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ORIGINAL ARTICLE

Green synthesis, characterization and hepatoprotective activity of silver nanoparticles synthesized from pre-formulated Liv-Pro-08 poly-herbal formulation

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Abstract

This study was performed to evaluate the possibility of synthesizing AgNPs by aqueous extract of pre-formulated Liv-Pro-08 polyherbal formulation and characterize the synthesized AgNPs. Moreover, their antioxidant potential, cytotoxicity and hepatoprotective activity using HePG2 cell line, and performed acute and sub-acute toxicity study on male wistar rats. The aqueous extract of Liv-Pro-08 reduces the $AgNO_3$ into AgNPs. It is primarily identified by the brown color formation in the reaction mix, and the predominant peak was found at 485 nm UV–visible spectrophotometer. The FT-IR analysis results showed that the synthesized phytomolecules capped AgNPs retain four significant functional group peaks correspond to various groups (aromatic amine, alkyne, etc.) of AgNPs. The SEM analysis states that the synthesized AgNPs were in spherical and cubic with 50–70 nm sized. As the Liv-Pro-08 contains significant antioxidant phytochemicals, it showed reasonable antioxidant and reduced power activity with the IC_{50} values of 711.00 µg mL and 613.75 µg mL correspondingly. The synthesized AgNPs showed an absence of cytotoxicity and possess significant hepatoprotective activity in hepatotoxin (CCl_4) exposed HePG2 cell line. The acute and sub-acute toxicity of synthesized AgNPs were studied in male wistar rats. The attained results showed the absence of acute toxicity. Surprisingly, in a sub-acute study, the biomolecule contents were increased in group IV (100 mg kg body weight) treatment than control (untreated). These results suggest that the Liv-Pro-08 synthesized and phytomolecules impregnated AgNPs might be considered for drug delivery-related processes and used as a cure for liver diseases.

Keywords AgNPs · Liv-pro-08 · SEM · FT-IR · HepG2 · Hepatoprotective

Introduction

The continuous spreading of modern and unhealthy lifestyles among people leads to several health issues (Narasimhan et al. 2016; Narayanan et al. 2021a). The frequent consumption of foods derived from modern farming activities minimize consumers' health conditions with the lowest immunity and reduced metabolic activity (Hill et al. 2014). The lowest immunity and poor metabolic activity leads to metabolic disorders such as liver cirrhosis, liver cancer, diabetes, etc. (Rani et al. 2016). According to Global Health Observatory data acquired from World Health Organization states, around 22.2 deaths/100000 populations are due to liver cirrhosis in India (Narasimhan et al. 2016). Globally, liver cirrhosis and disease responsible for about 2 million death per year by cirrhosis and viral hepatitis and hepatocellular carcinoma (Rani et al. 2016; Kandasamy et al. 2021; Whangchai et al. 2021). The liver cirrhosis and liver cancer attains the 11th and 16th place to cause death globally in human and they combined, they account for about 3.5% of death worldwide (Cotovio and Fernandes 2020). In another aspect, liver cirrhosis holds 20th place in causing disability or adjusted remaining life and even several years of life lost (Foreman et al. 2018; Narayanan et al. 2021b).

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Furthermore, these issues account for about 2.1% of global encumbrance (Balakumar et al. 2016; Anusha et al. 2021; Vijayan et al. 2020). The unhealthy lifestyle and excess consumption of alcohol are responsible for liver disease. Statistically, around 75 million populations have been found every year globally as alcohol-associated liver disease (Hill et al. 2014; Mathiyazhagan et al. 2015; Soman et al. 2020a). The frequent consumption of fast food and gem foods was responsible for 2 billion obese adults and 400 million diabetics (Cotovio and Fernandes 2020). These two risk factors can generate liver disease, cirrhosis, and hepatocellular carcinoma (Kumarasamy et al. 2020; Soman et al. 2020b). Apart from viral hepatitis, the drug (heavy dose or frequent consumption) induced liver wounds to increase the possible cause of acute hepatitis (Balakumar et al. 2016; Narayanan et al. 2021c). Hence the existing chemical drug formulation for liver disease cure can generate some side effects while consuming frequently (Borrelli et al. 2018). Finding a cure to protect the liver from the liver mentioned above is timely (Balakumar et al. 2016). Recently nanoparticle-based cure or medical research receiving more attention among the researchers to find an effective and side-effects-free cure to various diseases (Ravichandran 2010). Several reports are suggested that the nanoparticles are emerging as a key player with several applications in pharmaceutical, nutraceuticals, cosmetics, polymers, paints, surface coating, agriculture, medical sector, automobiles, environment, sensors development, etc. (Bhatia et al. 2016). Several types of nanoparticles such as silver, gold, titanium oxide, copper, chitosan, carbon, cerium, curcumin, etc. have been reported as various human welfare applications (Lugani et al. 2021).

Various chemical methods such as polyol method, micro emulsions, thermal decomposition, electrochemical synthesis, etc. have been used for nanoparticle synthesis and produce reproducible results (Ravichandran 2010). Nevertheless, in the chemical and physical synthesis process, the side reaction might occur during the synthesis that could reduce the quality and quantity of the nanoparticle yield (Thakkar et al. 2010; Narayanan et al. 2021d). Hence, the researchers find an alternative and eco-friendly source for nanoparticle synthesis; thus, the biological or green samples are preferred to synthesize the nanoparticles (Roy et al. 2019). Since the phytochemicals in the plant extract, such as flavonoids, terpenoids, and thiols (glucosinolates, allylic sulphides, indoles), have the potential to reduce elements such as silver, gold, titanium oxide, copper, chitosan, carbon, and so on (Thakkar et al. 2010; Lugani, et al. 2021). Since it is an oral drug, the dosage can be reduced to provide a margin of safety, the concept of AgNPs synthesis in preformulated Liv-Pro-08 polyherbal formulation study provided a valid note that minimum dose with maximum efficacy can be achieved.

