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Biogenic Synthesis, Characterization and Pharmacological Study of Silver Nanoparticles using *Enicostema axillare* Leaves

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Research Article

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Abstract

Background: Biogenic synthesis of silver nanoparticles with enhanced pharmacological effects is of great interest for the development of new antimicrobial agents. Current study reports the antioxidant and anti-inflammatory properties of silver nanoparticles showing better results than plant extract.

Results: The formation of synthesized nanoparticles, observed by UV-Visible spectroscopy exposed the Surface Plasmon Resonance at 449 nm and High Resolution Transmission Electron Microscopy (HR-TEM) analysis carried out and revealed the formation of AgNPs. Nanoparticles are spherical in shape having the size of 16 ± 2 nm.

Conclusions: The EAL-AgNPs prepared in this study demonstrate better pharmacological actions in *in-vitro* studies.

Keywords: *Enicostema axillare*; Biosynthesis; UV-Visible spectroscopy; Antioxidant activity

Introduction

Nanoparticles have attained strong scientific space for the application in multiple fields; this is due to the possibility of manipulating their basic structure and composition. These nanomaterials are thus capable to present solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine and water treatment [1]. Nanomaterials have a numerous properties that are not noticed in the bulk compounds [2]. Metallic nano-particles show size and shape-dependent properties with uses in fields of catalysis, sensing, optics, antibacterial activity and data storage [3]. Recently, synthesis of metal nanoparticles is a subject matter of research interest due to its wide potential applications in biomedical, optical and electronic fields [4].

Nanoscale therapeutics has been getting popularity over the past decade in the biomedical field, due to their advantageous applications, exclusively for tissue engineering, drug delivery, gene regulation, biosensors and diagnostics. Few examples of nanoscale materials that have been developed include micelles, dendrimers, liposomes, polymers, carbon nanotubes, gold and silver nanoparticles, peptide nanotubes and graphene oxide [5]. Amongst these, silver nanoparticles (AgNPs) have fascinated notice because of their uses in oxidative catalysis, antimicrobial activities, and others. In food industry several features such as food safety, packaging materials, nanosensors, nutrients delivery system,

bioavailability, new materials for pathogen detection and others have been addressed [6].

Silver particles exhibit antibacterial and antiseptic properties at the nanoscale. Silver nanoparticles are at present the mainly largely used agents in the fields of nanotechnology next to carbon nanotubes. It is pointed out in chronological literature that silver coins were used for wound injuries in war fields for fast healing. Silver nanoparticles have more anti-bacterial effects, which might be used as a strong healing in wound curing. Silver nanoparticles are competent to destroy more than 650 organisms, so are used as an effectual healing in burn, diabetic foot, skin disorders and some other infections [7].

The uses of nanoparticles are primarily reliant on their shape, size and functionalization. Thus, it is important to attain the required shape and size of nanomaterials with no any other significant difference and it is fundamentally dependent on the preparation method and precursors taken [8].

Biosynthesis of AgNPs using microorganisms, enzymes and plants or plant extracts have been recommended as cost effective, eco-friendly substitutes to chemical and physical methods. It is a green technology, as it does not involve any unsafe chemicals. So, an increasing need to build up an environmentally friendly method for nanoparticle synthesis without using toxic chemicals is gaining importance [9].

Creation of materials in nano size by utilizing microorganisms like bacteria, fungi and alga is of major significance, while still being a challenge in the field of nanotechnology when the appropriate NP size, shape and dispersion is sought. Evolutions made in biological methods endorse nanobiotechnology uses in a variety of fields of sciences [10]. Most important downside involved in biological synthesis of nanoparticles is pathogenicity of few of the organisms and prolonged reaction as the time required for completing the reaction usually ranges from 24-120h [11].

