EXPERIMENTAL ARTICLES

UDC 539:616.34 https://doi.org/10.15407/biotech12.03.024

TOXICITY OF SILVER NANOPARTICLES LOADED WITH *Pleurotus tuber-regium* EXTRACT ON RATS

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Received 16.01.2019
Revised 07.03.2019
Accepted 05.07.2019

The aim of the work was to provide synthesis and characterization of silver nanoparticles loaded with *P. tuber-regium* extract and to assess their acute toxicity and haemotoxicity activity on rats. Acute toxicity of silver nanoparticles was studied by up-and-down-procedure and haemotoxicity was analyzed by differential red bload cell (RBC) count. UV-visible spectroscopy analysis provided peak at 463.9 nm. Scanning electron microscopy analysis showed nanoparticles of 60.8 nm to 94.9 nm in size and spherical in shape. X-ray diffraction analysis showed 125.7 nm average sizes of the silver nanoparticles. Dynamic light scattering analysis provided the average diameter 71.4 nm and zeta potential of −11.2 mV of the synthesized nanoparticles. Fourier transform infrared spectroscopy analysis showed major transmission peaks at 3295.6 cm^{-1} corresponds to O-H stretch for alcohol and phenol 1602.2 cm^{-1} corresponds to N-H and C=C stretch for primary amine and conjugated alkene. Acute toxicity test showed no mortality, gross behavioural changes and decreased body weight. Significant increase in total RBC ($4.30 \pm 0.05 \times 10^6$/μL), phenotypic coefficient of variation paked cell volume (PCV: 26.46 ± 0.01%) and total white blood cell WBC ($8.12 \pm 0.27 \times 10^3$/μL) was observed in 400 mg/kg extract dose treatment group compared to control group. Thus, synthesized silver nanoparticles loaded with *P. tuber-regium* aquous extract had no acute toxic, haemotoxic effect but they showed dose dependent haematinic and immune modulation activity.

Key words: nanoparticles, health, mushroom.

In recent decade along with the globalization entire human population have been facing major problems such as climate change, poverty, invasion and increase rate of diseases, disorders and drug resistant pathogens, infection, etc. [1, 2]. Therefore, greater attention and effort has been paid to develop new therapeutics having least side effect with high efficacy to fight against challenges related to heath issues and sustainable development [3]. Crude or isolated substances of medicinal plants and fungi origin are getting more importance and wide range of recent research interest for the development of new drugs, but delivery and efficacy of synthetic and many herbal drugs is often limited because of site or target specific action of therapeutic molecules and they require few modifications such as changing the molecular structure of the drug or their proper distribution by incorporation in carrier system etc.[4, 5].

Recently nanotechnology has been extensively explored as a broad area in the field of modern pharmacology and medicine. Nanotechnology concerns the size of matters in the range between 1nm to 100 nm of drug, natural or synthetic polymer loaded

material act as carrier which have unique physicochemical properties such as ultra-small size, large surface to volume ratio, high reactivity and unique interactions with structural components such as core, emulsion to works as carrier and functional groups includes the therapeutic molecules and ligands for target location of biological systems, which have been significantly incorporated in pharmacy and medical research for the enhancement of pharmacological efficacy of drugs [6, 7].

Modern trends of application of nanotechnology in drug delivery system have been mainly focusing on synthesis of spherical metal nanoparticles within nano range. Silver nanoparticles (SNPs) have been broadly used due to its most important features such as easily synthesis and modification through biological root that would allow them to bind with ligands, antibodies, drugs and easily delivery [8, 9]. However, due to very tiny size silver nanoparticles, they may penetrate the basic biological membranes of normal cells, disrupting their normal functions and produce toxicity such as tissue inflammation, increased production of reactive oxygen species leads to cellular damage and death [10] and as well they may accumulate in specific organs such as such as the liver, kidney, spleen, myeloid tissue etc. and affect their normal function [11].

Mushrooms have been used as food supplement from times immemorial and have also been used for their medicinal properties as evident from ancient literature. Mushrooms belonging to *Pleurotus* sp. have been used as nutritional food and medicinal supplements during the past decades [12]. *Pleurotus tuber-regium* (*P. tuber-regium*) is a common edible gilled mushroom belonging to family Pleurotaceae and this mushroom has been using for treatment of pathogenic infection, renal and hepatic diseases, cancer, diabetes and other diseases [13]. It has been reported that mushroom *P. tuber-regium* contains various bioactive chemicals (mycochemicals) such as polysaccharides, lipopolysaccharides, proteins, peptides, glycoproteins, nucleosides, triterpenoids, lectins, lipids, etc. and their derivatives [14].

Several pharmacological analyses of *P. tuber-regium* extract have been done but application of this mushroom for the synthesis of nanoparticles and assessment of toxicological impact of synthesized silver nanoparticles using *P. tuber-regium* extract on rat model has not been explored yet. Therefore, present study was under taken for the synthesis of SNPs mediated by *P. tuber-regium* extract and to accesses the toxicological impact of synthesized SNPs on haematological parameters of rat because toxicity study may be explored further safe use of fungus mediated SNPS in the field of medicine and pharmacology.

Materials and Methods

Collection of P. tuber-regium

Fresh fruiting bodies of *P. tuber-regium* were collected by the corresponding author from Manas National park of Assam (26° 39′ 33.9264″ N and 91° 0′ 4.0644″ E). The collected fruiting bodies of *P. tuber-regium* were match and identified on the basis of morphology with museum specimen of *P. tuber-regium* by Plant Identification & Preservation Division of Department of Botany, Gauhati University, Assam where a voucher specimen (No. 832M) was deposited and rest of the fruiting bodies were brought to the Department of Zoology, Ranchi University, Ranchi, for experimental work.

Preparation of extract

Fresh mushrooms were washed and sterilized by treating with ethanol and washed again by distilled water. The mushrooms were dried in shade under room temperature for six to seven days, powdered and sieved. 50 g fine powder (50 g) of *P. tuber-regium* was subjected to Soxhlet extraction chamber and 300 ml of distilled water was used as solvent for aqueous extraction. The obtained extract was filtered, concentrated and dried in a rotary flash evaporator maintained at 45 °C.

Mycochemical screening

Qualitative mycochemical screening of the extract obtained from fruiting bodies of *P. tuber-regium* was done following protocol described by Arya et al. [15].

Test for carbohydrates

Presence of carbohydrate was determined by addition of few drops of Molisch's reagent to the test solutions (1 mg/ml extract), this was then followed by addition of 1 ml concentrated H_2SO_4 (98%) by the side of the test tube. The mixture was then allowed to stand for two minutes and then diluted with 5 ml of distilled water.

Test for glycoside

Glycoside was determined by addition of 1 mg/ml of extract to 3 ml of anthrone reagent and was mixed properly.

Test for proteins

Protein was estimated by addition of 0.5 mg/ml of the extract and 2 ml of Bradford's reagent were left for few minutes.

Test for alkaloid

Presence of alkaloid was determined by stirring of 1 mg/ml extract with 5 ml of 1% HCl on hot water bath and then filtered. 1 ml of the filtrate was taken individually into 2 test tubes and few drops of Dragendorff's reagent were added into the test tube.