Hence, this study's novel approach has been conducted to synthesize silver nanoparticle using pre-formulated Liv-Pro-08 poly-herbal formulation. Furthermore, the synthesized nanoparticles have been characterized by formal scientific analysis and subjected assess their hepatoprotective activity. The Liv-Pro-08 poly-herbal formulation has been previously proved (Vedanarayanan and Krishnan 2011) as medicinal value and act as a cure to non-alcoholic fatty liver disease and evidenced by in-vivo analysis in rats. Several literatures state that the silver nanoparticle could be a potential candidate for various medical and non-medical applications (Balakumar et al. 2016; Lugani et al. 2021). Hence, this research was designed to synthesize the silver nanoparticle using pre-formulated Liv-Pro-08 poly-herbal formulation and characterize and confirmed the synthesized silver nanoparticle by UV–visible spectrophotometer, Fourier-transform infrared spectroscopy (FT-IR), scanning electron microscope (SEM). Furthermore, assessed the hepatoprotective activity of synthesized silver nanoparticles by in-vitro (HepG2 cell line), and in-vivo study (Male albino rats of the Wistar strain).

Materials and methods

Brief profile of pre-formulated Liv-Pro-08 poly-herbal formulation

The Liv-Pro-08 poly-herbal formulation was used in this study to synthesize the silver nanoparticle. This Liv-Pro-08 was pre-formulated by Vedanarayanan and Krishnan (2011) and it comprised of seeds of *Nigella sativa* and *Entada pursaetha* and fruits of *Ficus glomerata* and these ingredients were naturally collected from Kolli hills, Namakkal district, Tamil Nadu, India.

Phytochemical profile analysis

The possible phytochemical components such as flavonoids, alkaloids, saponins, vitamin C, and total phenol contents, which could be involved in nanoparticle synthesis, were studied using qualitative and quantitative aspects of respective standard protocol (Akhtar and Mirza 2018).

Synthesis of silver nanoparticle (Ag NPs)

About 88 mL of 1 mM $AgNO_3$ was blended with 12 mL of aqueous extract of Liv-Pro-08 poly-herbal formulation (hereafter mentioned as Liv-Pro-08) and incubated at room temperature for 48 h and noted visible color changes as brown. The reaction mixture was then filtered through nylon mesh and subsequently screened by Millipore hydrophilic filter (0.22 µm) (Nayak et al. 2011). The filtered Ag NPs were stored for further characterization and application study.

Characterization of the synthesized AgNPs

UV–visible spectrophotometer analysis

The UV–visible spectrophotometer [Shimandzu (UV-1800), Double beam, Japan] analysis was performed to study the absorbance of color intensity of Ag NPs reaction mix with various nanometer ranges as 300–800 nm to confirm the reduction of $AgNO_3$ into AgNPs (Ahmed et al. 2016).

Fourier transform infrared spectroscopy analysis (FT-IR)

The FT-IR analysis was accomplished to identify the possible functional groups of biomolecules involved in the reduction and stabilization of $AgNO_3$ into AgNPs and formation of capping over the surface of AgNPs. The emission spectra were recorded using a Spectrum Two FT-IR Spectrometer (FTIR, Model L160000A, Perkin Elmer) with a wavelength range of 4000–400 cm-1 and the standard operating procedure. (Punithavathi et al. 2019).

Scanning electron microscope (SEM) analysis

The aqueous extract of Liv-Pro-08 synthesized An NPs was subjected to SEM analysis to study the nanoparticle surface morphology and size by following the typical operating procedure. Briefly, a high vacuum with different magnifications along with 5 kV was applied over the sample placed onto the glass slides (vacuum dried) (Alijani et al. 2019).

Antioxidant profile analyses by DPPH and reducing power assay

The free radical scavenging (antioxidant) activity of synthesized AgNPs was assessed by the standard DPPH (2,2-diphenyl-2-picrylhydrazyl hydratse) method. Briefly, 1 mL of DPPH stock solution was treated with various concentrations (50, 250, 500, 750, and 1000 µg mL) of AgNPs. Similarly, the reducing power assay was performed with 2.5 mL (each) of 0.2 M of phosphate buffer and 1% of potassium ferricyanide with fore mentioned concentration of AgNPs. This reaction mixture was kept over the water bath (50 °C) for 15–20 min and cooled instantly and blend with 2.5 mL of 10% trichloroacetic acid and spun for 10 min at 3000 rpm. Then the supernatant was mixed with 1 mL of 0.1% ferric chloride and incubated for about 10 min, and absorbance was read at 700 nm using a UV–visible spectrophotometer. The following formula was used to calculate the percentage of antioxidant and reducing power activity of AgNPs (Kharat and Mendhulkar 2016).

$$\text{DPPH scavenging effect (\%)} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Toxicity testing

In-vitro cytotoxicity analysis (MTT assay)

The cytotoxicity profile of synthesized AgNPs was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) analysis by following the standard protocol in HePG2 cell line (Adebayo-Tayo et al. 2019).

Culture maintenance of HePG2 cell line

The new HePG-2 cell line cultures were procured from National Centre for Cell Sciences (NCCS), Pune, India. The stock culture was maintained in Dulbecco's modified Eagle's Medium (DMEM) enriched with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU mL), streptomycin (100 µg mL) and amphotericin B (5 µg mL) and incubated at CO_2 incubator (5%) at 37 °C. The well-grown cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS), and dissociated cells were subjected to MTT analysis (Chen et al. 2014).

Determination of cytotoxicity of synthesized AgNPs by MTT assays

The 96 well plate method was used to determine the cytotoxicity profile of the synthesized AgNPs by following the standard protocol. The dissociated HePG2 monolayer cell concentrations were adjusted to 1.0×10^5 cells mL. Various concentrations (50, 2f50, 500, 750, and 1000 µg mL^{-1}) of AgNPs were added into each well-containing cells and incubated at CO_2 incubator (5%) at 37 °C for 24 h. Then the incubated plates were read at 540 nm, and calculated the percentage of cell viability.

$$\text{Percentage of cell viability (\%)} = \frac{\text{Absorbance (OD) of treated cell line}}{\text{Absorbance (OD) of untreated cell line}} \times 100$$

Hepatoprotective activity assay

Following the standard protocol, the hepatoprotective potential of synthesized Ag NPs was studied (Lee et al. 2012).

