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PREPARATION OF SILVER NANO PARTICLES FROM THE BARK OF ALSTONIA SCHOLARIS AND EVALUATION OF ITS ANTI-EPILEPTIC ACTIVITY

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ABSTRACT

Devil tree commonly known as alstoniascholaris belonging to family Apocynaceae. Alstonia species are used as medicinal agent for the treatment of various diseases. This study was conducted to develop an ecofriendly, cheap and effective procedure for the green synthesis of silver nanoparticles (AgNPs) form alstonia bark aqueous extract (ASAE) and evaluating their *in-vitro* anti epileptic activity. Addition of ASAE to $AgNO_3$ (1mM) solution kept on stirring at 60^oC resulted in the formation of alstonia bark Aqueous Extract Nano Particles (ASAENP). Synthesized nanoparticles are subjected to characterization by various procedures. UV-Visible spectroscopy showed absorption peak at 440nm which was absent in ASAE and $AgNO_3$. FTIR spectroscopy revealed the absorption peaks of different functional groups involved in the formation of ASAENP. Scanning Electron Microscopy (SEM) revealed average size of ASAENP as 115.95nm. 2 θ peaks at 10.60^o, 12.65^o, 13.02^o, 22.86^o and 25.50^o were identified by X-ray Diffraction studies (XRD). Stability of ASAENP was determined by zeta potential which was found to be -7.4mV. Successfully characterized ASAENP were evaluated for their *in-vitro* anti epileptic activities. Phenytoin was used as a standard.

KEYWORDS: Alstonia Scholaris, Silver Nanoparticles, Epilepsy, Pentylenetetrazole.

INTRODUCTION

Epilepsy is defined as group of neurological disorder characterized by epileptic seizures.[1] Epilepsy are mainly of two types partial and general seizures. Partial seizures can be further sub divided into simple and complex where only complex seizures can causes loss of consciousness. Generalised seizures are grouped into 6 major categories. Absence seizures (petit mall) are characterised by a partial loss of consciousness. Myoclonic seizures consists of very brief and sporadic arrhythmic movements. Tonic seizures consist of sudden stiffening movements involving the head, body or extremities that often occur during sleep. Clonic seizures are characterised by repeated, rhythmic motor movements often involving a large portion of the body as well as causing unconsciousness. Side effects of anti- epileptics include seizures, headache, joint pain, muscle aches, nausea, skin rashes, or diarrhoea, Sleep disturbance-insomnia, migraine headache, weight gain, suicide, sexual side effects, anxiety and restlessness, drowsiness or fatigue, dry mouth, sweating.[2,3]

Devil tree commonly known as *Alstonia scholaris* belonging to family Apocynaceae.[4] Alstonia species are used as medicinal agent[5] for the treatment of various diseases. Genus Alstonia is well known for its alkaloidal contents. Various Alkaloids like alstolactone, affinisine oxindole, lagumicine, 10-methoxycathafoline N (4)- oxide, alstomaline, 16-hydroxy alstonal, alstophyllal, 6-oxoalstophylline,6-oxoalstophyllal etc. have been reported along with two triterpenes, lupeol acetate and α-amyrin acetate from the title plant.[6,7] It has been reported to show antifertility activity in male albino rats.[8]

Distinctive property of nanoparticles is their large surface area to volume ratio. Silver, gold, palladium and platinum are the metals used in the synthesis but silver is superior among all the metals.[9] Physical and chemical methods used for the synthesis are harmful and expensive so there is a need of ecofriendly method for synthesis of nanoparticles.[10] Green synthesis is another approach for synthesis of nanoparticles increased enormously in current research because of safety, effectiveness and low cost. This innovative technique involves the reduction of Ag+ to Ago and stabilization of AgNPs by biomolecules which are present in plant extract.[11] Based on these benefits, present study was designed to use ASAE for the synthesis of silver nanoparticles and further evaluating their *in-vitro* anti-epileptic activity.

MATERIALS AND METHODS

Plant Material Collection and Extraction

Bark of Alstonia plant were collected from in and around areas of Sangareddy Dist. in Dec 2017. Plant material was authenticated at Botanical Survey of India, Hyderabad, Telangana, India. PRIP/PCOG/17-18/002 is the reference number given to herbarium and it is stored in dept. of Pharmacognosy, Pulla Reddy Institute of Pharmacy, Hyderabad, India. Freshly collected bark were washed under running tap water, dried in shade for 15 days, sliced in to small pieces and grinded to coarse powder. 20gm of powder was boiled with 200ml of double distilled water (DDW) for 1hr. After cooling to room temperature, mixture was filtered with whatman filter paper no.1 and filtrate is refrigerated (4^oC) for future use.