Test for steroid

Presence of steroid was determined by addition of 2 ml conccentrated H_2SO_4 (98%) with 2 mg/ml of extracts was mixed vigorously.

Test for triterpene

Triterpene was estimated by addition of 1mg/ml extract with one drop chloroform and concentrated H_2SO_4 (98%).

Test for phenol

Presence of phenol was estimated by phenolic-catechol method. Dilute aqueous extract (0.5 ml of 1:10 g/l) was pipette out in series of test tubes and volume was made up to 3 ml with distilled water. Folin-Ciocalteau reagent (0.5 ml) was added to each tube and incubated for 3 min. at room temperature and then sodium carbonate (20%; 2 ml) solution was added, mixed thoroughly and the tubes were incubated for 1 minute in boiling water bath.

Test for flavonoid

Flavonoid was estimated by dissolved 1mg ml extracts in water and later addition of 2 ml of the 10% aqueous sodium hydroxide and then addition of dilute hydrochloric acid as an indicator.

Test for tannin

Tannin was estimated by stirring 0.5 mg/ml of the extracts with 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate.

Test for saponin

Saponin was determined by heating 1 mg/ml extracts with alcoholic KOH and boiled for 1 min and cooled, and then the mixture was acidified with 1ml of concentrate HCl. Later few drops of 5% NaOH added drop wise.

Synthesis of SNPs (silver nanoparticles)

The synthesis of SNPs was done following slight modification of previous method of Kumar and Sinha [16]. 3ml (41 mg/ml) of *P. tuber-regium* aqueous extract was mixed with 197 ml of 0.1 M silver nitrate (169.87 g/mol) solution (i.e., 3.35 g $AgNO_3$/197 ml of distilled water) and incubated at 80 °C for 2hours using hot plate. During incubation the mixed solution was stirred continuously using magnetic stirrer bar. After transformation of pale yellow colour of mixed solution into dark brown colour, the mixed solution was cooled to room temperature and centrifuged at 15 000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with distilled water and obtained amount of synthesized SNPs was calculated in mg. Small amount of SNPs sample was kept in wet condition and rest of the SNPs sample was dried in the incubator at room temperature to get SNPs powder for characterization.

Characterization of silver nanoparticles

Synthesized SNPs powder and wet sample were characterized by Ultra Violet Visible (UV-visible) spectroscopy, scanning electron microscopy (SEM), X-Ray diffraction (XRD) analysis, dynamic light scattering (DLS) analysis and furior transform infrared (FTIR) spectroscopy [16].

UV-Visible spectra analysis

UV-Visible spectra analysis of SNPs wet sample was done by dilute 1 mg/ml of pure SNPs sample in 4 ml of deionised water and 1 ml of diluted sample was taken in standard quartz cuvette and placed in sample compartment. UV-Visible spectra analysis was done by using Parkin Elmer Lambda-25 UV-Visible spectrophotometer (PerkinElmer Inc., USA). The UV-Visible spectrophotometer was operated at 240V, 20 ± 2 °C, 60–70% humidity and light test specification at 200–800 nm wave length [17].

Scanning electron microscopy (SEM) analysis of SNPs

SEM analysis of SNPs powder sample was done using JEOL JSM-6390 LV (Japan) machine provided with Vega TC software. Thin layer of nanoparticles powder sample (1mg) was prepared on glass slide and then press on a carbon taped copper grid for SEM. Excess powder on surface of carbon taped copper grid was blown away with compressed air and the SEM grid was allowed to dry by putting it under a mercury lamp for 5 min and was coated with platinum using ion sputter [18].

X-ray diffraction analysis (XRD) of SNPs

XRD analysis of SNPs powder sample was done using a Rigaku-smartlab powered diffraction XRD machine with 40kV operating voltage and 15mA current, Cu-Kα X-rays of wavelength (λ) = 1.54056 Å and data was taken for the 2θ range of 10° to 90° with a step of 0.02°. The particle size was calculated by considering the peak at degrees and by using Debye-Scherrer formula [19].

Where, 'K' is a dimensionless shape factor or Scherrer constant (0.9), 'λ' is wave length of X-Ray (0.1541 nm), 'β' is FWHM (full width at half maximum), 'θ' is the diffraction angle and 'D' is particle diameter size.

Dynamic light scattering analysis

1mg/ml wet SNPs sample was diluted, filtered and 0.1mg/ml concentration nanopaticle colloidal solution was taken and ultrasonicated at 20% sonication amplitude with continuous mode for 882 second. The sonication was done for proper dispersion of nanoparticles in the solution. The dynamic light scattering for particle size and zeta potential analysis of nanoparticles was carried out using Malvern Nano ZS green badge) ZEN3500 (U.K.) zetasizer provided with zetasizer Nano software [20].

Fourier transform infrared (FTIR) spectra analysis

FTIR spectra analysis of SNPs powder sample was carried out using IPRresting-21 (Shimadzu Corp., Kyoto, Japan) in the diffuse reflectance mode operated at aresolution of 4 cm^{-1} in the range of 400 cm^{-1} to 4 000 cm^{-1} wave number. KBr was used as standard to identify the potential biomolecules present in fruiting body of *P. tuber-regium* extract are responsible for reducing and capping the reduced silver. The FTIR machine was operated at 25±5 °C, 60–70% humidity and 240 V AC [21].

Animals

Wistar albino rats (*Ratus norvegicus*) of 175 to 200 g body weight (BW) were obtained from the National Institute of Nutrition, Hyderabad, India. The rats were maintained under standard laboratory conditions at ambient temperature of 25 ± 2 °C and relative humidity at 50 ± 15%, with dark-light cycle of 12 hours. Rats were fed with a commercial pallet diet (Sadguru Shri Shri Industries Pvt. Ltd. Pune, India) and provided water *ad libitum*. The experiment was performed after prior approval of the Ethics committee of Ranchi University, Ranchi (Proceeding no. 46, page no. 137).

Acute toxicity test

According to OECD (Organisation for Economic Co-operation and Development) test guideline 425 (Up and Down procedure) limited test for *P. tuber-regium* extract mediated SNPs was performed at the test dose 2 000 mg/kg on male albino rat. 5 rats were taken and were fasted (3–4 hours) prior to dosing but were provided with water *ad libitum*. Single dose (2000 mg/kg body weight) of SNPs was administrated by gavage using stomach tube to single rat and rats were provided with food and water *ad libitum* after 2 hours. Similarly 4 other rats were treated with same dose of SNPs [22, 23].

Experiment design for haemotoxic study

Effects of *P. tuber-regium* extract mediated SNPs on haematological parameters of rats was studied by feeding low dose (LD) and high dose (HD) of SNPs. Fifteen fresh rats were distributed equally among three treatment groups (Group: 1, 2 and 3). Experimental design was done as followed by method of Oghenesuvwe et al. [24].

Group 1: Rats of this group were considered as control and were not treated with SNPs and received 1 ml distilled water orally for 7 days.

Group 2: Rats of this group were received 200 mg/kg body weight of *P. tuber-regium* extract mediated SNPs orally for 7 days and this group was considered as LD treatment group.

Group 3: Rats of this group were received orally 400 mg/kg body weight of *P. tuber-regium* extract mediated SNPs orally for 7 days and this group was considered as HD treatment group.

