Full length article

Polysaccharide-mediated green synthesis of silver nanoparticles from *Sargassum siliquosum* J.G. Agardh: Assessment of toxicity and hepatoprotective activity

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ABSTRACT

The current investigation aimed to determine the toxicity and hepatoprotective potential of silver nanoparticles (AgNPs) synthesized rapidly using sulfated polysaccharide extract from *Sargassum siliquosum*, a brown alga. The nanoparticles were characterized by UV–visible spectroscopy (UV–vis), scanning electron microscopy (SEM), fourier transform infra-red spectroscopy (FTIR), energy dispersive X-ray (EDX) alongside zeta potential measurement. SPR indicated prominent peaks at 420–460 nm. SEM and DLS showed that biosynthesized AgNPs were in the size range of 20–480 nm and 19.5 ± 6 nm to 471.7 ± 189.4 nm respectively. EDX spectrum indicated strong signal for AgNPs. Zeta potential was -47.4 ± 5.32 to -50 ± 3.49 mV indicating stable AgNPs. AgNPs up to 2000 mg/kg BW did not cause mortality to rats but caused minimal elevation of serum creatinine and blood urea nitrogen. Low dose was effective in revival of liver enzyme parameters to near normal in all intoxicated groups indicating the curing effects on paracetamol induced liver injury.

1. Introduction

Nanobiotechnology is one of the most promising areas in modern nanoscience and technology. The use of nanoparticles in synthesizing medical products is attracting attention in recent research because of their potential application as therapeutics, diagnostics, surgical devices and nano-medicine based antimicrobial agents [1–3]. Nanoparticles of metallic origin such as silver nanoparticles offer huge potential and benefits to nanotechnology, thus they have come under intense scrutiny due to toxicity issues which render them unsuitable for biomedical applications [4–6]. Metal nanoparticles and nanostructured materials can be prepared and stabilized by physical and chemical methods, but these methods are expensive and require the use of toxic and aggressive chemicals as reducing and capping agents [7,8].

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In recent years, rapid and green synthesis methods using biological extracts have shown a great potential in nanoparticle synthesis to overcome the reported toxic effects of nanoparticles that are synthesized in conventional approaches. The green synthesis of inherently safer AgNPs depend on the adoption of the basic requirements of green chemistry including solvent medium, good reducing agent and a non-hazardous stabilizing agent [9,10]. A number of living organisms are already well-known to elaborate nanostructured composites such as cyanobacteria, bacteria, fungi, algae and various extracts from plant materials [11,12]. Polysaccharides from marine algae such as *Sargassum* can be used as a bio-factory for synthesis of nanoparticles because these are highly stable, safe, not toxic and with known biological activities [13,14]. *Sargassum siliquosum* was selected for the study due to its high content of polysaccharides with reported biological activities [15,16]. Review of related literature revealed that the synthesis of nanoparticles using algae has been explored in number of little literature but mostly dealt with antibacterial activity. The aim of this study is to synthesize stable silver nanoparticles from sulfated polysaccharides from *Sargassum siliquosum* and assess their hepatoprotective potential in *vivo*. In particular, we reported the acute toxicity of these nanoparticles in rat model which may add new information on few literature dealing with in *vivo* toxicity of nanoparticles.

2. Experiment details

2.1. Sample collection and extract preparation

The brown seaweed *S. siliquosum* was collected from Bolo Beach, Alaminos Pangasinan. The specimen was authenticated at The Marine Science Institute of the University of the Philippines, Diliman. Collected samples were immediately washed thoroughly with tap water to remove extraneous materials and debris. The thalli were finally washed with sterile distilled water. The cleaned thalli were dried at ambient temperature without exposure to sunlight, then ground to fine powder using Wiley Mill and stored in airtight container at $4-10$ °C until use. The dried fine powdered 100 g was extracted in 500 mL distilled water at 100 °C for 4 h. The extracts were filtered through Whatmann No.41 filter paper, concentrated using rotary evaporator, lyophilized and stored in an airtight amber bottle until use.

2.2. Optimization and Biosynthesis of silver nanoparticles

The procedures for the synthesis were optimized to yield the final AgNPs for the assays. The first set up was made by the reduction of varying concentration of $AgNO_3$ ($2-10$ mM) with 6% (w/v) of extract. The second set-up is the reduction of 6 mM $AgNO_3$ with varying concentration of extract ($2-10$% w/v) and the pH adjusted from 2 to 10 using 1 M HCl and 1 M NaOH. Set-ups were maintained at room temperature, 25 °C, 50 °C, and at 121 °C 15 psi for 30 min. The formation of AgNPs was confirmed by the color change and periodic measurement of optical absorbance. Finally, the AgNPS used for toxicity assays were synthesized by autoclaving a mixture containing 6% extract, 6 mM $AgNO_3$ at pH8 for 30 min. The AgNP solution was centrifuged at 10,000 rpm for 15 min and the suspension was redispersed in sterile distilled water. The suspension was lyophilized for the assays. The different concentrations used in the assays were prepared by dissolving the lyophilized AgNPs in ultra-pure water.