Animals

Care and use of animals were carried out in accordance with CPCSEA guidelines. 150-200gm weight of female wistar rats was used. Work was approved by Institutional Animal Ethics Committee IAEC, Pulla Reddy Institute of Pharmacy, Hyd, Telangana, India.

Synthesis of ASAENP

380ml of 1mM $AgNO_3$ solution was prepared by using DDW, 20ml of aqueous extract was added slowly to it, reaction was carried out in a conical flask kept on stirring at a temp. of 60^oC for 8hrs. Change in the color of solution specifies the formation of ASAENP. Solution was centrifuged at 5000rpm to obtain the pellet of nanoparticles which was stored for future use.[12]

Characterization of ASAENP

UV-Visible spectroscopy and Visual identification of ASAENP

Color of ASAE and $AgNO_3$ solution was taken as control and change in the color of solution after addition of ASAE to AgNO $AgNO_3$ solution is the first indication for the formation of ASAENP. UV-visible spectroscopy was conducted in a range of 200-800nm by using UV-VIS Spectrophotometer (UV3000, LBINDIA).

FTIR spectroscopy of ASAE and ASAENP

This was recorded by using FTIR (BRUKER, ALPHA) was conducted in the range of 4000 to 500 cm^{-1}. It is used to determine the involvement of functional groups in the formation of ASAENP. ASAENP synthesized also contains some enzymes which are not capped on nanoparticles; the obtained ASAENP are removed by dissolving in DDW and centrifuged at

5000rpm for 15 min. Procedure is repeated for 3 times and final pellet obtained is dried in at 60^0C in hot air oven and used for characterization.

SEM studies of ASAENP

It determines the morphology of ASAENP by using SEM (ZEISS) operated at accelerating voltage 10.00kV, magnification 50.00 KX, working distance 7.0mm. A small amount of ASAENP was dropped on copper coated grid with carbon, blotting paper is used to remove the extra solution and gird was used for analysis.

XRD studies of ASAENP

It is used to identify the Crystallographic structure of ASAENP by using XRD (SHIMADZU, XRD7000) operated at scan speed – 4.0 deg/min, sampling pitch – 0.02 deg, preset time is 0.30 sec, 2 θ range from 10^0 to 80^0, 30mA current, 40kV voltage with Kα1 Cu radiation, λ = 1.54 A^0. Crystallographic structure of ASAENP was calculated by using peaks of XRD. Average size of ASAENP was assessed by using the Debye-Scherrer equation.

$$D = \frac{k\lambda}{\beta cos\theta}$$

Where D = average size of ASAENP, k = constant (0.94), λ = wavelength of X-rays (1.546Å), β = full width at half maximum (FWHM), θ = diffraction angle in degrees.

Zeta potential and Dynamic Light Scattering (DLS) studies of ASAENP

light scattering method was employed to determine the Stability of ASAENP for measuring zeta potential (HORIBA SCIENTIFIC, SZ100) operated at electrode Voltage - 3.4V, Conductivity - 0.199mS/cm, temperature (25^0 C) and Viscosity of dispersion medium (0.894mPa.s). Particle size distribution was estimated by DLS measurements.

RESULTS AND DISCUSSION

Visual identification and UV-Visible spectroscopy of ASAENP

Uv visible spectroscopy was employed to understand the formation of ASAENP is change of color of $AgNO_3$ solution from colorless to dark brown. Excitation of surface Plasmon resonance and reduction of Ag^+ to Ag^o are the two major causes for the change of color of solution (Figure 1). Temperature, time and stirring accelerated the reaction. UV spectra of ASAE, silver nitrate and ASAENP are presented in figure 2. Many studies reported the

characteristic peak of SNPs in the range of 400-450nm which was absent in ASAE and $AgNO_3$, whereas a peak at 440nm was successfully appeared in ASAENP spectrum.