Effect of SNPs on blood parameters

Haemotoxicity of *P. tuber-regium* extract mediated silver nanoparticles was studied on the basis of variations of different parameters such as total WBC (white blood corpuscles), total RBC (red blood coruscels), Hb (haemoglobin), PCV (packed cell volume), MCV (mean corpuscles volume), MCH (men corpuscles haemoglobin), MCHC (mean corpuscles haemoglobin concentration), PLT (platlet), LYM (lymphocyte), NEU (neutrophil), MON (monocyte), EOS (eosinophil) and BAS (basophil) of haematological indices. For analysis of haematological indices, blood samples were collected into sterile tubes containing EDTA and immediately analysed using Sysmex automated blood analyser — KX 21 Kobe, Japan.

Statistical analysis

Data were taken 5 times and results were expressed as a mean ± standard error of mean. Statistical analysis was performed using Student's *t*-test, $P < 0.05$ was considered as statistically significant. Entire statistical analyses were performed using full proof software WinSTAT.

Results and Discussion

The aqueous extract prepared from the *P. tuber-regium* is presented in Fig. 1. Result of mycochemical screening of aqueous extract of *P. tuber-regium* is presented in Table 1.

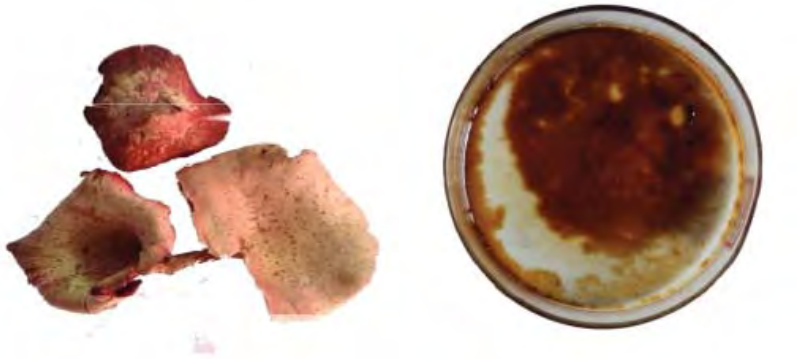

Fig. 1. **Fruiting bodies of *P. tuber-regium* and aqueous extract of *P. tuber-regium***

Table 1. **Screening of mycochemicls present in *P. tuber-regium* aqueous extract**

Mycochemical (*P. tuber-regium*)	Present / Absent in aqueous extract
Carbohydrate	+
Glycosides	+
Protein	+
Alkaloid	+
Steroid	+
Triterpene	+
Flavonoid	+
Tannin	+
Lipid	+
Saponin	+
Phenol	+

Note: "+" — present; "-" — absent.

*Fig. 2. **A*** **— $AgNO_3$ solution; ***B*** — **mixed $AgNO_3$ and extract; ***C*** — **colour transformation after 30 min; ***D*** — **colour transformation after 1 hour; ***E*** — **colour transformation after 2 hours**

Mycochemical screening of *P. tuber-regium* (Table 1) showed that the extract contains different mycochemicals such as carbohydrate, lipid, protein, tannin, saponin, tannin, flavonoid, phenol, etc. It has been reported that, preliminary biochemical screening of mushrooms provided the confirmation about the presence of biochemical such as saponins, tannins, glycosides, reducing sugar, alkaloid, flavonoid, terpenoid, etc. are associated with reduction of free radicals, possess therapeutic properties and reduces metallic ions [25, 26].

Initial confirmation about synthesis of silver nanoparticles mediated by *P. tuber-regium* extract is presented in Fig. 2. The result showed that initial pale yellow colour of mixed $AgNO_3$ and extract gradually transformed light brown to dark brown colour with progression of incubation time and temperature. Finally the mixed sample turned into unchanged dark brown colour which provided initial confirmation of synthesis of silver nanoparticles.

It has been reported, when extract of biological origin is mixed with silver nitrate solution and incubated along with increasing time period and temperature, then the silver ions are gradually change in to silver nanoparticles by the mycochemicals of extract and gradually the light colour of the mixed solution turns into dark brown colour [27, 28]. In the present experiment similar transformation of light yellow colour to dark brown of mixed solution correlates with the formation SNPs by the reduction of silver ions of previous studies.

UV-visible spectroscopy analysis is a most important primary technique used in nanotechnology for the confirmation of synthesis of silver nanoparticles. UV-visible spectrum analysis showed the peak at 463.88 nm (Fig. 3).

In nanotechnology UV- visible spectroscopy is used as primary technique to monitor the

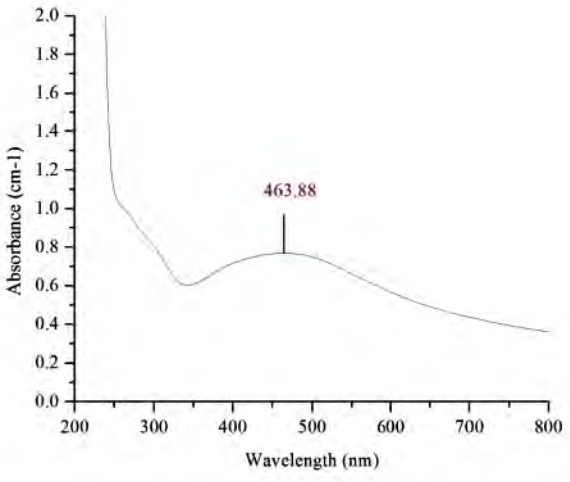

Fig. 3. **UV-visible spectrum of *P. tuber-regium* extract mediated silver nanoparticles**

formation and stability of nanoparticles with the help of the absorption spectrum [29]. UV-visible absorption spectroscopy, which showed peak at 463.88 nm corresponds to the surface plasmon resonance. Previous studies reported that formation and stability of silver nanoparticles mediated by extracts from biological sources such as plants and fungi shows maximum absorption 400–500 nm due to the surface plasmon resonance (SPR) for silver nanoparticles [30, 31]. In previous work variable UV-Visible spectra peaks were observed for synthesized silver nanoparticles from different mushroom extracts. Sujatha et al. reported the SPR for synthesized SNPs at 420 nm for *Ganoderma lucidum* and *Agaricus bisporus*, they also reported SPR for absorption spectra of *Plearotus florida* and *Pleurotus platypus* mediated silver nanoparticles at 435nm and 300 nm respectively [32]. In our study SRP at 463.88 nm of SNPs sample obtained from UV-visible spectra analysis and it provided confirmation of synthesis of SNPs mediated by *P. tuber-regium* extract.

Scanning electron microscopy analysis of SNPs sample provides the information about morphology and size in diameter of nanoparticles. Results of SEM analysis of *P. tuber-regium* extract mediated SNPs are presented in Fig. 4. The results showed synthesized silver nanoparticles were smooth and spherical shaped and were of 53.85 nm to 94.86 nm diameters. The SEM image also showed agglomeration of SNPs.

