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TRICHODESMA INDICUM NANOPARTICLES INHIBIT RUSSELL VIPER'S (DABOIA RUSSELLI) VENOM ACTIVITY – A STUDY

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ABSTRACT The Silver nanoparticles (Ag Np) are widely used nanoparticles in topical dressings for treatment of infection during burns, open wounds, and chronic ulcers. In the present study a medicinal plant ie*, Trichodesma indicum* which cures many diseases was selected for its extract as snake antivenom activity with the combination of silver. The silver and plant extract mixture prepared was said to be *Trichodesma indicum* silver nanoparticles (TiAgNps). It was characterized using UV-Vis spectroscopy, DLS, SEM and FTIR. These nanoparticles on UV absorption showed λ maximum at 440nm, the chemical analysis using FTIR has revealed the presence of Phenols, aromatics, aliphatic amines, carboxyl amines, amides and polyphenols, the SEM analysis showed the appearance spherical, triangular and cuboidal structures on 20, 10, and 2µm spaces, the density light scattering analysis further revealed that it exists as colloidal particle with the size of 87.2nm and with zeta potential of -32.6mV. Having of these properties the leaf extract particles found to inhibit enzymes of Russell viper *(Daboia russelli)* venom at 10µg/ml. However the fluorescent microscopy analysis of these two has revealed that the stem AgNps on comparison to leaf AgNps the formation of cell clumps were even not found. Therefore AgNps at 10µg/ml having various shapes and sizes inhibit the venom activities of Russell viper in blood systems. The *In vitro* antivenom activity studied was proved that the *T.indicum* AgNps can serve as alternative for antivenom activity of Russell viper.

KEYWORDS : Nanoparticles, SEM, FTIR, Russell viper and venom neutralization.

INTRODUCTION

Silver nanoparticles (AgNps) are increasingly used in various fields, including medical, food, health care, consumer, and industrial purposes, due to their unique physical and chemical properties. These include optical, electrical, thermal, high electrical conductivity, and biological properties (Gurunathan *et al* 2015; Li *et al* 2010; Mukherjee *et al* 2001). Many reports were documented on the biogenesis of silver nanoparticles using several plant extracts (Suman *et al* 2018, Vanaja *et al* 2014). The unique activities of physical and chemical properties of silver nanoparticles make excellent activities, such as antimicrobial, antivenomic and anti-inflammatory properties for many purposes in the medical field (Suman et al., 2018).

Medicinal plants used to play important role in the development of an impressive number of novel synthetic drugs. Traditional healers are using herbal medications since years to treat snake bites and several other diseases. Natural inhibitors of snake venoms play a significant role in the ability to neutralize the degradation effects induced by venom toxins (Kadali and Kindangi, 2015). Snake bites constitute a health problem in many tropical and sub tropical countries with an estimate of 2.5 million people envenomed each year worldwide. In India alone, 35,000-50,000 deaths were reported every year (Gutierrez *et al* 2010 and Mohapatra *et al* 2011). The major families of snakes in India are Elapidae, Viperidae and Hydrophidae (Shaw, George and Nodder, 1797). The most common poisonous snakes in the country are cobra *(Naja naja),* krait *(Bungarus caeruleus)*, Russell's viper *(Daboia russelli)* and saw-scaled viper *(Echiscarinatus)* (Bhavya *et al* 2014). Among all enzymes present in the snake venom, proteases, phospholipases, and hyaluronidases are reported to be medically important in developing effective antidotes (Santhosh *et al* 2013). In this perspective, several attempts have been made to develop snake venom antagonists from plants. In folk medicine, plant drug recipes are passed on to generations by oral tradition and are used as antidotes (Alam *et al* 2003).