2.3. Characterization of silver nanoparticles

The synthesized AgNPs were characterized by UV–visible spectrophotometer (Corona microplate reader SH-100 Hitachi Japan) recorded at 200–800 nm. FT-IR spectral analysis was carried out in the range of 4000–8000 cm^{-1} with a Spectrum RX-1 FT-IR spectrophotometer (Perkin-Elmer, USA) using KBR pellets. The morphology of the Ag nanoparticles was visualized using a field emission-scanning electron microscope (Carl Zeiss EVO MA 15). EDX analysis was performed in Oxford Inca Penta FeTX3 EDS instrument by drop coating AgNPs on carbon film. A zeta potential measurement of Ag nanoparticles was also carried out using Malvern Zetasizer Instrument.

2.4. Animals

Male and female Sprague Dawley rats were outsourced from the Department of Pharmacology, University of the Philippines, 5–6 weeks old and weighing approximately 180 g at commencement of experiment. The animals were housed in a protected access rodent facility, up to 5 animals per cage, in clear polycarbonate cages with stainless steel mesh lid and floor. Animal room controls were set to maintain temperature and relative humidity at (22 ± 2 °C and 55 ± 5% respectively. The room was lit by artificial light for 12 h each day. Drinking water was supplied ad libitum and animals were fed standard rat pellets. The study was conducted in compliance with the approved protocols of the University of Santo Tomas Institutional Animal Care and Use Committee (UST-IACUC) with permit from Bureau of Animal Industry.

2.4.1. Acute toxicity assays

Fifteen rats were randomly divided into 3 groups with 5 animals in each group. Group A served as normal control group. Groups B was administered orally with 2000 mg/kg BW synthesized AgNPs respectively. Both groups A and B were observed

for two weeks for changes in motor and behavior, tremors, convulsion, salivation, diarrhea, lethargy, sleep, coma and death. Group C was separately administered with 2000 mg/kg BW AgNPS and after 24 h, blood was collected in lithium-heparin tube and centrifuged. Plasma aliquots were subjected to evaluation of alanine amino-transferase (ALT), aspartate amino-transferases (AST), blood ureic nitrogen (BUN) and creatinine. All parameters were performed using the liquid-chemistry spectrophotometer technique (BM-Hitachi 911, Hitachi LTD., Tokyo Japan) at Hi-Precision Diagnostic, Philippines. After treatment, all animals were killed by cervical dislocation. The liver and kidney were harvested and fixed in 10% formalin for histopathological analysis.

2.4.2. Hepatoprotective assays

Twenty five rats were randomly divided into five groups with 5 animals in each group. Group A and B served as normal (saline) and control (paracetamol 2000 mg/kg BW on the seventh day only) groups respectively. Groups C, D and E received AgNPs (100 mg/kg BW, 200 mg/kg BW) and standard drug (silymarin 300 mg/kg BW) daily for six days and a single high dose of paracetamol (2000 mg/kg) on the seventh day respectively. On day 8, blood was collected by cardiac puncture under mild ether anesthesia. ALT and AST levels were estimated. The liver and kidney were collected for histopathologic analysis.

2.5. Statistical analysis

All statistical analyses were performed by SPSS 12.0 statistical package for Windows (SPSS, Inc. Chicago, I, USA). All values were expressed as mean $\pm$ SEM. Statistical difference among group was assessed by one-way ANOVA with Tukey's *post-hoc* test. Independent *t-test* was performed to compare values before and after treatment. Values were considered statistically significant at $p < 0.05$.

3. Results and discussion

Synthesis of silver nanoparticles was confirmed visually when the reaction mixture turned from light yellow to yellowish brown after autoclaving at 121 °C at 15 psi for 15 min (Fig. 1). Reaction mixtures were autoclaved because all preparations maintained at room temperature, 25 °C and 50 °C showed poor biosynthesis. This shows that biosynthesis of silver nanoparticles using polysaccharides from *S. siliquosum* requires high temperature and pressure.

Fig. 2 shows the UV–vis spectra of synthesized AgNPs obtained from different optimization parameters. A broad intense peak of different heights around 420–450 nm was observed from nanoparticles synthesized from 6% aqueous extract in varying AgNO$_3$ concentrations (2–10 mM) (Fig. 2(a)). Absorbance bands of different width were obtained in reaction mixtures of 6 mM AgNO$_3$ with varying concentration of aqueous extracts (2–10% w/v) (Fig. 2(b)). Optimal condition was obtained from biosynthesis reaction using 6% extract, 6 mM AgNO$_3$ at pH 8 showing a sharp intense peak between 420 and 450 nm (Fig. 2(c)).

