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Fabrication and Characterization of Plant Mediated Green Zinc Nanoparticles for Antileishmanial Properties

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***Abstract*: *Infectious diseases outbreak caused by different pathogenic bacteria and the emergence of antibiotic resistance microbial raise new concerns. This situation triggered pharmaceutical companies and researchers to seek for alternatives antimicrobial agents. The discovery of green nanoparticle in pharmaceutical area not only proven to have a strong efficacy as antimicrobial but also promote an eco-friendly compound where the production does not produce hazardous chemicals and waste. In the present study, metallic nanoparticles (Zn) were synthesized using plants extract from Viola yedoensis and Curcuma longa species. The green nanoparticles were successfully synthesized by incorporating plant extracts with the zinc salt with different molar concentrations. The mixture were prepared at different pH, zinc salt (ZnSO4) concentrations and interval times of incubation. The result showed 15 mM concentration of ZnSO4 at pH 7 and incubation at the room temperature for 24 hours were the best conditions for green nanoparticles production. The color intensity changes during the reduction reaction of ZnSO4 by plant extract to form zinc nanoparticles (ZnNPs) was measure using UV spectrophotometer. ZnNPs with the highest color intensity (viola-358nm; turmeric-375nm) were further characterized to determine the elements and morphology of the synthesized ZnNPs using FTIR, XRD and SEM. FTIR analysis demonstrated the presence of aromatic rings and strong bond of O-H and C-H indicating the presence of alcohol and alkene in turmeric ZnNPs. While for viola ZnNPs, the presence of aromatic ring and strong bond of amine showed the existence of alcohol and alkene. The average size of turmeric and viola ZnNPs measured by XRD and SEM were 24-28 nm with spherical shape and 42–60 nm with hexagonal shape, respectively. The biological activity of both ZnNPs were validated by conducting antimicrobial tests towards Leishmanial tropical in which ZnNPs have strong antileishmanial activities.*

Index Terms*: *Antileishmanial, Curcuma longa, cytotoxic assay, Green nanobiotechnology, Viola L.

I. INTRODUCTION

Metal nanoparticles synthesized by plants extracts (green synthesis) is growing into an important innovative form of biotechnology, especially in the enhancement of nutritive, absorptive and treatment power of plant compounds. This technology is biologically and environmentally safe; and easier than synthesis of metallic nanoparticles via chemical and physical ways. Previously, chemical and physical methods were applied in the process of synthesizing the metallic nanoparticles. However, those methods were energy exhaustive, capital intensive and involve toxic chemicals and non-polar solvent. The accumulative by-products from metallic nanoparticles manufacturing may cause various biological risk and environmentally instability [1]. Apart from the chemical and physical methods, there is also a biological method to produce metallic nanoparticle which is more environmentally acceptable, non-toxic and clean development. The production of metallic nanoparticle involve the use Prokaryote (bacteria, fungi and virus) and recently, Eukaryote (plant, algae, human cell) which only require energy from sunlight. The problem with this method is the elaborate of maintaining the cell culture and a slow synthesis rate [2]. Growing attention towards biosynthesis using plant extract satisfy the need for the development of a clean, reliable, biocompatible and eco-friendly process to synthesis nanoparticles. According to Zia-ur-Rehman et al [3] this method is comparatively more profitable than other organisms (microbes) for the synthesis of metallic nanoparticles since they are not required to be maintained in cell cultures and could be easily scale-up for beneficial products. In addition, nanoparticles synthesized from curative metallic salts with medicinal plant extracts as a reducing agent could have high therapeutic potentials, so it is important to investigate their effects on living cells, against pathogens and plant physiology. The basic mechanism in the synthesis of nanoparticle by plant extract is the reduction process that is mediated by some reducing agent. According to [4] phytochemicals like primary and secondary metabolism (antioxidant, flavonoid, polyphenol, carotenoid) are responsible for the reduction of metal oxide to metal nanoparticles. Nanoparticle production using plant extract has a special advantage that the plants are widely distributed, easily available much safer to handle. The use of metal nanoparticles has proved to be feasible for not only prevention but also treatment of infectious diseases. Instead, the metal nanoparticles also have the ability to withstand harsh process conditions and generally classified as safe materials for human being and animals. In particular, zinc oxide exhibits high catalytic and adsorption ability.