Scanning electron microscopy analysis of SNPs (Fig. 4) synthesized using *P. tuber-regium* extract were compared with previous SEM analysis of *Boswellia ovalifoliolata* extract mediated SNPs of spherical and 30–40 nm diameter and agglomeration among SNPs in dry state [33]. Result of SEM analysis of present study (i.e., large diameter of SNPs) can be correlated with previously SEM study of SNPs. XRD analysis is the most important characterization technique used in solid state chemistry and materials science studies. The information pertaining to phase formation, translational symmetry present and size and shape of the unit cell are obtained from different peak positions in the diffraction pattern of a *P. tuber-regium* extract mediated SNPs powder sample. The X-ray diffraction pattern of the *P. tuber-regium* extract mediated SNPs are presented in Table 2 and Fig. 5. The result shows various peaks but 4 major intensity peaks for particles were considered. By the use of XRD data and Scherrer formula SNPs of 105.78 nm to 142 nm with average particle size 125.73 nm were calculated.

X-ray diffraction pattern of a powder sample is supposed to be the fingerprint of that sample [34]. The information pertaining to phase formation, translational symmetry present and size and shape of the unit cell

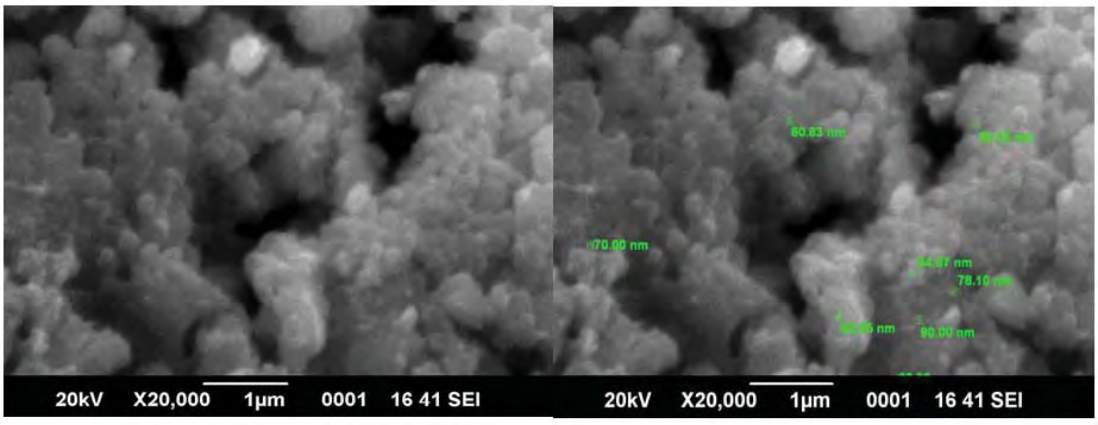

Fig. 4. **SEM images of *P. tuber-regium* extract mediated silver nanoparticles**

Table 2. **X-ray diffraction analysis for estimation of average particle size of powdered SNPs sample**

Copper K radiation: Wavelength λ (nm) = 0.154								
2θ of the major peaks (deg.)	θ of the peak s (deg.)	d-spacing (Å)	Intensity (cps)	FWHM of major peaks (β:deg.)	FWHM of the major peaks (β:rad.)	Size (Å)	Size (nm)	Avg. Size (nm)
35.45	17.72	2.52997	1951.02	0.0613	0.0010	1420.00	142.00	125.73
29.61	14.80	3.01432	1470.22	0.0677	0.0011	1266.7	126.67	
24.27	12.13	3.66428	853.31	0.066	0.0011	1284.9	128.49	
21.64	10.82	4.10279	735.88	0.0798	0.0013	1057.8	105.78	

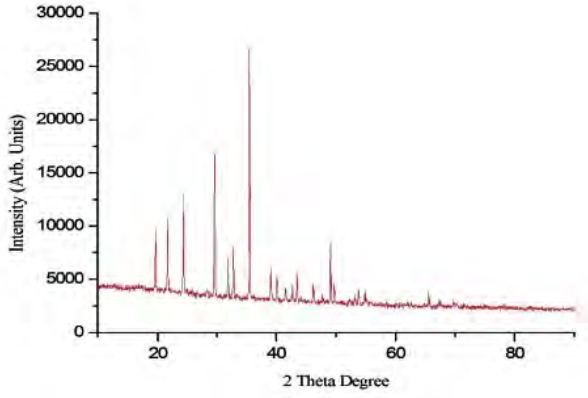

Fig. 5. **XRD peaks of particle size obtained from powdered sample of SNPs**

are obtained from peak positions of particles in the diffraction pattern of a sample. In present study average particle size 125.73 nm reflected four major peaks of silver particles with fcc type lattice and some additional unassigned peaks, which might be attributed to the formation of bio-organic phase acting as surfactant for the silver particles [19]. Mohanta et al. [35] synthesized nanoparticles mediated by from *Ganoderma sessiliforme* extract and they also analyzed the average size of nanoparticles were 45.26 nm using XRD. Previously it was also reported that nanoparticles of average 6nm were synthesized mediated by *Ganoderma lucidum* mycelia extract were analyzed by XRD [36]. Results of XRD analysis of present study cannot be correlated with the previous XRD analysis of SNPs powder samples and large particle size may be due to agglomeration of SNPs in dry sample and synthesized SNPs can only be called as particle because they had exceeded the diameter of 100 nm. Thus, further freshly prepared SNPs colloidal sample was analyzed by using DLS technique to know about proper dispersion, specific size of single particle in the sample and total number of particles within nanorange

Thus, further freshly prepared SNPs colloidal sample was analyzed by using DLS technique (Dynamic light scattering) to know about proper dispersion, stability, specific size of single particle in the sample and total number of particles present in the sample (in ml), that was taken for DLS analysis. Size and distribution of nanoparticles play a fundamental role in quality control for nanomaterial synthesis. It also basically associated diffusivity and passage of nanoparticles through cell membranes in the field of nanobiotechnology. In the present study size distributions by number, by intensity, by volume and zeta potential of synthesized *P. tuber-regium* extract mediated silver nanoparticles were analysed by DLS method. The results of DLS analysis are presented in Fig. 6–9. Cumulants mean (Z-average) of diameter of SNPs was 71.36 d. nm (diameter in nanometre). DLS analysis provided the average diameter of synthesized silver particles of average 58.71 d.nm were distributed 98% by intensity, particles of average 76.32 d.nm were distributed 93.6% by volume and particles of 19.85 d.nm were distributed 99.8% by number in 1 ml of synthesized nanoparticles colloidal sample suspension. Thus from the results obtained by DLS analysis it is better to say the synthesized particle as naoparticles. Which were previously called only particles according to the results of XRD analysis.