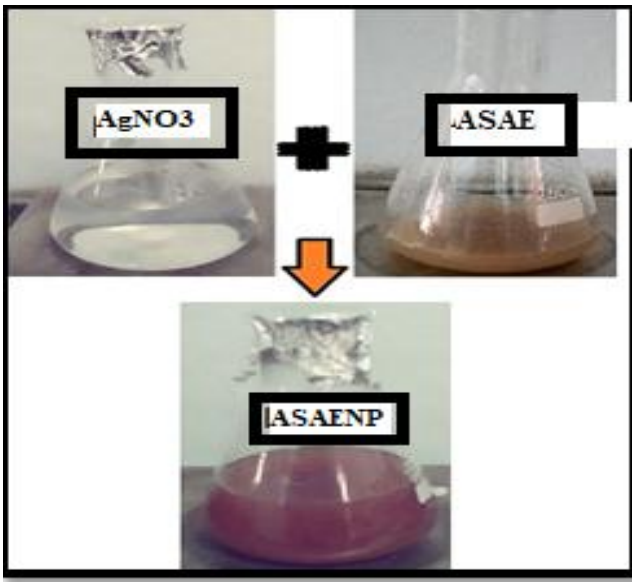

Figure 1: ASAE which is light brown in color when added to $AgNO_3$ solution 1mM it forms dark brown coloured ASAENP. Change of color indicates formation of nanoparticles.

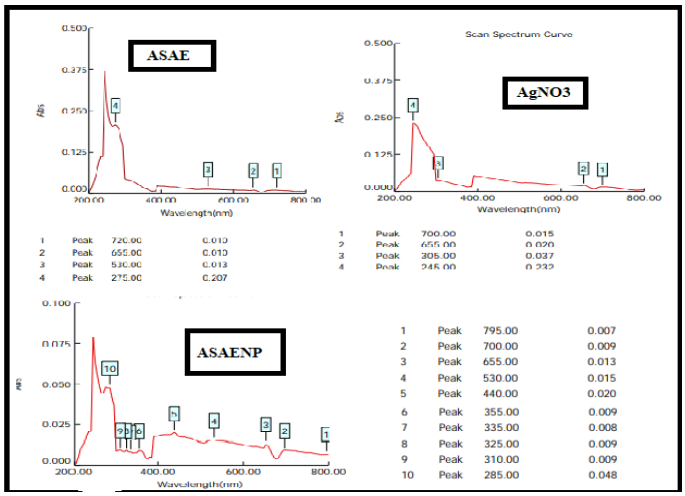

Figure 2: UV spectra of $AgNO_3$, ASAE and ASAENP.

FTIR spectroscopy of ASAE and ASAENP

FTIR spectroscopy was carried out to determine different functional groups involved in the formation of AgNPs depicted in figure 3. Different absorption peaks and their corresponding functional groups of ASAE and the shift in ASAENP are discussed in table 1. Proteins, polysaccharides and enzymes present in extract contains –OH group which undergoes stretching vibrations and produces peak at 3446.5 in ASAE, which is shifted to 3436.2 in

ASAENP indicating their role in the formation of SNPs. FTIR spectroscopy proved the capping of functional groups of different chemical constituents present in ASAE on AgNPs. ASAE absorption peaks at 1073, 1397 and 1635 and ASAENPabsorption peaks at 1248.7, 1270.5 and 1628 corresponds to carbon skeleton. ASAE peaks at 758.6, 780.6 and ASAENP absorption peaks at 1041.8 corresponds to flavonoids like structure.

Table 1: FTIR peak values and corresponding functional groups of ASAE and ASAENP.

S. No.	Peak in ASAE (cm^{-1})	Peak in ASAENP (cm^{-1})	Corresponding functional group
	3446.5	3436.2	Stretching vibrations of –OH group of phenolic acid compounds and carbohydrates with –H bonding.
	1635.1	1628.7	Stretching vibrations of C=C of alkenes
	1397.4	1384.2 & 1489.4	Stretching vibrations of C-O group of Carboxylic acids
	1073.9	1041.8 &1248.7	Stretching vibrations of C-N group of amines
	927.3	----	Stretching vibrations of C-H group of alkenes
	2094.2	---	Stretching vibrations of N=C=S of isothiocyanate
	758.6 & 780.6	---	Bending vibration of O-H group of polyphenol
	681.9	693.9	Stretching vibrations of C-Br
	585.1	596.5	Stretching vibrations of C-H group of aldehydes
	572.6	568.7	Bending vibrations of C-H group of alkanes