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Zinc oxide is usually used in astringent preparations for the relief of such minor skin irritations as those resulting from superficial cuts, allergies, insect bites, or fungal infections such as athlete's foot. Astringents in medicine cause constriction or contraction of mucous membranes or exposed tissues and are often used internally to check discharge of blood serum or mucous secretions EFSA [5]. There have been few reports on the biosynthesis of zinc oxide nanoparticles using plants such as Aloe Vera [6], Cinnamomumcamphora [7] and alfalfa [8]. Plant extracts have shown effective actions toward human health, with new bioactive compounds being extracted and screened every year. Turmeric (Curcuma longa) is an important medicinal plant that has prominent bioactive metabolites called curcuminoids, for instance curcumin (diferuloylmethane), bisdemethoxycurcumin and dimethoxy curcumin [9]. Previous work has shown that extracts from turmeric exhibited antifungal and antibacterial properties [10]. Other biological properties include beneficial effects against kidney and cardiovascular diseases, arthritis, cancer, and irritable bowel disease [11], Alzheimer's disease, [12] and diabetes [13]. Meanwhile, viola (*Viola yedoensis* (Makino)) is a medicinal plant with small violet flowers mostly found and distributed in the oriental area, especially in China. The whole dried plant (including the roots) is an important constituent of the traditional drug called “Zi Hua Di Ding”. The whole dried plant is boiled and consumed as a tea to treat toxic heat, swelling, sores, boils, snake bites, carbuncles, bronchitis, hepatitis, acute nephritis, appendicitis, and enteritis. Extracts from *V. yedoensis* have also been proven to inhibit the growth of human immunodeficiency virus (HIV), cancer and certain bacteria [14]. In this study, turmeric and viola were used to produce zinc nanoparticles (ZnNPs). The potential effects of the zinc nanoparticles against Leishmanial activities were then investigated. Leishmaniasis; a tropical disease commonly associated with poor socio-economic nations; is a disease caused by protozoan parasite Leishmania and is transmitted to humans through the bite of infected plebotomine sandflies [15]. To date, work in ZnNPs as antileishmanial agents is still very scarce [16],[17].

II. EXPERIMENTAL

A. *Material*

Viola and Turmeric plant powder were obtained from the local market in Islamabad, Pakistan. Zinc sulfate salt was purchased from Merck Millipore, USA. All samples were prepared using fresh double distilled water throughout the study.

B. *Plant Extraction*

Turmeric and viola plants were used as the capping and reducing agents for synthesizing green zinc nanoparticles. Plant powder were weighted for 1 g of each plant and were washed twice with tap water and twice in distilled water. The powder was then soaked for 24- 48 hours in 50 ml of distilled water. After the prescribed time, plant extracts were placed in a flask and heated on a hot plate at 60℃ with continuous stirring. The extracts were then filtered through Whatman filter paper (90 mm pore size) and centrifuged at 10,000 rpm for 10 min to obtain cell-free leaf extract. The fresh extract (supernatant) was collected in 250 mL Erlenmeyer flask and stored at 4℃ for further use within a week.

C. *Nanoparticles Synthesis*

Zinc sulfate solution was prepared with different concentrations (2.5 mM to 15 mM) by dissolving zinc sulfate in distilled water with different pH ranging from (pH 4 to pH 9). The freshly prepared plant extract was slowly added drop wisely into the salt solution on a magnetic stirrer at 80 °C for a certain period tested from 10 min to 24 hours to obtain a complex formation [18]. The formation of nanoparticles is indicated by a color change of solution mixture and color intensity. The complex formed after stirring was collected by centrifugation at 10 000 rpm for 10 min. Then, the complex was rinsed with water and then subjected to another centrifugation step at 5000rpm for 10 min. The separated complex was dried in an oven at 40 °C for 8 hours [19]. Table 1 lists the range of parameters tested to obtain optimized conditions for ZnNPs production.

Table 1 List of parameters used in the synthesis of green nanoparticles.

