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Invitro inhibition of *Daboia russelii* (Shaw & Nodder) venom with alginic acid-based silver nanoparticles

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Envenomation by Russell's viper [*Daboia russelii* (Shaw & Nodder, 1797)] is a major medical emergency in tropical countries. The antivenom therapy is a conventional remedy for such medical emergency, but it has limitations and side effects. Nanomedicine and nanotechnology are the most prospective areas of research in the current scenario. In the present study, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of crude*Daboia russelli venom* (VRV) was performed. Alginic acid-based silver nanoparticles (AgNP-ALG) were synthesized and characterized using UV-Visible spectroscopy, Dynamic Light Scattering (DLS), Scanning electron microscope (SEM), and X-ray diffraction analysis (XRD). SNPs have average hydrodynamic size of 80.30 nm with 0.271 PDI. X-ray diffraction analysis of AgNP-ALG, which confirmed the cubic crystal shape of silver. SEM studies of AgNP-ALG showed particle sizes ranging from 10 to 50 nm. Spectroscopic analysis showed a decrease in the absorbance intensity of venom upon interaction with AgNP-ALG, indicating interaction with venom proteins. From the data available from fluorescence spectroscopy, it is evident that viper venom preincubated with AgNP-ALG causes quenching of fluorescence intensity. The results obtained by direct hemolytic assay,proteolytic activity and blood clotting test revealed venom action inhibition due to silver nanoparticles. Thus, in the present study we have emphasized that silver nanoparticles inhibit the action of *Daboia russelli* venom *in vitro*.

Keywords: Alginic acid, Direct hemolytic assay, Proteolytic activity, Russell's viper, Silver nanoparticle, Snakebite envenoming

Snakebite envenomation is a deleterious condition worldwide with special reference to tropical and subtropical regions[1]. Globally, an annual occurrence of 4.5-5.4 million snakebites affects people, with 1.8-2.7 million individuals developing clinical toxicity. The associated death toll varies from 81,000 to 138,000[2]. Within the Indian subcontinent, the significant venomous snake species responsible for snakebite are the Saw-scaled viper, Common Indian krait, Spectacled cobra, and Russell's viper[3]. The characteristic indications of envenomation resulting from the bite of Russell's viper (*Daboia russelli*) encompass coagulopathy, local muscle damage, nephrotoxicity, and neurotoxicity[4]. Snake venom typically consists of components such as phospholipases A_2, metalloproteases, serine proteases, L-amino acid oxidases, phosphodiesterases, hyaluronidase, acetylcholinesterases, nucleases, and disintegrins[5]. Inspite of the presence of a host of enzymes and toxins, PLA2 attributes to induce neurotoxicity, coagulopathy, haemorrhage and other clinical conditions[6]. Treatment with antivenom is the only specific therapy but suffers from limitations. However, usefulness of immunotherapy is bounded by many factors such as the inappropriate hindrance in its administration, its availability and other side-effects that cause problems[7]. Alginic acid is a naturally occurring hydrophilic colloidal polysaccharide procured from the widespread species of brown seaweed (*Phaeophyceae*). It is a linear copolymer comprising primarily of residues of 1,4-linked D-mannuronic acid and 1,4 linked L-glucuronic acid. Contemporary experimental investigation has demonstrated that alginic acid has anti-anaphylactic action, immunomodulatory effect, antioxidant properties and anti-inflammatory effect[8].

Nanoscience and nanotechnology are swiftly advancing domains that hold substantial promise for the creation of innovative therapeutic mediators, clinical tools and gadgets in the arena of biomedicine. Silver nanoparticles emerging from the realm of nanotechnology stand out as a valuable contribution to humanity, particularly capturing the interest of

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pharmaceutical industries[9]. Earlier investigation reported interaction of bovine serum albumin with silver nanoparticles with hydrophobic interactions[10]. A substantial study has been conducted to emphasize the interaction between nanoparticles and proteins to develop protein-nanoparticle complex which could be used therapeutically in pharmaceutical industry. In another such study, synthetic polymer nanoparticles were designed and named as plastic antidote, which can neutralize the bee venom *in vivo*[11]. Gomes *et al.*[12] discussed the effectiveness of neutralization of venom using gold nanoparticles combined with 2-hydroxy 4 methoxy benzoic acid (HMBA) derived from the root of *Hemidesmus indicus*. This study corroborates the functionality of GNP-HBMA for neutralization of viper venom with enhanced protection. Present investigation explores the antisnake venom activity of Alginic acid-based silver nanoparticles in *in vitro* direct hemolytic assay, proteolytic activity, blood clotting test and the interaction of venom with silver nanoparticles was explored in ultraviolet visible spectroscopy analysis and fluorescence spectroscopic study.

Materials & Methods

Collection of venom samples

Freeze-dried venom powder of Russell's viper was commercially purchased from the Irula Snake Catcher Cooperative Society, Kancheepuram, Chennai and maintained at 4°C. The dry weight of the venom was used (mg/mL, w/v). 0.9 % *w/v* saline was used to dissolve the venom and it was further spinned at 3000 rpm for 20 min, before collecting the supernatant.

Chemicals

Alginic acid from brown algae (*Macrocystis pyrifera*) and silver nitrate (AgNO3) were obtained from Sigma-Aldrich Chemicals, USA while other chemicals were procured from Merck. Deionized water, was used for the formulation of all buffers.

Sodium Dodecyl Sulphate- Polyacrylamide gel electrophoresis (SDS PAGE) of venom

SDS-PAGE was conducted according to Laemmli, 1970. The crude venom (5 mg) was dissolved in sample buffer and loaded on a 12% gel, where electrophoresis was carried out under reducing conditions at 60 V for a period of 2 hours. Coomassie Brilliant Blue R-250 was used for protein visualization and the analysis of protein bands were performed using the Bio-Rad Gel Doc EZ Gel Imaging System.