The reduction of Ag^{+} to Ag^{0} occurs by combinations of biomolecules such as proteins, polysaccharides, and flavonoids. Certain biological preparation of metal and their alloy nanoparticles is nontoxic, eco-friendly and a inexpensive technology for the large-scale production of well-characterized nanoparticles. Though, study of the plant systems as another potential nature nanofactory has amplified attention in the biosynthesis of nanoparticles [12]. The utility of different parts of the medicinal plant in conventional medicinal system viz., Ayurveda, Siddha and Unani to treat a variety of illnesses is in vogue for several centuries. Medicinal plants act as another source for treating several sicknesses as their usage is increasing day by day [13]. Natural products are the most chief source for drugs and drug discovery. The WHO calculated approximately that about 65% of the World's populations are mostly relying on natural products derived from plants for their primary health care systems and most of them are from developing countries, the remaining 35% are mostly from developed courtiers who are also used natural products indirectly to maintain a good health [14].

Botanical Description

Figure 1: *Enicostema axillare* whole plant, *Enicostema axillare* flower.

Enicostema axillare, as shown in Figure 1, is ecofriendly and an important medicinal crop. *Enicostemma axillare*belongs to family *Gentianaceae*. It is also called as *Vellarugu* in Tamil, Chota chirayata in Hindi, Mamejavo in Gujarati and Nagajivha in Bengal. It is a glabrous perennial herb attaining height of 15-20 inch with sessile lanceolate leaves and is found all over India up to a height of 1500ft [15].

The entire plant is used in medicine as digestive, anti-inflammatory, liver tonic, antimalarial, antipyretic and as a laxative. According to ayurvedic literature survey, the fresh juice of leaves has been used as a bitter tonic, to control arthritis, in typhoid fever and as cooling agent. The plant is conventionally used in the healing of hepatic diseases and as a blood purifier. It also acts as ethnomedicine for snakebite. The plant paste is applied on boils. The leaves are fed to cattle to increase appetite [16].

Phytochemicals of the Plant

The extracts of *Enicostema axillare* have shown higher phenol and flavanol content. Alkaloids, tannins, phenols,

flavanols, proteins and amino acids are present in the extracts. Steriods present in ethanolic and methanolic extracts. Saponins and oils are present only in ethanolic extract. Therefore ethanolic extract has shown higher antioxidant activities due to the presence of these phytoconstituents [17]. Glycoside has been isolated from the plant. The plant has ophelic acid. The preliminary phytochemical investigation showed the existence of alkaloid, flavanoid, glycoside and tannin in extract of the plant [18]. Methanolic extract of *Enicostemma axillare* is a wealthy source of vitamin C and vitamin E [19]. Proteins and amino acids were present in chloroform, methanol and water extracts [20].

In phytochemical study of different parts of *Enicostemma axillare* of plants showed presence of cardiac glycosides in leaf and root, reducing sugar higher in leaf, steroids relatively higher in leaf and flower, high levels of terpenoids seen in flower, most amounts of alkaloids found in leaves. On comparing the results, it is fairly interesting to note that flavonoids, alkaloids and saponnins are reasonably less in entire plant [21]. Swertiamarin is a secoiridiod glycosides was found to contain a major constituents of the extract isolated from *Enicostemma axillare* linn [22,23].

In this article, we report biosynthesis of stable AgNPs using *Enicostema axillare* leaves (EAL) extract using microwave assisted method and carried out pharmacological evaluation of their antioxidant and anti-inflammatory activities by *in vitro* methods.

Materials and Methods

Chemicals

Silver nitrate ($AgNO_3$), Diclofenac, DPPH and Ascorbic acid were purchased from Sigma-Aldrich, India. All solutions were prepared in Millipore water and all apparatus were rinsed with aqua regia (3:1 solution of HCl, HNO_3) and then washed with Millipore water before use. All reagents and solvents used in this study were of guaranteed reagent grade.

Preparation of EPL Aqueous Extract by Microwave Method

Enicostema axillare is a shrub belonging to the family Vitaceae. *Enicostema axillare* leaves (CRL) were collected on 4th December, 2017 from the lands in Mekkilarpatty, Aundipatty in Theni (DT). The plant was identified with the help of Mr. P. Packiaraj, Assistant professor of Botany, Saraswathi Narayanan College, Madurai. About 10 g of the *Enicostema axillare leaves* were thoroughly washed and were soaked in 100 mL of Millipore water and irradiated with Microwave for 120 seconds. The extract was then filtered and used for further experiments. The extract was centrifuged at 3000 rpm for 15 minutes to separate any plant debris and stored at 4°C before adding to the silver nitrate solution.