Zinc sulfate concentration (mM)	Time	pH
2.5	10 min	4
1	15 min	5
5	25 min	6
7.5	40 min	7
10	60 min	8
15	24 Hour	9

D. *Characterization of ZnNPs*

The synthesized ZnNPs were characterized using different techniques including UV-visible spectroscopy, XRD (x-ray diffraction), FTIR (Fourier Transform infra-red) and SEM (Scanning electron microscope). An amount of 1 ml of colloidal sample solution was dissolved in distilled water and its colour intensity was measured using UV spectroscopy at the wavelength between 200 – 700 nm. FTIR spectroscopy was performed in the region of 400–4000 cm^{-1} units spectral range using Perkin-Elmer 1425X FTIR spectrometer with KBr pellet technique for detection of surface functional groups. About 5 mg of samples was dried and mixed with 245 mg of potassium bromide (KBr) to form pellets prior to FTIR analysis. Next, the crystalline structure of the synthesized ZnNPs was investigated by using X-ray diffraction, for which the samples were washed, dried and then a thin film of nanoparticles was formed by evaporation. A small amount of colloidal solution was placed on a glass slide for XRD analysis at the wavelength of 1.5406 A˚ for 2 hours at the temperature between 20 to 80 ℃ in continuous scanning mode. The SEM analysis of synthesized nanoparticles was measured using JEM 3145 LV (JOEL, Germany) equipment [20]. Samples were prepared by dissolving 2 mg of synthesized ZnNPs in 100 µl of tetryhydrofuran. The mixture was vortexed for 100 minutes and sonicated for 30 minutes at the room temperature. A drop of sample was then placed on small piece of glass slide on the rotating machine and was dried for SEM analysis.

E. *Anti-Promastigote Assay*

Leishmania tropica (L. tropica) KWH23 was incubated at 23℃ in M199 media (pH 7.2) supplemented with sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS) and 25 mM HEPES Buffer (4-(2-hydroxyethyl)-1-piper-as-neethanesulfonic acid). The concentration of seeding cells was fixed at 1×10^7 cells/ml. The culture was incubated at 22-25 ℃ for promastigotes growth and proliferation.

A stock solution of biologically synthesized ZnNPs of voila and turmeric was prepared by suspending 1 mg/mL nanoparticles in distilled water. The mixture was vortexed for 1-2 min for complete dispersion of aggregates, and subjected to sonication (Ultrasonic Cleaner, DSA100-XN) for 10 min. M199 Medium containing culture promastigotes were suspended to yield 1×10^6 cell/ml in each well of 96 wells plate. The synthesized nanoparticles were serially added into each well. The final volume was attuned to 1000 µl with M199 media. Cells were then exposed to a light-emitting diode (LED) at 25 ℃ for 10 min followed by incubation in the dark at 25-27 ℃. Control leishmanial culture was not treated with nanoparticles solution or any irradiation. The viability of promastigotes was analysed using the Neubauer chamber (MARIENFELD, Germany) under a microscope (micros, AUSTRIA, MC700). All reactions were performed in triplicates. The reactions were conducted from mid of November to end of December; when the external temperature was between 23 - 26 ℃, which is favourable for the growth of *L. Tropica* [21].

F. *Cytotoxicity Study*

Haemolysis assay was conducted to evaluate the cytotoxicity of synthesized nanoparticles on human blood. Fresh human blood was collected from healthy volunteer (O, A+ and B+ group) in vacutainer. Blood was centrifuged at 3000 rpm for 3 minutes to separate the erythrocytes, afterward washed with phosphate buffer for 2-3 times. Erythrocytes solution was diluted 10:90 with phosphate buffer solution (PBS). 100µl of the dilution was added to each Eppendorf tubes and treated with serial solutions of nanoparticles and their pure extracts. Negative control for the experiment was the red blood cells suspended in phosphate buffer, without treated with nanoparticles. As for positive control, red blood cells were lysed with 0.1% triton X-100. Reaction was performed in photo-irradiated with LED for 10 minutes and then samples were incubated in the dark at 37oC for 3 hours. Then, samples were centrifuged at 6000rpm for 10 minutes to separate the hemoglobin. Finally, absorbance of hemoglobin was measured at 576nm by UV-visible spectrophotometry. These experiments were performed in triplicates. Percentage hemolysis was calculated by using Eq. 1.

$$Haemolysis = \frac{OD\ at\ 576nm\ in\ nanoparticles\ solution - OD\ at\ 576nm\ ub\ PBS}{OD\ at\ 576nm\ in\ 0.1\%\ Triton\ X\text{-}100 - OD\ at\ 576nm\ in\ PBS} \times 100 \quad (1)$$

All the experiments were performed in triplicates and results are presented as mean ± standard deviation (SD). For the evaluation of significant differences, the Student's t-test was done by using SPSS software and P-values < 0.05 were measured as statistically significant difference.