Dynamic light scattering (DLS) is also known as photon correlation spectroscopy (PCS) and has been widely used for analysis of nanoparticles size in liquid phase, particle

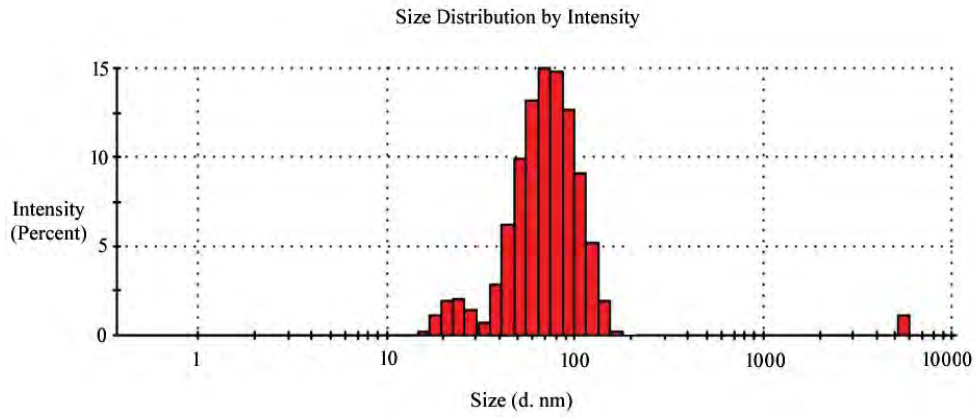

Fig. 6. **DLS size distribution by intensity of *P. tuber-regium* extract mediated SNPs**

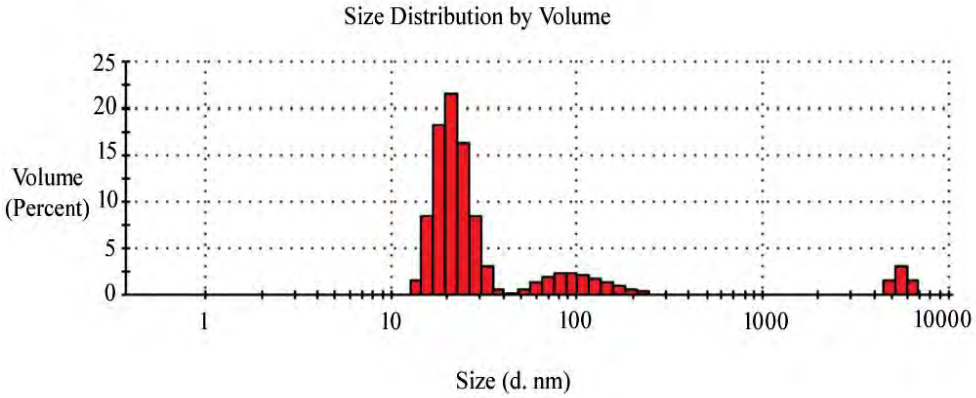

Fig. 7. **DLS size distribution by volume of *P. tuber-regium* extract mediated SNPs**

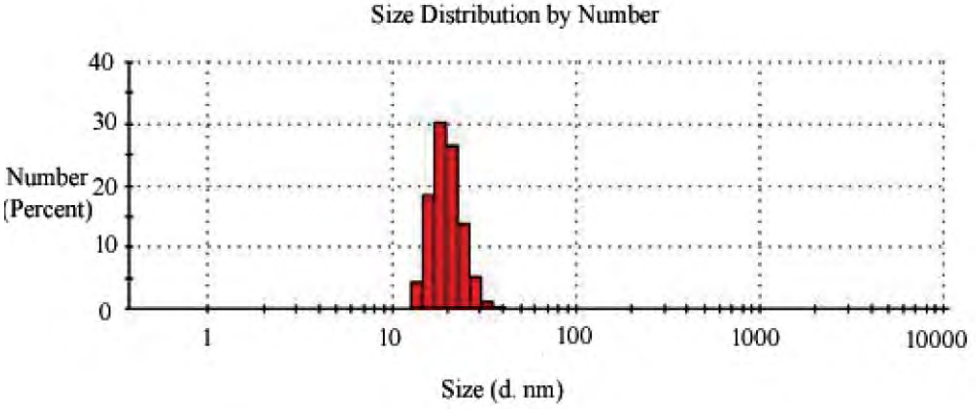

Fig. 8. **DLS size distribution by intensity of *P. tuber-regium* extract mediated SNPs**

shape, colloidal stability, and surface coating [37, 38]. The result of DLS analysis of nanoparticles distribution by intensity in the colloidal solution depends upon the rate of fluctuation of intensity of the laser beam by the particles of different size bean [39]. Fluctuation of intensity corresponds constant motion of particles is due to Brownian motion i.e. quick motion small particles and slow motion of large particles in liquid environment due to random collision among them and provides fundamental size of the nanoparticles [20]. Although, an intensity distribution is the fundamental size distribution generated by DLS which is further converted to a volume distribution and the volume distribution further converted to a number distribution by inbuilt software of nanozetasizer [20]. The

DLS size distribution by volume analysis of nanoparticles represents the total volume of particles of different size bins [39, 40]. The DLS size distribution by number analysis of nanoparticles represents the total number of particles of different size bins [16, 39].

DLS analysis also provides the zeta potential (ZP) of synthesized nanoparticles. In present study zeta potential of synthesized nanoparticles (−11.2 mV) is presented in Fig. 9.

Zeta potential is the electrostatic charge distribution, develops in liquid layer or capping materials on surface (stern layer) of the nanoparticles and diffuse layer present outside the stern layer which gives the potential stability of the particles in colloidal system presented in hypothetical Fig. 10 [20, 40].

It has been reported nanoparticles dispersion with ± 10 to 20 mV are moderately stable [41]. However, ZP does provide indications on colloid stability and colloid stability depends on the sum of van der Waals attractive forces and electrostatic repulsive forces due to the Electric double layer of nanoparticles. It has also been reported that some nanoparticles are very highly stable even they have very low ZP such as silica nanoparticles. Even in the present study ZP of freshly prepared sample of SNPs mediated by *P. tuber regium* extract was −11.2 mV, which correlates with stability of nanoparticles with low ZP of freshly prepared sample of previous studies and theories [42]. The ZP value of nanoparticles may be positive or negative but in present study ZP of *P. tuber-regium* extract mediated SNPs was exist within -25mV which correlates with even distribution of SNPs and the efficiency of the capping biomolecules of the extract to stabilize the nanoparticles in colloid solution [43, 44]. Final confirmation of capping biomolecules present

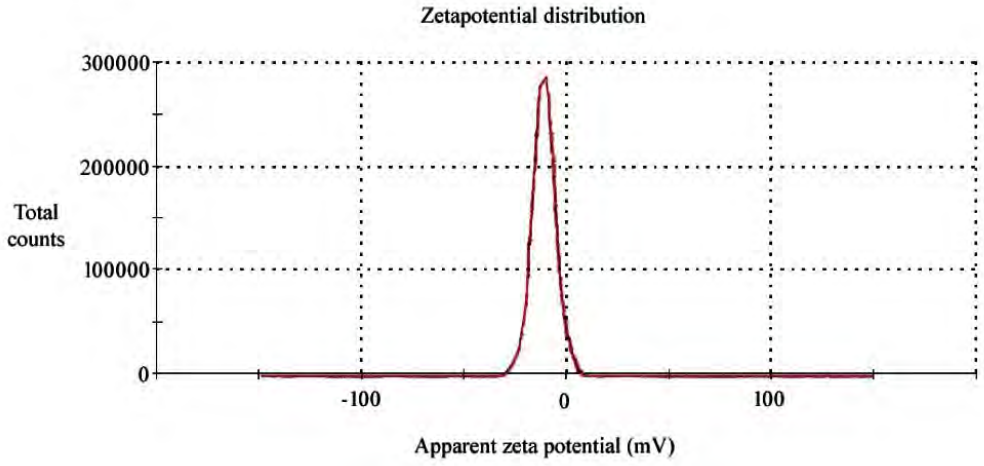

Fig. 9. **Zeta potential distribution of *P. tuber-regium* extract mediated SNPs**