Green Synthesis of EAL-AgNPs

For the phytosynthesis of AgNPs, about 1 mL of EAL aqueous extract was added to 30 mL of 2.5×10^{-4} M $AgNO_3$ aqueous solution and kept in microwave oven at Micro level 60% with continuous microwave irradiation for 90 seconds. Rapid reduction of Ag^+ ions to Ag^0 was observed by the change in the color of the solution from yellowish brown to dark brown colour of EAL-AgNPs synthesized were taken up for further study.

Characterization of EAL Extract-AgNPs

Characterization of nanoparticles is very significant to comprehend and control nanoparticles synthesis and applications [24]. The reduction of pure Ag^+ ions was monitored by measuring the UV- Vis spectrum of the reaction medium at 5 hours after diluting a small aliquot of the sample into distilled water. UV-Vis spectral analysis was done by using Perkin Elmer Lambda 35 UV-Vis spectrophotometer. To remove any free biomass residue or compound that is not the capping ligand of the nanoparticles, the residual solution of 100 ml after reaction was centrifuged at 3000 rpm for 10 min and the resulting suspension was redispersed in 10 ml distilled water. The centrifuging and redispersing process was repeated three times. Thereafter, the purified suspension was freeze dried to obtain dried powder. Finally, the dried nanoparticles were analyzed by JASCO FT-IR 400.

Scanning Electron Microscopic (SEM) analysis was done using VEGA3 TESCAN machine. Thin film of the sample were prepared on a glass plate by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min. The morphology and composition of the prepared Ag NPs were examined by High Resolution Transmission Electron Microscopy (HR-TEM) of AgNPs were obtained from JEOL JEM-2100 operating at 200 kV. The sample was prepared by dropping the silver NPs solution onto the form var - coated copper g rid and dried in air naturally.

Antioxidant Assays

The synthesized AgNPs were evaluated for antioxidant activity using following methods as follows. The average of the results for each experiment was calculated. *Enicostema axillare* leaves extract was tested as control and L-Ascorbic acid (AA) was used as reference.

DPPH Radical Assay

The DPPH free radical scavenging assay was performed by Liyana-Pathirana and Shahidi method. 200 µL of 0.1 mM DPPH prepared in methanol was added to 100 µL of the plant extract. The resulting mixture was incubated at room temperature in the dark for 15 minutes. Absorbance was observed at 517 nm. BHT was taken as a positive control. The experiment was carried out in triplicates and percentage inhibition of the DPPH radical scavenging activity was calculated.

% Inhibition= $((A_0-A_1)/A_0)*100$ Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample [25].

Estimation of Reducing Power

The reducing power of the plant extract was estimated by the method of Oyaizu [26]. 500 µL of 0.2 M phosphate buffer (pH - 6.6), 500 µL of ferricyanide (1% w/v) and 200 µL of both the extract selected for the study was added. The above prepared mixture was incubated at 50°C for 20 minutes followed by the addition of 500 µL of TCA (10% w/v). The resulting mixture was then centrifuged at 3000 rpm for 10 minutes. 500 µL of the supernatant was collected and to it 500 µL of milli-Q water was added along with 100 µL of ferric chloride (0.1% w/v). Absorbance of the solution was observed at 700 nm against blank. Percentage inhibition was calculated for the determining reducing power.