Briefly, separated HePG2 cells were treated with 40 mM CCl_4 (hepatotoxic agent) in 96 well plate and incubated for 1.5 h and the hepatoprotective activity of AgNPs were studied with various concentrations (50, 250, 500, 750, and 1000 µg mL) of AgNPs blended with HePG2 cell line individually and incubated at CO_2 incubator (5%) at 37 °C for 12 h. A similar dosage of silymarin was used as the positive control. After incubation, each well of each concentration containing 96 plates was read at a microplate reader at 540 nm. Triplicates were performed, and positive and negative controls were appropriately maintained.

Assessment of HePG2 cell line functionality

The active metabolism activity of AgNPs treated HePG2 (2×10^4 well) cell line was studied by assessing the quantitative analysis was performed to understand the viability and metabolic nature of cells at various concentrations (50, 100, 150, 200, 250 µg mL) of Ag NPs treated cells by assessing Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), and Alanine aminotransferase (ALT) by following the protocol of Huang et al. (2012).

Acute and sub-acute toxicity study by animal model

Acute toxicity

The acute and sub-acute toxicity of synthesized AgNPs were studied on 3–4 weeks of male albino rats of the Wistar strain (weighing 140 ± 15 g), procured from Sri Venkateswara Enterprises, Bangalore. The standard maintenance protocol was followed to raise the rats (Almansour et al. 2016). The animal ethical committee of Periyar University had approved the animal study as per the guidelines (Approval number: PU-IAEC/2018/M1/01 & PU-IAEC/2020/M1/02). The acute toxicity was performed as the OECD guidelines 423 (OECD 2001). The study was designed into two portions, Phase I (Observation made on Ist day) and IInd Phase (Observation for the next sequent 14 days). From 12 h before until 3 h after the oral administrations, animals were kept without food and water access. The animals received AgNP crude extract of Liv-Pro-08 at 100 mg/kg orally (maximum dose for acute study as per OECD, 2001 guidelines). Observations were made and recorded systematically 1, 2, 3, 4, 8 and 24 h after dose administration for physical and characters changes such as appearance, activeness, gait, reaction to stimulus (sound, touch, and light), lacrimation, salivation, piloerrection, stimulation, depression, and convulsions. They were kept under observation for up to 14 days, and the body weights were recorded on the 8th and 14th day.

Sub-acute toxicity study

A sub-acute toxicity study (28 days) was performed to assess the toxicity of AgNP extract of Liv-Pro-08. Rats were given a regular diet for 1 week to be adapted to vivarium conditions and then randomly divided into four groups ($n = 6$ per group): group I served as control, group II, III, and IV were administered orally with crude AgNP extract of Liv-Pro-08 in various doses, ranging from 25, 50, 100 mg/kg by for 28 days, respectively (Table 1) (Nghilokwa et al. 2020).

At the end of the experimental period, rats were fasted overnight and sacrificed by cervical dislocation under mild chloroform anesthesia. The liver was dissected out, washed in ice-cold saline, blot-dried, and weighed. A 10% w/v homogenate was prepared in 0.1 M phosphate buffer, pH 7.4, and used for the biochemical analyses. Liver tissues were preserved in 10% buffered formalin solution for biomolecules examination by following the protocol of Manga-González et al. (2004).

Results and discussion

The phytochemical ingredients of plant extracts are the most important factors involved in reducing various metals into nanoparticles. Similarly, the phytochemical ingredients such as flavonoids, alkaloids, saponins, vitamin C, and total phenol contents of Liv-Pro-08 were studied. The qualitative analysis results state that the significant quantity of phytochemicals such as flavonoids (19.71 ± 0.91 mg g), entire phenol content (13.16 ± 0.31 mg g), saponins (15.11 ± 0.27 mg g), alkaloids (16.47 ± 0.32 mg g), and vitamin C (2.47 ± 0.21 mg g). The presence of this significant volume of metals reducing phytochemicals in Liv-Pro-08

Table 1 Animal model and grouping (Sub-acute study)

Treatment groups	Treatment conditions
Group-I	Control group (normal diet with ad libitium)
Group-II	Rats were fed with Liv-Pro-08 synthesized AgNPs 25 mg/kg body weight for 28 days
Group-III	Rats were fed with Liv-Pro-08 synthesized 50 mg/kg b.wgt for 28 days
Group IV	Rats were fed with Liv-Pro-08 synthesized 100 mg/kg b.wgt for 28 days
Acute study	Rats were fed with Liv-Pro-08 synthesized 100 mg/kg b.wgt for once