Synthesis and characterization of Alginic acid-based silver nanoparticles

Synthesis of silver nanoparticles

Alginic acid was weighed and treated with distilled water and expressed at concentration 20 mg/mL. The concentration of Alginic acid to metal ion were varied at concentration (10:1,20:1.40:1,60:1), respectively. One mM AgNo3 was optimal for the for the synthesis[13]. Alginic acid-based silver nanoparticles (AgNP-ALG) were prepared by mixing 200 µL of 1 mM of $AgNO_3$ and 9.8 mL Alginic acid solution (pH 10). The mixture was subjected to heating at 90°C with stirring at 500 rpm and an observable transformation of the colour of the solution to dark brown provided a visual confirmation of nanoparticle formation.

pH *for formation of* AgNP-ALG

pH was varied from 5 to 10 with a difference of 1 to estimate the optimal pH of AgNP-ALG.

Effect of concentration of AgNo3

$AgNO_3$ @0.5-4 mM concentration were studied. Concentration of $AgNO_3$ for synthesis of AgNP was selected by UV–vis absorption spectroscopy.

Optimum temperature for the formation of AgNP-ALG

The temperature was varied from 20 to 80°C with a difference of 10°C to see the effect on the formation of AgNP-ALG.

Characterization of Alginic acid-based silver nanoparticles

Ultraviolet–visible (UV–Vis)absorbance spectroscopy analysis

The synthesis of silver nanoparticles was corroborated by UV-Visible spectrophotometer. 1mL of sample was tested after 4 h. The optical density (OD) was derived over a wide spectrum of wavelengths from 300 to 700 nm with the help of a UV–visible spectrophotometer (Shimadzu1800, Kyoto, Japan) and the graph was devised on the basis of the OD readings by automated software UV Probe.

Particle size and zeta potential analysis

The particle size and size distribution (polydispersity index, PDI) were determined by dynamic light scattering using a Malvern Instruments ZS Zeta sizer Nanoseries, Malvern instrument Nano Zs 90.

Fourier-transform infrared analysis

AgNPs were air dried and pelleted with potassium bromide (KBr) in the ratio of 1:10 and provided for FTIR spectroscopic measurement using FT-IR spectrometer (Nicolet IS5, Thermo Scientific).

X-ray powder diffraction studies

The XRD analysis was conducted to evaluate the crystalline nature of AgNP-ALG. A thin film of AgNP-ALG was set up on a glass plate and provided for X-ray diffraction studies using a powder X-ray diffractometer. ("X" Pert PRO PAN alytical, Netherlands).

Thermogravimetric analysis (*TGA*)

The thermal analysis (TGA) of the biosynthesized silver nanoparticles was conducted by using a thermal system DT-60H thermal analyser from Perkin Elmer. The TGA-DTA thermograms were acquired in the 30-900°C temperature range under nitrogen atmosphere at the rate of 10°C/min.

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDAX) analysis of AgNP-ALG

The AgNPs were drop coated on an aluminium foil and dried. The structure and the dimension of nanoparticles were viewed using SEM (Oxford INCA Penta FETX3). The elemental composition of AgNP-ALG were studied using with EDAX.

Venom inhibition study of *Daboia russelli venom:*

UV-Visible spectroscopic study

The absorbance maxima for viper venom and venom incubated with alginic acid and silver nanoparticles were estimated on Shimadzu UV-VIS Spectrophotometer in a wavelength range of 200-600 nm. Path length was set at 10 mm[14].

Fluorescence spectroscopic study

Fluorescence spectroscopy involves analyzing fluorescence signals emitted by fluorescent molecules or fluorophores. This dynamic technique is employed to study molecular-level interactions and is widely utilized in molecular interactions study, such as protein-protein, protein-ligand, and protein-nanoparticle interactions. The intrinsic fluorescence characteristics of crude viper venom and venom incubated with Alginicacid andAlginic acid-based silver nanoparticles (AgNP-ALG) were recorded on fluorescence spectrophotometer (Hitachi, F-7000) by using quartz cuvette. Before the experiment, excitation wavelength (280 nm), emission wavelength (281 nm to 600 nm), slit width (10 nm), and PMT voltage (600 V) were set[10].

Direct hemolytic assay and neutralization studies

Direct hemolytic assay induced by *Daboia russelli* venom and subsequent neutralization studies were carried out *in-vitro* by using the Red blood cell (RBC) assayas described by Oguiura *et al*;2011[15]**.** Goat blood was collected from a local slaughter house and ethylene diaminetetraacetic acid (EDTA) was added to prevent coagulation. Following this, 5 mL of whole blood was gently spinnedat 900 rpm for 10 minutes. The clear supernatant was discarded following centrifugation and the resultant pellet was washed thrice with physiological saline solution (0.9% *w/v*) and reconstituted in the same. Next, 0.5 mL of reconstituted RBC was mixed with 5 mL phosphate buffer solution (pH 7.4) mixture and used as control. For 100% haemolysis, 0.5 mL of reconstituted RBC was mixed with 5 mL of double distilled water. For the test samples, 5mL of VRV /Alginic acid / AgNP-ALGwas mixed with 0.5mL of reconstituted RBC. All the sample aliquots were incubated at 37°C for 1hr and then centrifugedfor 20 minutes at 2000 rpm. The supernatants thus obtained were taken in fresh tubes for measuring the OD at 540nm in an Ultraviolet-visible spectrophotometer. Distilled water was used as blank control. The haemolysis was estimated by the formula

$$\frac{\text{Experimental sample} - \text{Control sample}}{\text{100\% hemolysis (RBC + D.Water)}} \times 100$$