% Inhibition= $((A_0-A_1)/A_0)*100$ Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Nitric Oxide (NO) Scavenging Activity

NO scavenging activity of sample was determined by adding 400 µL of 100 mM sodium nitroprusside, 100 µL of PBS (pH-7.4) and 100 µL of different concentration of plant extract. This reaction mixture was kept for incubation at 25°C for 150 minutes. To 0.5 mL of above solution, 0.5 mL of Griess reagent was added (0.1 mL of sulfanilic acid and 200 µL (naphthyl) ethylenediamine dichloride (0.1%) w/v)). This was kept on incubation at room temperature for 30 minutes, and finally absorbance is observed at 540 nm. All the reactions were performed in triplicates, and their percentage inhibition was calculated by the following formula:

% Inhibition= $((A_0-A_1)/A_0)*100$ Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

In-Vitro Anti-Inflammatory Activity

The Human Red Blood Cell (HRBC) Membrane Stabilization Method: The blood was collected from healthy human volunteer and equal volume of Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) was mixed with it and centrifuged at 3,000 rpm for 10 min. The obtained packed cells were washed with normal saline and a 10% HRBC suspension was made. Various concentrations of EAL-AgNPs were prepared (5, 10, 15, 20 and 25 mcg/mL) using distilled water. A mixture of 1 ml of phosphate buffer, 2 ml of hypo saline and 0.5 ml of HRBC suspension (of above said various concentrations) was made. It was incubated for 30 min at 37°C min and centrifuged at 3,000 rpm for 20 min. The absorbance of supernatant solution was measured spectrophotometrically at 560 nm [27]. Diclofenac sodium was taken as standard drug. The experiment was repeated three times. The percentage (%) of HRBC membrane stabilization or protection was calculated using the following formula [28].

$$\text{Percent protection (\%)} = \frac{100-\text{OD of drug treated sample x } 100}{\text{OD of conrol}}$$

Results and Discussion

UV- Visible Spectral Analysis

The UV-Vis spectrophotometer would be used to scrutinize size and shape controlled nanoparticles in aqueous solution. Metal nanoparticles possess free electrons, which yield a surface plasmon resonance (SPR) absorption band, due to the mutual vibration of electrons of metal nanoparticles in resonance with light wave. The appearances of the peaks show the characteristics of surface plasmon resonance of silver nanoparticles [29]. The UV-Vis spectra of the silver nanoparticles shows a dark brown color due to a well defined surface plasmon band centered at around 449 nm at different time interval as shown in Figure 2 [30].

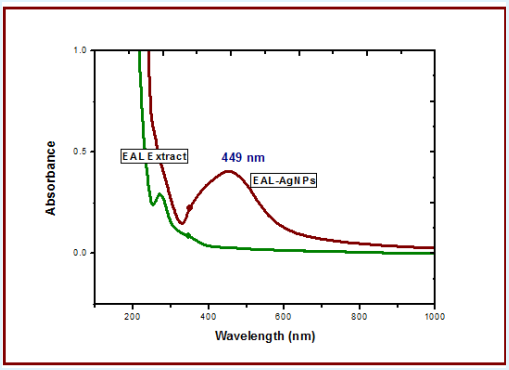

Figure 2: UV- Visible spectrum of EAL extract and EAL Silver Nanoparticles.

FT-IR Spectroscopy

Various plant metabolites, including terpenoids, polyphenols, sugars, alkaloids, phenolic acids, and proteins, play an important role in the bioreduction of metal ions, yielding nanoparticles. Flavonoids are a large group of polyphenolic compounds that comprise several classes: anthocyanins, isoflavonoids, flavonols, chalcones, flavones, and flavanones, which can actively chelate and reduce metal ions into nanoparticles. Flavonoids have various functional groups capable of nanoparticle formation. It has been hypothesized that the tautomeric transformations of flavonoids from the enol-form to the keto-form may discharge a reactive hydrogen atom that can reduce metal ions to form nanoparticles [31].

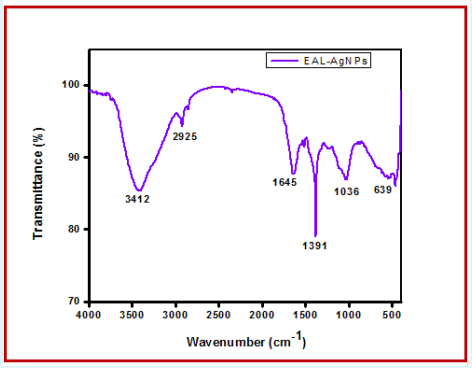

Figure 3: FT-IR spectra of biologically synthesized silver nanoparticles using EAL extract.

