Nyctanthes arbor-tristis mediated silver sulphide nano particles: Synthesis, characterization and anti-inflammatory activities

Richa Kothari, Harleen Rathore and Akanksha Chaturvedi

DOI: https://doi.org/10.22271/chemi.2020.v8.i2a.8751

Abstract
Silver sulphide nanoparticles (Ag$_2$S) are engineered nanoparticles with promising applications in industrial processes and have been used in infrared sensors, Solar cells optical filters and biomedical applications. Due to their low solubility Ag$_2$SNPs have been shown to be less toxic than AgNPs and Ag⁺ using aqueous extract of Nyctanthes-arbor-tristis leaves in the synthesis of silver sulphide nanoparticles is considered as green, eco-friendly, low priced biotechnology that gives importance over both chemical and physical method. In the present study the aqueous extract of fresh leaves of N. arbor tristis extract was used for green synthesis of silver sulphide nano particles plays a dual in nature in both reducing and stabilizing Ag$^{2+}$ S NPS. In UV-Visible absorption, Fourier transform (FI-IR), XRD, Transmission electron microscopy (TEM), EDX and Scanning electron microscopy (SEM) were achieved for confirming and characterizing the biosynthesized Ag$_2$SNPs. TEM images of Ag$_2$SNPs detected the spherical Ag$_2$ S NPS shape with diverse size range within 1.486-6.69nm. FT-IR analysis demonstrated the presence of free amino groups in addition to sulphur containing amino acid derivative actives as stabilizing agent as well as the presence of either sulphur or phosphorous functional groups which possibly attaches silver. In this study, biosynthesized N-Ag$_2$S NPs exhibited anti-inflammatory activity against standard aspirin drug using protein denaturation, protease inhibition assay and heat induced haemolysis method as well as cytotoxic effects against skin cancer cell line A-431 cells gives IC50 (20.11µg/ml),Haemolytic activity of Ag$_2$SNPs was investigated and confirm as being non-toxic to human RBCs in low concentration.

Keywords: Silver sulphide nano particles, n yctanthes arbor-tristis leaves, eco-friendly, anti-inflammatory activity and haemolytic activity

1. Introduction
Nowadays nano science is a rapidly developing field contributed to produce a wide variety of various synthesized metal nano particles (MNPs). Due to unique physiochemical properties of MNPs and the shapes, a promising scientific area of research appeared for biochemical applications in biomedicine, environmental bioremediation, optical and electronic fields as well as usage in drug delivery and bioimaging [1, 2]. Literature revealed that MNPs possess increased electrical conductivity, roughness and the ability to strength metals and alloys [3]. Silver is the noble metal in bio fabrication of Ag$_2$SNPs due to its wide spectrum of bactericidal and fungicidal activities as well as its ability to coordinate with various ligands and bio molecules in microbial cells. Silver which has been widely used in controlling microbial proliferation as well as curing wound healing due to its anti-inflammatory effect [4, 5]. Silver salts have been applied to inhibit the microbial infections subordinated with various medical devices like catheters, wound dressing, orthopaedic and cardiovascular implants [6]. Silver sulphide nano particles have opened new various disciplines in biomedical protocols because this showed reactivity of nano silver sulphide particles was attributed to their layer surface area-to-volume ratios [4]. Synthesis of Ag$_2$SNPs was attained physically and chemically by various approaches but these nanoparticles have some disadvantages as being expensive in addition to their hazard effects on the environment due to its very less solubility. But, green synthesis of Ag$_2$SNPs via leaf extract through green chemistry concepts to produce silver nanoparticles with increased stability [7]. Green synthesis of metallic nano particles by naturally biodegradable e components including polysaccharides, biopolymers vitamins, plant extracts.
and microorganisms represent sustainable resources in biosynthesis of metallic nanoparticles [8, 9]. Subsequently, using plant extracts could induce the required reduction for metal nanoparticle synthesis. Providing an eco-friendly low-priced technology as well as simplicity in scaling up for high production [10]. Plant based silver sulphide nanoparticles synthesis is feasible, eco-friendly as well as possessing catalytic activity for degrading different organic pollutants as an azo dyes [11]. Currently, bacteria signify a main treat facing medical remediation since the appearance of anti-biotic resistant bacterial strain as a consequence of some complicated influences of the evaluation and spread of resistance mechanism [12]. Recently, drug resistance had been emerged as a complicated remediation problem according to the over usage of the anti biotics and drugs in treating infectious disease in addition to the harmful effects and drawbacks associated with the antibiotics as an immune-suppression hypersensitivity and allergic effects. Consequently, developing new anti-microbial drugs for treating microbial pathogens and therapeutic anti-microbial agents of plant origin have high remediation effects [13]. The new developing discipline, nano technology, stimulated the production of metallic nano particles especially Ag₂SNPs characterized by low toxic influences to human and high bactericidal potential may be used as an alternative to antibiotic drugs exhibiting effects on multi drug resistant bacteria [14]. The existence of protein caps on the metallic nanoparticles support both stabilization and binding to the bacterial cell wall leading to increments in binding and the absorption of drug on patient cells [15]. The mechanism of mode of action of Ag₂SNPs anti-bacterial potential is expressed on the basis of disturbing bacterial cell permeability, cellular respiration as well as penetration inside the bacterial cell causing damage via reacting with DNA and protein (phosphate and sulphate containing compounds) [16].

2. Materials and methods

2.1 Materials
Silver nitrate and all other chemicals were purchased from Thermo Fisher scientific India Pvt. Ltd. (Mumbai). Leaves of Nyctanthes arbor-tristis were collected from the medicinal garden of ITM university, Gwalior.