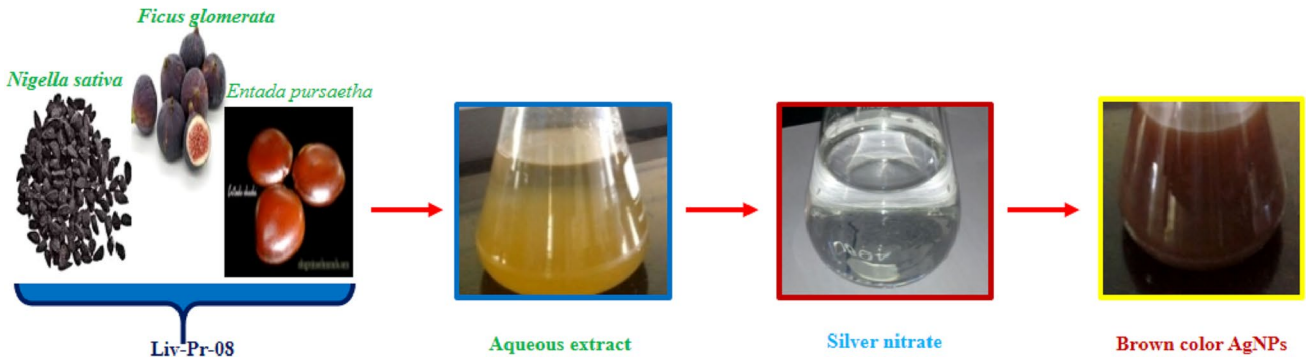

Fig. 1 Biosynthesis of silver nanoparticles from Liv-Pro-08formulation

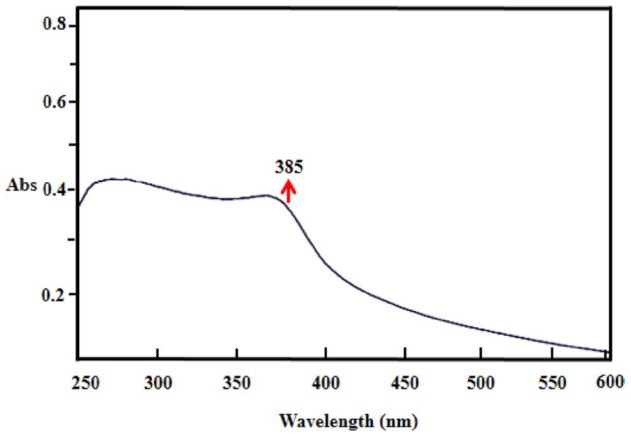

Fig. 2 UV–Visible spectrophotometer analysis of AgNPs synthesized by Liv-Pro-08

states that it could execute the metal reduction and synthesize the nanoparticles.

The metal-reducing and stabilizing potential of phytochemicals (metal reducing) enriched Liv-Pro-08 polyherbal formulation was subjected to nanoparticles synthesis. While during the synthesizing process, the nanoparticle synthesis from $AgNO_3$ was primarily confirmed by the development of brown color in the reaction mix (Fig. 1). The reduction of $AgNO_3$ into AgNPs by Liv-Pro-08 was confirmed by UV–visible spectroscopy analysis by reading the absorbance at 300–800 nm. A single predominant peak was found at the 485 nm that represents that the existence of AgNPs reduced from $AgNO_3$ with the short duration (10–15 min) of reaction time due to the existence of surface Plasmon resonance (Sun et al. 2002) and its electromagnetic field (Chen et al. 2005) aid the reduction process over the surface of $AgNO_3$ and yielded AgNPs (Fig. 2).

FT-IR analysis

The functional groups that exist over the surface of the phytomolecules capped AgNPs were studied through FT-IR analysis. About 4 predominant peaks were found at 3463.84 cm^{-1} corresponding to the aromatic amine group (N–H stretch symmetric vibration). Subsequent absorption peaks were observed at 2061 cm^{-1} and 1636.50 cm^{-1} attributes to the C≡C stretch and C=C stretching vibrations which corresponding to alkyne and alkene functional groups, respectively (Baghizadeh et al. 2015) and also corresponds to carbonyl, alcohol, ethers, and esters functional groups (Rajaram et al. 2015). Furthermore, the peak observed at 539 cm^{-1} corresponds to Ag–O stretch vibration (Fig. 3). These functional groups could be responsible for the stabilization of AgNPs. Another study revealed the predominant peaks of AgNPs synthesized by green extracts at 3439.16, 2922.29, 2854.13, 2360.07, 2342.06 1734.23, 1636.01, 1457.49, and 1057.74 cm^{-1} (Apriliani et al. 2020). A similar kind of FT-IR spectra band pattern was reported in AgNPs synthesized by *Tephrosia tinctoria* (Rajaram et al. 2015).

SEM analysis

The surface morphology of AgNPs synthesized by Liv-Pro-08 showed that the semi-spherical and partially cubic shaped AgNPs with smooth surfaces and edges. Furthermore, the size was found in a range of 50−70 nm. Obviously, the silver nanoparticles synthesized by plant extracts are preferably spherical, and size is in the range of 10–100 nm (Pandian et al. 2015). In this study, the cubic-shaped particles were in 50 nm size, and spherical-shaped nanoparticles were 60–70 nm shaped (Fig. 4). Similarly, Khodashenas and Ghorbani (2019) reported that the cubic-shaped AgNPs synthesized by plant extracts were in the size range of 50 to

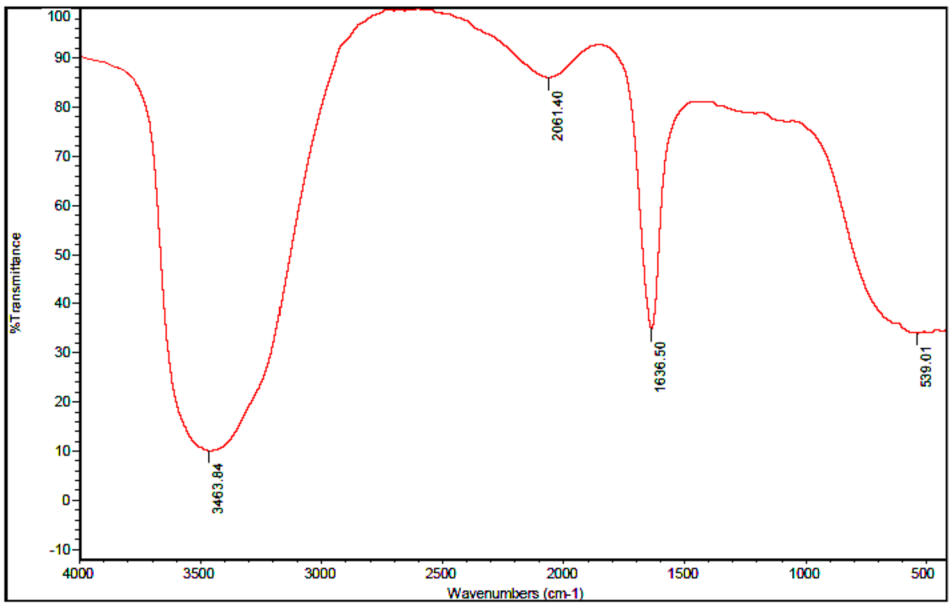

Fig. 3 FT-IR analysis of AgNPs synthesized by Liv-Pro-08

60 nm. Most of the silver nanoparticles synthesized from plant sources are in the field of 2–100 nm and in some cases, it might reach up to 150 nm (Geoprincy, et al., 2013). Thirunavoukkarasu et al. 2013 reported that AgNPs synthesized from Desmodium gangeticum leaves showed spherical-shaped particles with the size ranges from 18 to 39 nm.