III. RESULTS AND DISCUSSION

A. *Detection of ZnNPs Formation*

The visual colour change observed while mixing the zinc sulfate and plant extract solution is a preliminary detection for ZnNPs formation. During nanoparticles synthesis, the color changed from light brownish yellow to dark brown for viola extract (Fig. 1) and bright yellow to brown for turmeric extract (Fig. 2); indicating reduction of Zn SO4. The best response was obtained at the concentration of 15mM ZnSO4 at pH 7 solution mixture and 60 min mixing time. The absorbance peaks for turmeric and viola green zinc nanoparticles was found to be at 375 nm and 358 nm respectively. This observation was similar to previous reports [22],[23]. While, their crude extract was observed to be at the peaks of 420 nm and 541 nm respectively (Fig. 3).

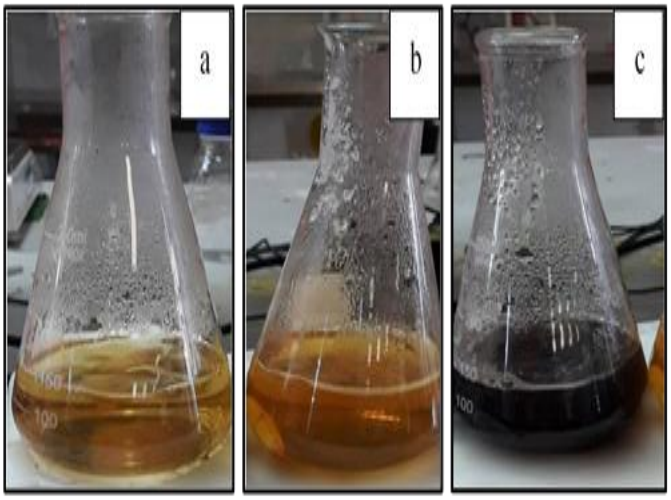

Fig. 1 Change in colour intensity of ZnNPs formation using Viola extract; (a) light brownish yellow, (b) brownish and (c) dark brown.

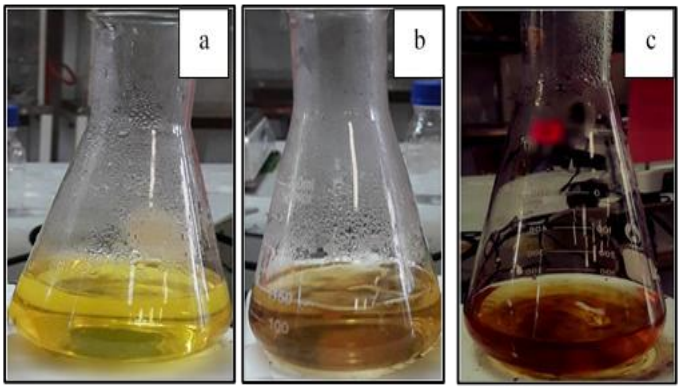

Fig. 2 Change in colour intensity of ZnNPs formation using turmeric extract; (a) light yellow, (b) Brownish yellow and (c) brown.

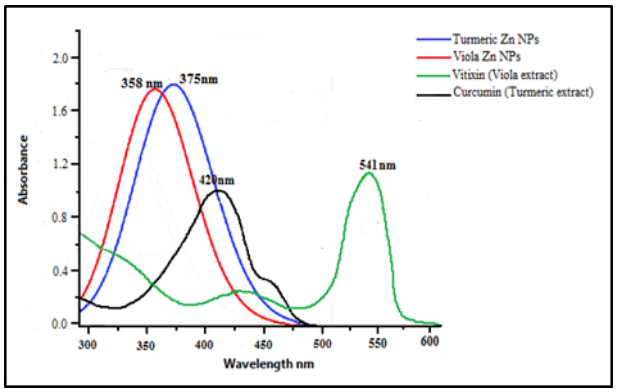

Fig. 3 Absorbance peak of crude extract of Viola and Turmeric was compared with ZnNPs for turmeric and viola using a spectrophotometer.