2.2 Preparation of nyctanthes-arbor leaf extract
Fresh leaves of Nyctanthes Arbor plant were collected early in the morning and cleaned by washing several times with running water and subsequently with distilled water. Leaves were dried at room temperature in shade until all the moisture was lost (12-14 days). Dried leaves were then ground to yield coarse powder, 20gm of which was boiled in 100ml of double distilled water for 15 minutes. The aqueous extract of leaves was then cooled, filtered using what’s man no. 1 filter paper and stored at 4 °C for further use. Dried leaves were stored in an air tight container at room temperature for subsequent cycles of extract preparation.

2.3 Synthesis of Silver sulphide nanoparticles through green approach
0.01M Aqueous solution of silver nitrate and thio-semi carbazide hydrochloride 0.01m was prepared in separate beakers by adding 10ml distil water. The pH of the solution was maintained at 12 and the solution was stirred on magnetic stirrer continuously for about 1hr, then add 10ml of aqueous extract of Nyctanthes arbor-tristis leaf extract was added in aqueous solution of silver nitrate with different quantities and stirred for 30 minutes then solution of thio-semicarbazide hydrochloride (10ml) is added slowly drop wise and further stirred for another 30 minutes at 60 °C. A black precipitate resulted which was then dried at 60 °C overnight. Prior to drying, the precipitate was centrifuged at 4000 rpm for 20minutes and washed twice with sterile de-ionized water. Complete conversion of silver sulphide nanoparticles (Ag₂SNPs) takes place during drying. However, the synthesis conditions were optimized for the current reaction by varying various parameters involved in synthesis. Various concentrations of silver nitrate, from 0.01M to 0.03M, were used as substrates. Leaf extract was added to 10ml of silver nitrate solution in volumes ranging from 10ml to 30ml. The thio-semicarbazide hydrochloride was added to silver nitrate solution in volumes ranging from 10ml to 30ml and was maintained at pH 12. Finally, reaction temperature was maintained at 60 °C.

2.4 UV-Visible Spectroscopy
For UV-Visible Spectroscopy the resultant Ag₂ SNPs nanoparticles from each of the reaction was re suspended in equal amount of sterile de-ionized water and spectrum scans were performed using UV-Visible Spectrophotometer Perkin-Elmer UV/Vis Lambda 25 in the wavelength range of 200-700 nm.

2.5 FT-IR Spectroscopy
Fourier transform infra-red (FT-IR) spectroscopy helps to establish the presence of various phytochemical constituents involved in reduction and stabilization of biosynthesis silver sulphide nanoparticles. FT-IR spectrum for dried and powdered Ag₂SNPs was obtained using Perkin Elmer FT IR spectrophotometer Frontier using the technique of Attenuated Total Reflectance (ATR) in the range of 400-500cm⁻¹.

2.6 Fluorescence spectroscopy
Fluorescence spectroscopy is a spectroscopic method used to analyze the fluorescence properties of a sample by determining the concentration of an analyte in a sample. This technique is widely used for measuring compounds in a solution, and it is a relatively easy method to perform fluorescence spectrum for dried and powdered silver sulphide nanoparticles was obtained using Perkin Elmer LS-55, Fluorescence spectrometer.

2.7 X-ray diffraction
Washed and dried samples of Ag₂SNPs was used for XRD analysis using ultimo IV (Rigaku, Japan) at the wavelength of 1.5406 A°.XRD was performed in the range of 20-80 degrees at 40KV and 15 mA with a divergence slit of 10 mm in 2θ continuous scanning mode.

2.8 EDX study
This technique determines the elemental composition of a sample. In this study it was used to confirm the presence of silver in the particles as well to detect the other elemental compositions of the particles. Beside identification of the elements present in the sample by the use of EDX it is also possible to estimate their concentration. The particle solution was diluted 100-fold in water and a drop of 10μL diluted solution was placed on a carbon stub and air-dried. The EDX spectrum was obtained at an acceleration voltage of 20 kV and collected for 19 s. Mapping was completed using pseudo-colors to represent the two-dimensional spatial distribution of energy emissions of the chemical elements present in the
sample. Analysis was done using JEOL JSM 6360 equipped with an EDX (energy dispersive X-ray) analyzer.\(^{(17)}\)

2.9 Transmission electron microscopy
For TEM analysis, Ag\(_2\)SNPs nano powder was suspended in sterile deionised water, sonicated for 15 minutes and diluted to yield slightly turbid suspension. The suspension was then coated onto a copper grid and allowed to dry Morgang 268 D, FEI TEM studies at an accelerating voltage of 200 kV. The results were visualized using Olympus Siviewer.

2.10 Scanning electron microscopy
Recently, the field of nano science and nano technology has provided a driving force in the development of various high resolution microscopy techniques in order to learn more about nanoparticles sing a high beam of high energy electron to probe objects on a very high fine scale.\(^{(18, 19, 20)}\) Among various electron microscopy techniques, SEM is a surface imaging method, fully capable of resolving, different particle sizes, Size distribution, nanoparticles shapes and the morphology of the synthesised particles at the micro and nanoscale.\(^{(21, 22, 23, 24)}\) For SEM the Ag\(_2\)SNPs nano powder was suspended in sterile de ionized water and sonicate for 15 minutes prior to analysis using Hitachi-PU 5.0 KV 7.8mm x 120 K, LA30 (U). The combination of SEM with energy dispersive X-ray spectroscopy (EDX) can be used to examine silver sulphide powder morphology and also conduct chemical composition analysis.