Antioxidant analysis

The phytochemical analysis results suggest that the aqueous extracts of Liv-Pro-08 contain a significant volume of antioxidant components that capped over the surface of the synthesized silver nanoparticle scavenge the free radicals (Kharat and Mendhulkar 2016). Figure 5 showed that the increased concentration of AgNPs scavenges the free radicles and significantly reducing potential in a dose-dependent manner. The IC_{50} value of DPPH and reducing power assay of Liv-Pro-08 synthesized AgNPs showed as 711.00 µg mL and 613.75 µg mL correspondingly statistical significance of difference at $> p$ 0.003 and $> p$ 0.003. At this concentration, the AgNPs inhibit or delay cell damage possibly caused by various oxidants such as ROS, free radicals, RNS, and other unstable molecules by removing the oxidant molecules (Apak et al. 2016; Nallanthighal et al. 2017).

This AgNPs capped Liv-Pro-08 could react with oxygen and nitrogen atom of the free radicles and convert it into less toxic or non-toxic components and enhance the viability of cells (Baghizadeh et al. 2015). The antioxidant mechanisms could vary depending on the type of antioxidants, source, specific molecule target, etc. (Sun et al. 2002; Chen et al. 2005). The various types of antioxidant active defense mechanisms are reported in the literature are by stimulating the synthesis of significant intracellular enzymes such as superoxide dismutases, reductases, peroxiredoxins, glutathione peroxidases, catalases, etc. (Thirunavoukkarasu et al. 2013) and extracellular defenses through the synthesis of transferrin, bilirubin, α-keto acids, uric acid, etc. and significant nutritional supplements such as vitamins (C, E, A, D, etc.) and elements (Se, Fe, Zn, etc.) (Rajaram et al. 2015).

Cytotoxicity and hepatoprotective activity analysis

The MTT assay analysis results state that the synthesized AgNPs showed no significant activity against the HePG2 cell line at all concentrations. Hence unable to cause or induce any damage to liver cells while consuming it as a cure for diseases. The in-vitro hepatotoprotective potential of AgNPs was studied with HePG2 cell line (Fig. 6), since the antioxidant and cytotoxicity (MTT assay) results suggest that as it possess and free radicles scavenging activity and no significant cytotoxicity activity on normal HePG2 cell line. Hence the CCl_4 exposed HePG2 cell line was treated with various concentrations (50, 250, 500, 750, and 1000 µg mL) of AgNPs.

The results stated that the significant hepatoprotective activity was found as dose dependent, compared with standard positive control drug silymarin. Figure 7 depicted that the dose-dependent hepatoprotective activity was statistically significant at $> p$ 0.005 (Fig. 6). The hepatotoxin such as CCl_4 can induce lipid peroxidation, reduce enzyme activity involved in the scavenging process and enhance the development and accumulation of free radicles (Girish et al.

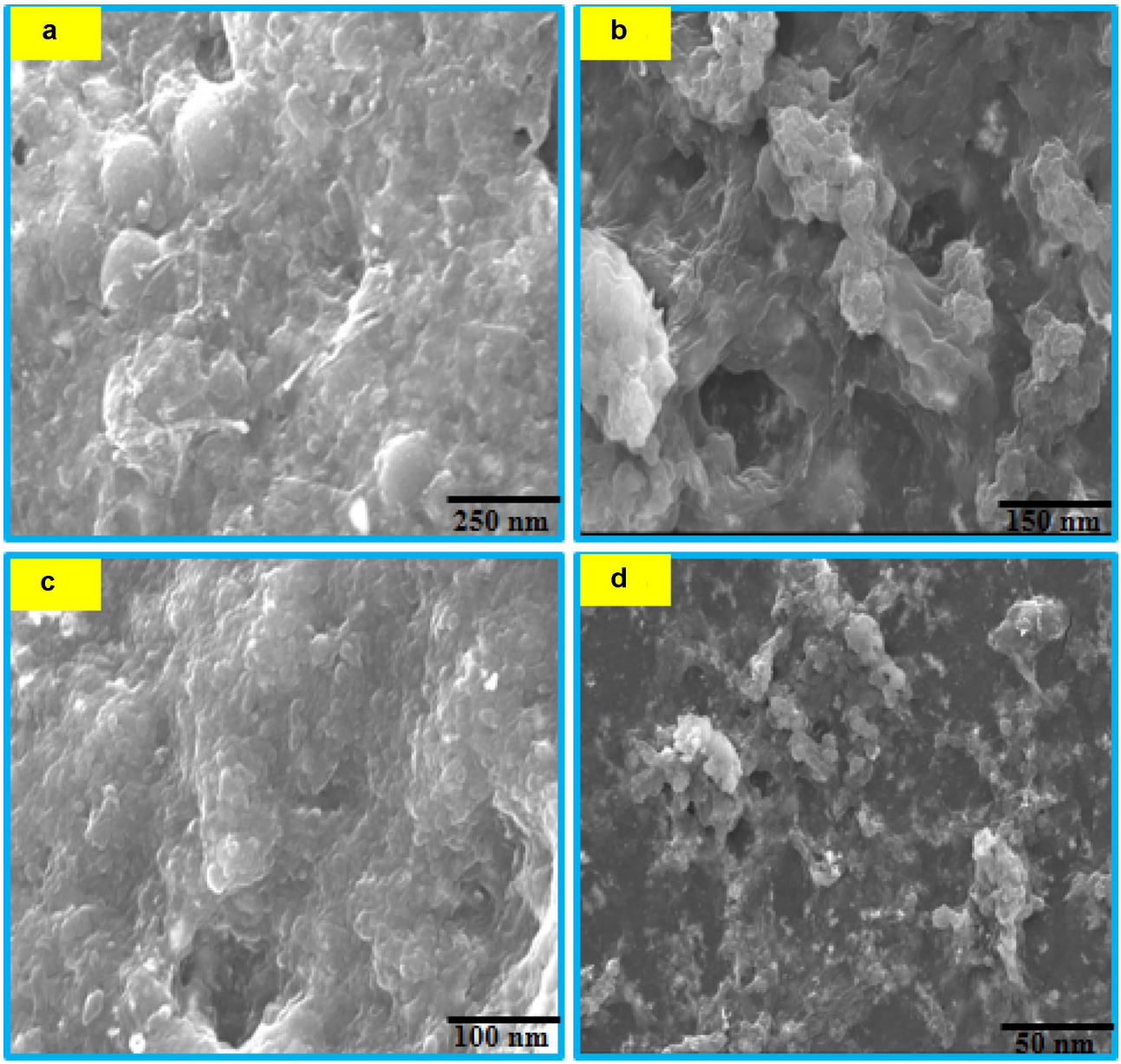

Fig. 4 SEM images of Liv-Pro-08 synthesized AgNPs. **a**: 250 nm, **b**:150 nm, **c**: 100 nm, **d**: 50 nm

