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

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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_2SNPs 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 biosynthesised Ag_2SNPs. TEM images of Ag_2SNPs 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 IC5O (±0.11µg/ml).Haemolytic activity of Ag_2SNPs was investigated and confirm as being non-toxic to human RBCs in low concentration.

Keywords: Silver sulphide nano particles, *nyctanthes* 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_2SNPs due to it wide spectrum of bactericidal and fungicidal activities as well as its ability to coordinate with various ligands and bio molecules in microbial cells. Sliver 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_2SNPs 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_2SNPs 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 Ag_2SNPs 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 surface leading to increments in binding and the absorption of drug on patient cells [15]. The mechanism of mode of action o Ag_2SNPs 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 (Ag_2SNPs) 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 Ag_2 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. FI-IR spectrum for dried and powdered Ag_2SNPs was obtained using Perkin Elmer FI 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 Ag_2SNPs was used for XRD analysis using ultimo IV (Rigaku, Japan) at the wavelength of 1.5406 A^0.XRD was performed in the range of 20-80 degrees at 40KV and 15 mA with a divergence slit of 10 mm in 20/θ 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 as 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_2SNPs 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_2SNPs nano powder was suspended in sterile de ionized water and sonicate for 15 minutes prior to analysis using Hitchi-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_2SNPs 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_2SNPs. 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:

$$\%\text{Inhibition protein denaturation} = \{\frac{100-\{A1-A2\}}{Ao} \times 100\}$$

Where A_1=Absorbance of sample
A_2 =Absorbance of biosynthesised Ag_2SNPs
Ao= 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_2SNPs 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

$$\%\text{ protein inhibition} = [100-\{ A1 - A2/_{Ao} \}] \times 100$$

Where A_1= absorbance of the sample
A_2 = absorbance Ag_2SNPs
Ao= absorbance of the positive control

2.11.3 Heat induced haemolysis method

The anti-inflammatory activity was done by the heat induced haemolysis method [27, 28] 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 (P^H=7.2). Equal volume of (200µl) Ag_2SNPs 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 acetylsalicylic acid (aspirin) drug was taken as control.

3. Results and discussion

The present study reports the use of fresh leaves of Nyctanthes 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 Nyctanthes 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 Nyctanthes 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 cheeking the availability of flavonoids, alkaloids, glycosides, steroids, phenols, saponins, terpenoids, cardiac glycosides and tannins.[29] The qualitative estimation of leaf extract was performed and the results showed the availability of various which are present in Table 1.

Table 1: The qualitative estimation of phytoconstituents present in Nyctanthes arbor-tristis leaf extract

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 Triterpenoids	+
12.	Carbohydrates	+
13.	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 Ag^{+} ions and $NO^{-}_{3\ ions}$. When freshly prepared aqueous leaf extract of Nyctanthes Arbor-testis mixed with aqueous solution of silver nitrate and the solution of thio-semicarbazide hydrochloride. The chemical functional groups are present in leaf extract immediately interact with Ag^{+} ions and reduces it to its zero valent state 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 Ag_2SNPs in resonance with a light wave.

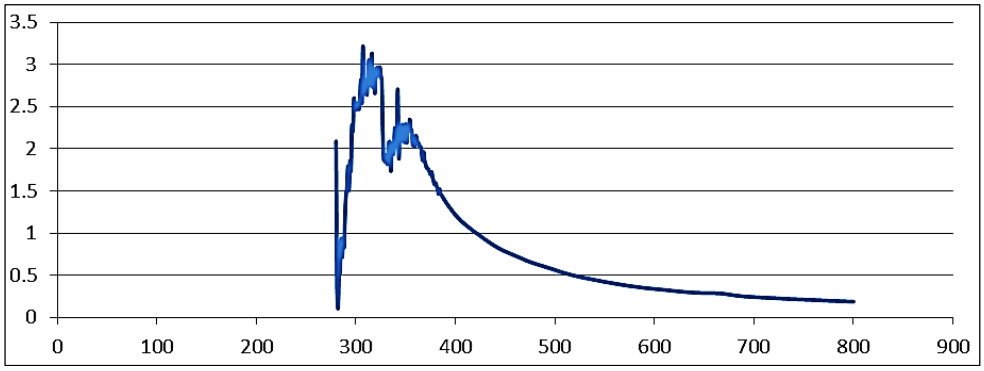

Fig 2: UV-Visible spectra of biosynthesised silver sulphide nano particles (1:1 ratio)