2.11. In-Vitro anti-inflammatory activity

2.11.1 Inhibition of protein denaturation
Inhibition of protein denaturation by aqueous extract og crude plant extract and biosynthesised Ag\(_2\)SNPs was evaluated by the method of Mizushima et al.\(^{(25)}\) with slight modification. 500µl of 1% of bovine serum albumin was added to 100µl of crude plant extract and biosynthesised Ag\(_2\)SNPs. This mixture was kept at room temperature for 10 minutes followed by heating at 51 °C for 20min The resulting solution was cooled down to room temperature and absorbance was recorded at 660nm. Acetyl salicylic acid (aspirin) was taken as positive control. The experiment was carried out in triplicates and the percentage inhibition for protein denaturation was calculated using the following equation:

\[
\%\text{Inhibition of protein denaturation} = \left(\frac{100 - \left[\frac{A_1 - A_2}{A_0}\right]}{A_0}\right) \times 100
\]

Where \(A_1=\text{Absorbance of sample}\)
\(A_2=\text{Absorbance of biosynthesised Ag}_2\text{SNPs}\)
\(A_0=\text{Absorbance of positive control (Aspirin)}\)

2.11.2 Protease inhibition assay
Inhibition of trypsin by the aqueous extract of crude plant extract and biosynthesised Ag\(_2\)SNPs was evaluated by the method of Sakat et al.\(^{(26)}\) 100ml of bovine serum albumin was added to 100µl of crude plant extract and biosynthesised Ag\(_2\)SNPs. This solution was incubated at room temperature for 5 minutes. Reaction was inhibited by the addition of 250µl trypsin followed by centrifugation. The supernatant was collected and absorbance was observed at 210nm. The experiment was out in triplicates and the percentage inhibition of protease inhibition was calculated as

\[
\%\text{protein inhibition} = \left(100 - \left\{\frac{A_1 - A_2}{A_0}\right\}\right) \times 100
\]

2.11.3 Heat induced haemolysis method
The anti-inflammatory activity was done by the heat induced haemolysis method using a methanol as a standard. 5ml blood sample was collected from healthy human and centrifuged at 3000 rpm for 15 minutes. Cells were washed with saline. Equal volume of (200µl) Ag\(_2\)SNPs of various concentrations (25%, 50%, 100% concentration) and 400µl 10% RBC suspension was transferred in centrifuged tube and kept on water bath for 20 minutes. The absorbance at 520 nm was monitored by using UV Spectrophotometer at 10 minutes intervals. Saline containing acetysalicylic acid (aspirin) drug was taken as control.

3. Results and discussion
The present study reports the use of fresh leaves of Nycanthes arbor-tristis for synthesis of silver sulphide nanoparticles, which was free from any external stabilizing accelerating agents and does not require continuous stirring of aqueous extract of fresh leaves of Nycanthes arbor-tristis. The particles formation of black coloured particles is a clear indication of the formation of silver sulphide nanoparticle in the different ratios of the mixture.

3.1 Phytochemical screening of the aqueous extract of fresh leaves of Nycanthes arbor-tristis
The freshly prepared leaf extract was subjected to qualitative phyto chemical screening which was done to understand the presence of phytochemical constituents of the leaf extract chose for the study. For this some specific tests were performed to evaluate the presence of particular phytochemicals. The test performed for checking the availability of flavonoids, alkaloids, glycosides, steroids, phenols, saponins, terpenoids, cardiac glycosides and tannins. The qualitative estimation of leaf extract was performed and the results showed the availability of various which are present in Table 1.

| Serial No. | Phytoconstituents     | Availability in aqueous extract |
|------------|-----------------------|---------------------------------|
| 1          | Flavonoids            | +                               |
| 2          | Alkaloids             | +                               |
| 3          | Glycosides            | +                               |
| 4          | Steroids              | +                               |
| 5          | Phenols               | +                               |
| 6          | Terpenoids            | +                               |
| 7          | Saponins              | -                               |
| 8          | Resins                | +                               |
| 9          | Tannins               | +                               |
| 10         | Cardiac Glycosides    |                                 |
| 11         | Phyto sterols and    |                                 |
| 12         | Triterpenoids         | +                               |
| 13         | Carbohydrates         | +                               |
| 14         | Fixed oil and fats    | -                               |

3.2 Visual characterization
Silver nitrate solution has turned black in colour, which indicated the formation of silver sulphide nanoparticles (fig.1). According to literature, there are three phases of metallic nano particles synthesis via green approach. The first
phase is activation phase which indicates metal ion reduction and then their nucleation process starts. The second phase is considered as the growth phase, which involves the aggregation of biosynthesized small metallic nano particles. The last phase is the termination phase which facilitates the final shape and geometry of biosynthesized nano particles. In the bio-reduction process of silver nanoparticles, crystalline silver nitrate salt is dissolved in doubly distilled water. Due to ionic nature of silver nitrate salt, it immediate dissociates into $\text{Ag}^+\text{ ions and NO}_3\text{ ions}$. When freshly prepared aqueous leaf extract of Nyctanthes Arbor-testis mixed with aqueous solution of silver nitrate and the solution of thiosemicarbazide hydrochloride. The chemical functional groups are present in leaf extract immediately interact with $\text{Ag}^+$ ions and reduces it to its zero valent state $\text{i.e. Ag}$. Which leading to the formation of silver sulphide nanoparticles followed by the growth phase, leaving behind the remaining components as by product $[30]$. **Fig 1**: Biosynthesised Silver sulphide nano particles from Nyctanthes arbor-tristis leaf extract

### 3.3 UV-visible spectroscopic analysis

UV-visible spectroscopy is a very useful and reliable technique for the primary characterization of synthesised silver sulphide nano particles which is also used to monitor the biosynthesis and stability of silver sulphide nanoparticles in aqueous solution.$[^31]$ Sulphide nanoparticles are known to exhibit a UV-visible absorption maxima in the range of 400-500nm.$[^32]$ The result obtained from UV-visible spectra showed the absorption peak approximately at 450nm for freshly prepared leaf extract, 400nm for 1:1 ratio, 425nm, for 1:2 ratio and 685nm for 1:3 ratio indicating the formation of silver sulphide nanoparticles. A broad absorption peak at 400nm is due to the surface plasmon resonance absorption band along with free electronic vibrations of $\text{Ag}_2\text{SNPs}$ in resonance with a light wave.**Fig 2**: UV-Visible spectra of biosynthesised silver sulphide nano particles (1:1 ratio) **Fig 3**: UV-Visible spectra of biosynthesised silver sulphide nano particles (1:2 ratio)
3.4 Fourier transform (FT-IR) spectroscopic analysis

FT-IR is able to provide accuracy, reproducibility, and also a favourable signal to noise ratio. By using FT-IR Spectroscopy, it becomes possible to detect small absorbance changes on the order of $10^{-3}$, which helps to perform difference spectroscopy, where one could distinguish the small absorption bands of functionally active residues from the large background absorbance of the entire molecule [33, 34].