In our study we had selected one of the medicinal plants ie*, Trichodesma indicum* R. Br. belongs to the family *Boraginaceae*. It is commonly known as Adhapushpi. This is a perennial medicinal herb distributed in tropical and subtropical Asia, Africa and Australia. In India, it is available on roadsides and stony dry wastelands. In Indian traditional medicine the *Trichodesma indicum* is acrid and bitter tasting, and considered to be thermogenic, emollient, alexeteric, anodyne, anti-inflammatory, carminative, constipating, diuretic, depurative, ophthalmic, febrifuge and pectoral. The whole plant traditionally used as a carminative, laxative, diuretic, depurative and febrifuge. Flowers are sudorific and pectoral, and Leaves depurative. The roots, leaves and flowers are used for medicinal purposes. Leaves and flowers are edible (Ram Krishna and Chattrerjee 2016). The whole plant have antitussive activity (Srikanth *et al* 2002), anti diarrheal (Perianayagam *et al* 2005) and anti-inflammatory properties (Perianayagam *et al* 2006), insecticidal, metal chelating (Anusha *et al* 2014)and corrosive inhibitor (Alarmal Mangai 2013) Insecticidal activity (Khan *et al* 2008) and as veterinary medicine. It is a multidrug plant used to reduce or cure inflammation, pain, osteoarthritis and conjuctivitis. The plant is used for expulsion of dead fetus abortion, inhibition of diarrhoea, reduction of sulfur dioxide-induced cough reflex and also to treat breast cancer(Verma *et al* 2010). The leaves of this plant are used to treat cancer (Ali 2008).The leaves and roots of *Trichodesma indicum* are effective against snake bites, urinary diseases and used as diuretic. The leaf paste of *T. indicum* is used to treat stomach disorders, and intestinal worms in cattle, the treatment of mastitis and for uterine prolapsed (Vanitha *et al* 2015). Roots are made into paste with water and applied externally to swollen joints, inflammations and skin injuries, for diarrhea, dysentery, cough, cold and fever in folk medicine (Periyanayagam *et al* 2012)

The present work was aimed to isolate the compounds of *Trichodesma indica* to synthesize silver nanoparticles, characterize their structures, and study antivenomic activity of silver nanoparticles (TIAgNps) using various methods on Russell viper venom.

MATERIALS AND METHODS:

Procurement of chemicals:

All the chemicals used in the present study were of Analytical Grade (AR) and were obtained from Sigma (St. Louis, MO, USA), Fischer (Pittsburg, PA, USA), Merck (Mumbai, India), Ranbaxy (New Delhi, India), and Qualigens (Mumbai, India) scientific companies

Collection, Preparation and AgNP synthesis:

The Indian medicinal plant, *Trichodesma indicum,* was collected from Sri Venkateswara University campus Tirupathi, Andhra Pradesh, India. Fresh and healthy plants were collected and rinsed with tap water followed by distilled water to remove all the dust and unwanted visible particles. Each part of the plant were separated, like, stem, root, flower etc and were cut into small pieces and shade dried at room temperature. About 10gms of each of these finely incised leaves and stems of *Tricodesma indicum* were weighed separately and transferred into beakers containing 1000 mL of distilled water, and boiled for about 20 min. The extracts were then filtered thrice through Whatman No. 1 filter paper to remove particulate matter and collected clear solutions of them, and then refrigerated (4°C) for further experimentation.

In the typical synthesis of silver nanoparticles, 100 mL of leaf and stem extracts, separately, were treated with 900 mL of 1 mM silver nitrate solution and stored at room temperature after vigorous mixing. The reactions were carried out in darkness (to avoid photoactivation of $AgNO_3$). Subsequently the synthesis of silver nanoparticles was initially identified by brown colour formation and further monitored by measuring UV-Vis spectra of the reaction mixture (Vanaja *et al* 2014).

UV-Visble Spectroscopy:
The reaction mixture of leaf and stem were subjected to UV-Vis Spectrophotometric measurements to ascertain molecules that absorb ultraviolet or visible light. Formation of silver nanoparticles was detected by spectroscopy because the colored particle solutions showed a peak at 440nm.

Fourier transform infrared spectroscopy (FTIR) analysis:
Analysis of FTIR to the dried Ag Nps was carried out through the potassium bromide (KBr) pellet (FTIR grade) method in 1:100 dilution ratio and spectrum was recorded in range from 3500-1000cm^{-1}. This analyte was used for the study of functional groups which were present in the sample mixture.

Scanning Electron Microscope (SEM):
Standard protocols were employed for the preparation of *Trichodesma indicum* AgNp and were subjected to the structure determination of nano particle using SEM.

Dynamic Light Scattering (DLS):
Physicochemical characterization of prepared nanomaterials is an important factor for the analysis of biological activities using radiation scattering techniques such as Particle size and Zeta potential of AgNps. The particle size of the synthesized AgNps was detected using intensity and laser diffraction which are poly-dispersed in mixture solution. The stability was further confirmed by Zeta potential of the particles.

Collection of Venom
Lyophilized snake venom of Russell's viper (*Daboia russelli*) was of gift from Prof. K. Kemparaju, Department of Biochemistry, University of Mysore, Manasagangotri, Mysuru.