2009). This free radicle accumulation collapses the membrane's lipids, reducing the functional integrity of hepatic mitochondria, resulting in liver damage (Akhtar and Mirza 2018). The antioxidants (phytochemicals of Liv-Pro-08) capped AgNPs reduce the accumulation of free radicles by oxygen and hydrogen atoms of radicles and convert them as non-toxic and protect the HePG2 cell line from CCl_4toxicity. This potential suggests that these AgNPs synthesized from Liv-Pro-08 might be considered for drug formulation to use as a cure for liver cirrhosis or disease (hepatopathies) (Al-Dbass et al. 2012).

Effects of Liv-Pro-08 synthesized AgNPs in metabolic activity of HePG2 cell line

The hepatoprotective potential of AgNPs synthesized from Liv-Pro-08 protect the HePG2 cells to reduce the toxicity of hepatotoxin through enhancing the cell metabolic activity by stimulating the various enzyme activity such as Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), and Alanine aminotransferase (ALT) which neutralizing the toxicity impacts of CCl_4 in HePG2 cell line toxicity. The IC_{50} values of AgNPs on the enzymatic activity of AST, ALP,

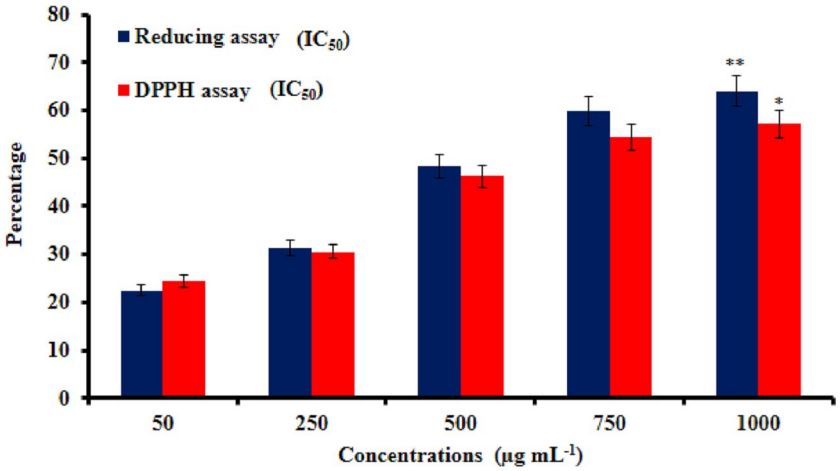

Fig. 5 Antioxidant properties of AgNPs synthesized by Liv-Pro-08 through DPPH and reducing power assay. The mentioned values are the mean and standard error ± SE of triplicates. **: indicates the statistical significance of difference at > p 0.005.*: indicates the statistical significance of difference at > p 0.003

and ALT were found as 141.51, 231.69, and 182.05 µg mL (Fig. 8) and these were statistically significant at the range at > p 0.005 and > p 0.003. Each enzyme has an individual significant role in cell metabolic activity, example the AST stimulate the reversible transfer of significant amino group among the aspartate and glutamate and act as an important enzyme in amino acid metabolism in liver cells, kidney cells, etc. (Nallanthighal et al. 2017). The ALP is another significant enzyme involved in protein metabolism in liver cells. The conversion of alanine to pyruvate, which is involved in the cellular energy production process, is mediated by the ALT enzyme. (Soman et al. 2020a,b). These most essential enzymatic activities in HePG2 cell line have been properly balanced under the stress of hepatotoxin.

Acute toxicity analysis

The toxicity nature of Liv-Pro-08 synthesized crude AgNPs was assessed by in-vivo study on male albino rats with a dosage of 100 mg/kg of body weight. About various parameters (Table 2) such as appearance, activeness, gait, reaction to stimulus (sound, touch, and light), lacrimation, salivation, piloerrection, stimulation, depression, and convulsions were studied by acute toxicity analysis (1 day with different time intervals, 8th and 14th day analysis).. The results attained emphasise no visible toxicity impacts noted on both AgNPs treated albino rats and as well as control (untreated) rats. Similarly, the silver nanoparticle synthesized from extracts of *Azadirachta indica* showed no significant toxicity effect on rats (Ahmed et al. 2016). These results suggest that the Liv-Pro-08 synthesized AgNPs unable to cause any harmful effects on rats. Hence it can be considered a suitable carrier to carry and deliver a cure for liver cirrhosis or disease at a limited concentration of 100 mg/kg of body weight. The excess dosage of AgNPs could cause severe damage to various organs as in the rank of lung > spleen > liver > kidney > thymus > heart (Bergin et al. 2016), hence the optimized dosage could be considered for drug delivery purpose.

Sub-acute toxicity analysis

The sub-acute toxicity study was performed to evaluate the impact of Liv-Pro-08 synthesized AgNPs treated rats by assessed the biomolecules such as protein, Albumin, total bilirubin, urea, creatinine, cholesterol, and triglycerides. The obtained results were tabulated in Table 3 and state that absence of significant adverse effects on treated rats. Fortunately, the increased concentration (100 mg/kg of body weight) of AgNPs treatment showed substantial positive changes in the biomolecule synthesis process in dose-dependent manner. Since, in group IV (100 mg/kg of body weight) treated rats the biomolecules (protein (7.60 ± 0.32), Albumin (3.96 ± 0.40), total bilirubin (0.56 ± 0.04), urea (33.31 ± 0.30), creatinine (1.38 ± 0.02), cholesterol (63.44 ± 1.67), and triglycerides (44.04 ± 0.11 mg mL)) quantities were significantly increased compared to group I (control) (Table 3).