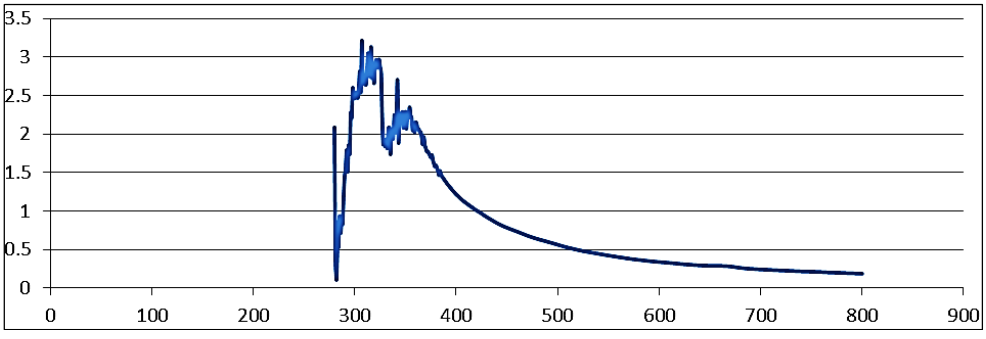

Fig 3: UV-Visible spectra of biosynthesised silver sulphide nano particles (1:2ratio)

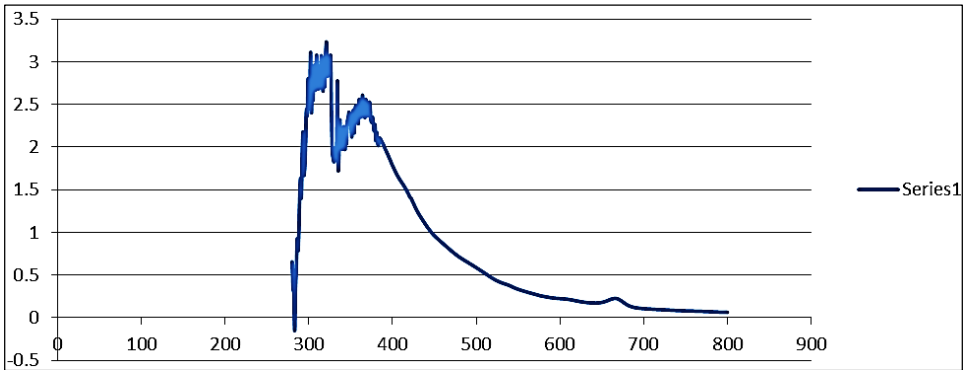

Fig 4: UV-Visible spectra of biosynthesised silver sulphide nano particles (1:3 ratio)

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 resides 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 Ag_2SNPs. 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	1:3ratio
1.	3859cm^{-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 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)

Fig 7: FT-IR spectra of biosynthesised silver sulphide nano particles (1:3 ratio)

The strong bands at 3859, 3825, and 3901 cm^{-1} may results from the N-H stretching vibrations. The prominant bands at 2995, 2865, 2126, 1635.48, 1532, 1152 cm^{-1} can be assigned as absorption bands of-C=H,-OH,-SH,-N=C=N,-C=O and-S=O stretching vibrations. Such bands are arised 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.[35] 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 [36]. 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 [37]. 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_2S 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_2S NPs synthesized using Nyctanthes arbor-tristis *leaf* extract are also reported to be luminescent with emission band at 561 and 600 nm [38].