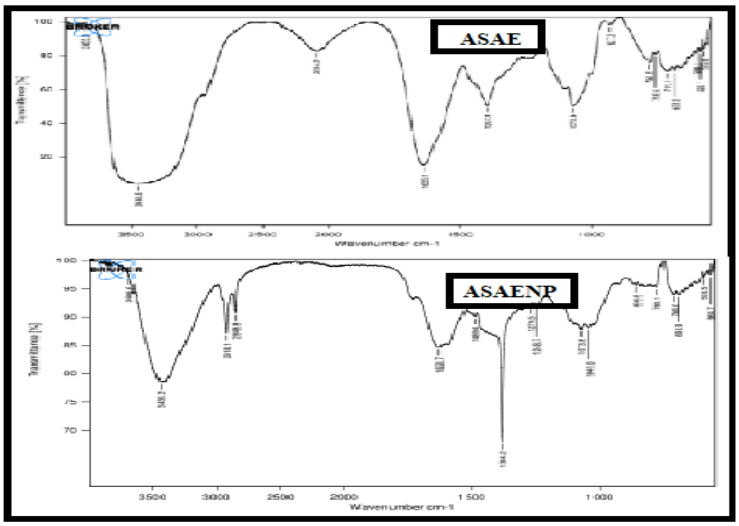

Figure 3: FTIR spectra of ASAE and ASAENP.

SEM studies of ASAENP

Morphology of surface of ASAENP is presented in figure 4. AgNPs identified by SEM analysis have a size ranging from 72.9nm to 144.9 nm with an average size of 115.95nm and

are spherical in shape. Thus SEM results strongly confirm the role of ASAE as reducing and capping agent in the synthesis of ASAENP.

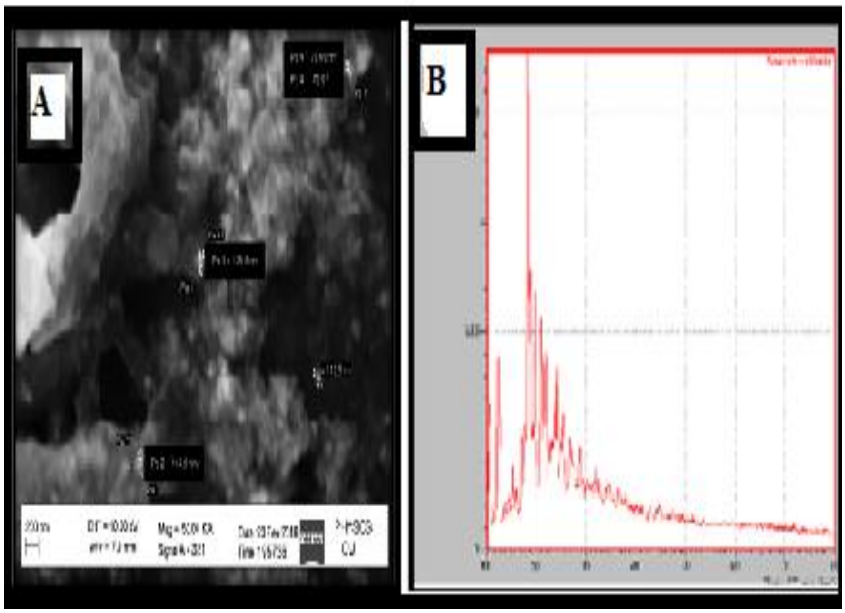

Figure 4: A: SEM micrograph of ASAENP at 200nm; B: XRD pattern of ASAENP.

XRD of ASAENP

2 θ peaks at 10.60°, 12.65 °, 13.02 °, 21.79 ° and 25.50 °corresponds to 187, 253, 197 and 109 planes of Bragg's reflection of silver. Figure 4 and Table 2 represent the data of XRD analysis. These planes clearly indicate the face centered cubic structure of silver. NM is the average size of ASAENPs.6.3nm is the average size of ASAENP$_s$.

Table 2: Size of ASAENP by using Debye-Scherrer equation.

S. No.	2θ (deg)	D (angle)	FWHM (deg)	Int. I (cps deg)	Size (nm)
1.	10.60	8.33688	0.4961	4542	5.73
2.	12.65	6.98985	0.6192	6284	2.37
3.	13.02	6.79417	0.1534	859	10.35
4.	22.86	0.4400	0.3868	1282	8.05
5.	25.50	3.48971	0.3061	2152	5
		Average size of ASAENP			6.3

Zeta Potential Studies of ASAENP

Zeta potential was conducted to determine the stability of ASAENP. Mean Zeta potential was found to be -7.4mV. Negative value indicates the capping of constituents present in ASAE on surface of ASAENP. Moreover, negative charge also proves the stability and thus preventing them from agglomeration.[13] Average particle size measured by DLS was found to be 354.1

nm (Figure 5). Size detected by DLS was higher than SEM analysis because SEM measures physical size of particle without capping agent where as DLS measures size of particle along with capped biomolecules.[14]