Proteolytic activity

Anti-proteolytic potential of the alginic acid-based silver nanoparticles (AgNP-ALG) was investigated by the protocol adopted in earlier studies by Tan *et al*.[16]. The methodology was modified and employed in the existing study to compute the proteolytic activity of *Daboia russelli* venom (VRV). Two mL of 1% casein suspended in 0.25 M sodium phosphate buffer (pH 7.75) and 0.1 mL of venom (2 µg) in physiologic saline were mixed and incubated for 1 h at 37°C. The undigested casein was precipitated and the reaction was stopped by addition of 2 mL of 5% trichloroacetic acid. A centrifugation of the sample was carried at 10,000 rpm for 10 min, the absorbance of the supernatant was determined at 280 nm in a UV spectrometer. One unit of proteolytic activity was defined as the increase of 0.001 absorbance units at 280 nm per hour. Anti-proteolytic activity Alginic acid /AgNP-ALG was also evaluated against *Daboia russelli* venom. All experiments were performed thrice and the results presented as average values with standard errors.

Pro-coagulant activity

The pro-coagulant activity of alginic/alginic acid-based silver nanoparticles (AgNP-ALG) was evaluated following the method described in an earlier study by Theakston & Reid[17]. The VRV was

dissolved in 100 µLphosphate buffer solution (pH 7.2) before adding citrated mice blood to the solution followed by incubation at 37°C. The coagulation time was calculated by establishing the Minimum Coagulant Dose (MCD) of venom *i. e.*, the least dose of venom which initiated RBC coagulation within 60 s. To evaluate the pro-coagulant activity, Alginic /AgNP-ALG was incubated for 30minutes along with the MCD concentration of venom. A mixture of 0.1 mL of phosphate buffer along with 0.3 mL of citrated blood was used as control and the clotting time was estimated.

Statistical analysis

Results are shown as Mean ± SD. Results were analyzed using one way ANOVA. The difference was given as statistically significant at P <0.05 are compared to venom control.

Results and Discussion

pH of synthesized alginic acid-based silver nanoparticles (AgNP-ALG)

The optimum concentration of alginic acid for nanoparticle synthesis was 20 mg/mL. The pH of the solution medium influences the size and texture of the synthesized nanoparticle. In the present study, we have observed that AgNP-ALG was optimally synthesized at pH 10. The optimized solution of nanoparticles was kept in the dark for 60 days at 8±2°C and the stability of the synthesized nanoparticle was determined by UV-spectral analysis. An earlier report has shown that an alkaline environment is necessary for AgNP synthesis which corroborates our present findings[18].

SDS polyacrylamide gel electrophoresis (SDS-PAGE) of venom

SDS-PAGE was used to characterize of *Daboia russelli* venom proteins using Gel documentation system. Seven distinct bands were observed in the 135 kDa region and in 75, 63,48,35, 25 and 20 kDa (Fig. 1). In similar study, eight protein bands (six major bands and two minor bands) were examined on SDS-PAGE in reducing condition subsequent to staining with Coomassie brilliant blue[19].

Synthesis and characterization of alginic acid-based silver nanoparticles

Optical analysis

When Alginic acid was added to silver nitrate solution, followed by pH adjustment and heating, the colour of the reaction instantaneously changed from colourless to brownish. The intensity of the brown

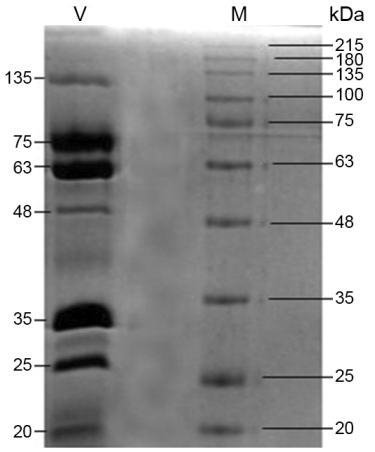

Fig. 1 — SDS- polyacrylamide gel electrophoresis (SDS- PAGE) of *Daboia russelli* venom, [V: *Daboia russelli* venom sample; M: molecular mass markers (kDa)]

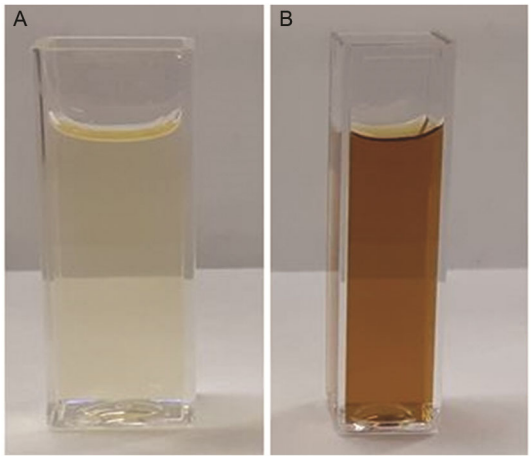

Fig. 2 — Optical analysis of colour alteration in (A) Alginic acid; and (B) Alginic acid-based silver nanoparticles (AgNP-ALG).

colour augmented quickly and there after remained stable for the subsequent three hours (Fig. 2). It is well documented that AgNP colloids possess brown coloration due to their distinctive excitation of surface plasmon in the range of 400-490 nm[20]. This transition of the solution from colourless to brown designates the formation of AgNPs[21].