These results were correlated with the cytotoxicity results (no cytotoxicity found) study on the HePG2 cell line. Furthermore, it correlated with hepatoprotective activity on HePG2 cell line, since the biomolecule stimulating mechanism and potential of Liv-Pro-08 synthesized AgNPs could minimize the toxicity of hepatotoxin. Similarly, the AgNPs synthesized from *Momordica charantia* showed significant positive impacts on insulin secretion in male wistar rats subjected for sub-acute toxicity (Shanker et al. 2017). Fasting insulin levels in the AgNP- and ZnO NP-treated diabetic rats restored the levels significantly ($p < 0.001$) to normalcy as compared to the diabetic control group Hence these results confirmed that the increased volume of crude Liv-Pro-08 synthesized AgNPs (as dose-dependent manner) could be used as a possible carrier for a cure for liver diseases or cirrhosis.

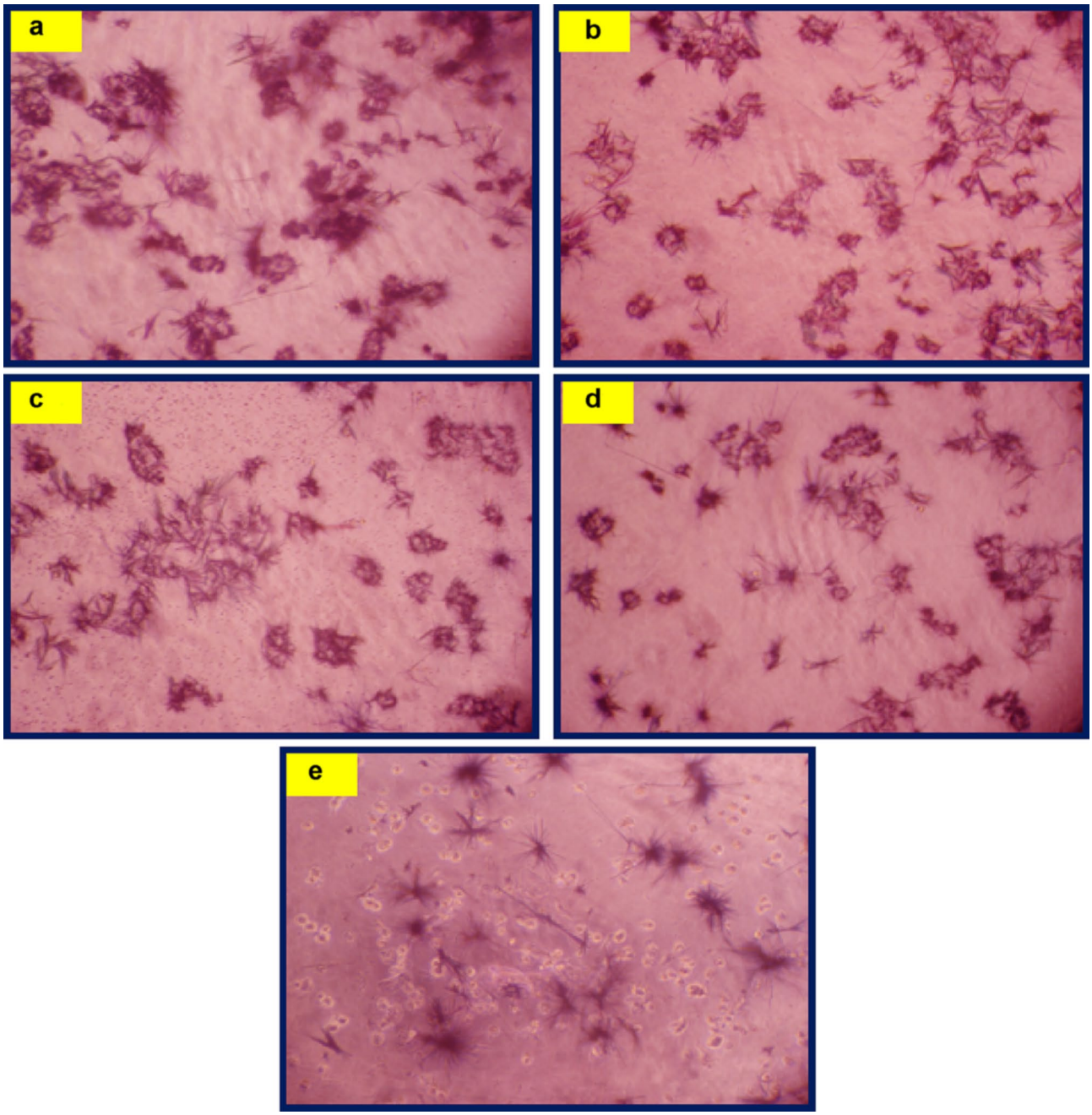

Fig. 6 Hepatoprotective activity of Liv-Pro-08 synthesized AgNPsin HePG2 cell line. **a**: 50 µg mL, **b**: 250 µg mL, **c**: 500 µg mL, **d**: 750 µg mL, **e**: 1000 µg mL

Conclusion

The phytochemical analysis of Liv-Pro-08 polyherbal formulation states that it contains the most significant volume of flavonoids (19.71 ± 0.91 mg g), total phenol content (13.16 ± 0.31 mg g), saponins (15.11 ± 0.27 mg g), alkaloids (16.47 ± 0.32 mg g), and vitamin C (2.47 ± 0.21 mg g). These constituents are formerly reported as fine antioxidants that could reduce the AgNO3 into AgNPs by the development of brown color confirmed it and the reduction were established by the recorded predominant peak at 485 nm in UV–visible spectrophotometer. Furthermore, the most significant 4 functional groups peaks (3463.84 cm^{-1}, 2061 cm^{-1}, 1636.50 cm^{-1}, and 539 cm^{-1}) correspond to aromatic amine, alkyne, alkene groups were noted in the AgNPs impregnated (capped) with phytomolecules of Liv-Pro-08. The shape and size of the synthesized AgNPs were found as spherical and cubic shaped with 50–70 nm size