FT-IR measurements were carried out to identify the presence of possible biomolecules in aqueous extract of Nyctanthes Arbor-tristis. Which are responsible for capping ability to efficient stabilization of Ag₂SNPs. The main IR frequencies of various ratios of silver sulphide nanoparticles are shown in table 2.

**Table 2: Major FT-IR frequencies of various ratios of biosynthesised silver sulphide nanoparticles.**

| Serial No. | 1:1 ratio | 1:2 ratio | 1:3 ratio |
|------------|------------|------------|------------|
| 1.         | 3859 cm⁻¹  | 3825       | 3901       |
| 2.         | 2995       | 2995       | 3001.76    |
| 3.         | 2862       | 2865       | 2286       |
| 4.         | 1845       | 1869       | 1845       |
| 5.         | 1635.48    | 1647       | 1522       |
| 6.         | 1563       | 1532       | 1496       |
| 7.         | 1481       | 1025       | 1384       |
| 8.         | 1077       | 844        | 1078       |
| 9.         | 877        | 745        | 708        |
| 10.        | 704        | 543        | 573        |
| 11.        | 522        | 352        | 436        |
| 12.        | 359        | 360        |            |

**Fig 4:** UV-Visible spectra of biosynthesised silver sulphide nano particles (1:3 ratio)

**Fig 5:** FT-IR spectra of biosynthesised silver sulphide nano particles (1:1 ratio)

**Fig 6:** FT-IR spectra of biosynthesised silver sulphide nano particles (1:2 ratio)
The strong bands at 3859, 3825, and 3901 cm\(^{-1}\) may result from the N-H stretching vibrations. The prominent bands at 2995, 2865, 2126, 1635.48, 1532, 1152 cm\(^{-1}\) can be assigned as absorption bands of C=H, -OH, -SH, N=N, -C=O and S=O stretching vibrations. Such bands are arisen due to water soluble compounds such as flavonoids, alkaloids, and polyphenols are presents in Nyctanthes Arbor-tristis leaves. Biological components are interact with silver nitrate solution along with thio-semicarbazide hydrochloride solution via these functional groups and mediates their self-reduction to silver sulphide nanoparticles. Previous studies have revealed that terpenoids are often associated with formation of nanoparticles by dissociating eugenol-OH group protons, thus generating structures that can be further oxidised leading to the reduction of metal ions and ultimately the formation of nanoparticles. The flavonoids showed tautomeric shift from keto to enol form, as a result of this, nascent hydrogen gas released which caused the reduction of Ag\(^+\) ion into zero valent silver sulphide nanoparticles The presence of plants sugar in leaf extract, oxidises hydroxyl group into aldehyde group and again aldehydic group converted into carboxylic group which was responsible for the reduction of metal ion into metallic nanoparticles. In the same way different functional groups have different reactions mechanism for biosynthesis of nano metallic nanoparticles. The extract reaction mechanism behind the synthesis of nano particles is still unknown and this area thus needs further elaboration.

3.5 Fluorescence study
Biosynthesized AgNPs are reported to exhibit visible photoluminescence and their fluorescence spectra are shown in Fig (8, 9, 10). The optimized Ag \(\rightarrow\) S NPs were found to be luminescent with three emissions at 550,750nm for 1:1 ratio, 590,900nm for 1:2 ratio and 525, 890nm for 1:3 ration, for an excitation at 350 nm. When Ag\(_2\)SNPs were excited at 550nm, it showed excitation at 590 nm, the excitation of 550nm is of high intensity in comparison to another one. The luminescence at 250 nm and 300 nm may be due to the presence of phytoconstituents or antioxidants present in the leaf extract. The Ag \(\rightarrow\) S NPs synthesized using Nyctanthes arbor-tristis leaf extract are also reported to be luminescent with emission band at 561 and 600 nm.
3.6 Powder X-Ray diffraction analysis

X-ray diffraction (XRD) is a popular analytical technique which has been used for the analysis of both molecular and crystal structures\(^{40}\), qualitative identification of various compounds\(^{40}\), quantitative resolution of chemical\(^{41}\), measuring the degree of crystallinity\(^{42}\), isomorphous substitution\(^{43}\) and the particle size\(^{44, 45}\). XRD is a non-destructive technique with great potential for the characterisation of both organic and inorganic crystalline materials\(^{46}\). This method has been used to measure phase identification, conduct qualitative analysis and to determine structure imperfections in samples from various disciplines, such as geological polymer, environmental, pharmaceutical and forensic science. The crystalline structure nature of Ag\(_2\)SNPs was confirmed by the analysis of the X-Ray diffraction diagrams.

| Table 3: X-Ray diffraction peaks (2θ values) of various ratios of biosynthesised silver sulphide nanoparticles |
|---|---|---|
| 1:1 | 1:2 | 1:3 |
| 38.139 | 38.2 | 38.12 |
| 44.57 | 44.5 | 44.17 |
| 64.45 | 64.59 | 64.4 |
| 77.402 | 77.419 | 77.3 |
| 81.44 | 1.180 | 0.81 |

The estimated particle size was below 100nm (Calculated by using Debye-Scherer equation) the width of the peaks obtained in XRD pattern is cognate to the crystalline size of the particle. The small size of nanoparticles indicated the high surface area and high surface area to volume ratio.\(^{47}\)

\[
D = \frac{0.9 \lambda}{\beta \cos \theta}
\]

Where D = Crystalline size

\(\lambda\) = wavelength of X-Rays

\(\beta\) = Full width at half maximum of the diffraction peak

\(\theta\) = Bragg’s angle
The average crystalline size of various ratios of biosynthesised silver sulphide nano particles are as follows in table 4.