B. *FTIR*

FTIR spectra give an impression on the vibrational and rotational modes of the proposition of a molecule, and hence a consistent technique for characterization and identification of the substances. Molecular vibrations and sensation give rise to IR bands only if they make a change in the dipole moment of the molecule in relation to the quantum mechanics for energy and its molecular absorption. The absorbance variations are due to the variation of the spectral profile of the two samples. Turmeric extract (Fig. 4) showed absorption bands at 1880, 3446 cm-1 with ketone C=O and C=N amine stretch respectively, while for the green nanoparticles, the stretches could be seen at 3300- 3650 cm-1 indicating O-H stretching vibrations of aromatic rings and strong bond for the alcoholic group which confirms the presence of nanoparticles. The change between extract and its nanoparticles could also be observed at 3000 - 3000 cm-1 where nanoparticles showed stretches for alkanes C-H with medium bonding.

Whereas, for viola extract, a small band at 1200 - 1300 cm-1 representing C-O stretching vibrations of carboxylic acid was observed. Peaks at 1550 and 1650 cm-1 indicate polyols (phenolic acid and flavonoids), terpenoids, and protein compounds, which are abundant in viola extract. Meanwhile, the nanoparticles showed clear increased peak intensity at 1550 and 1650 cm-1 for C-N stretching vibrations of aromatic rings, strong bond for amines and a clear band for C-O indicating strong acyl group. Stretch between 3000 - 3001 cm-1 indicated the presence of aromatic rings and strong bond for hydroxyl group O-H and C-H which confirms the presence of alcohol and alkanes in the synthesized ZnNPs while being absent in the extract solution. FTIR spectra of ZnNPs showed the absorption band at 3739 cm-1 ,3429 cm-1, 2490 cm-1, 1637 cm-1, 1252 cm-1, 1056 cm-1, 675 cm-1 and 456 cm-1, respectively. The peaks in the region between 600 and 400 cm-1 are allotted to M–O(Zn-O). The band at 457 cm-1 confirms stretching vibrations of ZnNPs.

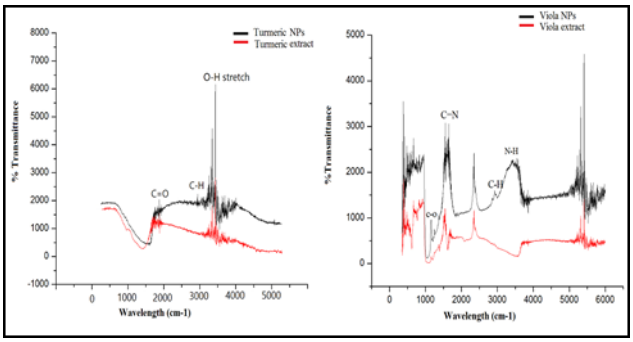

Fig. 4 FTIR spectrum for turmeric and viola crude extracts and their ZnNPs.

C. *X-ray Diffraction (XRD)*

The crystalline size and structural properties of the zinc nanoparticles were examined using Powder X-ray diffraction. The XRD was carried out with Cu Ka radiation (k = 0.1540 nm), and 2Theta ranges from 200 to 800. The XRD pattern of bio-synthesized zinc nanoparticles from viola and turmeric are shown in Fig. 5. The peak positions which indexed the planes 100, 002, 110, 102, 110,103, 112 and 201 with for 2h values of 31.71º, 34.38º, 36.21º, 45.21º, 47.48º, 56.51º, 62.80º, 67.88º and on 69.01º respectively. All peaks have confirmed the Zinc NPs holds hexagonal shapes for viola NPs while spherical for turmeric (wurtzite structure) by comparison with JCPDS card No.89-7102. Same results were found by Elumalai E. K. et al., [24]. The sharp and narrow diffraction peaks indicate that both products are well-crystallized. The mean crystalline size (D) of the particles was determined from the XRD line broadening measurement using Eq. (2).

$$D = 0.89\lambda / (\beta \cos\theta) \quad (2)$$

Where,

λ = The wavelength (Cu Kα) radiation; β = The full-width half-maximum (FWHM) of the ZnNPs (101) line or respective diffraction peak; θ = The diffraction angle.