FT-IR spectra were recorded for *Enicostema axillare* extract and synthesized silver nanoparticles to identify the possible biomolecules responsible for the reduction of $AgNO_3$ into AgNPs. FT-IR spectrum of *Enicostema axillare* shows different major peaks positioned at 3412, 2925, 1645, 1391, 1036, and 639 cm^{-1} (Figure 3). The presence of peak at 3416 cm^{-1} could be ascribed to O–H group in polyphenols or proteins/enzymes or polysaccharide. A small peak positioned at 2925 cm^{-1} may be due to CH-stretching of alkanes. A sharp intense band observed at 1645 cm^{-1} can be due to the stretching vibration of the (NH)=O group. The observed band at 639 cm^{-1} is due to a-glucopyranose rings deformation of carbohydrates. The band positioned at 1036 is due to C–N stretching vibration of aliphatic amines. On the other hand, FT-IR spectrum of the synthesized AgNPs shows the presence of major peaks at 3412 and 1645 cm^{-1} which are associated with OH–stretching vibrations and stretching vibration of the (NH)=O group, respectively. The (NH)C=O groups within the case of cyclic peptides are involved in stabilizing the nanoparticles. Thus, the peptides may play an important role in the reduction of $AgNO_3$ into Ag nanoparticles [32].

Scanning Electron Microscopy (SEM)

The shape of the synthesized silver nanoparticles was analyzed by SEM. Figures 4 and Figure 5 show the results of surface morphological and nanostructural studies using SEM and EDS images. The result (Figure 4) showed monodispersed spherical silver nanoparticles of varying sizes and shapes. Overall, the synthesized EAL-AgNPs are spherical in shape, and well dispersed with low agglomeration. EDS analysis gives a qualitative as well as quantitative status of the elements that may be involved in the formation of nanoparticles. The elemental profiles of the synthesized nanoparticles for the EAL-AgNPs, showing a higher count at 3 keV due to the silver, confirm the formation of silver nano-particles (Figure 5). In general, metallic silver nanocrystals show a characteristic optical absorption peak at approximately 3 keV due to their surface plasmon resonance. The elemental analysis of the silver nanoparticles shown in figure 5 reveals the highest proportion of silver followed by O, and Na. Mg is also present in trace amounts [33].

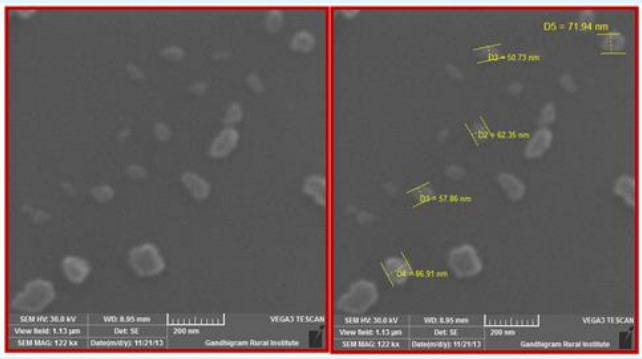

Figure 4: SEM image of the synthesized silver nanoparticles.

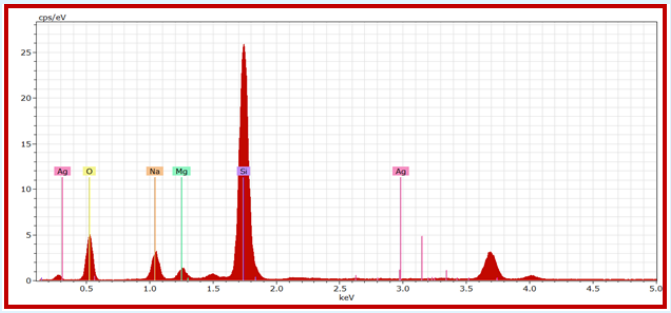

Figure 5: EDS graph of EAL-Silver nanoparticles

Transmission Electron Microscopy (TEM)

The morphology and size study of the synthesized EAL - AgNPs were done by high resolution transmission electron microscopy (HRTEM). The samples used for HRTEM observations were prepared by dispersing the particles in de-ionized water under sonication of 30 minutes, then placing a drop of the dispersion onto a copper grid coated with a layer of amorphous carbon. Figure 6 shows the HRTEM images of EAL–AgNPs at two different magnifications. The HRTEM images divulge that the particles are spherical in shape supporting the SEM observation. The images also shows that the synthesized synthesized nanoparticles are distributed uniformly and spheres are interconnected with each other. From this micrograph it is noticed that the particles have an average size of about 16 ± 2 nm [34].