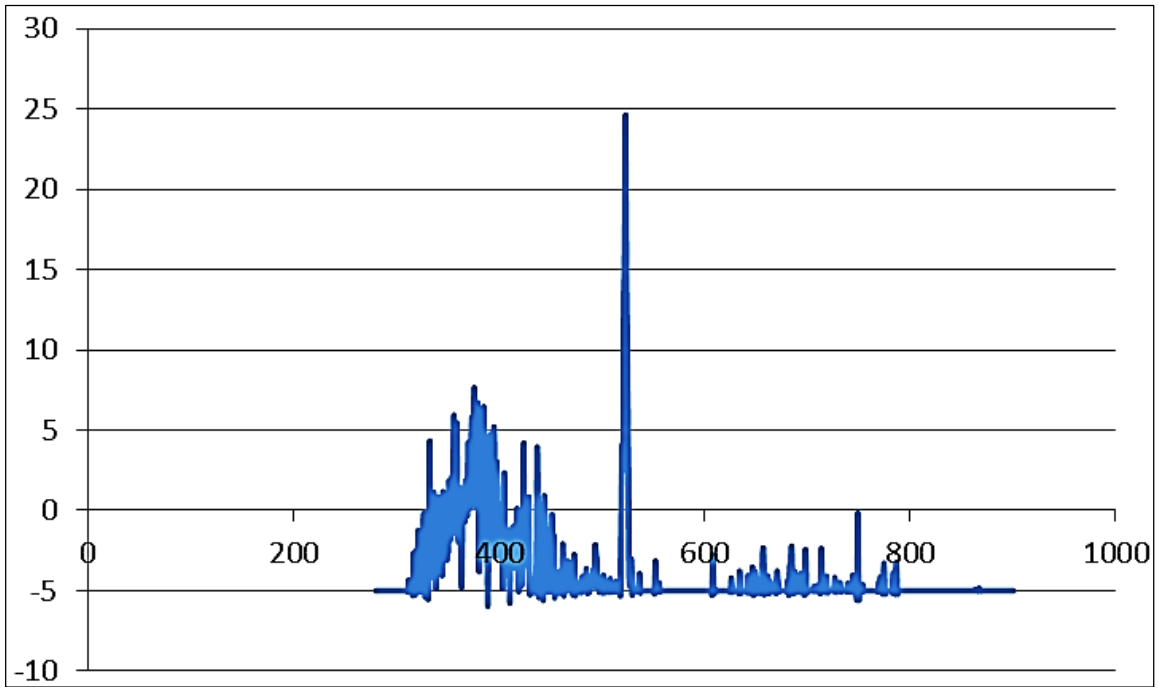

Fig 8: Fluorescence spectra of biosynthesised silver sulphide nano particles 1:1 ratio