Ultraviolet–visible (UV–Vis)absorbance spectroscopy analysis

The UV–Vis spectra of the synthesized alginic acid-based silver nanoparticles (AgNP-ALG) demonstrated the maximum peaks at 420 nm, as shown in Fig. 3. In a previous study, silver nanoparticles synthesized by *Allium cepa* extract exhibited surface plasmon resonance peak at 438 nm[9]. The colour of the reaction mixture started changing to yellowish brown after 3 h, indicating the generation of silver nanoparticles, due to the reduction of silver

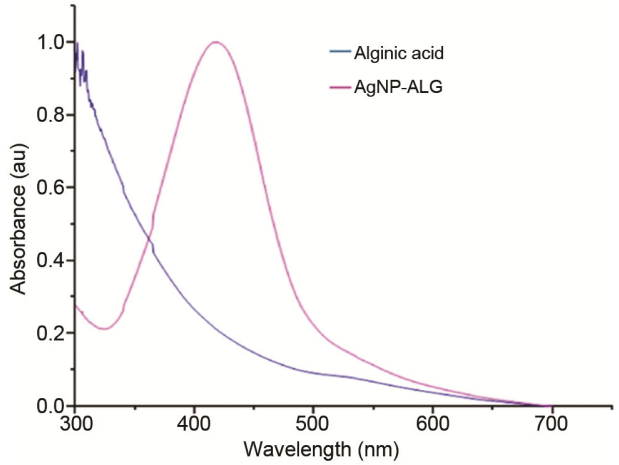

Fig.3 — UV-Visible absorption spectra of silver nanoparticles

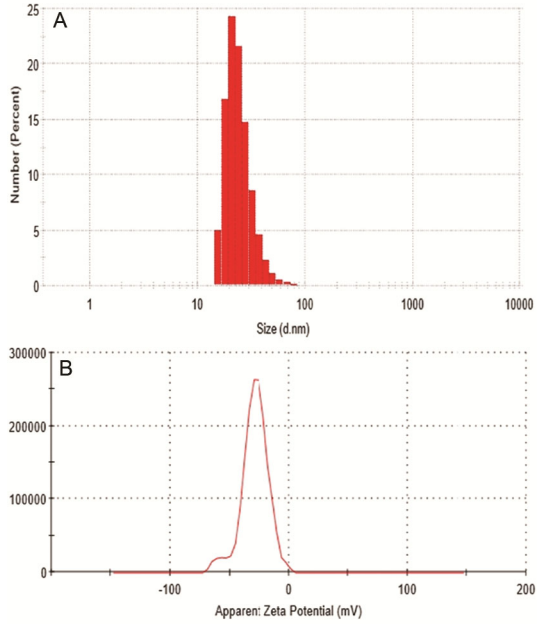

Fig 4. — (A) Dynamic light scattering (DLS) pattern and (B) zeta potential distribution of alginic acid-based silver nanoparticles (AgNP-ALG).

metal ions Ag^{+} into silver nanoparticles Ag^{0}*via* the active molecules present in alginic acid. This color is attributed to the excitation of surface plasmon resonance (SPR). As shown in Fig.3, a characteristic and well-defined SPR band for silver nanoparticles was obtained at around λ max 420 nm.

Particle size and zeta potential analysis

The hydrodynamic diameter of AgNP-ALG was found 80.30 nm with Polydispersity Index 0.271. The zeta potential of AgNP-ALG was found -28.5 mV (Fig. 4 A & B). Dynamic Light Scattering (DLS) is applied to establish the surface distribution outline of small particles in suspension, and it quantifies its hydrodynamic diameter, thereby estimating the fluctuation of scattered light intensity of small sized particles[22]. Number of peaks of particle size characterizes its monodispersive or polydispersive character. In the current investigation, low poly-dispersity index corroborates the development of monodispersive AgNP-ALG. A high positive or a high negative zeta potential induces repulsion force between the particles, which makes the particle more stable. In the present study, zeta potential of silver nanoparticle was to be found -28.5 mV. A negative potential value supports long term stability, good colloidal nature and high dispersity of AgNPs due to negative-negative repulsion.

Fourier-transform infrared (FTIR) analysis

The FTIR analysis was performed to confirm the involvement bio-molecules associated with the synthesis of nanoparticles. FTIR spectra showed several characteristic peaks at 3424 cm^{-1} which signified the presence of hydroxyl ($^{-}$OH) group. Peak at 2918 cm^{-1} signified the presence of C-H stretching (alkane), peak at 2162 cm^{-1} signified the presence of C≡C bending (alkyne), peak at 1621 cm^{-1} signified the presence of C-H stretching (aromatic compound), peak at 1413 cm^{-1}signified the presence ofS=O stretching (sulphate), peak at 1028 cm^{-1}signified the presence of C-F stretching (fluro compound) ,peak at 621 cm^{-1}signified the presence of C-I stretching (halo compound) (Fig. 5A).Comparable FTIR spectra for polysaccharide-catalyzed nanoparticles were achieved for green alga extract from *Botryococcus braunii*[23].

X-ray powder diffraction studies

X-ray diffraction (XRD) spectra was recorded to verify the crystallinity of obtained Alginic acid-based silver nanoparticles (AgNP-ALG).X-ray diffraction pattern of AgNP-ALG had Bragg's reflections of (111), (200), (220), (311), (222), (331), (420) and (422) planes, which confirms the cubic crystal structure of silver (Fig. 5B).The synthesis of silver nanoparticles with sharp bands of Bragg peaks which implied stabilization of the synthesized nanoparticles by the reducing agents[24].