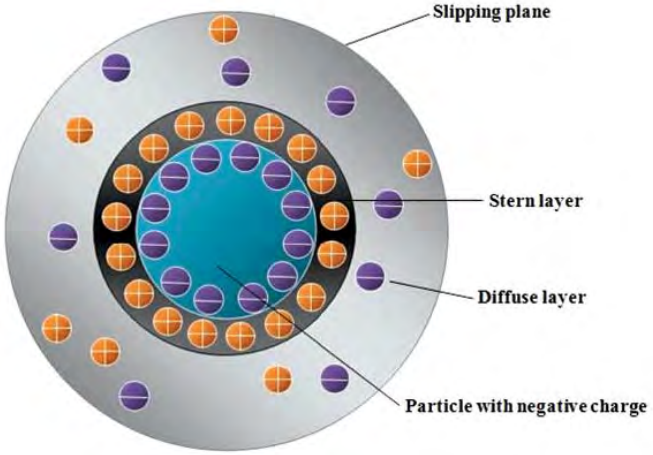

Fig. 10. **Dielectric potential exists at the boundary of a nanoparticle**

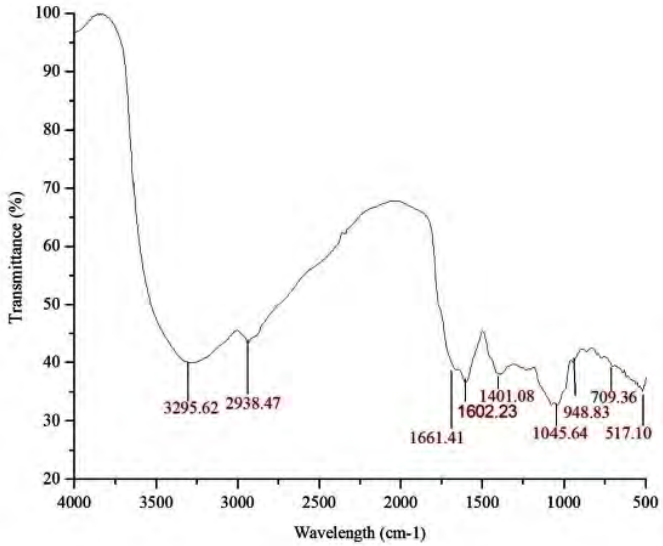

Fig. 11. **FTIR analysis spectrum of *P. tuber-regium* extract mediated SNPs**

on the surface of SNPs was analyzed by FTIR spectroscopy (Fig. 11). FTIR analysis provides confirmation of presence of biomolecules by analysing the functional groups and provides the confirmation about capping tendency of therapeutic molecules of extracts present on the surface of synthesized nanoparticles [30, 45]. Result of FTIR spectroscopy analysis of synthesized nanoparticles sample is presented in Fig. 11. The result showed different transmission peaks for different biomolecule.

3295.62 cm^{-1} corresponds to O-H stretch for alcohol and phenol, 2938.47 cm^{-1} corresponds to C-H stretch for alkane, 1661.41 cm^{-1} corresponds to C=O stretch for amide, 1602.23 cm^{-1} corresponds to N-H and C=C stretch for primary amine and conjugated alkene, 1401.88 cm^{-1} corresponds to O-H stretch for carboxylic acid, 1045.64 cm^{-1} corresponds to C-N and S=O stretch for amine and sulphoixde respectively, 709.36 cm^{-1} and 517.10 cm^{-1} corresponds to C-X stretch for halo alkanes. Gurunathan et al. [33] synthesized gold nanoparticles (AuNPs) extract of *G. lucidum* and reported strong bands of FTIR spectra at 602, 1096, 1201, 1388, and 1636 cm^{-1} correspond to the amide polypeptides or proteins which served as capping agents in AuNPs and make them stable in colloidal solution. Zhu and Tan [46] also reported FTIR spectra analysis of crude extract of *G. lucidum* and reported the presence of biochemicals such as terpenoids and polysaccharide showed peaks at 1 150 to 1 000 cm^{-1} and 1 760 to 1 600 cm^{-1} corresponds to terpenoids, polysaccharide and carbonyl compounds. In present study the infrared spectrum transmission peaks of *P. tuber-regium* extract mediated SNPs sample provided the transmission peaks which provided confirmation about functional groups of mycochemicals such as phenols, amins and other compounds (Fig. 11). FTIR analysis of SNPs in present study supports the capping tendency of mycochemicals and correlates with previous FTIR analyses. Thus, it can be said that mushroom mycochemicals capped on the surfaces of SNPs further carry by the SNPs to the specific sites of the body where, the mycochemicals show their activities [33, 45].

Medicinal mushrooms have been used since centuries for the treatment of different diseases [47] and according to WHO, application of herbs and mushrooms for the therapy of diseases ethnomedicinally should be evaluated and explored for health safety and toxic effects [48, 49]. Experimental animals have been used as important tools in non-human research models for scientific purposes. Dose calculation, stock solution preparation and amount of drug to be dissolved in adequate volume of vehicle (solvent that acts as a medium in which a drug is dissolved) must be calculated before treatment according to the BW of experimental model animal [24]. In present study dose of SNPs and volume of stock solution for limit test doses (2 000 mg/kg) for acute toxicity study, LD (200 mg/kg) and HD (400 mg/kg) for haemotoxic activity were calculated according to BW of experimental animals. The calculated volume of vehicle and doses of SNPs for limit test doses, LD and HD for acute toxicity and haemotoxicity are presented in Table 3. Behavioural pattern and

Table 3. **Amount of SNPs and vehicle (distilled water) used in limit test doses (2 000 mg/kg), control, LD (200 mg/kg) and HD (400 mg/kg)**

Treatment groups	Calculated dose of SNPs (mg)	Equivalent dose of vehicle (ml)
Group-1	0.0 ± 0.0	1.77
Group-2	36.36 ± 0.87	1.82 ± 0.04
Group-3	73.68 ± 2.40	1.85 ± 0.06
Limit test group	375.2 ± 19.34	1.88 ± 0.09

Table 4. **Observation of behavioural patterns of rats in groups treated with *P. tuber-regium* extract mediated SNPs**

Treatment groups and Time interval	Group-1			Group-1			Group-2			Limit test group		
Parameters	30 min	24 Hrs.	7th Day	30 min	24 Hrs.	7th Day	30 min	24 Hrs.	7th Day	30 min	24 Hrs.	7th Day
Fur & skin	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N
Breathing	I	N	N	I	N	N	I	N	N	I	N	N
Somatomotor activity & behaviour pattern	N	N	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	Y	N	N	Y	N	N
Convulsions & tremors	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Itching	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Coma	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Mortality	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF

Note: "N' — normal; "NF" — not found; "I" — Increase; "Y" — found.

mortality due to treatment of limit test dose in acute toxicity study and LD and HD of SNPs in haemotoxicity study were presented in Table 4.

