leaves extract are not only cheap and renewable but also act as reducing agent which convert the toxic effects of silver metal solution to beneficial and disease curing. It was observed that the overall effect was enhanced by the use of silver nanoparticles from lemon leaves extract due to probably their essential oil components and synergetic effect, however further studies are imperative.

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