Thermogravimetric analysis (TGA)

The thermal behaviour or thermal stability of synthesized silver nanoparticles was investigated *via* TGA analysis at high temperatures. The TGA and DTA spectra that are shown in Fig. 5C. in the temperature range of 30-900°C were recorded on

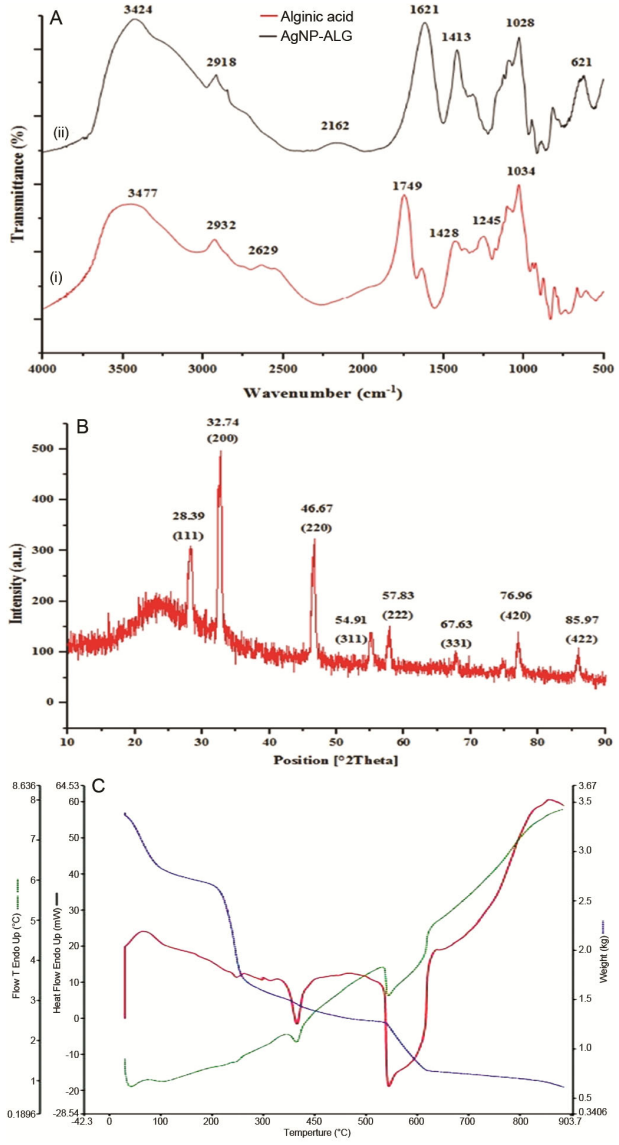

Fig.5 — (A) Fourier transform-infrared (FT-IR); (B) X-ray diffraction; and (C) Thermogravimetric analysis of (i) Alginic acid (ALG) and (ii) Alginic acid-based silver nanoparticles (AgNP-ALG)

thermal system under nitrogen atmosphere at a heating rate of 10°C/min. According to the TGA curve, the study weight loss occurred in the temperature range of 200-650°C. In TGA studies exhibited weight loss at 200,300, 550, and 650°C. The first slight weight loss up at 200°C was due to physically adsorbed water molecules on the surface of the silver nanoparticles. The major weight loss occurred up to 350°C and was due to the decomposition and evaporation of organic substance on the surfaces of the silver nanoparticles as surface stabilizing agents.

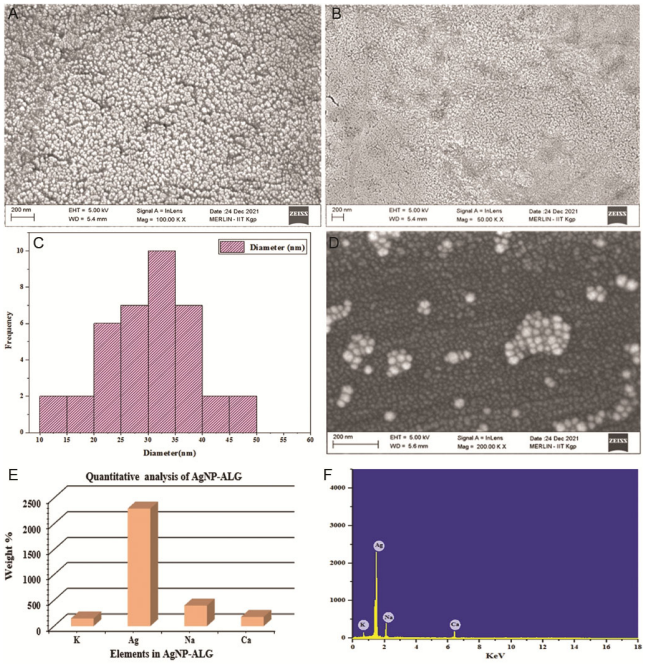

Fig. 6 — SEM images of alginic acid-based silver nanoparticles (AgNP-ALG). (A) image acquired at a magnification of 50 KX; (B) image acquired at a magnification of 100 KX; (C) image acquired at a magnification of 200 KX; (D) Particle size distribution from SEM; and (E & F) Energy dispersive X-ray analysis (EDAX) of alginic acid-based silver nanoparticles (AgNP-ALG) for elemental analysis

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDAX) analysis of AgNP-ALG

The SEM studies of AgNP-ALG exhibited a particle size ranging from 10 to 50 NM. The EDAX analysis provided elemental analysis and specified the amount of Ag (in percentage) which was denoted by the sharp peaks in the resultant graph (Fig. 6). SEM is a surface imaging technique competent of resolve different particle sizes, size distributions and surface morphology of synthesized nanoparticles[25].

