Supplementary Data

Crest to Troughs Cellular Drifting of Green Synthesized Zinc Oxide and Silver Nanoparticle

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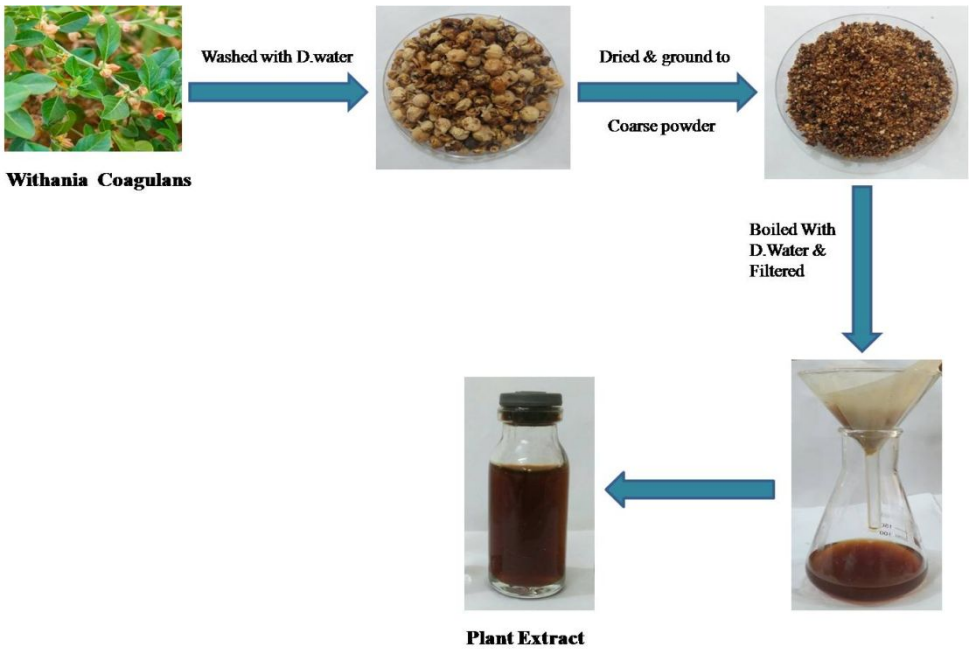
Materials and Methods

Materials and chemicals: Table S1.

Reagent	**Supplier (By Sigma)**
Distilled water	H_2O CAS Number 7732-18-5
Ethanol	Ethyl alcohol $CH3CH_2OH$ CAS Number: 64-17-5 Purity ≥99.9%
Zinc Acetate	Zinc acetate dehydrate $Zn\ (CH_3CO_2)_2.2H2O$ CAS Number 5970-45-6 Quality level: 200
Silver nitrate	Silver nitrate AgNO3 CAS Number:7761-88-8
Agar	Agar-agar $(C_{12}H_{18}O_9)_n$ CAS Number 9002-18-0 Quality level: 200
Ciprofloxacin	Ciprofloxacin $C_{17}H_{18}FN_3O_3$ CAS Number: 85721-33-1

	Purity ≥98%
iodonitrotetrazolium (INT)	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride $C_{19}H_{13}ClIN_5O_2$ CAS Number 146-68-9 ≥97.0% (calc. on dry substance, NT) Quality level: 200
Bacteriological Peptone	Peptone CAS Number 91079-38-8
Dextrose	Dextrose $C_6H_{12}O_6$ CAS Number: 50-99-7
DPPH	(2, 2-diphenyl-1-picrylhydrazyl) $C_{18}H_{12}N_5O_6$ CAS Number 1898-66-4 Quality level: 100
Ascorbic acid	Ascorbic acid $C_6H_8O_6$ CAS Number 50-81-7
Fish Feed (Soyabean Flour)	Protein ~52% (85+% dispersible and 1% fat.) CAS Number S9633

Preparation of plant extract of *Withania Coagulans*: (Schematic Figure S1)