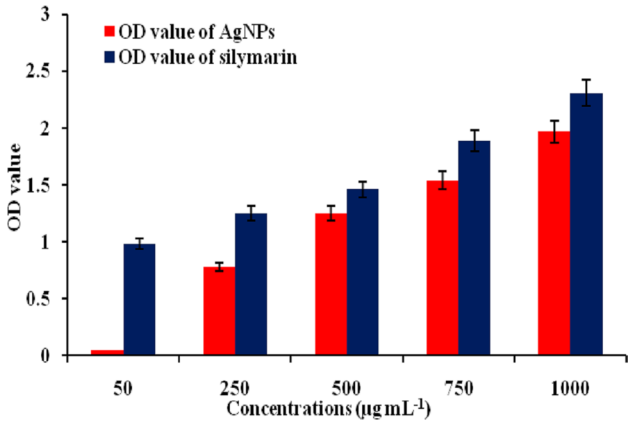

Fig. 7 Hepatoprotective activity potential of AgNPs. The mentioned values are mean and standard error (± SE) of triplicates. **: indicates the statistical significance of difference at > *p* 0.005

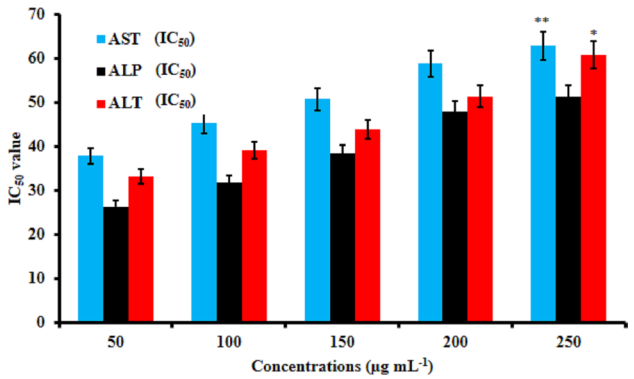

Fig. 8 Determination of metabolic activity of cell by enzyme analysis. The mentioned values are mean and standard error (± SE) of triplicates. **: indicates the statistical significance of difference at > *p* 0.005.*: indicates the statistical significance of difference at > *p* 0.003

by SEM analysis. The phytochemicals impregnated AgNPs possess significant antioxidant (DPPH) and reducing power potential and their IC_{50} values were found as 711.00 µg mL and 613.75 µg mL correspondingly. Fortunately at tested concentration the synthesized AgNPs showed a lack of cytotoxicity on HePG2 cell line. Moreover, surprisingly it showed hepatoprotective activity as dose-dependent manner in CCL_4 (hepatotoxin) treated HePG2 cell line and it was almost similar to the activity of hepatoprotective drug silymarin. Since the Liv-Pro-08 synthesized AgNPs showed reduce or protect the hepatotoxicin toxicity by enhancing the cell enzyme synthesis which involved in various metabolic processes such as amino acid (AST), protein (ALP), and energy production (ALT) mechanisms. The acute and sub-acute toxicity results of I–IV group studies showed that the synthesized AgNPs showed a lack of toxicity on male wistar rats and enhance the biomolecules synthesis such as protein (7.60 ± 0.32), Albumin (3.96 ± 0.40), total bilirubin (0.56 ± 0.04), urea (33.31 ± 0.30), creatinine (1.38 ± 0.02), cholesterol (63.44 ± 1.67), and triglycerides (44.04 ± 0.11 mg mL)). These results finally concluded that the Liv-Pro-08 synthesized and phytomolecules impregnated AgNPs showed significantly medicinally valuable results and suggested that it could be considerable for drug delivery-related processes and used as a cure for liver diseases after purification study. The characterization study confirmed that the nanobased Liv-pro-08 polyherbal formulation outlayed the quantum of AgNPs synthesised; additionally, the preformulated polyherbal formulation comprehensively validated the repair and restoration of hepatic damage, thereby providing effective hepatoprotection. Furthermore, it is declared to be safe and environmentally friendly for both humans and the environment.

Table 2 Morphological and behavioral nature of rats in acute toxicity study with AgNPs of Liv-Pro-08

Parameter	1 h	2 h	3 h	4 h	8 h	24 h	8th day	14th day
Appearance	N	N	N	N	N	N	N	N
Activeness	P	P	P	P	P	P	P	P
Gait	N	N	N	N	N	N	N	N
Reaction to stimulus								
Sound	++	++	++	++	++	++	++	++
Touch	++	++	++	++	++	++	++	++
Light	++	++	++	++	++	++	++	++
Lacrimation	A	A	A	A	A	A	A	A
Salivation	A	A	A	A	A	A	A	A
Piloerrection	A	A	A	A	A	A	A	A
Stimulation	A	A	A	A	A	A	A	A
Depression	A	A	A	A	A	A	A	A
Convulsions	A	A	A	A	A	A	A	A

N Normal, *P* Positive, ++: Good

Table 3 Assess the effect of AgNPs of Liv-Pro-08 on biochemicals/biomolecules parameters in control and treated rats in sub-acute toxicity study

Biochemical parameters	Control (Group I)	Doses of AgNPs mg/kg body weight		
		25 mg (Group II)	50 mg (Group III)	100 mg (Group IV)
Protein (g/dL)	7.14 ± 0.44	7.02 ± 0.45	7.44 ± 0.39	7.60 ± 0.32
Albumin (g/dL)	3.65 ± 0.13	3.39 ± 0.19	3.80 ± 0.34	3.96 ± 0.40
Total bilirubin (mg/dL)	0.35 ± 0.04	0.41 ± 0.05	0.51 ± 0.04	0.56 ± 0.04
Urea (mg/dL)	27.40 ± 0.34	29.31 ± 0.31	31.47 ± 0.25	33.31 ± 0.30
Creatinine (mg/dL)	1.01 ± 0.04	1.09 ± 0.05	1.25 ± 0.02	1.38 ± 0.02
Cholesterol (mg/dL)	62.11 ± 2.19	61.87 ± 2.11	62.50 ± 2.34	63.44 ± 1.67
Triglycerides (mg/dL)	43.23 ± 0.21	42.14 ± 0.22	43.35 ± 0.22	44.04 ± 0.11

The mentioned values are mean and standard error (± SE) of triplicates

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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