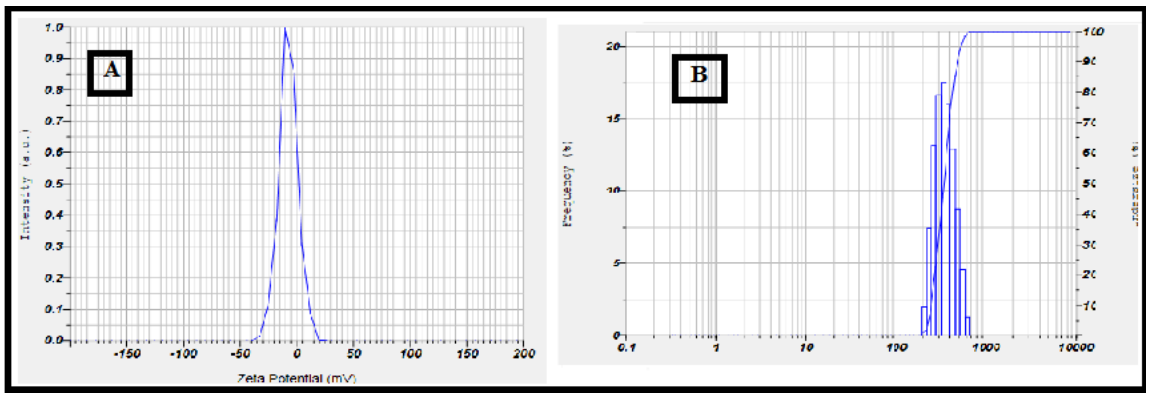

Figure 5: A: zeta potential of ASAENP; B: Size distribution of ASAENP with maximum intensity at 354.1nm.

***In-vitro* anti epileptic activity**

Effect of ASAENP on PTZ induced epilepsy in female wistar rats

20 female wistar rats were divided into 4 groups. Group1 received tween 80 10%v/v, group 2 received anti-epileptic drug phenytoin (40mg/kg, I.P.) and group 3 and 4received ASAENP 80 and 160 mg/kg BW, P.O respectively. One hour after, all the groupsreceived PTZ injection (60mg/kg, i.p). The seizure behavior was evaluated by placing ratsindividually in plexi glass boxes and seizure behaviors were observed for 60 min asfollows 59, 60, 61, 62. Stage –0 no response; Stage-1 ear and facial twitching; Stage-2 myoclonic jerks (MJ); Stage-3 clonic fore limb convulsion; Stage-4 generalized clonic seizures; Stage-5 generalized tonic - clonic seizures (GTCS) or death within 60 minutes. Latency to theonset of myoclonic jerks and generalized clonic-tonic seizures stage were measured.

Table no. 3 Effect of SNGMAE on PTZ induced epilepsy in female wistar rats.

Experimental animals	Stage 1 (sec)	Stage 2 (sec)	Stage 3 (sec)	Stage 4 (sec)	Stage 5 (sec)
Negative control	99.00 ± 0.4082	242.5± 3.227	107.5± 3.663	117.5± 3.227	1087± 2.056
Positive control (phenytoin 40mg/kg)	20.25± 0.6292***	72.25± 1.931***	68± 0.9129***	55± 1.291***	286.8± 1.109***
ASAENP (80mg/kg)	20.75± 0.8539***	105.3± 2.287***	58.75± 1.493***	63± 1.291***	285.3± 2.626***
ASAENP (160mg/kg)	16.25± 0.8539***	56.5.± 1.555***	49.25± 1.25***	58.75± 1.493***	204.5± 1.936***

All values are expressed as Mean ± SEM, n=4, analysed by One way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test; ***p<0.001, ****p<0.0001 as compared to control group; ns= non-significant.

CONCLUSION

The nanoparticle of aqueous bark extract of alstoniascholaris demonstrated the anti epileptic properties and shows the less toxicity in the animals at the dose used. With the view of the result obtained from the study presents, the green synthesis as safe, cheaper for the production of nanoparticles. Various method of characterization proved the formation of ASAENP. UV-Visible showed the peak at 440nm, FTIR identifies the different functional group capping on ASAENP, SEM and DLS determines the formation of particle size, crystalline structure is identified by XRD. Stability is proved by zeta potential. The data obtained in this study proves the inhibitory potential of ASAENP. However, further in depth studies are required to be conducted in terms of *in-vitro* and *in-vivo* procedures to develop ASAENP as potent contender with high therapeutic efficacy and low side effects for the treatment of epilepsy.

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