Schematic Figure S1. Schematic sketch of plant extract preparation

Synthesis of Zinc Oxide NPs (ZnONPs) S1

Zinc Oxide NPs was prepared with of concentrations of Zinc Acetate 0.02M and 3ml concentration of *Withania Coagulans* plant extract. To synthesize 0.02M ZnO NPs, 0.2g Zinc Acetate + 50 ml distilled water was taken in a beaker and 3ml of *Withania Coagulans* plant extract was added. The pH was maintained at 12 by using 2M solution of NaOH. The solution was kept at 90°C for 5 hours on a hotplate stirrer. Color change was occurred indicating that the NPs are synthesized. The UV-visible spectroscopy further confirmed the formation of zinc oxide nanoparticles. After this the solution of NPs was centrifuged at 6000 round per min (RPM) for 20 minutes at room temperature. The pellets were separated with the help of sucker fixed on the burette. The pellet was transferred into a 100ml beaker and kept in incubator at 37°C for 24 hours to dry. The dried powder was removed from the beaker and stored in Eppendorf tubes for further use and its application on fish.

Synthesis of silver NPs (Ag-NPs): S2

Synthesis of Ag-NPs by using 1Mm solution of silver nitrate and 10ml of plant extract both are added in 500ml reagent bottle and placed on the hot plate for 90mintues for heating and stirring at 90°C .The change

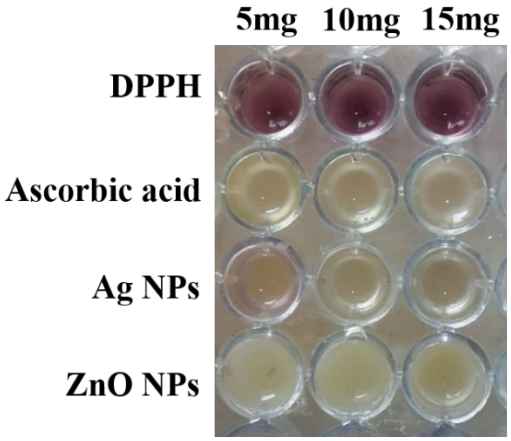
in colour of silver nitrate from faint light to colloidal brown indicates the formation of Ag-NPs (Ahmed *et al*., 2016). The UV-visible spectroscopy further confirmed the formation of Ag-NPs. After this the solution of NPs was centrifuged at 6000 RPM for 15 minutes at 4°C. Supernatant and pellets ware separated with the help of sucker fixed on the burette. The pellet was transferred into a 100ml beaker and kept in incubator at 100°C for 24 hours to dry. The dried powder was removed from the beaker and stored in Eppendorf tubes for further use and for application on fish.

Anti-oxidant: S3.

The DPPH solution was made by adding 6.7 mg of DPPH (2, 2-diphenyl-1-picrylhydrazyl) to 20 ml of methanol. Concisely, DPPH upto 70 µl solution was added in 96 well plates, 10 µl of ZnO NP and Ag NP (10, 30 and 50 mg/ml) were added separately. After an incubation time of 1 h, the absorbance was taken at 370 and 408 nm respectively. Reducing amplitude of signal at the selected wavelength confirmed a great radical scavenging activity. Ascorbic acid was working as positive standard while DPPH solution minus samples as negative control. The percent scavenging of DPPH, was considered by the following formula:

$$\% \text{ Scavenging} = [AB_C - AB_S / AB_C\}] \times 100$$

Where "AB_S" and "AB_C" represent to absorbance of sample and absorbance of control.