The color change interference is an easy way to confirm the formation of nanoparticles as a result of a decrease in size with an increase in excitation of the outer surface electrons known as plasmon resonance [17,18]. The SPR band at 420 indicated that the formation of AgNPS had occurred [19]. Many factors are responsible for the formation of nanoparticles such as temperature, incubation time and pH. Similar results showing synthesis of nanoparticles on alkaline pH were obtained from different plant families [20], a green alga *Ulva lactuca* [21], 3D guar gum networks [22] and *Brucea javanica* [23].

FT-IR spectrum of polysaccharide extract of *S. siliquosum* shows peaks at 3515, 2800, 1630, 1420, 1140, and 619 cm^{-1}. The strong band at 3515 cm^{-1} corresponding to O-H broad stretching of high concentration of alcohols or phenols; the band at 2800 cm^{-1} corresponding to C-N stretching of any R-N=C=S; the bands at 1630 cm^{-1} corresponds to stretching of C=N; the band sat 1420 and 1140 cm^{-1}, correspond to asymmetric stretching vibration of organic sulfate; the band at 619 cm^{-1}
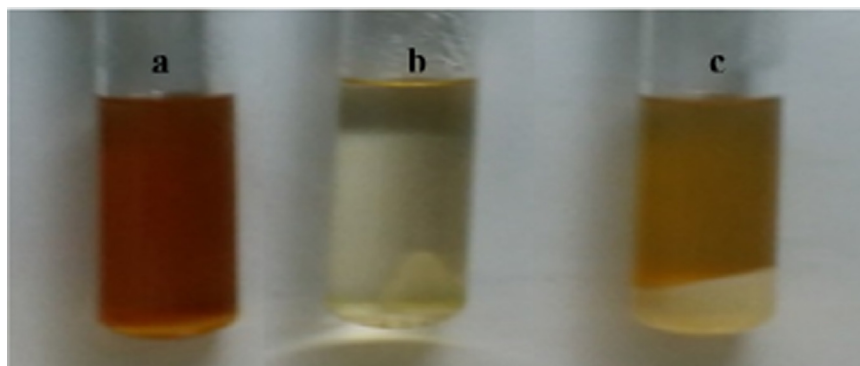

Fig. 1. Synthesis of silver nanoparticles. (a) Synthesized silver nanoparticles from 6% aqueous extract +6 mM AgNO$_3$ solution at ph8, (b) 6 mM AgNO$_3$ solution only and (c) 6% aqueous extract only. Reaction mixtures were prepared by autoclaving at 121 °C, 15 psi and 30 min.
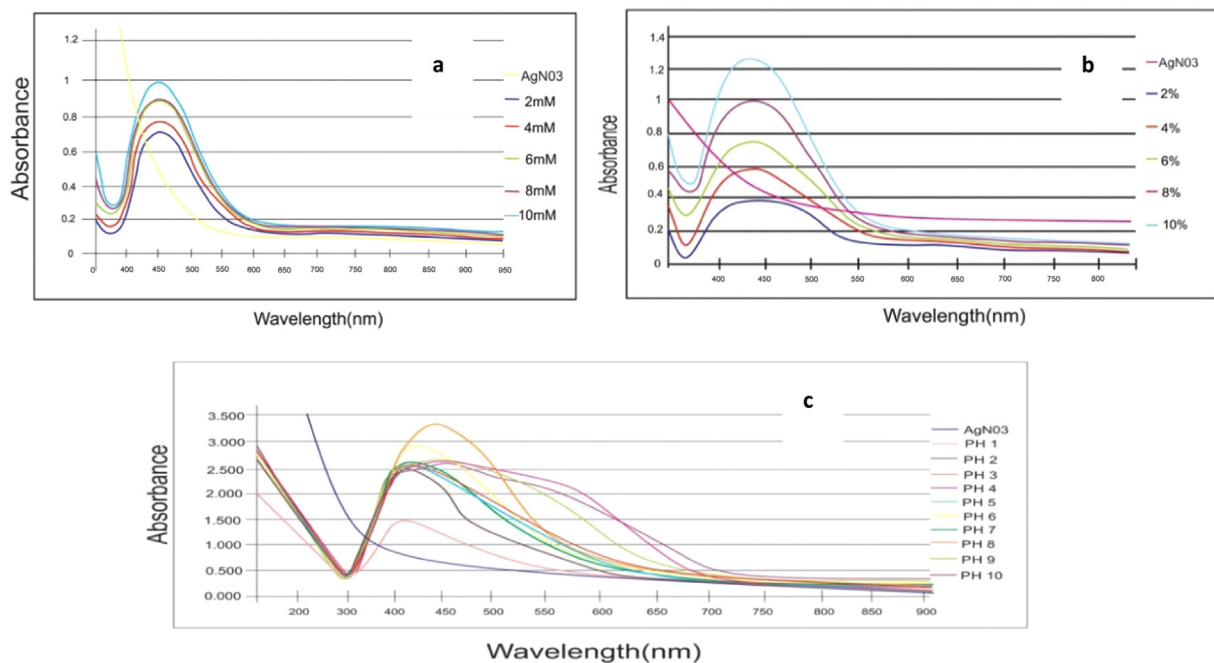
Fig. 2. UV–vis spectrum analysis/Surface Plasmon Resonance (SPR) of synthesized nanoparticles from (a) $2-10$ mM AgNO$_3$ reduced by 6% (w/v extract); (b) $2-10$% (w/v extract) reduced by 6 mM AgNO$_3$, and (c) 6 mM AgNO$_3$ reduced by 6% (w/v extract) with the pH adjusted from 1 to 10.
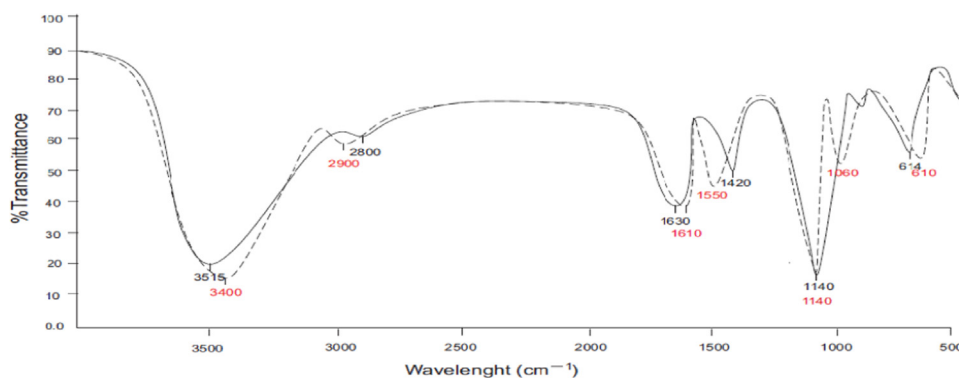