Previous researches reported that woody mushrooms and metal nanoparticles were toxic to experimental animals [50]. It has also been reported that, doses of drug (Sylimarin tablet) and salt (Alloxan monohydrate) and volume of vehicle were calculated for 200 mg/kg and 400 mg/kg BW selected doses for experimental animals [51, 52]. Therefore, dose determination and acute toxicity of *P. tuber-regium* extract mediated SNPs in rat model was performed [22] before study of impact of SNPs on haematological of rats. The results of acute toxicity study showed (Table 3 and Table 4) mortality, convulsions and tremors, itching, comma, salivation and somatomotor activity were not found due to toxicity during the entire experiment. Breathing and sleeping effects were observed in animals within 30 min time interval of administration of SNPs (Table 4). An increase in breathing and sleeping within 30 minutes from treatment time period in animals treated with SNPs and became normal after 30 min. An increase in breathing and sleeping within 30 min from treatment were associated with short-term stress in rats [53, 54]. It has been reported that, as the acute oral toxicity study is necessary to determine the safer dose range to manage the clinical signs and symptoms of the drugs [23] and the toxic outcomes of drugs such as decrease body weight, clinical signs and symptoms which are principal observations among various toxicity indicators [55]. It has been reported that, acute toxicity study is necessary to determine the safer dose range to manage the clinical signs and symptoms of the drugs [23] and the toxic outcomes of drugs such as decrease body weight, clinical signs and symptoms are principal indicators among the various toxicity indicators [48]. In present study insignificant increase in body weight (BW) was observed in SNPs LD and HD treated animals, however insignificant decrease in BW

Table 5. **Impact of *P. tuber-regium* extract mediated silver nanoparticles on body weight of rats. Values presented mean** ± SEM

Treatment groups	Initial BW (g)	Final BW (g)
Group-1	182.2 ± 3.16	186 ± 2.89*
Group-2	181.8 ± 1.66 NS	185.2 ± 1.88 NS
Group-3	184.2 ± 2.83 NS	187 ± 2.63 NS
Limit test Group	187.6 ±4.32	186.4 ± 3.50 NS

Note: "BW" — body weight; "NS" — not significant and "*"- significant at $P \leq 0.05$ when compared final BW with initial BW.

of animals treated with limit dose was observed (Table 5).

Thus, insignificant increase in BW revealed that, different doses of *P. tuber-regium* SNPs were not associated with any adverse acute toxic effect and various metabolites of *P. tuber-regium* extract capped on the surface of SNPs associated with the normal processing of lipids, carbohydrates and protein metabolism inside animals body because these nutrients play a major role in different physiological functions of the body [56, 57].

Haematological parameters are usually associated with health status and are of diagnostic importance in clinical assessment and they are good indicators of physiological, pathological, nutritional and immunological status of body [58, 59]. Results of impact of SNPs on haematological parameters are presented in Table 6.

Significant increase in PCV was observed due to increase RBC count, Hb concentration and due to enhancement of erythropoisis in myeloid tissues of body, proper absorption of iron from food and their incorporation in haemoglobin molecules during their synthesis [60].

In the present study significant increase in PCV, WBC count, nutrophil, monocyte and basophil count was observed (Table 6).

400 mg/kg dose of SNPs significantly ($P = 0.04$) increased total RBC count ($4.30 \pm 0.05 \times 106/\mu L$) in rats of group-3 compared to group-1 but 200 mg/kg dose showed insignificant ($P = 0.33$) increase in total RBC count in group-2 compared to group-1. Similarly 200 mg/kg dose of SNPs significantly ($P = 0.0003$) increased PCV (26.42±0.01%) in group-2 and 400mg/kg dose of SNPs significantly ($P = 0.00003$) increased PCV (33.12±0.22%) in group-2 compared to group-1. Both 200 and 400 mg/kg doses of SNPs showed insignificant increased in Hb level and MCV in rats of group-2 and group-3 compared to group-1 but only 400 mg/kg dose of SNPs significantly ($P = 0.0003$) increased MCHC (24.03±0.02 g/dL) in rats of group-3 compare to group-1. MCV and MCHC are measures of the average volume or size of a red blood cell and average concentration of the haemoglobin inside the RBC respectively. Low MCV is associated with iron deficiency, microcytic anaemia, vitamin B_{12} deficient, etc. but high MCV is associated with macrocytic RBC [61]. Significant decrease level of MCHC is an indication of synthesis of abnormal haemoglobin, failure of blood osmoregulation and plasma osmolarity and oxygen carrying capacity of the RBC [62 Waggiallah] but significant increase of MCHC is associated with hereditary spherocytosis [63]. Significant increase in PCV was observed due to increase in total RBC count and insignificant increase in Hb concentration occurred due to significant increase in MCHC which were directly reflected the significant increase in total RBC count and PCV. It has been reported that increase in Hb concentration occurs due to free hemoglobin in blood massive hemolysis and increase in MCV occurs due to increase in average size of RBCs and both increase in Hb concentration and MCV reflects the toxicity impacts of drugs. It has also been reported that enhancement of erythropoisis in myeloid tissues of body, proper absorption of iron from food and their incorporation in haemoglobin molecules during their synthesis increase the RBC count, PCV and MCHC [60].

It has been reported that, significant increased platelet count in mice model is associated with inflammation and abnormal bleeding induced by toxic biochemicals such as tannins, administered drugs or alternative medicines etc. [60]. Thus, 200 mg/kg and 400 mg/kg doses of SNPs did not show any adverse toxic impact in RBC indices and were safe.

Table 6. **Impact of *P. tuber-regium* extract mediated SNPs on hematological parameters of rat. Values presented mean** ± SEM

Haematological parameters	Treatment groups		
	Group -1 (Control)	Group — 2 (LD of SNPs)	Group — 3 (HD of SNPs)
RBC $\times 10^6$/μL	4.21 ± 0.56	4.24 ± 0.01NS	4.30 ± 0.05*
Hb g/dL	11.66 ± 0.45	11.82 ± 0.09 NS	12.03 ± 0.012 NS
PCV%	26.30 ± 0.04	26.42 ± 0.01*	26.46 ± 0.01*
MCV μL	93.01 ± 0.35	93.02 ± 0.33 NS	93.01 ± 0.40 NS
MCHC g/dL	23.71 ± 0.11	23.81 ± 0.05NS	24.03 ± 0.02*
WBC $\times 10^3$μL	6.81 ± 0.08	7.59 ± 0.05*	8.12 ± 0.27*
NEU%	23.27 ± 0.81	24.51 ± 0.36*	25.44 ± 0.79*
MON%	7.21 ± 0.09	7.43 ± 0.02*	7.66 ± 0.09*
LYM%	32.88 ± 0.20	33.05 ± 0.10 NS	33.12 ± 0.22 NS
EOS%	4.43 ± 0.01	4.58 ± 0.22NS	4.78 ± 0.23*
BAS%	0.73 ± 0.01	0.75 ± 0.006*	0.75 ± 0.004*
PLT%	339.40 ± 2.28	340.86 ±1.78 NS	341.08 ± 2.25 NS

Note: "*" — $P \leq 0.05$; "NS" — not significant at $P \leq 0.05$ when compared the values of LD and HD treatment groups with control.