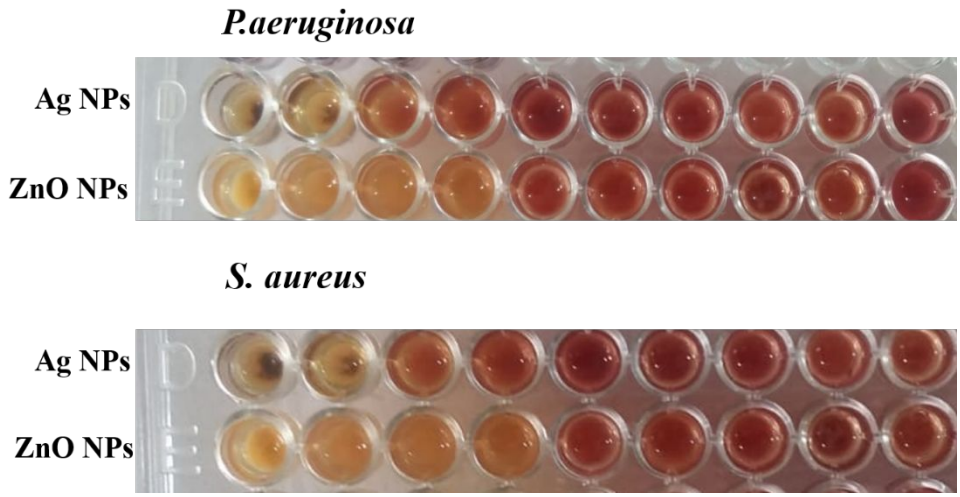
Antibacterial Activity: S4.

The antibacterial activity of ZnO NP and Ag NP was studied against gram negative (*P.aeruginosa, Staphylococcus aureus* strains obtained from B.V, Hospital Bahawalpur) bacteria at different

concentrations by well diffusion method. The activity was carried out by spreading and diffusion method, followed by measuring of zones of inhibition. Bacterial strains were initially obtained and grew in liquid broth media. Then, the agar was dissolved in distilled water, autoclaved and then naturally cooled down to the agar plates. Later, the calculated amounts of ZnO NP and Ag NP were added in the petri plates and left for solidification. After solidification, diffusion of fresh cultured media of the respective strain on petri plates was done and left for applicable time so that the culture can absorb the NPs and left overnight at 37 °C in incubator. Next day the growth of the bacteria was monitored. ZnO NP and Ag NP were active, and they kill all the bacterial strains. In diffusion method, the agar plates were prepared as mentioned above, after solidifying the plates spread the bacterial strains on agar plates. Then, the prepared paper discs were taken and dip in ZnO NP and Ag NP. The dipped paper discs were stuck on plates one by one and incubated overnight at 37 °C in incubator. After 24hr the growth of the bacteria was monitored and inhibition in growth confirmed the active nature of ZnO NP and Ag NP.

Minimum Inhibitory Concentration (MIC): S5.

Minimum Inhibitory Concentration (MIC) was conducted to calculate the minimum concentration of synthesized ZnO NP and Ag NP which is used to constrain the growth of a micro-organisms. MIC test for ZnO NP and Ag NP was executed in 96 well Microtiter plate using broth micro-dilution method against bacterial strains, *S. aureus* and *P.aeruginosa.* For this examination 100 µl of broth culture was decanted in 10 wells of 96-well plate then 10 µl of 0.5M ZnO NP and Ag NP suspension was added in the first well and serially diluted to achieve the concentrations in the range 2000 µg–0.0195 µg/ml. Then 20 µl of the bacterial inoculum was added in each well. Similar method was used for ZnO NP and Ag NP. The Microtiter plates were positioned in the incubator at 37 °C over night. Then on next day, 50 µl of p-Iodonitrotetrazolium Violet (INT) was added in all wells and incubated them at 37 °C for 30 minutes, this step is used to inquire the growth of bacteria as well as to calculate MIC values. After the incubation period, MIC value were determined which is the lowest concentration with no visible growth.



Fish collection and acclimatization: S6

In this study, seventy Rahu (*L.rohita)* fish were purchased from Punjab Government fish hatchery Bahawalpur having average weight 120 ±6.7 and length 19.7±0.7. Fishes were acclimatized for 15 days in a glass aquarium. Dimensions measuring of aquarium were 92 × 46× 46 cm (L x H x W) with a total volume of water was 120L. Each aquarium was well aerated system and after 48 hours 50% water was changed from each aquarium. Fish were fed with a commercial fish food (3% body weight).