Fig. 3. FTIR analysis of (−) sulfated polysaccharide and (−) synthesized silver nanoparticles from 10 mL reaction mixture containing 6% aqueous extract (w/v), 6 mM AgNO$_3$ solution at pH8.

for inorganic sulfate group (Fig. 3(a)). The FTIR spectrum of *S. siliquosum* formed AgNPs shows peaks at 3400, 2900, 2850, 1610, 1550, 1140, 1060, and 616 cm^{-1}. Strong band at 3400 cm^{-1}. corresponding to N-H stretching vibration of primary amines, the band at 2850 cm^{-1} corresponding to C-N stretching of any R-N-N=C=S. Another band observed at 1610 cm^{-1} is attributed to the stretching vibration of (NH) C=O and indicate that a member of nitro compounds is involved in sta-bilizing the nanoparticles (Fig. 3(b)). Moreover, the appearance of new peaks at 1550 cm^{-1} confirmed that reduction has been carried out by hydroxyl groups present in the brown alga. The band at 1140 and 1060 cm^{-1} indicates the role of sulfate group in the reduction. A similar observation has been reported from *Stoechospermum marginatum* [24].

The results support the theory that the phenolic compounds and protein are essential in the formation and stabilization of nanoparticles. These functional groups may encapsulate the surface of silver ions as capping agents that stabilized the nanoparticles, but the exact mechanism involved in the formation of Ag nanoparticles is still debated. In addition, polyols such as terpenoids, flavones and polysaccharides that reduced silver and chloroaurate ions may assume the same role in the synthesis of nanoparticles from *S. siliquosum* [25,26].