The calculated crystallite size for the powder particles of ZnNPs from viola is about 60- 42 nm and for ZnNPs from turmeric is 24-28 nm. The diffraction peaks of ZnNPs from turmeric is at 2θ = 36.19° with corresponding to lattice plane (101). The results with the same line were also reported by others researcher such as [25] the images clearly showed the presence of secondary metabolites capping which is assigned to bio-organic compounds present in the plant extracts which are confirmed by the sharp reflections in the XRD spectrums of both samples. The XRD pattern of bio-synthesized NPs shows that all the peaks have confirmed ZnNPs from viola held hexagonal shape and ZnNPs from turmeric was in spherical shape (wurtzite structure) in comparison to JCPDS card no. 89-7102 [26].

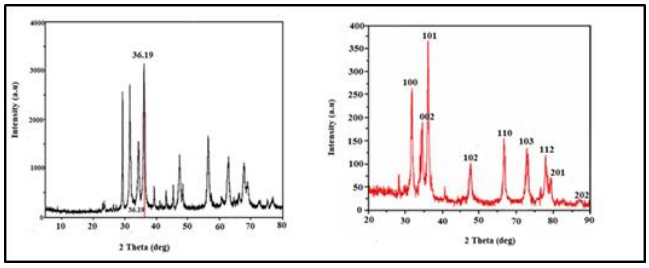

Fig. 5 XRD results for turmeric and viola nanoparticles.

D. *SEM Result*

The morphology (shape, size, and microstructure) of the ZnNPs was observed using scanning electron microscopy (SEM) Model No. JEM 3145 LV, JOEL, Germany. The SEM image of ZnNPs is shown in Fig. 6. It shows that the synthesized ZnNPs exhibited varieties of shapes such as spherical by ZnNPs from turmeric and hexagonal by ZnNPs from the viola. The synthesized ZnO nanoparticles were agglomerated with a particle size ranging from below 60- 42 nm for ZnNPs from viola and 24-28 nm for ZnNPs from turmeric. Besides, the morphology of the nanoparticles were generally in random and not uniform, the spherical and hexagonal (wurtzite) shapes are in agreement with the result obtained from XRD examination pattern of nanoparticles and to those results codded in the previous literature [27]. The hexagonal shape of nanoparticle formed with

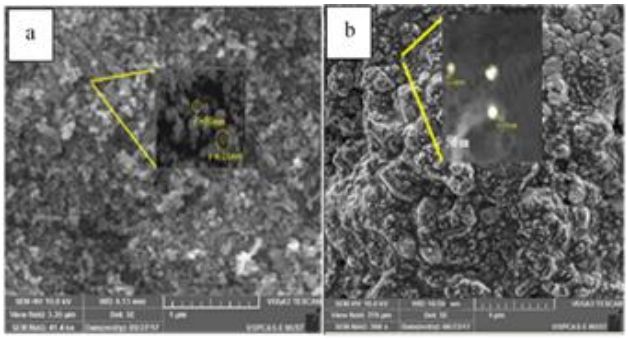

Fig. 6 SEM images of a. viola ZnNPs with size range between 48-60nm and b. Turmeric ZnNPs with the size range between 24-28 nm

r = 30nm and some with r = 21 nm and thus having a diameter range from 42-60 nm (Fig. 6). While the spherical ZnNPs showing r = 12nm and r = 21nm with diameter 24- 28 nm (Fig. 6). The images also show the presence of zinc nanoparticles agglomeration with high surface energy that mostly occurs when synthesis was carried out.

E. *Antileishmanial Activity*

The efficacy of ZnNPs against Leishmania infection was analyzed on the strain of Ltropica KWH23. Viable cells were counted microscopically using Neubauer chamber at 40X. The viability of promastigotes cells was checked in both control and test groups.

Based on Fig. 7, ZnNPs viola and Zn NPs turmeric maximum suppressive effect on parasites was observed at high concentration of 10 µg/µl suggesting that the both ZnNPs can be considered as suitable candidates for leishmaniasis treatment. However, viola and turmeric extracts in their pure forms showed better effect compared to their nanoparticles. The ceased effects in the nanoparticle form could be due to capping of the effective secondary metabolites moieties.