**Table 4: Average particle size of various ratios of silver sulphide nano particles**

| S. No | Various ratios (AgNO₃+TSC+Leaf extract) | Average particle size in nm |
|-------|----------------------------------------|-----------------------------|
| 1.    | 1:1                                    | 1.486 nm                    |
| 2.    | 1:2                                    | 6.09 nm                     |
| 3.    | 1:3                                    | 6.69 nm                     |
3.7 Transmission Electron Microscopy

TEM image silver sulphide nanoparticles solution providing information on the morphology and the size of the nanoparticles shown in the fig. -14, 15. It exhibits that the particles are well dispersed, crystalline in nature. The particle size in all 3 ratios were ranging from 1.48nm to 6.69nm. The TEM images shows that nano particles are not combined but are separated by equal distances between the particles, which was confirmed by microscopy visualizing under higher resolution. TEM images confirms that shape of silver sulphide nano particles are spherical in shape. The TEM images explains that the silver sulphide nano particles are bounded with the phytochemicals of the leaf extract.

![Fig 14: TEM Images of biosynthesised silver sulphide nano particles](image)

3.8 Scanning electron microscopy

SEM is a surface imaging analysis method, fully capable of resolving different particle sizes, size distributions, nanomaterial shapes and surface of morphology of the synthesized nanoparticles at the macro and nano scale (48, 49, 50, 51). The combination of SEM with energy- dispersive X-Ray spectroscopy (EDX) can be used to examine silver sulphide nano particles morphology and also conduct chemical composition analysis. The SEM images of the Ag₂SNPs as shown in fig.-15. The formation of silver sulphide NPs, as well as their morphological dimensions in the SEM study demonstrated that the average size was _1.48-6.69nm_ with inter particle distance. It is seen that Ag₂SNPs of different shapes were obtained in leaf extract being used as reducing and capping agents formed approximately, tubular and cuboidal Ag₂SNPs respectively. This may be due to the availability of different quantity and nature of capping agents present in leaf extract.

![Fig 15: SEM Images of biosynthesised silver sulphide nano particles](image)

3.9 EDX studies

Fig.16 shows the EDX spectrum of the biosynthesized silver sulphide nano particles nanoparticles. Silver (Ag) signal comes from the Ag₂S nanoparticles and the atomic percentage of silver is 5.75%. Except for Ag, there were also some other peaks. The atomic percentages of Carbon (C), Oxygen (O), and Sulphur are 28.42%, 26.22%, and 2.10% respectively. The carbon (C) signal came from the adsorbed components of the leaf extract as well as coating material of the instrument. The signals of O and S may be due to adsorption of leaf extract over Ag₂S NPs. The signal of O may partly be coming from the atmosphere or-OH from the NaOH used for pH adjustment. Except carbon, other elements have a very low atomic percentage compared to silver, and suggest the formation of pure Silver sulphide nano particles. The signal from the EDX studies confirms the presence of silver. It indicates that the reduction of silver nitrate and thiosemicarbazide hydrochloride solution in silver sulphide nano particles using fresh leaf extract of Nyn --------. The weight composition of silver is 5.75% and the atomic composition is then calculated as 1.29% respectively. The other impurities is found such as carbon, oxygen, sulphur was identified because of the interaction with the leaf extract during processing.

Ident-Spectrum Date: 11/5/2019 11:28:15 AM HV: 15.0kV Puls th: 1.24k cps
3. And growing size of inflammatory responses of Ag₂SNPs are still limited. The leaves of Nyctanthes arbor-tristis exhibited significant Anti-proteinase activity. The percentage of inhibition was observed in Nyctanthes arbor-tristis leaf extract. The standard drug Aspirin showed maximum proteinase inhibitory action 92.8%. The Nyctanthes arbor-tristis leaf extract was observed as crude leaf extract 87% and different ratios of Ag₂SNPs were observed as ~95.7%, 94.3% and 99.24% respectively.

### Table 6: Inhibition of Anti-proteinase activity

| S. No. | Samples               | Protection ±SD (%) | P value | Error value |
|--------|-----------------------|--------------------|---------|-------------|
| 1.     | Crude leaf Extract    | 87%                | 0.01    | 0.087       |
| 2.     | Ag₂SNPs (1:1)         | 95.7%              | 0.01    | 0.095       |
| 3.     | Ag₂SNPs (1:2)         | 94.3%              | 0.01    | 0.094       |
| 4.     | Ag₂SNPs (1:3)         | 99.24%             | 0.01    | 0.099       |
| 5.     | Standard drug (Aspirin)| 88±0.24            | 0.01    | 0.088       |
| 6.     | Control               | No inhibition      | —       | —           |

3.10.3 In vitro Heat-induced haemolysis method
The In vitro anti-inflammatory activity was studied for leaf extract and different ratios of biosynthesized Ag₂ SNPs particles. The results are summarised in table 2.

### Table 7: In vitro anti-inflammatory activity by heat-induced haemolysis method

| S. No. | Samples | Protection ±SD (%) | P value | Error value |
|--------|---------|--------------------|---------|-------------|
| 1.     | Crude leaf extract    | 50%                | 0.01    | 0.050       |
| 2.     | 1:1                 | 42%                | 0.01    | 0.042       |
| 3.     | 1:2                 | 13%                | 0.01    | 0.013       |
| 4.     | 1:3                 | 48.3%              | 0.01    | 0.048       |

4. Discussion
The synthesis of silver sulphide nanoparticles by green, unique and eco-friendly pathway using the natural extract of Nyctanthes arbor-tristis fresh leaves as an effective anti-inflammatory agent. The UV-visible spectra as a function of time showed increasing absorbance bands indicating gradual reduction as well as nucleation and growing size of nanoparticles. The absorption spectra of biosynthesised Ag₂ SNPs have been known to be antimicrobial, but the anti-inflammatory field Ag₂ SNPs have been observed as 95.7%, 94.3% and 99.24% respectively.