Experimental design and exposures of NPs:

The acclimatized fish were randomly divided in to 7 groups, control and six were treated. Each group contains one replicated aquarium. Ten specimens were present on each aquarium. The fish group was control (Tap water free of zinc oxide and silver nanoparticles), 1mg/l0g ZnONPs, 2mg/l ZnONPs, 3mg/l0g ZnO Nps 1mg/l0g Ag-NPs, 2mg/l0g Ag-NPs and 3mg/l0g Ag-NPs respectively designated as treatment, T1, T2, T3, T4, T5 and T6 aqueous Exposure of NPs and its exposure period were 96h and15days. ZnONPs and Ag-NPs were added to the fish feed in a falcon tube and sonicated in a bath type sonicator (100W, 40 KHz) to scatter the particles for 30 min. (Wang *et al*., 2011; Karthigarani and Navaraj, 2012). The sonicated NPs expose to the different treatment in respective doze and control group without any doze. During the experiment each aquarium contains 60L water and well aerated system.

Hematological studies: S7

Hematological parameter analysis performed through hematological analyzer from local commercial laboratory.

Biochemical studies: 3ml Blood sample were collected without anticoagulant (EDTA) in yellow cap vacutainer at the day of sampling. The Vacutainer were shaking for mixing of blood with the coagulant material present in the Vacutainer. After shaking the Vacutainer were leave for 15-30 minutes for clotting without disturbing at room temperature. After 15-30 minutes the clot was formed that lied on the bottom of Vacutainer and the serum formed a layer on the top of the Vacutainer. The serum separated by help of the pipette into the Eppendorf tubes. After that the serum was stored into Eppendorf tubes at 2-8^{0}C temperature for biochemical analysis.

Assessment of LFT Enzymes: The serum was analyzed for ALT, ALP, total protein, globulin, Albumin and bilirubin through semi-automatic analyzer (Biosystems BTS350, Barcelona, Spain) using commercial kits

Histology of fish: The fishes were treated for 15days. After the treatment fishes were collected from aquarium with the help of hand net and instantly anesthetized with chloroform. Anesthesia was applied through gills. When fishes were properly anesthetized. Total weight and body length of fishes were measured before dissection. Afterwards the fishes of all doses groups were dissected to remove organs such as liver and gills. After removal then these organs were fixed in formalin buffered solution. Different grades of ethanol were used for preserved tissues. After ethanol tissues were treated with xylene and embedded with wax. Thick tissue sections up to 5 microns were cut with the help of rotary microtome. Hematoxylin, eosin and PAS stains were used for staining. After staining the slides were dried and observed under high resolution microscope. Microscopic Images were taken with the help of digital camera.

Composition of neutral buffered formalin:

Sodium hydrogen phosphate	6.5 g
Sodium dihydrogen phosphate	4.0 g
Formalin	100 ml
Distilled water	900 ml

Histological slides were prepared by using usual protocol followed by preservation, dehydration, clearing, embedding, cutting, staining and mounting. Various histological deformities were detected in slides and micrographs were reserved using computer supported microscope.

Histological examination: For histological analysis preservation of organs (Liver and gills) in 10% formalin was done. Following protocol was adopted.

Method for tissues processing:

Procedure	**Chemicals applied**	**Duration (hours)**
Washing	Flowing water	07
Dehydration process	70% Alcohol	06
	80% Alcohol	04
	90% Alcohol	04
	Absolute Alcohol (1)	04
	Absolute Alcohol (2)	03
Clearance	Alcohol and xylene (1:1)	0.3
	Xylene: (1)	0.3
	Xylene: (2)	0.3
Filtration	Paraffin: (1)	01
	Paraffin: (2)	01
	Paraffin: (3)	01
Embedding	Paraffin wax	Instantly

After embedding tissues were detached from molted wax in a block. After removing the bubbles paraffin wax was allowed to harden. During hardening process trimming of blocks was performed with the assistance of knife or scalpel from paraffin wax then it was allowed to fix on wooden block for sectioning.