Prepartion of RBC cells from Human Blood:
Human blood was collected in glass tubes containing citrate as anticoagulant. The blood samples were centrifuged at 800rpm for 10mins and plasma was carefully removed. The pellet of red blood cells were washed thrice using PBS buffer (0.15MNaCl,10mM phosphate buffer pH 7.0) and then suspended in a fresh PBS buffer at a density of $1.2 X10^9$ cells/mL monitored by a Neubauer chamber. To this 1% of egg albumin was added (Habermann and Neumann 1954; Gutierrez *et al*, 1988) and the resultant mixture was considered as substrate to snake venom PLA_2s in the haemolytic assay.

Blood Agar diffusion method:
Petri plates and other requirements were wrapped in paper and autoclaved before the experiment. Nutrient agar was prepared and blood was added to the nutrient agar after autoclaving and cooling it to 45°C, and poured in to the plates in laminar air flow and allowed to solidify. After solidification, wells were made by using sterilized glass tube. The wells were loaded with the venom separately in one plate, PBS as control in one plate, plant extracts of leaf and stem on each plate to observe protective action against venom and plant extract, venom in one plate. The zones formed after 24hrs were measured for each plate and recorded.

Phospholipase (PLA_2) inhibition
Inhibition of PLA_2 was evaluated using egg yolk as substrate in 1% agarose plates according to the method described by Gutierrez *et al* 1988. The PLA_2 inhibition was calculatedby measuring the zone of clearance in the presence and absence of plant extract. PLA_2 activity of venom in absence of plant extract served as control.

Heamolytic activity:
The venom was preincubated with various concentrations of extracts for 1hour at 37°C. Hemolytic inhibition was determined as Boman and Kaletta, 1957, with slight modifications of Gopi *et al* 2016.

RESULT AND DISCUSSION
UV-Spectroscopy analysis UV-Vis Spectroscopy is useful and reliable technique for the primary characterization of AgNps in simple, fast, sensitive, and selective mode for different types of colloidal NPs, Formation of silver nanoparticles was detected by using spectroscopy because the colored nanoparticle solution on light absorption showed a peak in between 400-450 nm. (Fig.1) however the maximum light absorption of AgNPs were found at λ440nm.

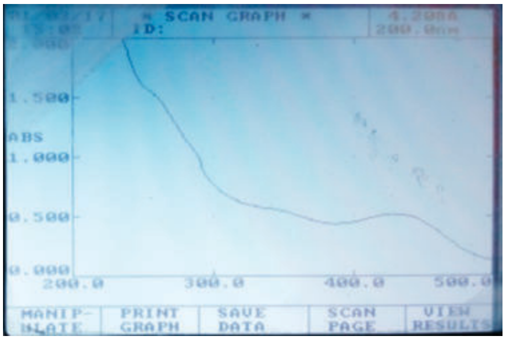

Fig: 1 UV-Vis absorption spectra of aqueous silver nanoparticles of *T.indicum* leaf extract

Fourier transform infra red (FTIR) analysis of biosynthesized AgNps using leaf extract of *T.indicum*
The use of FTIR spectrum has become a versatile tool for analysis of plant products in the fields of biochemistry, molecular biology (Schwinte *et al* 2008), ecology (Lang *et al* 2009), physiology (Oliveira *et al* 2008) and agriculture (Artz *et al* 2008). The leaves and stem of *T.indicum* FTIR analysis results have proved that they consists of aromatic compounds, aliphatic amines, carboxyl groups, amide bonds of proteins, polyphenolics, alcohols and phenols.

From the results of **Figure 2 and Table 1** on FTIR analysis of TIAgNps showed peaks at 536, 672, 1352, 1433, 1482, 1630, 2106, 2353 and 3326 cm^{-1}. The peaks at 536 and 672 cm^{-1} were due to C-H bond aromatic compounds (Litvin *et al* 2013), the peak at 1352 cm^{-1} was corresponding to C-N stretching vibrations of aliphatic amines (Sanghi and Verma 2009), the peak at 1433 cm^{-1} was corresponding to C-O stretch of carboxyl groups, the peak at 1482 cm^{-1} was due to amide band of proteins (Kora *et al* 2012), the peak at 1,630 cm^{-1} was corresponding to the N-H bend of primary amines, the peak at 2106 cm^{-1} was assigned to C-O group of polyphenolics, the peak at 2353 cm^{-1} was corresponding to C–O stretch vibrations of proteins (Ashok *et al* 2010), and the peak at 3,326 cm^{-1} was related to stretching vibrations of O-H bonds of alcohols or phenols (Thirunavoukkarasu *et al* 2013).