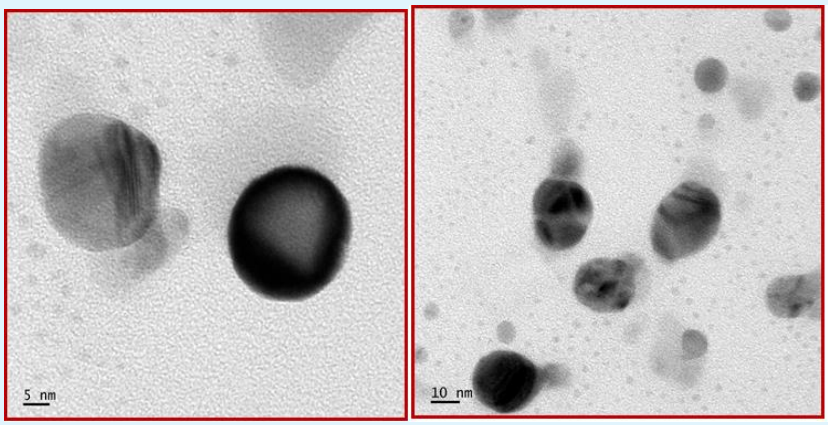

Figure 6: TEM images EAL- Ag nanoparticles with homogeneous size distribution. *In vitro* studies.

Oxidative stress and inflammation are connected pathophysiological environments that have a main role in many chronic disease. Lately, agents with antioxidant activity have been occupied in the development of new therapeutic strategies. Hence, it seems that management of oxidative stress by agents with antioxidant capacity may have a positive therapeutic effect on chronic diseases including disorders associated with inflammation. On the basis of recent studies biosynthesized silver nanoparticles (Ag-NPs) appear to have the potential to improve inflammation and oxidative stress [35].

Antioxidant Activity

Oxidation is a crucial biological process in many living organisms for the making of energy; but, the uncontrolled production of oxygen derived free radicals. Reactive oxygen species (ROS) caused damage of complex cellular molecules such as carbohydrates, proteins, lipids and DNA. This resulted in the appearance of many health problems like cancer, cardiovascular diseases, liver diseases, renal failure, inflammatory problems, and aging in general.

Antioxidants are agents that, in one way or another, limit the harmful effects of these oxidant reactions. These limitations can engage scavenging free radicals or preventing radical formation and hence can improve the immune defense and lower the opportunity of diseases occurrence. The investigation for new antioxidants is of huge significance to avoid the side effects and diseases caused by synthetic ones.

In this study, antioxidant activity of the synthesized AgNPs was investigated using 3 different assays because evaluation of antioxidant activity cannot be carried out accurately by single general method. DPPH scavenging capacity test is the best choice for the measurement of antioxidant activity because of its stability (in radical

form), simplicity, and fast assay. Antioxidants, on interaction with DPPH, either transfer electrons or hydrogen atoms to DPPH, thus neutralizing the free radical character and decreasing its absorbance [36].

In reducing power assay, the presence of antioxidants in samples would result in the reduction of Fe^{3+} to Fe^{2+} and the amount of Fe^{2+} complex was monitored by measuring the formation of blue colour at 700 nm. Increase in absorbance at 700 nm indicated an increase in reductive ability. The reducing ability of the EAL aqueous extract, EAL-AgNPs and the standard drug are shown in Table 1. The results of study of the EAL aqueous extract, EAL-AgNPs and the standard drug at concentrations of 5, 10, 15, 20, and 25 µg/ml, as discussed in the case of antioxidant activities, showed that the increase in absorbance of the reaction mixture showed an increase in the reducing power indicating the AgNPs were having a lesser reducing power than the standard. It is evident from figure 7 that the reducing powers of the EAL aqueous extract, AgNPs and the standard drug were increasing with increase in concentration.