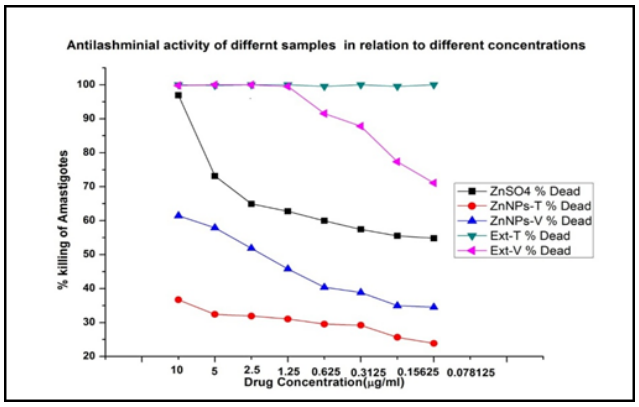

Fig. 7 Antileishmanial activity of NPs against Leishmania tropica (KWH23L).

F. *Hemolysis (Cytotoxicity) Analysis*

In the present investigation, the results shows that the least % death of the erythrocytes, was observed for ZnNPs from viola which has about 30% haemolysis values even at its highest concentrations of 500 µg/µl. ZnNPs from turmeric shows haemolysis percentage of 70% at its highest concentrations 500 µg/ µl. Whereas, Zinc salt shows maximum haemolysis (60%) at the very low concentration (50 µg/µl). Overall observation shows that the synthesized green NPs are safer at their specific concentrations as compared to their pure extracts. Taken together, at certain doses, the green synthesized ZnNPs were observed to be effectively killing the pathogens but is safe for human use.

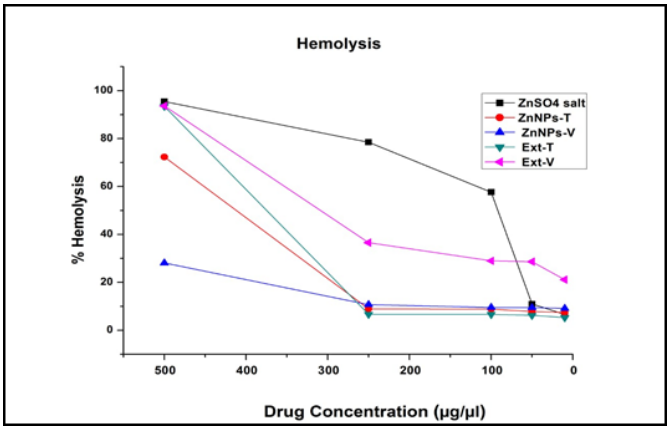

Fig. 7 Cytotoxicity (haemolysis assay) of ZnNPs.

IV. CONCLUSION

The present study reports two types of green and safe Zinc nanoparticles with antileishmanial activity. UV and FTIR confirmed the nanoparticles while SEM and XRD analysis showed the morphology as spherical with a particle size of 24-28nm for turmeric and hexagonal with 42-60 nm particle size for viola green nanoparticles.

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AUTHORS PROFILE

Safia Gul graduated in pure Science from University of Baluchistan in 2001 and continued her master's from the same institution in Plant Sciences and Molecular Biology in 2003 and got honored by taking Gold medal in whole province. She was offered lecturer ship in the newly opened and a very first women's university of the province SBKW (Sardar Bahadur Khan women's University Quetta). She joined the university and taught at master level and conducted many research projects on water quality and environmental problems. She obtained her M.Phil. in plant and agriculture biotechnology and genetics, from UOB, CASVAB (Centre for Advanced Studies Vaccinology and Biotechnology) in 2014 and started serving as Assistant Professor. At the same year she joined the IIUI (International Islamic University Islamabad) for her Ph.D. in nanobiotechnology, and joined IIUM (international Islamic University Malaysia) for her research on cancer cell lines. She is an active researcher and potential teacher with special interest on medicinal plants, biotechnology engineering and ecology at SBKWU and she is member of Alumni committee. She has published more than 15 papers in National and International Journals. She is young scientist aiming for integration of impressive research facilities being a pioneer in developing of new technologies and advancement of older ones to benefit humanity.

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