**Fig 16:** EDX spectra of silver sulphide nano particles which shows the presence of Ag, S, C, O Elements.
SNPs were rerecorded against ethanol in order to monitor the formation and the stability of nano particles. The colour change of the solution (leaf extract + AgNO₃+ Thiosemicarbazide hydrochloride) was first recorded through visual characterization. The colour change following incubation is due to the formation of plasmon at the colloid surface, indicating the synthesis of Ag₂SNPs.²⁸ The effect of leaf extract quantity, silver nitrate and thiosemicarbazide hydrochloride concentration on biosynthesis of nanoparticles have been studied. Result reveal that surface plasmon resonance absorption band increases with increased quantities of reactants.

A typical FT-IR spectrum of the obtained silver sulphide nanoparticles to identify the potential biomolecules present in the leaf extract responsible for the reduction and also the capping agent responsible for the stability of the bio reduced silver sulphide nano particles was associated with absorption bands, indicating various stretching modes including C-H, N-H, O-H, C≡C, C=N, C=O and C-O:⁵⁷,⁵₈,⁵₉. From FT-IR analysis, it is prove that the silver sulphide nano particles are capped with phytochemicals with various functional group of organic molecules such as a flavonoids, saponins, steroids, terpenoids, tannins or phenols, which give characteristic peak in the spectrum. The typical XRD pattern is compatible with cubic phase of silver with corresponding to the various diffraction peak to the FCC structure (JCPDS file: No. 89-3722). No other characteristic peaks were found, indicating the high purity of the prepared silver sulphide nano particles. The most intense peaks of Ag₂SNPs were chosen to calculate the average crystalline particles size. Thus we selected the (111) lattice planes of silver sulphide nano particles. The intense and narrow diffraction peaks reveal that the crystalline nature of the synthesised nano particles.⁶⁰ Distribution, surface morphological and nano structural studies of synthesized Ag₂SNPs have been investigated using HR- TEM combination with EDX. It has been cleared that the biosynthesised silver sulphide nano particles were crystalline aggregated, spherical in shape with varied size, such variation in size is common when using biological systems for the synthesis.⁵⁵

The large particles size of the nano particles maybe due t the FCC structure of metals. They have the tendency to nucleate and grow into twinned particles with their high surface bounded by the lowest every facets. Silver sulphide nano particles have their tenancy to agglomerate due to their high surface tension of ultra-fine nano particles. The fine particle size of silver sulphide nano particles enhances the nano particles activities. The EDX spectra f bioreduced silver sulphide nanoparticles indicated that the silver sulphide nano particles contain pure silver sulphide without oxide. The ability of silver sulphide nano particles to inhibit protein denaturation has been investigated for potential anti-inflammatory action mechanism. It is very well documented that protein denaturation is involved in arthritic reactions and development of tissue damage during inflammation.⁶¹,⁶²,⁶³,⁶⁴,⁶⁵,⁶⁶ Results showed that synthesized silver sulphide nanoparticles were effective in inhibiting thermally induced protein denaturation at all tested concentrations, indicating their capability of controlling protein denaturation involved in the inflammatory process. Thus the inhibition of protein denaturation, protease inhibition and hemolytic activities of silver sulphide nanoparticles synthesised from Nycanthes arbor-tristis leaf extract clearly establish their anti-inflammatory potential and therefore can be considered as potential drug of the anti-inflammatory drug. However, one should try to further figure out other action mechanism responsible for this activity via other detailed experimentations.

5. Conclusion

The concept of nanomedicine has risen to be the future of medicine excellent advantages of using silver sulphide nanoparticles to treat inflammation have many benefits such as low cost, high efficacy, eco-friendly. Silver sulphide nanoparticles mediated from Nycanthes arbor-tristis leaf extract have been obtained and characterized from various biophysical techniques like UV visible, FT-IR, XRD, TEM, SEM-EDX etc. Synthetic optimisation shows increases in plasmon resonance bands with the Silver nitrate, thiosemicarbazide hydrochloride and fresh leaf extract concentration as well as pH. The synthesized nanoparticles found to be crystalline spherical in shape and biofunctionalized with organic molecules. The biosynthesized Ag₂ SNPs exhibited significant inhibition of protein denaturation, protease Inhibition and hemolytic activity, indicating a strong anti-inflammatory potential of nanoparticles, hence, could be considered as a potential source of the anti-inflammatory drug.

6. Acknowledgement

Richa Kothari thanks the management of ITM University, Gwalior for granting a mobility Grant in 2019. The authors are thankful to the SAIF Chandigarh for spectral analysis. Authors also thankful to the multi-disciplinary laboratory of ITM University Gwalior for providing technical support.

7. Footnotes

7.1 Disclosure: The authors report no conflicts of interest in this work

8. Graphical Method

9. References

1. Bilal M, Rasheed T, Iqbal HMN, Hu H, Zhang X. Silver nanoparticles: Biosynthesis and antimicrobial potentialities. Int. J Pharmacol. 2017; 13:832-845.
2. Sharma A et al. Algae as crucial organisms in advancing nanotechnology: a systematic review. J Appl Phycology. 2015; 28:1759-1774.
3. Rittner MN, Abraham T. Nanostructured materials: An overview and commercial analysis. JOM. 1998; 50:37-38.
4. Chaloupka K, Malam Y, Seifalian AM. Nanosilver as a new generation of nano product in biomedical applications. Trends Biotechnol. 2010; 28:580-588.
5. Lansdown AB. A pharmacological and toxicological profile of silver as an antimicrobial agent in medical devices. Adv Pharmacol., 2010.

6. Wijnhoven SW et al. Nano-silver: A review of available data and knowledge gaps in human and environmental risk assessment. Nanotoxicology. 2009; 3:109-138.