Section cutting by microtome: After this the tissues were embedded on the wooden blocks and then by using microtome 5µm thin tissue sections were cut. After cutting, tissues were stretched along with the ribbons on a Fischer slide being warmed at 60º C and then fixed to already clean albumenized glass slides and later on these slides were placed in incubator to remove the bubbles for about 12 hours.

Process of Staining: In histological analysis hematoxylin stain and Eosin stain were prepared.

Preparation of Hematoxylin stain: List of chemicals that were used in hematoxylin preparation are

Chemical	**Quantity**
Hematoxylin	1gram
Double distilled water	100ml
Ammonium alum	50gram
Sodium iodate (50gm)	0.2gram
Citric acid	1ml
Chloral hydrate	50gram

Method

- 2gram of hematoxylin was mixed in 100ml of ethanol
- For synthesis of ammonium alum solution dissolve 3gram of alum in distilled water and then boiled it.
- Then we added Ammonium alums solution into the solution of hematoxylin.
- Glycerol and sodium iodate were added to the solution carefully.
- Last but not least acetic acid was mixed thoroughly.

Preparation of Eosin Y stains: For the formation of Eosin Y stains 1gram of water soluble Eosin Y was weight in the digital weight balance. The 80ml of double distilled water was taken in the beaker and 320ml of 95% alcohol was added and in the last 0.4ml acetic acid was mixed with solution and Eosin Y stains were formed.

Method: Firstly eosin is dissolve in water and then adds it into 95% alcohol (1:4, Eosin: Alcohol) Acetic acid (0.4ml) was added into final solution. Eosin staining intensity is increased by acetic acid. When stain becomes cloudy it becomes ready to use. If stain is clear then add few drops of acetic acid to make stain cloudy. The solution should be standardized by staining the control slides.

Staining method for tissue sections: During the staining of the tissue sections, firstly tissue was dehydrate by using a Xylene for 3minutes in a twice. Then tissue was clear by the using of absolute alcohol for 2minutes in a twice and then 70% alcohol was used. For the washing of tissue flowing water used for 5minutes. The tissue was stain at the slides used a hematoxylin stain for 12minutes. During washing and staining may be some water remaining in the tissue therefore again dehydration of tissue take place through acidic alcohol just only 2 dips of tissue in the solution. Then again washing is done by the running water for 5minutres. Dehydration was done by the 70% alcohol for 3minutes. The staining was done by the Eosin Y stain for 3minutes and dehydration was done by the absolute alcohol for 2minutes and in last tissue was clear through the xylene.

Hematological parameters (Mean ± SE) of fresh water fish *L. rohita* of exposure ZnO and Ag NPs (15days, n=10) Table S2

Treatment's control		**ZnO NPs**			**Ag-NPs**	
		T1	**T2**	**T3**	**T4**	**T5**
RBC	2.00±0.18^{a}	1.34±0.03^{b}	1.005±0.00^{cd}	0.62±0.03^{d}	0.99±0.045^{c}	1.27±0.03^{b}
HGB	9.76±0.30^{a}	7.53±0.15^{b}	6.95±0.07^{b}	5.10±0.84^{c}	7.00±0.10^{c}	8.40±0.30^{b}
HCT	30.20±1.73^{a}	19.90±2.26^{b}	13.05±0.63^{bc}	7.35±0.35^{cd}	11.73±0.55^{c}	25.73±0.85^{b}
MCV	152.26±22.55^{a}	135.96±23.87^{a}	129.90±7.21^{a}	117.60±0.98^{a}	118.18±5.87^{c}	201.70±9.73^{b}
MCH	45.70±7.00^{d}	49.03±11.63^{d}	69.15±0.21^{bc}	82.10±18.24^{a}	70.56±4.20^{a}	65.73±1.06^{b}