Both 200 mg/kg and 400 mg/kg doses of SNPs modulated most of the WBC indices except lymphocytes and eosinophils (Table 6). 200 mg/kg dose of SNPs significantly ($P = 0.0000$) increased total WBC count ($7.59 \pm 0.05 \times 10^3$ μL) in rats of group-2 compared to group-1 and 400 mg/kg dose of SNPs significantly ($P = 0.0000$) increased total WBC count ($8.12 \pm 0.27 \times 10^3$ μL) in rats of group-3 compared to group-1 ($6.81 \pm 0.08 \times 10^3$ μL). Significant increase in neutrophil, monocyte and basophil were observed in both group-2 and group-3 compared to group-1. Only 400 mg/kg dose of SNPs showed significant ($P = 0.01$) increase in eosinophil but a insignificant increase was observed lymphocyte in rats of group-2 and group-3 compared to rats of group-1. Mushrooms have been known to possess immunostimulatory property and increase the WBC count [64]. Lymphocytes are primarily responsible for humoral antibody formation but significant increase in lymphocyte is observed due to acute and chronic infection and lymphoma or leukemia [65]. Eosinophils are primarily assigned the function of detoxification and phagocytosis, however, their phagocytic capacity is limited [66]. Neutrophils are known to express receptors that specifically recognise microorganisms and efficiently ingest and destroy these pathogens [67]. It has been reported that significant increase in neutrophils, monocyte and basophils ocuurs due to modulation of defence mechanism of body by fungal protein, polysaccharides and other biochemicals [60, 64]. Thus, in the present study did not show adverse toxic effect on WBC indices but the conjugated mycochemicals of SNPs stimulate and help to strength the defence system of the body. Previously it was reported that rats feed with mushroom extract of edible *Plurotus* species (*P. ostreatus* and *P. pulmonarius)* enhance the total RBC and WBC count, haemoglobin concentration and PCV in treatment group compare to non treatment group [68]. Previously impact of *Pleurotus florida* on haematological parameters such as RBC count, Hb concentration and MCV of cadmium toxicity rats fed with *Pleurotus florida* supplement diet was studied and reported *P. florida* can prevent cadmium toxicity, maintain these parameters and enhance the level of these parameters [69]. Thus, in the present study increased level of RBC, Hb and PCV are associated with non toxic and beneficial impacts of *P. tuber-regium* extract mediated SNPs. Hence, present study correlates with beneficial effect of mushroom studied previously. Present study also supports the beneficial impacts of mushroom and non toxic impact of synthesized SNPs mediated by mushroom *P. tuber-regium*.

Thus, in this study *P. tuber-regium* extract help in synthesis of SNPs of average diameter of 71.36 nm. 2000 mg/kg dose of SNPs do not posses acute toxicity and mortality. 200 mg/kg and 400 mg/kg doses of SNPs are safe and do not possess haemotoxicity and the conjugated mycochemicals present on the surface of SNPs are effectively carried and delivered by the SNPs which help to improve the RBC indices and defence system of the body. Further assessment of impact of synthesized SNPs mediated by medicinal mushroom extract on hepatic and renal profile will provide final nontoxic clarification of SNPs.

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The work was funded according to the competitive target research program of the Ranchi University, India.
The authors declare no conflict of interests.

ТОКСИЧНІСТЬ ЕКСТРАКТУ *Pleurotus tuber-regium*, ЩО МІСТИТЬ НАНОЧАСТИНКИ СРІБЛА, НА ЩУРАХ

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Метою роботи було забезпечити синтез і дати характеристику наночастинок срібла, опосередкованих екстрактом *P. tuber-regium*, та оцінити гостру токсичність і гемотоксичність синтезованих наночастинок срібла на щурах. Гостру токсичність наночастинок срібла вивчали методом «up-and-down-procedure», а гемотоксичність — за диференціальною кількістю еритроцитів. УФ-спектроскопічний аналіз показав наявність максимума за 463,9 нм. Аналізом, виконаним сканувальною електронною мікроскопією, встановлено, що наночастинки мають розмір від 60,8 до 94,9 нм і сферичну форму. Рентгеноструктурний аналіз показав, що середній розмір наночастинок срібла становить 125,7 нм. Аналіз динамічного розсіювання світла виявив, що середній діаметр дорівнює 71,4 нм, а дзета-потенціал синтезованих наночастинок — 11,2 мВ. Аналіз за допомогою інфрачервоної спектроскопії з Фур'є-перетворенням показав, що основні піки пропускання за 3295,6 $см^{-1}$ відповідають розтягуванню O-H для спирту і фенола, а 1602,2 $см^{-1}$ відповідає розтягуванню N-H і C=C для первинного аміну і сполученого алкену. Тест на гостру токсичність не виявив смертності, грубих змін поведінки і зниження маси тіла. Значне збільшення загальної кількості еритроцитів ($4,30 \pm 0,05 \times 10^6$/мкл), фенотипічного коефіцієнта варіації — PCV (26,46 ± 0,01%) і загальної кількості лейкоцитів ($8,12 \pm 0,27 \times 10^3$/мкл) спостерігалося для 400 м/кг групи лікування порівняно з контрольною групою. Таким чином, синтезовані наночастинки срібла, опосередковані екстрактом *P. tuber-regium*, не спричинювали гострої токсичної та гемотоксичної дії, однак виявляли дозозалежну гематенічну та імунну модуляцію.

Ключові слова: наночастинки, гемотоксичність, гриби.

ТОКСИЧНОСТЬ ЭКСТРАКТА *Pleurotus tuber-regium*, СОДЕРЖАЩЕГО НАНОЧАСТИЦЫ СЕРЕБРА, НА КРЫСАХ

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Целью работы было провести синтез и дать характеристику наночастиц серебра, опосредованных экстрактом *P. tuber-regium*, а также дать оценку активности острой токсичности и гемотоксичности синтезированных наночастиц серебра на крысах. Острую токсичность наночастиц серебра изучали методом «up-and-down-procedure», а гемотоксичность — по дифференциальному количеству эритроцитов. УФ-спектроскопический анализ обнаружил максимум при 463,9 нм. Анализ, выполненный с помощью сканирующей электронной микроскопии показал, что наночастицы имеют размер от 60,8 до 94,9 нм и сферическую форму. Рентгеноструктурный анализ показал, что средний размер наночастиц серебра составляет 125,7 нм. Анализ динамического рассеяния света выявил, что средний диаметр составляет 71,4 нм, а дзета-потенциал синтезированных наночастиц — 11,2 мВ. Анализ с помощью инфракрасной спектроскопии с Фурье-преобразованием показал, что основные максимумы пропускания при 3295,62 $см^{-1}$ соответствуют растяжению O-H для спирта и фенола, а при 1602,2 $см^{-1}$ соответствуют растяжению N-H и C=C для первичного амина и сопряженного алкена. Тест на острую токсичность не выявил смертности, грубых изменений поведения и снижения массы тела. Значительное увеличение общего количества эритроцитов ($4,30 \pm 0,05 \times 10^6$/мкл), фенотипического коэффициента вариации — PCV (26,46 ± 0,01%) и общего количества лейкоцитов — ($8,12 \pm 0,27 \times 10^3$/мкл) наблюдалось для 400 мг/кг группы лечения по сравнению с контрольной группой. Таким образом, синтезированные наночастицы серебра, опосредованные экстрактом *P. tuber-regium*, не обладали острым токсическим и гемотоксическим действием, однако проявляли дозозависимую активность относительно гематенической и иммунной модуляции.

Ключевые слова: наночастицы, гемотоксичность, грибы.

The work was funded according to the competitive target research program of the Ranchi University, India. The authors declare no conflict of interests.