7. Mohanpuria P, Rana NK, Yadav SK. Biosynthesis of nanoparticles: technological concepts and future applications. J Nanopart Res. 2008; 10:507-517.

8. Huang J et al. Biosynthesis of silver and gold nanoparticles by novel sundried Cinnamonum camphora leaf. Nanotechnology. 2007 18:105-104.

9. Shukla AK, Iravani S. Metallic nanoparticles: green synthesis and spectroscopic characterization. Environ Chem Lett, 2017, 1-9.

10. Saxena A, Tripathi R, Zafar F, Singh P. Green synthesis of silver nanoparticles using aqueous solution of Ficus benghalensis leaf extract and characterization of their antibacterial activity. Mater Lett. 2012; 67:91-94.

11. Rasheed T et al. Catalytic potential of bio-synthesized silver nanoparticles using Convolvulus arvensis extract for the degradation of environmental pollutants. J Photochem Photobiol B. 2018; 181:44-52.

12. Holmes AH et al. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 2016; 387:176-187.

13. Jain D, Daima HK, Kachhwaha S, Kothari S. Synthesis of plant-mediated silver nanoparticles using papaya fruit extract and evaluation of their anti-microbial activities. Dig J Nanomater Biostruct. 2009; 4:557-563.

14. Song JY, Kim BS. Rapid biological synthesis of silver nanoparticles using plant leaf extracts. Bioprocess Biosyst Eng. 2009; 32:79.

15. Zhang Y, Cheng X, Zhang Y, Xue X, Fu Y. Biosynthesis of silver nanoparticles at room temperature using aqueous aloe leaf extract and antibacterial properties. Colloids Surf A Physicochemical Eng Asp. 2013; 423:63-68.

16. Ghosh S et al. Synthesis of silver nanoparticles using Dioscorea bulbifera tuber extract and evaluation of its synergistic potential in combination with antimicrobial agents. Int J Nanomedicine. 2012; 7:483.

17. Majumdar R, Bag BG. Maity Acacia nilotica (Babool) leaf extract mediated size-controlled rapid synthesis of gold nanoparticles and study of its catalytic activity Int. Nano Lett. 2013; 3:53.

18. Pawley J. The development of field-emission scanning electron microscopy for imaging biological surfaces. Scanning. 1997; 19:324-336.

19. Wang ZL. Transmission electron microscopy of shape-controlled nanocrystals and their assemblies. J Phys. Chem. B. 2000; 104:1153-1175. Doi: 10.1021/jp993593c.

20. Yao H, Kimura K. Field emission scanning electron microscopy for structural characterization of 3D gold nanoparticles on super lattices. In: Méndez-Vilas A, Díaz J, editors. Modern Research and Educational Topics in Microscopy. Formatex Research Center, Badajoz, Spain: 2007:568-575.

21. Fissan H, Ristig S, Kaminski H, Asbach C, Epple M. Comparison of different characterization methods for nanoparticle dispersions before and after aerosolization. Anal. Methods, 2014; 6:7324-7334.

22. Johal MS. Understanding Nanomaterials. CRC Press; Boca Raton, FL, USA, 2011.

23. Hall JB, Dobrovolskaia MA, Patri AK, McNeil SE. Characterization of nanoparticles for therapeutics. Nanomed. Nanotechnology. Biol. Med. 2007; 2:789-803.

24. Ranter BD, Hoffman AS, Schoen FJ, Lemons JE. Biomaterials Science-An Introduction to Materials in Medicine. Elsevier; San Diego, CA, USA, 2004.

25. Rao TA. Records the botanical survey of India, 1961, 2

26. Harborne JB. Phytochemical Methods, 3rd ed. New York: Chapman and Hall Inst, 1998.

27. Savith VS. Philip D. Spectrochim Acta A, 2014,1185-26

28. Sakat S, Juvekar AR, Gambhire MN Int. J. Pharm. Pharm. Sci. 2010; 2:146.

29. Senguttuvan J, Paulsamy S, Kathnika K. Phytochemical analysis and evaluation of leaf and o parts of the medicine herbs. Hypochoeris radiacta L. for invito antioxidant activities Asian Pac. J Trop Biomed. 2014; 4(1):359-367.

30. Cerda JS, Gomez HB, Nofiezc GA, Riveroa IA, Poncea YG, Lopez LZ. A green synthesis of nanoparticles using native cyclexdextrins as stabilizing agents, journal of Saudi chemical society. 2017; 21:341-348.

31. Sasty M, Patil V, Sanikar SR. Electrostatically controlled diffusion of carboxylic acid derivatized silver colloidal particles in thermally evaporated fatty amine films. J Phys. Chem. B. 1998; 102:1404-1410.

32. Sasty M, Mayya KS, Bandyopadhyay K. pH dependant changes in the optical properties of carboxylic acids derivative silver colloidal particles colloids surf. A. 1997; 127:221-228.

33. Vogal R, Siebat F. Vibrational spectroscopy as a tool for probing protein functions. Curr. Opin. Chem. Biol. 2000; 4:518-523.

34. Kim S, Bav BA. Reaction-induced FT-IR. Spectroscopic studies of biological energy conversion in oxygenc photosynthesis and transport. J phys. Chem. B. 2001; 105:4072-4083.

35. Ganesh Babu MM, Gunase Karan P. Production and structural characterization of crystalline silver nanoparticles from Bacillus (ereus isolate, colloids surface, B: Biointerace. 2009; 74:191-195.

36. Single AK, Tolat M, Singh DP, Srivastava ON. “Biosynthesis of gold and silver nanoparticles by natural precursor clove and their functionalization with amino group. J Nanoparticles Res. 2012: 12:1667-1675.