Venom inhibition study of *Daboia russelli* venom

UV-Visible spectroscopic study

The crude viper venom displayed absorbance maxima at 278 nm, a characteristic attribute indicative of the presence of proteins. Typically, proteins exhibit absorbance maxima within the range of 275 nm to 280 nm in the UV spectrum. The absorption in the UV range is primarily attributed to the content of tyrosine (Tyr) and tryptophan (Trp) in proteins, along with a smaller contribution from phenylalanine (Phe) and disulfide bonds. The λ max of viper venom (278 nm) was seen to be shifted towards lower wavelength (269 nm–265 nm) alongside increasing absorbance intensity upon incubation with alginic acid and alginic

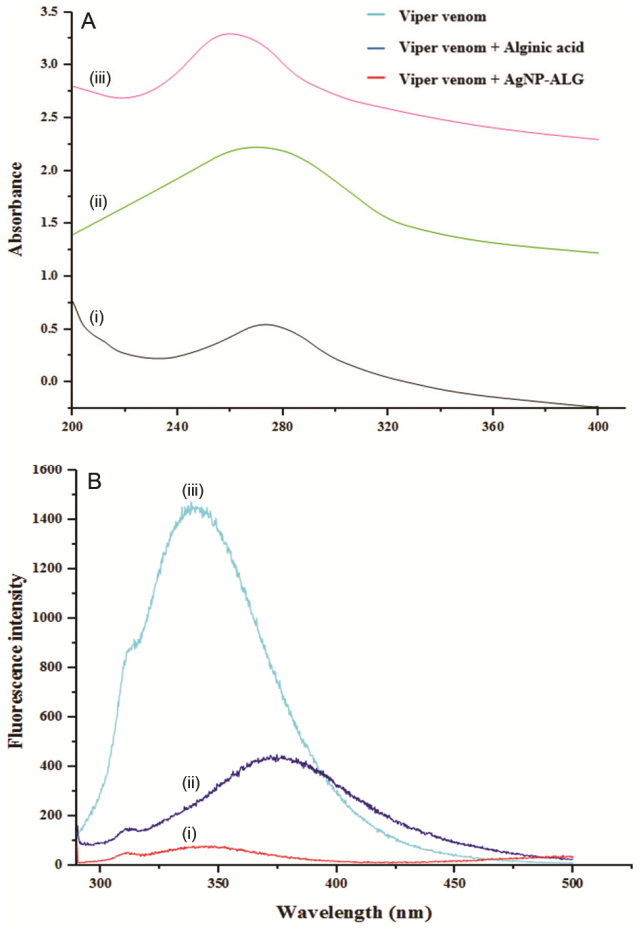

Fig. 7— (A) UV Visible absorbance spectra; and (B) Fluorescence spectra of (i) *Daboia russellii* venom; , (ii) *Daboia russellii* venom in presence of Alginic acid; and (iii) *Daboia russellii* venom in the presence of alginic acid-based silver nanoparticles (AgNP-ALG).

acid-based silver nanoparticles (AgNP-ALG). This signifies the interaction between silver nanoparticle and venom protein (Fig. 7A).Similar findings were reported by Jaiswal & Dongre[26]. They studied biophysical interactions between silver nanoparticle-albumin interface and curcumin.

Fluorescence spectroscopic study

Fluorescence spectroscopy is utilized for analysing fluorescence signals emanating from fluorescent molecules or fluorophores, offering a dynamic approach to studying interfaces at the molecular level. This technique is broadly employed in the investigation of various interactions, including those involving protein-protein, protein-ligand, and protein-nanoparticle interactions. In a current study, we have studied the interactions between venom proteins (crude venom) and silver nanoparticles. The crude

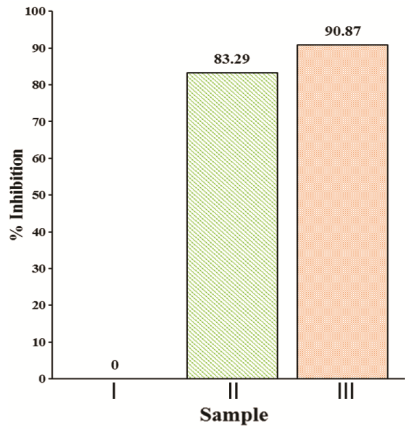

Fig. 8 — Percent inhibition of direct hemolysis with alginic acid and alginic acid-based silver nanoparticles (AgNP-ALG)

Table 1 — Direct hemolytic activity (*Daboia russelli* venom)

OD of hemolysis DRV (1 µg)	OD of Control (RBC + PBS)	OD of RBC + D. Water (100% hemolysis)	% of Hemolysis
3.562	0.04	3.675	95.83

viper venom exhibited a peak emission at 349 nm with heightened intensity, which quenched once upon interaction with silver nanoparticles (Fig. 7B). Quenching is reported as the dimunition in the fluorescence intensity of a fluorophore in the attendance of a quencher. It is influenced by various molecular interactions between the quencher (silver nanoparticles) and the fluorophore (crude venom), involving processes such as excited state reactions, molecular rearrangements, energy transfer and molecular collisions. In this context, we suggest that the interaction between silver nanoparticles and venom led to the formation of a ground state complex. Hingane *et al.*[27] examined a quenching in fluorescence intensity when Russell's viper venom interacted with silver nanoparticles, using fluorescence spectroscopy.

Direct hemolytic assay and neutralization studies

Direct hemolytic activity of venom

In the studies on direct hemolysis of *Daboia russellii* venom, the crude venom could lyse the RBC's and the percentage of hemolysis was found to be 95.83%. RBC + distilled water served as 100% hemolysis and RBC + PBS served as control. (Table 1) (Fig. 8).

Direct hemolysis neutralization assay

In neutralization assay, it was observed that the alginic acid, when incubated with venom, provided 83.29% protection whereas when the venom was incubated with AgNP-ALG, it provided 90.87%

protection against venom mediated haemolysis (Table 2).

Neutralization of Proteolytic activity:

Alginic acid-based silver nanoparticles could effectively neutralize the proteolytic activity of the *Daboia russellii* venom at its initial proteolytic dose 2 µg and DRV produced303 units of proteolytic activity.Alginic acid-based silver nanoparticles could effectively neutralize the proteolytic activity of the *Daboia russellii* venom at its initial proteolytic dose 2 µg and DRV produced303 units of proteolytic activity.Alginic acid provided protection against proteolytic activity of DRV. Additionally, it was observed that AgNP-ALG substantially neutralized the DRV-induced proteolytic activity(Table 3).