The surface morphology of synthesized nanoparticles was clearly illustrated from the SEM images. The nanoparticles were roughly spherical in shape with sizes ranging from 20 nm to 480 nm (Fig. 4(a)). The size variation may be due to the presence of other organic components in the polysaccharide which are involved in reducing and stabilizing the nano-particles during their forming stage [27]. Generally, sub-micro particles with size below 1000 nm are acceptable nanometric
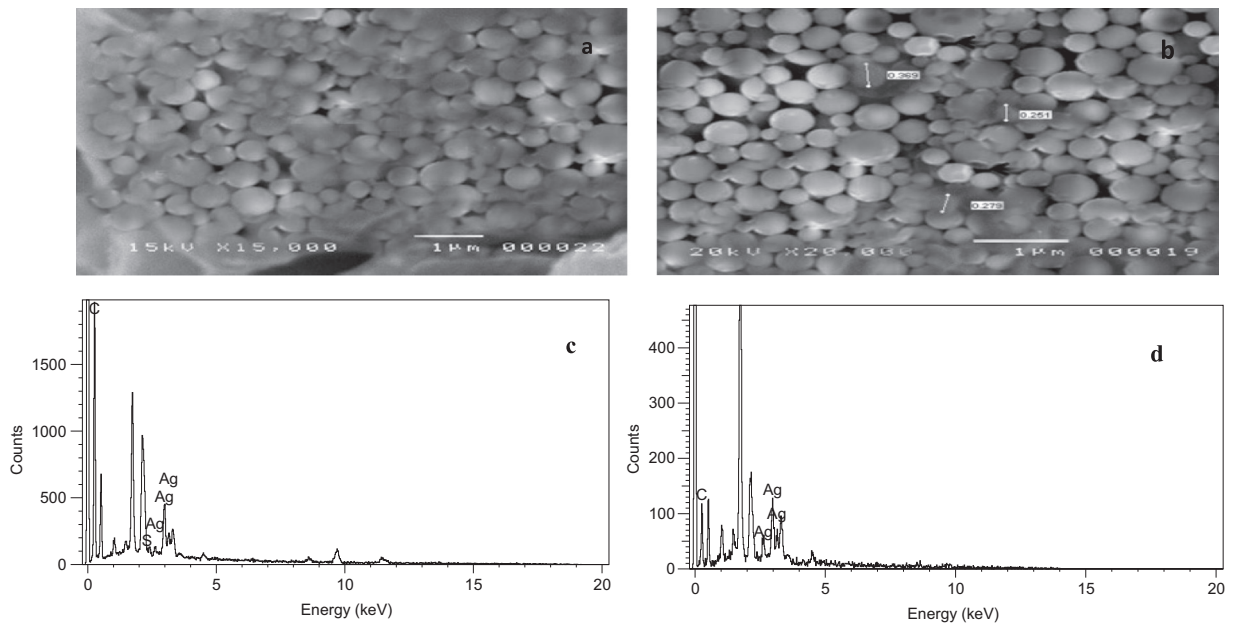
Fig. 4. SEM image and EDX spectra of synthesized nanoparticles. (a) Spherical shaped nanoparticles at 15,000 × and at (b) 20,000 ×, (c) EDX spectrum of nanoparticles after 24 h and (d) one week incubation in an amber bottle at room temperature.
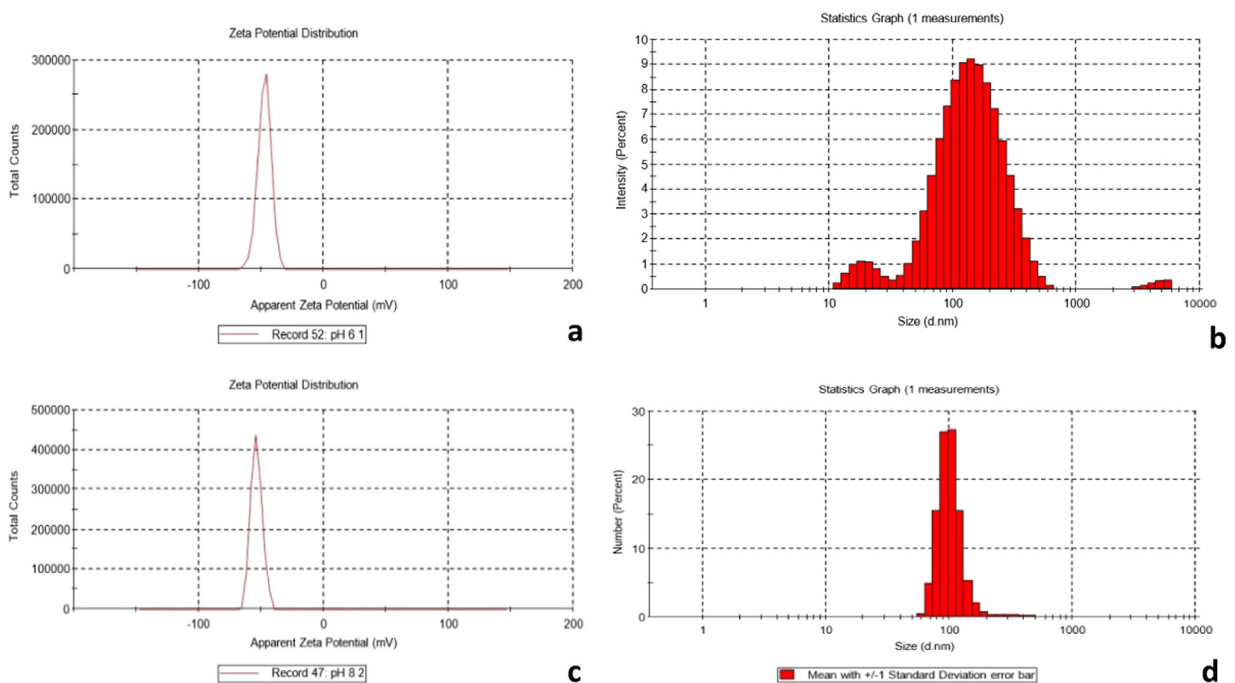

Fig. 5. Mean surface zeta potential and size distribution intensity of the silver nanoparticles synthesized from *S. siliquosum* at (a,b) pH 6 and (c,d) pH 8.