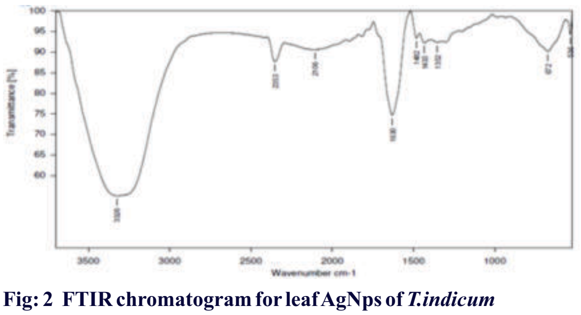

Fig: 2 FTIR chromatogram for leaf AgNps of *T.indicum*

Table.1 FTIR Spectral peaks of *T.indicum* leaf nanoparticles with their respective functional molecules

Frequency cm-1	Bonds	Functional groups
536, 672	C-H bond	aromatic compounds
1352	C-N stretching	aliphatic amines
1433	C-O stretching	carboxyl groups
1482		amide band

1630	N-H bend	amines
2353	C–O stretching	proteins
3326	O-H bonds	alcohols or phenols

The FTIR analysis has revealed that the TIAgNps composed of aromatic and aliphatic molecules with the functional groups of hydroxyl, peptides, carboxyl, carbonyl and amines.

Scanning Electron Microscope (SEM):

The field of nanoscience and nanotechnology are provided with a driving force in the development of high-resolution microscopy techniques in order to know more about nanomaterials using a beam of highly energetic electrons to probe objects on a nano scale. Among various electron microscopy techniques, SEM is a surface imaging method, fully capable in resolving different particle sizes, size distributions, nano material shapes, and particle surface morphology of synthesized particles at the micro and nano scales. The results of SEM on synthesized silver nanoparticles of plant were shown in Figure 3. As shown in Figure **(3)**, the SEM analysis has confirmed that the size range of particle was 22 - 40nm, a clear indication of the formation of silver nanoparticles with the beam waves of 20µm (3A), 10µm (3B), and 2µm (3C). The AgNps formed had spherical, triangular and cubic structures. This may be due to availability of different quantity and nature of capping agents. The sizes shapes and radius were supported by the shifts and difference in areas of the peaks obtained on FTIR analysis.

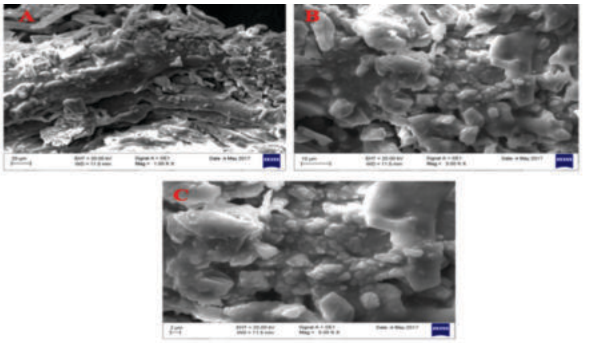

Fig:3: Scaning electron microscopy *T.indicum* leaf images of silver nanoparticles.

The Dynamic Light ScatteringParticle size and Zeta potential analysis of the biosynthesized AgNps of leaf extract of *T.indicum*

Dynamic light scattering (DLS) is a method that depends on the interaction of light with particles when scattered from a laser beam that passed through a colloid solution and used to determine particle size and size distributions in aqueous or physiological solutions (Murdock *et al* 2008; Fissan *et al* 2014). The particle size of the leaf AgNps was detected by (DLS) technique found to be polydispersed with the sizes of 40-100 nm and the average size or hydrodynamic radius of AgNps was found to be 87.2nm (Figure 4A). The difference in the sizes of the AgNps suggests that they were capped by proteins and polyphenolics compounds of different molecular weights to confer stability. Stability was further determined by Zeta potential measurements.

Zeta potential is the important measurement to determine the stability of TIAgNps. It reveals the electrostatic repulsion forces present among similarly charged nanoparticles in a colloidal medium. In this study the zeta potential value of the synthesized leaf AgNps was calculated as -32.6mV(Fig 4B). This high negative value confers good colloidal nature, long-term stability and high dispersity of AgNps without any aggregation. The long term stability is very essential for the biomedical applications of AgNps.