The results obtained support earlier studies, proteolytic and hemorrhagic activities were inhibited by ethyl acetate extract of *Eclipta prostrata* against Malayan pit viper venom[28]. Alginic acid-based silver nanoparticles also reported antiproteolytic action and has the capacity to synergistically function against the proteolytic action of *Daboia russellii* venom.

Pro-coagulant activity:

Normal coagulation was produced when blood was mixed with PBS and $CaCl_2$. *Daboia russellii* venom increased coagulation time. Alginic acid and Alginic acid-based silver nanoparticles (AgNP-ALG) provided significant protection against VRV mediated procoagulant activity (Table 4 and Fig. 9). In neutralization studies, no clot formation appeared.

Snake envenomation attributes to significant death globally every year[1]. Various investigations have been used to interpret the mechanism of action of different venom antidotes. The present advances in the field of protein-nanoparticle interactions, nanoparticle is conceived to interact with venom. The present study, thus investigates the interface between crude venom and silver nanoparticles employing different biophysical methods. Fluorescence spectroscopy deals with fluorescence signals emanating from flourescent molecules. It is dynamic technique to study the interactions at molecular level. It is being extensively used to study the protein-protein, protein-ligand, protein-nanoparticles interactions. We have analysed interactions of venom proteins (crude venom) with AgNP-ALG. Other bio-macromolecules present in venom like lipids and saccharides are essentially non-fluorescent. Proteins exhibits intrinsic fluorescence because of the presence of three amino acids viz. phenylalanine, tyrosine and tryptophan. Out of which tyrosine and tryptophan has high quantum yield and thus can give good fluorescence signals. Both amino acids get excited at 280 nm wavelength. Since these amino acids are very sensitive to change in microenvironment, they can be used to study the change in conformation of proteins, denaturation, substrate binding and ligand binding with protein. Enzymatic toxins present in snake venom like phospholipase A2, acetylcholinesterase, proteases, vipoxinetc contain tyrosine and tryptophan residues[29].

In the present study, the crude viper venom exhibited a peak emission at 349 nm with heightened intensity which quenched once upon interaction with silver nanoparticles. Earlier, the relative intrinsic fluorescence intensities of *Crotalus durissus* venom was monitored and detected the quenching in

Table 3 — Antiproteolytic activity of alginic acid and alginic acid-based silver nanoparticles (AgNP-ALG)

Groups	Proteolytic activity (Unit)	% inhibition of proteolytic activity
DRV	303±0.1*	-
DRV + Alginic acid	134±0.05*	55±0.05
DRV + AgNP-ALG	67±0.03**	77±0.07

[DRV, *Daboia russellii* venom. Results are expressed as mean± SE. **P* <0.05 between DRV group and DRV + Alginic acid group. ***P* <0.05 between DRV + Alginic acid group and group of DRV + AgNP-ALG]

Table 4 — Effect Alginic acid-based silver nanoparticles (AgNP-ALG) on neutralization of pro coagulant activity

Group	Time for formation of clots (s)
Control (PBS 7.4 + $CaCl_2$ 0.2 M)	44±0.26
DRV (20 µg)	211±0.87
DRV + Alginic acid (20 mg/mL)	37±0.01
DRV (20 µg)+AgNP-ALG (3 mg/mL)	18±0.03

[DRV, *Daboia russellii* venom]

Table 2 — Inhibition of hemolytic activity of venom by alginic acid and alginic acid-based silver nanoparticles (AgNP-ALG)

OD of hemolysis	OD of Control (RBC + PBS)	OD of RBC + D. Water (100% Hemolysis)	% of Hemolysis	% Inhibition
Daboia russellii venom (DRV) (1 µg) + Alginic acid (20 mg/mL)				
0.501	0.04	3.675	12.54	83.29
Daboia russellii venom (DRV) (1 µg) + AgNP-ALG (3 mg/mL)				
0.222	0.04	3.675	4.95	90.87

[AgNP-ALG, = Alginic acid-based silver nanoparticles; D. Water, Distilled Water; OD, Optical density; PBS, Phosphate buffer solution]

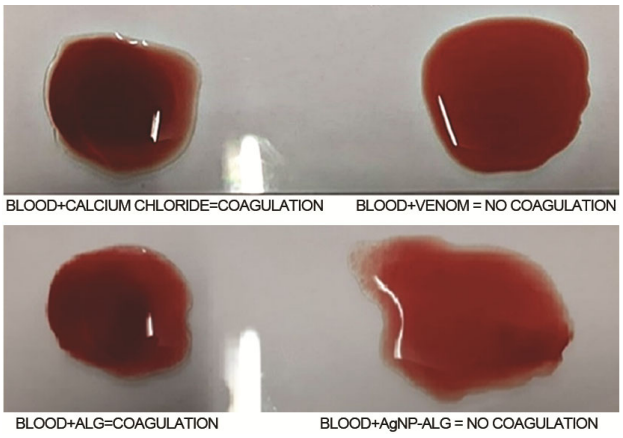

Fig. 9 — Effect of alginic acid and alginic acid-based silver nanoparticles (AgNP-ALG) on neutralization of pro coagulant activity in comparison to control

florescence intensity when venom interacted with the Quercetin with the assistance of fluorescence spectroscopy[30]. In the UV-Vis studies with crude venom, theλ max of viper venom was 278 nm which was transferred to a lower wavelength (269-265 nm) upon incubation with Alginic acid or AgNP-ALG. Absorbance by protein in UV range is principally owing to its tyrosine (Tyr) and tryptophan (Trp) content along with phenylalanine (Phe) and disulfide bonds to small extent. It is responsive to the adjustment in the microenvironment of these amino acids or conformational transformation in polypeptide. This shift in spectra was result of increase in a polarity of solvent. This is indicative of interaction between silver nanoparticle and venom protein. This interaction might have resulted in conformational changes of venom proteins with an increase in polarity of solvent, shifting amino acids in the hydrophobic environment of the protein silver nanoparticle formed a ground state complex with the venom proteins. Comparable results were provided earlier[10,29]. Zeta potential is the potential difference existing between the particle surfaces and dispersing liquid. Most of the proteins and polypeptides (phospholipase A2, proteases, etc.) present in venom form complexes (homo/ heterodimers).