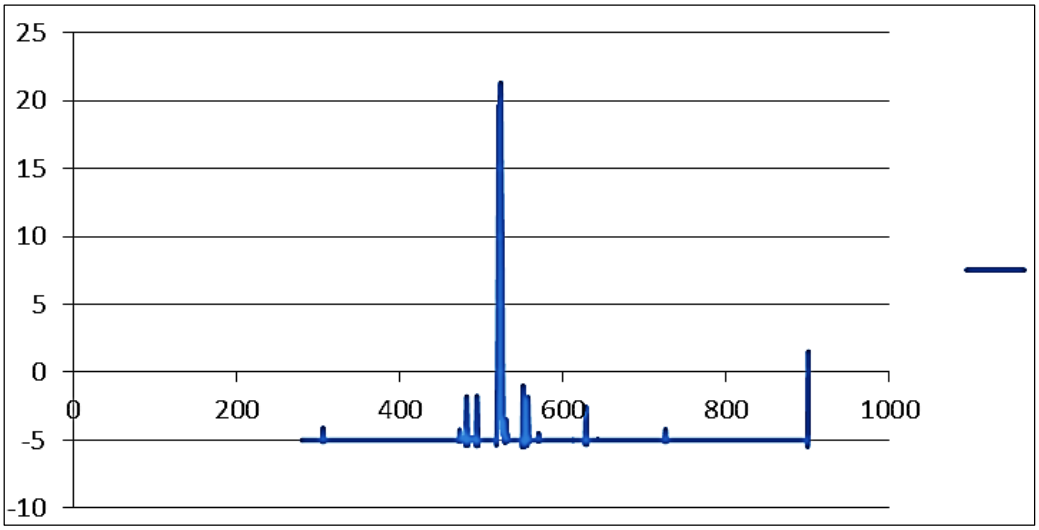

Fig 9: Fluorescence spectra of biosynthesised silver sulphide nano particles 1:2

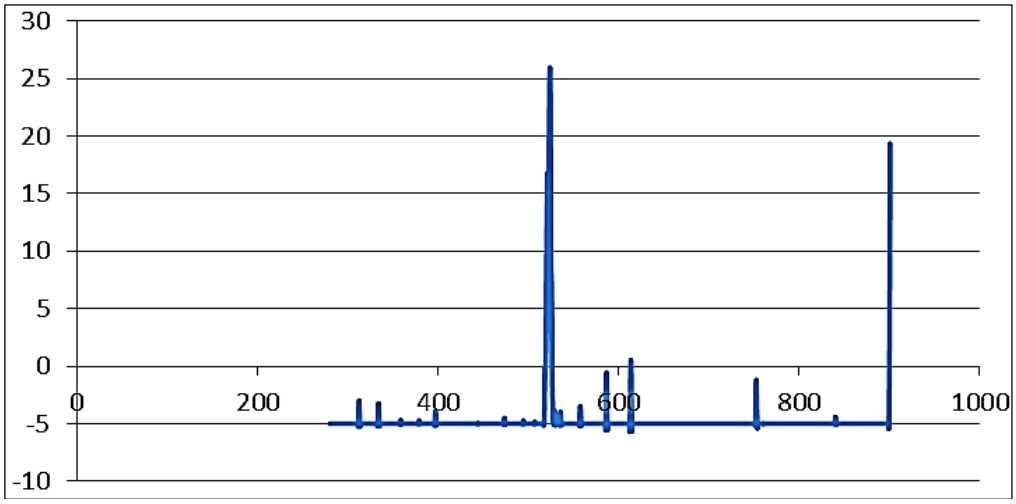

Fig 10: Fluorescence spectra of biosynthesised silver sulphide nano particles 1:3

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[39]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_2SNPs 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
44.457	44.5	44.17
64.45	64.59	64.4
77.402	77.419	77.3
81.44	1.180	0.81

The four different peaks 38.12, 44.5, 64.4and 77.3 are indeed as 111, 200, 220, 311 phases of FCC silver (JCPDS file Np. 89-3722). From the full width at half maximum of diffraction peaks (111) is employed to calculate the average crystalline size using Debye- Scherer's equation. i.e.,

$$D = \frac{0.9\,X\,\lambda}{\beta\cos\theta}$$

Where D = Crystalline size

$\boldsymbol{\lambda}$= wavelength of X- Rays
$\boldsymbol{\beta}$= Full width at half maximum of the diffraction peak
$\boldsymbol{\theta}$= Bragg's angle

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]

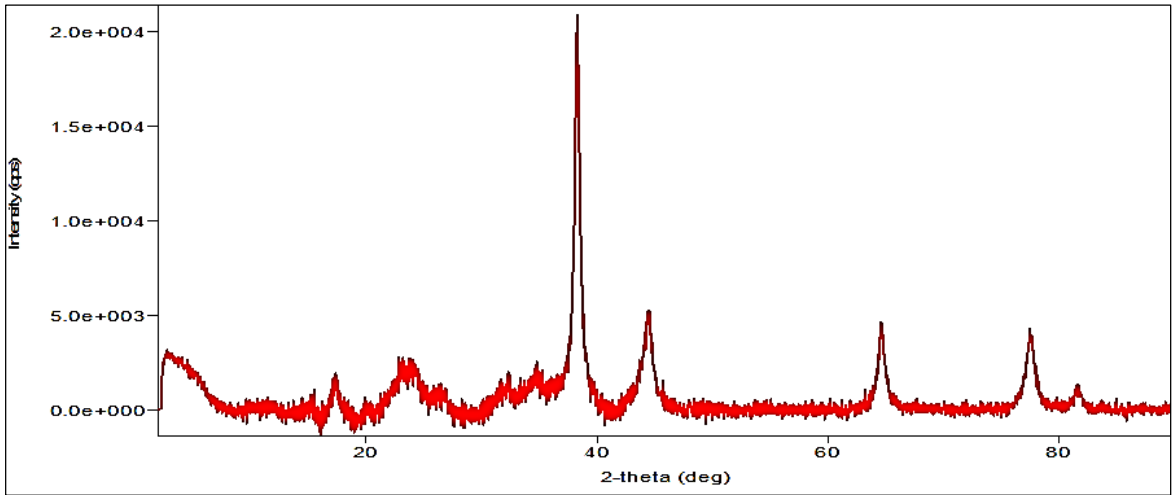

Fig 11: X-Ray diffraction pattern of silver sulphide nano particles (1:1 ratio)