carriers used to deliver drugs or biomolecules [13]. EDX analysis revealed peaks at ∼3 keV, which confirms the presence of elemental silver in the AgNPs (Fig. 4(c) and (d)) [28,29]. Similar peaks were obtained from the AgNPs synthesized from *Artemisia nilagirica* and *Alfalfa* [30,31]. A silver concentration of 36.24% and 24.65% was recorded after 24 h and one week of incubation at room temperature. The peaks of carbon and sulfur confirm the presence of sulfated polysaccharides on the surface of AgNPs. EDX analysis provides the quantitative and qualitative status of elemental silver involved in the formation of AgNPs [32].
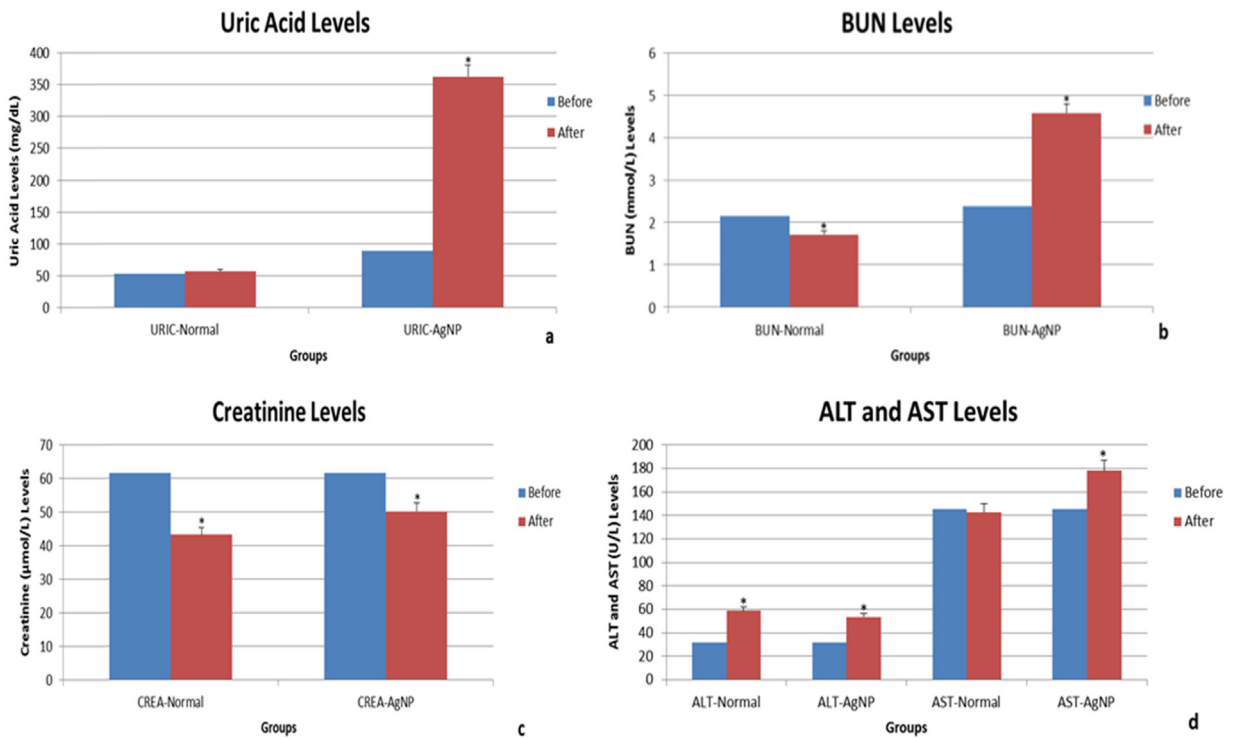
Fig. 6. Mean biochemical indices of kidney and liver of rats before and after treatment with 2000 mg/kg BW AgNPs. (a) Uric Acid; (b) BUN; (c) creatinine; (d) AST and ALT. *$p < 0.05$ significantly different after treatment. Error bars represent $\pm$ SEM.
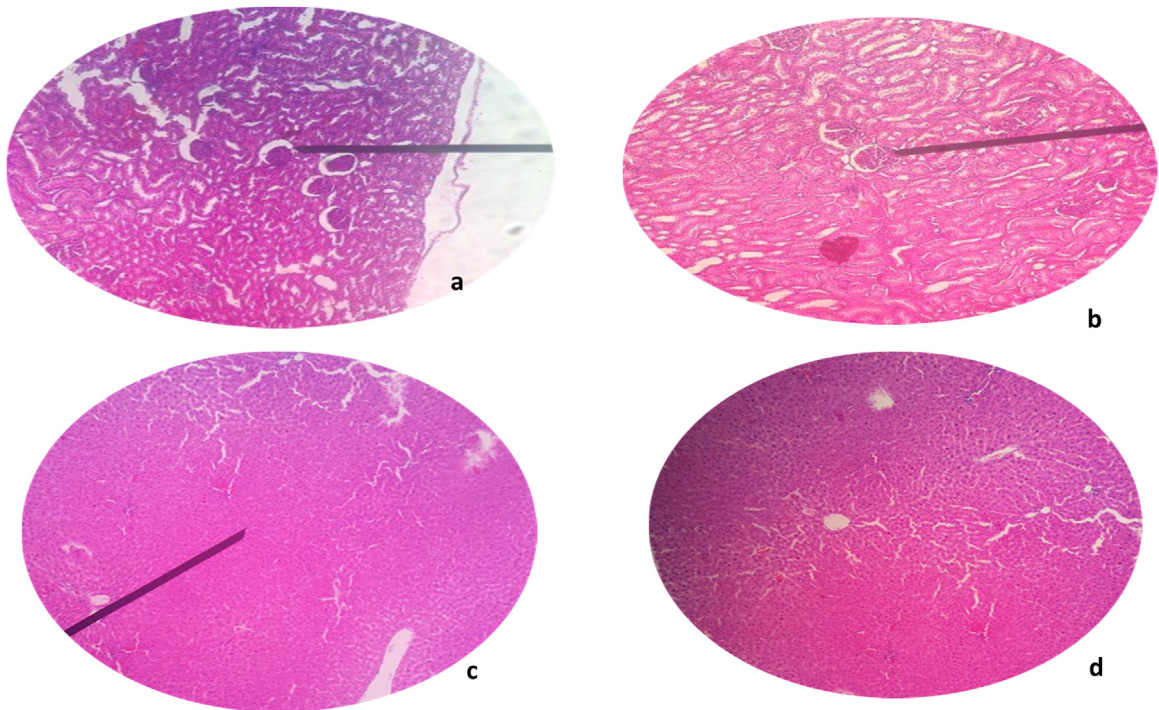