In Nitric Oxide radical scavenging studies of the EAL aqueous extract, EAL-AgNPs and the standard drug were increasing with increase in concentration and it was found to be higher for AgNPs when compared to EAL as well as the standard shown in Table 1. In the present study, nitric oxide generated from sodium nitro prusside, at physiological pH 7.4 liberates nitrate which gets converted to nitrite which further forms nitrite ions on contact with air. The nitrite ions when diazotized with sulphanilic acid and coupled with naphthylethylene diamine formed the pink color complex, which was measured at 546 nm [37].

S. No.	Test drug	Concentration	% of Inhibition		
		µg/ml	DPPH Radical Scavenging method	Reducing Power Assay	Nitric Oxide Radical Scavenging method
1	EAL	5	77.8	79.5	77.3
		10	78.3	80.3	78
		15	79.1	81.9	78.9
		20	80.7	82.1	80.4
		25	81.9	83.4	81
2	EAL-AgNPs	5	92.4	85.2	88.5
		10	93.8	86.5	89.8
		15	94.3	87.6	90.5
		20	95.5	89.3	91.7
		25	96.2	92.5	93.1
3	Ascorbic acid (std)	5	91.2	84.5	89.8
		10	92.8	85.2	90.5
		15	93.6	86.7	91.9
		20	94.5	87.3	92.7
		25	95.8	88.6	93.8

Table 1: *In vitro* Antioxidant assay of EAL, EAL-AgNPs and the standard by DPPH, H_2O_2 and Nitric Oxide Assay methods.

The results showed that AgNPs have higher antioxidant activity as compared to onion extract or ascorbic acid and their activity increased with concentrations.

Anti-Inflammatory

In the present study the percentage of efficiency of anti-inflammatory at different concentration ranging from 5 mcg/mL to 25 mcg/mL, was studied for silver nanoparticles from leaves extract of *Enicostema axillare* by HRBC membrane stabilizing method using diclofenac as the standard. The result of the study for extract and silver nanoparticles from *Enicostema axillare* was shown in Tables 2 and 3 respectively.

S. No	Concentration mcg/mL	Absorbance	% of Inhibition
1	5	0.9723	83.79
2	10	0.8632	85.61
3	15	0.7526	87.43
4	20	0.6872	88.54
5	25	0.5932	90.11

Table 2: Inhibition Efficiency for EAL aqueous Extract.

S. No	Concentration mcg/mL	Absorbance	% of Inhibition
1	5	0.7236	87.94
2	10	0.6932	88.44
3	15	0.5372	91.04
4	20	0.4632	92.28
5	25	0.3125	94.64

Table 3: Inhibition Efficiency for EAL - Silver Nanoparticles.

The anti-inflammatory effects of extract increases with their concentrations to similar events. The standard drug Diclofenac possessed a scavenging effect of 96.95% at the concentration of 25 mcg/mL (Table 4).

S. No	Concentration mcg/mL	Absorbance	% of Inhibition
1	5	0.5632	90.61
2	10	0.4726	92.12
3	15	0.3257	94.57
4	20	0.2976	95.04
5	25	0.1826	96.95

Table 4: Inhibition efficiency for Standard (Diclofenac) solution.

Conclusion

This study has revealed that the green synthesis of silver nanoparticles using *Enicostema axillare* leaves extract has resulted in the formation of biologically active silver nanoparticles of size 16 ± 2 nm. The synthesised silver nanoparticles are capped by the phytochemicals of *Enicostema axillare* and show significant antioxidant and anti-inflammatory effects. In conclusion combining the benefits of phytomedicine with nanomedicne can result in the formation of more efficient silver nanoparticles with minimal toxic effects. This finding suggests a novel pharmacological rationale for the treatment of various inflammatory disorders.

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Conflicts of Interest

None of the authors have any conflicts of interest to declare.

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