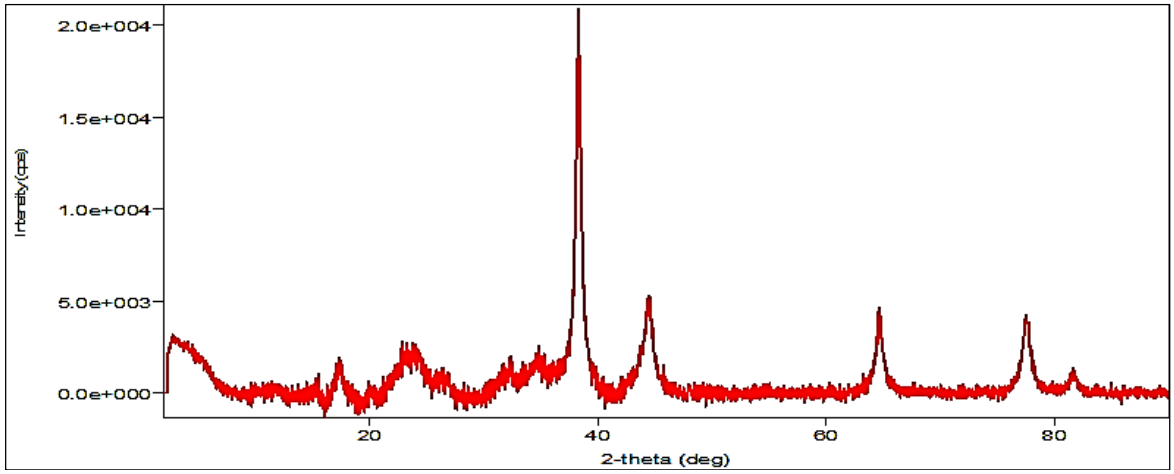

Fig 12: X-Ray diffraction pattern of silver sulphide nano particles (1:2 ratio)

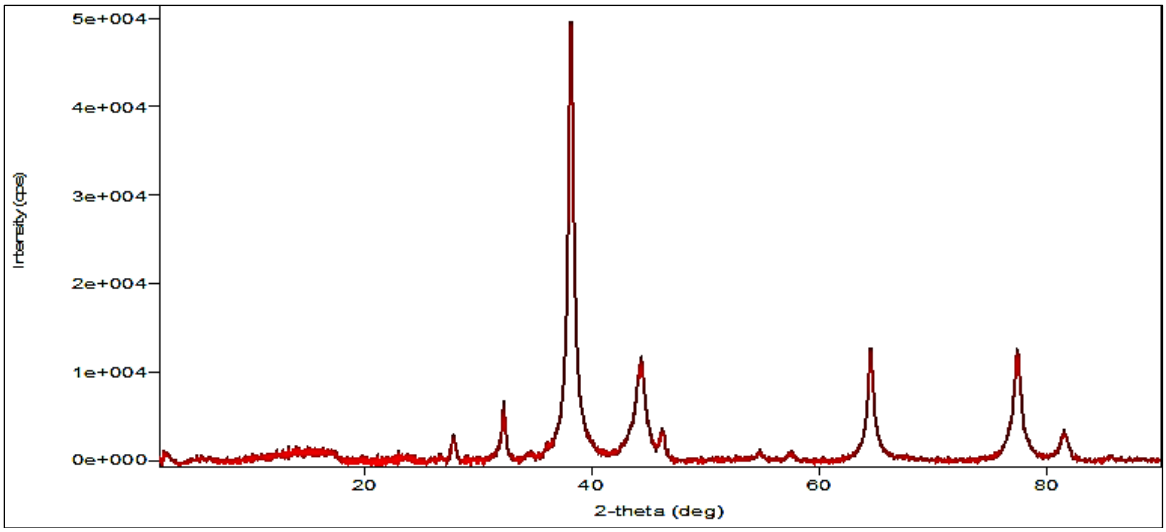

Fig 13: X-Ray diffraction pattern of silver sulphide nano particles (1:3ratio)

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 ($AgNO_3$+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.