Fig. 7. Section of kidney and liver of rats. (a, b) Kidney of normal group and rats exposed to AgNPs (2000 mg/kg BW) respectively; (c, d) liver of normal group and rats exposed to AgNPS (2000 mg/kg BW).

The size obtained in DLS studies are the hydrodynamic size ((z-average diameter)) of the particles when they are dispersed in a liquid medium. Fig. 5(a) shows the mean hydrodynamic size of synthesized nanoparticles were 19.5 ± 6–132.1 ± 7.73 nm

Table 1
Effects of paracetamol, silymarin and AgNPs on hepatic marker enzymes.

Experimental groups	Aspartate aminotransferase (AST)		Alanine aminotransferase (ALT)	
	Before	After	Before	After
A. Normal	197.25 ± 1.51	216.92 ± 15.97	49.96 ± 0.92	61.35 ± 3.56
B. Paracetamol (2 g/kg BW)	197.02 ± 1.50	6846.60 ± 452.11[*a]	45.64 ± 2.49	3951.77 ± 246.07[*a]
C. AgNPs (200 mg/kg BW)	216.70 ± 15.94	389.19 ± 6.94[*]	47.23 ± 1.63	100.08 ± 11.76[*]
D. AgNPs (100 mg/kg BW)	216.84 ± 15.92	179.95 ± 10.34[*]	46.18 ± 2.42	62.98 ± 3.96
E. Silymarin (300 mg/kg BW)	212.81 ± 15.21	117.89 ± 7.94[*]	39.73 ± 0.49	62.91 ± 4.35[*]

Values are expressed as mean $\pm$ SEM for five rats per group.
Units: AST, ALT μmoles of pyruvate liberated/min/mg proteins.
Groups C, D and E were administered AgNPs and silymarin for six days respectively+a single dose of Paracetamol (2 g/kg BW) on the seventh day. The symbols (*) represents significant difference in liver indices before and after treatment, and (*a) significantly different among groups after treatment at $p < 0.05$.

and 117.21 ± 32.18–471.7 ± 189.4 nm for nanoparticles synthesized from pH 6 and pH 8 respectively. Surface zeta potential is another essential parameter for characterizing the stability of nanoparticles in aqueous environment. The surface zeta potential of the nanoparticles synthesized at pH 6 and pH 8 were 47.4 ± 5.32 mV and -50.0 ± 3.49 mV respectively. Both nanoparticles have a PDI value of more than 0.5 referring to broad distribution that results to aggregation of particles. Particles with zeta potentials greater than ± 30 mV and less than -30 mV are considered stable for colloidal dispersion in the absence of stearic stabilization [19]. Many literatures have concluded that pH plays a very important role in the formation of nanoparticles and their stability [33].

3.1. Acute toxicity study

Exposure to AgNPs (2000 mg/kg BW) did not result to any mortality, abnormal reactions or behavior in rats until the end of the experiment. Fig. 6 shows the effect of AgNPs on blood biochemical parameters BUN, creatinine, ALT and AST of rats. AgNPs significantly reduced the level of creatinine but level of BUN, uric acid, ALT and AST was significantly increased after administration of AgNPs. A significant variation in the result of kidney and liver indices of normal rats before and after the experiment was also observed. The recorded variation in liver enzyme parameters could be due to age, sex, diets and especially with the higher bone metabolism in younger one [34]. Considering the zero mortality, absence of abnormal reactions and minimal effect on kidney and liver indices, we concluded that the synthesized AgNPs possess low toxicity [35].