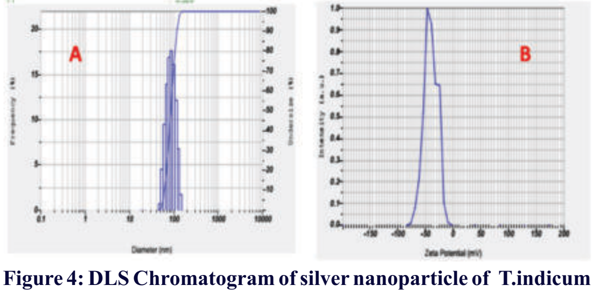

Figure 4: DLS Chromatogram of silver nanoparticle of T.indicum leaf extract, particle size(A) and Zeta potential measurement(B)

After isolation, preparation, analysis and characterization of AgNps the following aspects were performed to confirm its inhibitory activity on Russell viper venom using blood as sample.

Hemolytic inhibition

Hemolytic activity is a distinct feature of snake venoms that is greatly induced by multicomplex molecules like metalloproteases, PLA_2 and more specifically, cardiotoxins and cytotoxins of venom (Osorio *et al* 1989 and Fletcher *et al* 1991). The RBC from direct hemolytic activity of snake venom as cardiotoxins and cytotoxins can induce lysis of human erythrocytes however it is possible that the phytochemicals of plants could inhibit these toxins of the venom.

Enzymatic and inhibition studies using TIAgNps has revealed that the *T.indicum* was able to inhibit, phospholipase A_2 (Table:2) and hemolytic enzymes present in the Russell viper venoms at 10 µg/mL concentrations with a promising inhibiting effect of Russell viper toxins.

Table 2: In vitro inhibition studies Russell viper venom constituents by *T.indicum* AgNps.

Constituent (µg/ml)	% Inhibition
Leaf Alone	95.95
Russel Viper Alone	17.24
TI Stem+R.Viper	89.89
TI Leaf+R.Viper	90.25

Blood Diffusion:

The hemolysis of blood was tested for Russell viper venom using zones of lysed blood on blood mixed in agar plates. The results of Figure 5 have shown that the Russell viper had hemolysis and the concentration was taken from the haemolytic inhibition assay. The three different concentrations ie, 10, 20 and 30µgs were tested. The protection for Russell viper for leaf extract was at 10µg where as for stem extract at 20µg. The zone of diameter was measured for three concentrations in millimeters. Among all the concentrations Russell viper + stem showed more of diffusion. All the concentrations of leaf and stem showed inhibition of neutralization of venom on blood agar plates (Figure 5 A, B, C and Table.3).

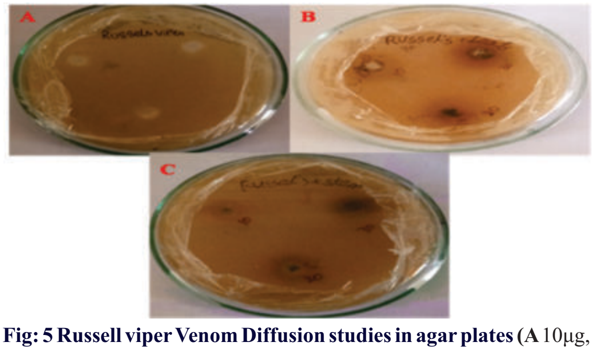

Fig: 5 Russell viper Venom Diffusion studies in agar plates (A 10µg, B 20µg and C 30µg)

Table: 3 Antivenomic effect analysis of *T.indicum* AgNps on blood agar plates by measuring the Zone of inhibition (in mm**)**

Sample	Concentrations (µg/ml)		
	10	20	30
Control	0.7	0.7	0.7
Control+Stem	0.9	1.0	1.4
Control+Leaf	0.6	1.2	1.3
Russell viper +stem	1.1	1.1	1.4
Russell viper + Leaf	0.8	0.9	0.9

The blood discs which had been impregnated with a series of leaf and stem AgNps were placed on agar surface in a petri plates. The agar plate was then incubated at 37°C for 18 to 24 hours. After the incubation, the plates were examined for inhibition zone. The inhibition zone was then measured. The test was repeated thrice to ensure reliability. Then the blood agar plates were taken for fluorescent microscopy and observed the changes that were occurred in control (Fig 6A), Russell viper toxins (Fig 6B) and *T indicum* AgNps (Fig 6C & D). From Figure 7 it was observed that in the absence of dye to the erythrocytes and the changes observed under the fluorescence were normal intact erythrocytes (Control, Fig 7A), erythrocyte blebbing on venom treatment (Fig 7B), erythrocyte protection in leaf AgNps (Fig 7C) and stem AgNps (Fig 7D) . These two microscopic studies reveal that the results of hemolysis were similar in change of morphology of erythrocytes.