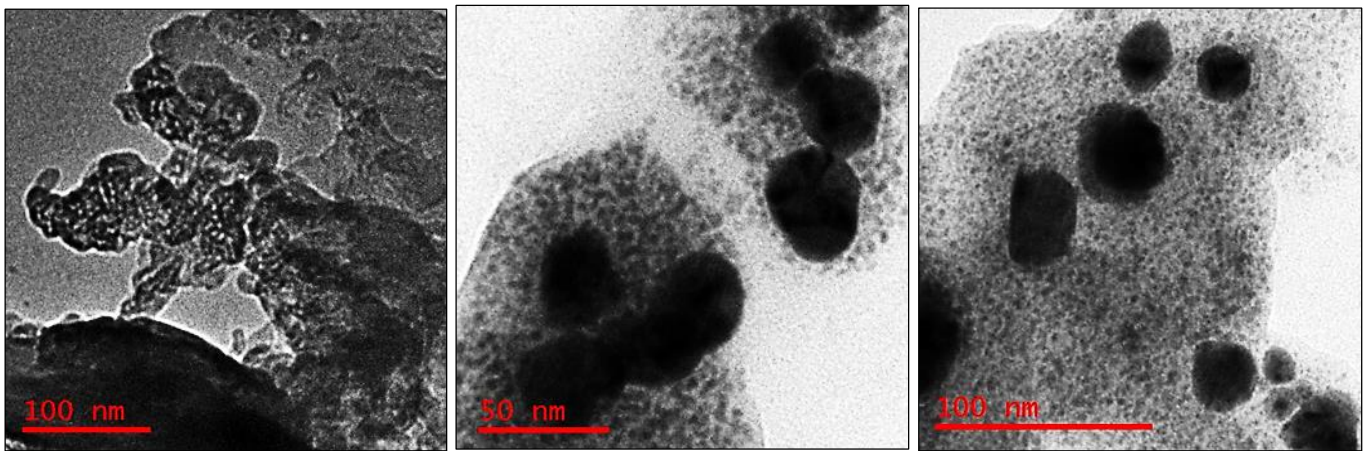

Fig 14: TEM Images of biosynthesised silver sulphide nano particles

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 Ag $_2$SNPs 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 Ag $_2$SNPs of different shapes were obtained in leaf extract being used as reducing and capping agents formed approximately, tubular and cuboidal Ag $_2$SNPs respectively. This may be due to the availability of different quantity and nature of capping agents present in leaf extract

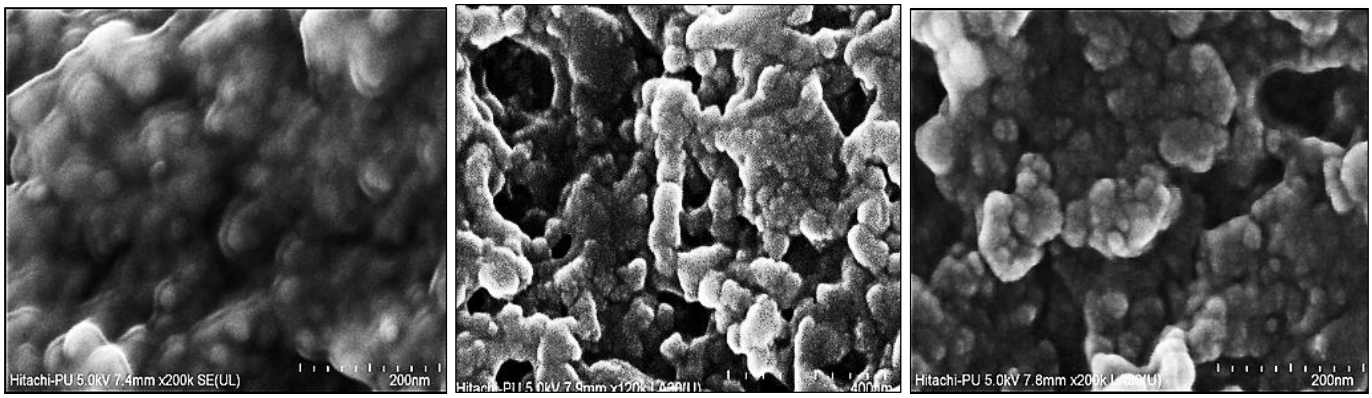

Fig 15: SEM Images of biosynthesised silver sulphide nano particles

3.9 EDX studies

Fig.16 shows the EDX spectrum of the biosynthesized silver sulphide nano particles nanoparticles. Silver (Ag) signal comes from the Ag_2 S 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 Ag_2 S 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: 1.24kcps