Fig. 7 shows the tissue sections of liver and kidney of normal and rats exposed to AgNPs. Administration of rats with AgNPs (2000 mg/kg BW) caused moderate toxicity to kidney cells. This was seen as diffuse tubular epithelial necrosis, very few atrophied glomeruli and foci of lymphoid infiltrates in the interstitium of cortex in rats (Fig. 7(b)). Kidney section of untreated rats showed normal renal organization (Fig. 7(a)). On the other hand, high dose of AgNPs did not cause toxic effect to liver cells of rats. Liver section of rats exposed to AgNPS showed normal organization of hepatocytes, intact cell membrane, without necrotic evidences analogous with the liver section of untreated group (Fig. 7(c) and (d)).

3.2. Evaluation of Hepatoprotective property

Table 1 shows the effect of AgNPS on the liver enzyme parameters of rats exposed to toxic level of paracetamol (2000 mg/kg BW). As expected, paracetamol induced significant hepatocellular damage causing a marked increase in ALT and AST levels ($p < 0.05$). Pre-treatment of rats with AgNPs significantly prevented this damaging effects of paracetamol and revived levels of ALT and AST to near normal comparable to normal and silymarin-treated groups ($p < 0.05$). Level of ALT and AST of rats pretreated with AgNPs was significantly lower than liver indices of rats treated with paracetamol alone.
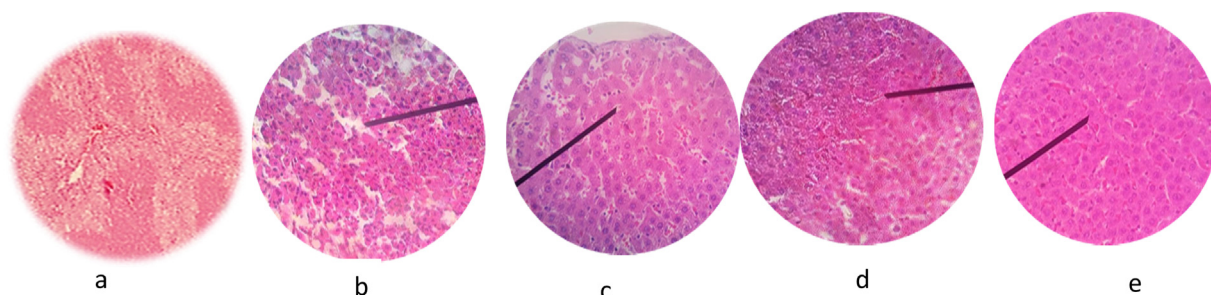

Fig. 8. Liver sections of rats. (a) Paracetamol (2000 mg/kg BW); (b) AgNPs (200 mg/kg BW); (c) AgNPs 100 mg/kg BW; (d) Silymarin (300 mg/kg BW) and (e) normal rats.

Paracetamol was chosen because of its predictable toxicity at doses higher than 150 mg/kg causes liver damage and ne-phrotoxicity in both humans and animals [36,37]. The observed elevation of liver transferases is often the first biochemical abnormality detected in most model of Paracetamol-induced hepatic injury [38,39].

Fig. 8 shows liver sections of rats treated with paracetamol, silver nanoparticles and silymarin. Paracetamol induced widespread hepatocellular necrosis, congested blood vessels, cytoplasmic vacuolation and loss of hepatocyte in-dividualization (Fig. 8(a)). On the other hand, rats treated with silver nanoparticles showed mild hepatocellular degen-eration. This shows that silver nanoparticles from *S. siliquosum* can control the damaging effects of paracetamol in liver cells (Fig. 8(b) and (c)). Silver nanoparticles synthesized rapidly using Andrographis paniculata at low dose (25 mg/kg BW) have been observed to display curing effects on CCl_4 induced liver injury [40]. However, the hepatoprotectant potential of bio-synthesized nanoparticles is not comparable to Silymarin which maintained hepatocytes in near normal and healthy condition (Fig. 8(d) and (e)).

4. Conclusion

To conclude, silver nanoparticles were synthesized from *S. siliquosum* using green chemistry. The formation of silver nanoparticles was confirmed by UV spectra, FT-IR, EDX-SEM, and zeta potential measurement studies.

The nanoparticles were effective in maintaining liver enzyme parameters to near normal in all intoxicated groups in-dicating the homeostatic effects on paracetamol induced liver injury. The observed hepatoprotective effect is quite inter-esting but the apparent toxic effect on kidney should be given attention. The toxicity study of silver nanoparticles from *S. siliquosum* is the first report on this algal species and can expand the knowledge base of very limited literature on in vivo toxicity of nanometric carriers synthesized using green chemistry.

Conflict of interest

There is no conflict of interest regarding the publication of this research article in any journal between the authors and the host institutes.

Acknowledgment

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