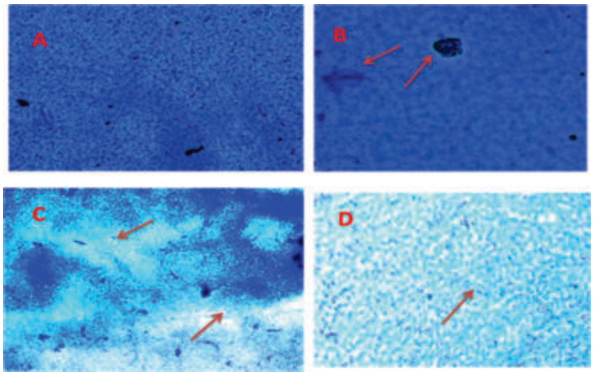

Fig:6 Fluorescent Microscopic analysis blood cells stained with Giemsa dye and mixed with effectors of Venom.

Note: A) Control: Cells are intact and not lysed as that of venom, B) Russell viper's venom: The arrows point outs the Clump of cells as smear, C) Russell viper's venom plus leaf AgNps: Some of the cells are ruptured, and D).Russels viper venom plus stem AgNps: The arrow indicated represents Cells intactness

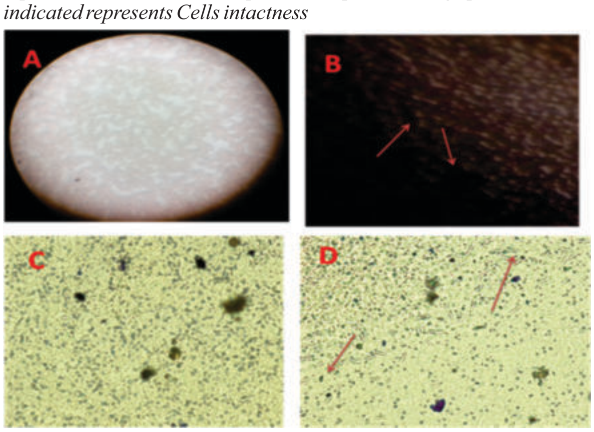

Fig:7 Microscopy of Blood agar in absence of dye and fluorescence

Note*: A) Control blood Cells were normal and intact in freshly prepared slide, B) Russell viper venom plus blood : Cells size is normal but blebbing is seen, C) Russell viper venom plus leaf AgNps : Cells are normal and protection against venom was found and D) Russell viper venom plus stem AgNps : Cells identity found.*

CONCLUSION:

The plant, T. indicum according to our results conclude that its extracts can form into nanoparticles with Ag metal ion to have a size of 22-40nm in different crystal shapes that contained functional moieties of carboxyl, phenolic, alcoholic, amino, peptidyl and carbonyl. All these functional moieties and least sized particles can participate in the protection of RBC from the haemolysis that caused by snake venom. Further these results are in accordance with Alam and Gomes (2003) and Gopi et al (2016). Hence, our results conclude that T. indicum as a plant can serve as an antivenom principle to protect the human and animals.

Much attention was drawn for the benefits of plant-based formulations and their bioactive molecules/compounds for the treatment of infections, inflammations and other diseases. Earlier researchers studied on the neutralization parameters of snake venom using blood by plant molecules have compromised our results with respect to their anti-venom action on the medicinal plant extracts. Our results and procedures applied shall show that the efficacy of a plant AgNps against the intoxication of venom. The present study shows that the *Trichodesma indica* with its sliver nanoparticles could inhibit snake venom activity by the inhibiting of phospholipases and venom other proteiases. In conclusion, the observations confirmed that the nanoparticle extracts of *T.indicum* possesses potent snake venom neutralizing properties. Thus it may be used as an alternative treatment to serum therapy and as a rich source of potential inhibitors of toxins.

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