37. Shiv Shanar S, Ahamad A, Paskha R, Sajady MJ. Bioreduction of chloroaurate ions by geraun leaves and its endophytic fungus yield gold nanoparticles of different shapes, Metre chem. 2003; 13:1822-1846

38. Ahmed S, Saifullah Ahmad M, Swami BL, Ikram S. Green synthesis of silver nanoparticles using Azadirachta indica aqueous leaf extract. Journal of Radiation Research and Applied Sciences. 2016; 9(1):1-7.

39. Das R, Nath SS, Chakdar D, Gope G, Bhattacharjee R. Preparation of silver nanoparticles and their characterisation J Nanotechnology. 2009; 5:1-6.

40. Waseda Y, Matsubara E, Shinoda K. x-ray Diffraction Crystallography: Introduction, Examples and solved Problems. Springer Verlag: Berlin, Germany, 2011.

41. Invavasive I. Physical stability studies of miscible amorphous solid dispersion J Pharm, Sci. 2011; 99:4005-4012.

42. Carbal M, Pediosa F, Margarido F, Noaureia C. A End-of lofe Zn-MnO₂ batteries: Elcrod materials characterisation Envior. Technol. 2013; 34:1283-1295.
43. De, A, Mukhopadhyay AK, Gangadharan S, Sinha MK, Bosu D. Characterization of microplasma sprayed hydroxyapatite coating J Therm spray Technol. 2009; 18:578-592.

44. Amanias D, Pae FA, Carlos LD, Rocha J. Chiral microporous rare earth silico-germanates: synthesis, structures and photoluminescence properties, Microporous mesoporous Mater. 2013; 166:50-58.

45. Singh DK, Pandey DK, Yadav RR, Singh D. A Study of ZnO nanoparticles and ZnO- EG nanofluid J Exp Nanosui. 2013; 8:567-57.

46. Das R, aLI e, hamid SB current application of X-Ray powder diffraction-A, review Rev. Adv. Mater. Sci. 2014; 38:95-109.

47. Venkateswarlu K, Sreekanth D, Sandhyarani M, Muthupandi V, Bose AC, Rameshbabu N. X-ray peak profile analysis of nanostructured hydroxyapatite and fluorapatite. International Journal of Bioscience, Biochemistry and Bioinformatics. 2012; 2:389-393.

48. Fissan H, Ristig S, Kaminski H, Asbach C, Epple M. Comparison of different characterization methods for nanoparticle dispersions before and after aerosolization. Anal. Methods. 2014; 6:7324-7334.

49. Johal MS. Understanding Nanomaterials. CRC Press; Boca Raton, FL, USA, 2011.

50. Hall JB, Dobrovolskaia MA, Patri AK, McNeil SE. Characterization of nanoparticles for therapeutics. Nanomed. Nanotechnol. Biol. Med. 2007; 2:789-803. Doi: 10.2217/17435889.2.6.789.

51. Ranter BD, Hoffman AS, Schoen FJ, Lemons JE. Biomaterials Science-An Introduction to Materials in Medicine. Elsevier; San Diego, CA, USA, 2004.

52. Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: Molecular and cellular mechanisms. J Investing. Dermatol. 2007; 127:514-525.

53. Wong CK, Cheung PF, Ip WK, Lam CW. Intracellular signaling mechanisms regulating toll-like receptor-mediated activation of eosinophils. Am J Respir. Cell Mol. Biol. 2007; 37:85-96. doi: 10.1165/rcmb.2006-0457OC.

54. Broughton G, Janis JE, Attinger CE. The basic science of wound healing. Plast. Reconstr. Surg. 2006; 117:12s-34s. Doi: 10.1097/01.prS.0000225430.42531.e2.

55. Witte MB, Barbul A. General principles of wound healing. Surg. Clin. N. Am. 1997; 77:509-528. Doi:10.1016/S0039-6109(05)70566-1.

56. Mulvaney P. Surface Plasmon spectroscopy of nano sized meta particles. Langmuir. 1196; 12(3):788-800.

57. Jyoti K, Baunthiyal M, Singh A. Characterization of silver nanoparticles synthesised using urtica dioica Linn, leaves and their synergistic effects with antibiotics J Radiot Res Appl Sci. 2016; 9(3):217-227.

58. Tarchewski M, Cwmegga HK, Tuckeman R, Baurreccka S, FI-TR study f CO2 and H2O/CO2 nano particles and their temporal evaluation at 80 k, J Phys Chem. 2005; 109(15):3337-3343.

59. Gayathri S, Ghosh OSN, Sathish Kumar S. Investigation of physiochemical properties of Ag doped ZnO nano particles prepared by chemical route Appl Sci. Lett. 2015; (1):8-13]

60. Wang P, Hauang B, Lou Z et al. Synthesis of highly efficient Ag2AgCL plasmonic. Photocatalyst with arious structure, chemistry. 2010; 16(2):538-544.

61. Awward AM, Salem NM, Ibrahim 2M, Abdeen AO, Phytochemical silver/silver chloride nano particles usin Albiza julibrissim flowers extract, Adv. Matwe Latt. 2015; 6(8):726-730.

62. Annamalai J, Nallamuthu T. Green Synthesis of silver nanoparticles: characterization and determination of antibacterial potency. Appl. Nano Sci. 2006, 259-265.

63. Opie EL. On the relation of necrosis and inflammation to denaturation of protein, J experiment med. 1962; 115:597-608.

64. Umapatly E, Ndebia EJ, Meeme A et al. An experimental evaluation of Albunea setosa aqueous extract on membrane stabilization, protein denaturation and white blood cell migration during acute inflammation J Med. Plants Res. 2010; 4:789-795.

65. King AE, Critically HO, Kelly RW. Presence of secretary leukocyte protease inhibitor in human endometrium and first trimester decidua suggest and antibacterial protective role. Mil. they reproduce. 2000; 6(2):191-196.

66. Make S, Mc William AS, Kita H. Trypsin induces activation and inflammatory mediator release from human eosinophils through protease activated receptor-2. J Immunol. 2001; 167(11):6615-6622.