Venom has strong adsorption on negatively charged nanoparticle and change its alpha helical structure. Destruction of venom protein structure would result in suppression of activity of venom proteins. Nanoparticles bear negative charges; higher negative charge will cause more repulsion force between the particles along with more stability. Venom protein emits fluorescence when excited at its maximum absorbance. After conjugation with nanoparticles, they show fluorescence quenching activity[31]. The foremost physiological manifestation of envenomation encompasses neurotoxicity that produces paralysis, haemotoxicity which comprises of toxins that forms the basis of coagulation aberrations (including hemorrhagic and haemolytic toxins), myolysis ensuing muscle degeneration and resultant renal malfunction, and a direct nephrotoxic effect[32]. Haemolysis represents the most commonly employed initial toxicity assessment.Of the diverse venom components, there are only a small number of that are of significance to this study. Among these are lipase enzymes, such as phospholipases, which act to cleave phospholipids. The vital component is phospholipases found in snake venoms include phospholipase As and phospholipase B, with a particular report of phospholipase C being found in *Bothrops alternates* venom[33]. Proteolytic activities leading to degradation of protein structures e.g., basement membranes of blood vessel and extracellular matrix components. Because the hemorrhagic activity of snake venoms has been ascribed to the presence of hemorrhagic metalloproteinases[34].

In the present study, we have investigated the direct haemolytic and proteolytic activity of *Daboia russelli* venom. The protection offered by Alginic acid against proteolytic activity of venom was up to 55%. AgNP-ALG on the other hand was also observed to significantly neutralize the VRV induced proteolytic activityproviding upto 77% inhibition of the proteolytic action. Alginic acid-based silver nanoparticles exhibit antagonistic potential against proteolytic activities of *Daboia russelli* venom could be considered significantly strong, as shown in Fig. 8. This could be due to the neutralization of snake-venom metalloproteases and hemorrhagic toxins of *Daboia russelli* venom by alginic acid and Alginic acid-based silver nanoparticles (AgNP-ALG). Silver nanoparticles should bind close to the enzyme's catalytic zinc, causing direct blockage. The results obtained here support other previous studies showing that when the zinc atom of hemorrhagic metalloproteinases was removed or chelated, their proteolytic and hemorrhagic activities were inhibited[35].

Alginic acid andalginic acid-based silver nanoparticles (AgNP-ALG) provided significant protection against DRV mediated procoagulant activity in the present study. Venom induced

consumption coagulopathy (VICC) is a pivotal systemic condition that arises due to snake envenoming with procoagulant toxins, includes envenoming by many *Viperid* snakes, certain elapids[36]. Earlier SDS-PAGE has provided an inexpensive and effective technique for characterising proteins from the venoms of *Bothrops jararaca*, *Bothrops atrox* and *Bothrops erythromelas* in their native forms, as well as to preserve their proteolytic activities and immunoreactivity[37]. In the present study we have documented 7 bands in 135 kDa region and in 75, 63, 48, 35, 25 and 20 kDa. Based on the molecular weights on SDS-PAGE, clusters of venom protein bands could be categorized in to protein families as given in the manual on snake venom protein components[38]. When compared with the characteristic venom protein profiles given in the manual on snake venom protein components, these banding patterns revealed various protein families, such as, phosphodiesterase, L-amino acid oxidase, metalloprotease, serine protease, cysteine-rich secretory proteins (CRISPs), phospholipase A2, etc., which could be recognized for the venoms. Further *in vitro* and *in vivo* studies with animal models can help to evaluate the biological efficacy of AgNP-ALG and the mechanistic approach to develop a tool to counteract viper venom.

Conclusion

Snakebite envenoming, a neglected tropical disease, requires immediate consideration. The solitary medically approved treatment entails the application of monoclonal antibodies, specifically antisnake venom. However, this approach is associated with limitations and adverse effects, such as anaphylactic shock, skin rashes, nausea leading to vomiting, fever, and serum sickness. As the use of nanoparticles in the pharmaceutical field continues to grow, and considering the findings from present study nanoparticles appear to be a promising alternative to antisnake venom (ASV). Results obtained by UV-Visible spectroscopy showed the formation of ground state complex between viper venom and silver nanoparticles (SNPs). Fluorescence spectroscopic modes revealed the interaction between silver nanoparticles and crude viper venom with decrease (quenching) in the fluorescence intensity. Venom components might have adsorbed on the surface of SNPs making Viper venom proteins more compact in nature results in modification of its activity. The functional activity of the SNP-venom complex was studied by proteolytic activity and Pro-coagulant activity. Significant reduction in the proteolytic activity of venom was observed in the presence of silver nanoparticles compared to crude venom. This represents an initial step in the development of a therapy based on nanoparticle-mediated inhibition of snake venom. Systematic investigation of this approach holds potential for significant outcomes. The current study systematically investigated, the interaction between silver nanoparticles and crude viper venom through various biophysical, biochemical assays, and *in vitro* neutralization studies. It can be concluded that alginic acid-based silver nanoparticles (AgNP-ALG) may provide supplementary strategy against snake venom in the near future.

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Conflict of interest

Authors declare no potential competing interests.

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