MCHC	32.43±5.20[b]	35.90±5.14[b]	53.35±3.18[b]	69.75±14.91[a]	59.70±0.0[a]	32.70±1.90[b]
WBC	166.26±1.98[d]	196.50±2.49[c]	223.70±0.42[b]	141.00±6.50[a]	228.56±4.49[a]	219.46±3.85[a]
PLT	35.33±4.50[d]	54.66±2.51[c]	76.00±1.41[b]	95.00±8.48[a]	82.66±2.21[b]	47.00±2.00[c]
LYM	94.65±0.07[d]	96.65±0.07[c]	98.25±0.07[b]	98.55±0.07[a]	97.15±0.15[c]	98.11±0.07[b]
MON	1.8±0.0[d]	0.95±0.07[c]	1.15±0.07[b]	1.00±0.00[a]	1.15±0.00[b]	1.10±0.00[b]
NET	3.55±0.07[d]	2.40±0.14[c]	0.60±0.00[b]	0.45±0.07[a]	1.70±0.07[c]	0.79±0.07[b]

Different superscript indicate significantly difference at (P < 0.05).

Enzymological parameter exposure of ZnO and Ag NPs on the *L. rohita* fish: Table S3

Different parameter after 4 day exposure of ZnO and Ag NPs on the *L. rohita* fish

Treatments		**ZnO NPs**			**Ag-NPs**		
Control		**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
ALT	12.56±0.96[d]	30.52±1.05[c]	40.47±0.9[b]	47.73±0.44[a]	30.63±1.47[b]	20.40±1.07[c]	58.03±1.33[a]
ALP	8.96±0.30[d]	26.36±0.62[c]	37.43±0.75[b]	43.77±0.54[a]	26.10±.61[c][b]	32.44±1.24[b]	45.05±1.46[a]
T.P	4.49±0.04[a]	3.71±0.02[b]	3.38±0.01[c]	3.04±0.07[d]	3.71±0.03[b]	3.03±0.07[d]	3.02±0.02[c]

Different superscript indicate significantly difference at (P < 0.05).

After 15day exposure of ZnO and Ag NPs on the *L. rohita* fish

Treatments	Control	ZnO N Ps			Ag-NPs	
		T1	T2	T3	T4	T5
ALT	12.33±0.15 d	25.05±0.21 b	40.13±0.25 a	43.15±0.35 c	42.25±0.2b	45.31±0.37a
ALP	8.70±0.20 d	27.63±0.13 c	40.15±0.44 b	45.60±0.53 a	41.95±0.39b	47.84±0.45a
T.P	4.49±0.04a	3.97±0.03 b	2.10±0.02 c	1.62±0.01 d	2.10±0.01b	1.63±0.02a

Different superscript indicate significantly difference at ($P < 0.05$).

Protein parameters:

Protein parameters after 4 day exposure of ZnO and Ag NPs on the *L. rohita* fish **Table S4**

Treatments Control		ZnO NPs			Ag-NPs		
		T1	T2	T3	T4	T5	T6
GLOBULIN	2.32±0.16 a	2.29±0.02 a	2.21±0.02 a	2.04±0.13 a	2.28±0.02 a	2.04±0.13 a	2.01±0.02 a
ALBUMIN	2.10±0.01 a	1.41±0.02 b	1.17±0.01 c	1.03±0.05 d	1.43±0.01 b	1.03±0.05 c	1.01±0.01 c
BILIRUBIN	0.73±0.02 a	0.58±0.01 c	0.46±0.03 d	0.62±0.01b	0.59±0.01c	0.47±0.02 d	0.44±0.03d

Protein parameters after 15 day exposure of ZnO and Ag NPs on the *L. rohita* fish

Treatments	**Control**	**ZnONPs**			**Ag-NPs**	
		T1	**T2**	**T3**	**T4**	**T5**
GLOBULIN	2.3±0.16 [a]	2.26±0.01 [a]	1.31±0.02 [b]	1.02±0.01c	1.30±0.01[b]	1.02±0.01[c]
ALBUMIN	2.10±0.02 [a]	1.71±0.01 [b]	0.80±0.02 [c]	0.60±0.03 [d]	0.80±0.01[b]	0.61±0.02[c]
BILIRUBIN	0.72±0.03 [a]	0.55±0.02 [b]	0.44±0.01 [c]	0.39±0.01 [c]	0.43±0.02[b c]	0.39±0.01[c]