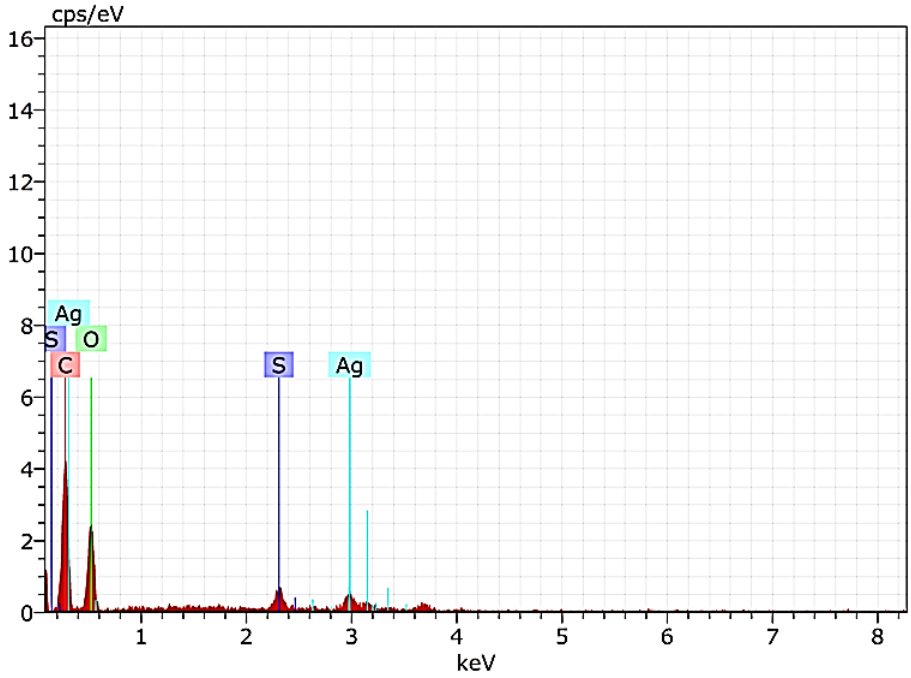

Fig 16: EDX spectra of silver sulphide nano particles which shows the presence of Ag, S, C, O Elements

Spectrum: test 5163

Element Series unn. C norm. C Atom. C Error (3 Sigma) [wt. %] [wt. %] [at. %] [wt. %]

Carbon K-series 28.42 45.48 57.38 15.15
Oxygen K-series 26.22 41.96 39.74 15.49
Sulfur K-series 2.10 3.37 1.59 0.47
Silver L-series 5.75 9.20 1.29 0.97

Total: 62.49 100.00 100.00

3.10 Anti-inflammatory activity

Inflammation is an early immunological response against foreign particles by tissue; which is supported by the enhanced production of pro-inflammatory cytokines, the activation of the immune system, and the release of prostaglandins and chemo tactic substances such as complement factors, interleukin-1 (IL-1), TNF-alpha [52,53,54,55]. Among several anti-inflammatory agents, Ag_2SNPs have recently played an important role in anti-inflammatory field Ag $_2$ S NPs have been known to be antimicrobial, but the anti-inflammatory responses of Ag2SNPs are still limited.

3.10.1 Inhibition of protein denaturation activity

The Nyctanthes arbor-tristis leaf extract were effective in inhibiting induced albumin denaturation. Nyctanthes arbor-tristis leaf extract was observed as crued extraction was __85%____ and biosynthesized Ag2SNPs in different ratios were 92.7% for 1:1, 97.7% for 1:2, and 84.4% for 1:3. And 88.8% for standard. Aspirin was used as a standard anti-inflammatory drug as shown in table 5.

Table 5: Inhibition of protein denaturation activity

S. No.	Samples	Protection ±SD (%)	P value	Error value
1.	Crude Leaf Extract	85%	0.01	0.085
2.	Ag2SNPs(1:1)	92%	0.01	0.092
3.	Ag2SNPs (1:2)	97.76%	0.01	0.097
4.	Ag2SNPs (1:3)	84.4%	0.01	0.084
5.	Standard	88%	0.01	0.088
6.	Control	No inhibition	—	—

3.10.2 Inhibition of Anti-proteinase activity

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 Ag_2SNPs 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 Ag_2 SNPs particles. The results are summarised in table 2.

Table 7: *In vitro* anti-inflammatory activity by heat-induced haemolyses 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 ant 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 Ag_2

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 +$AgNO_3$+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 Ag_2SNPs.[56] 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-I R analysis, it is prove that the silver sulphide nano particles are caped 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 point corresponding 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 Ag $_2$SNPs 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 Ag_2SNPs has 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 Nyctanthes 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 Nyctanthes 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, thio-semicarbazide 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 Ag_2 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.

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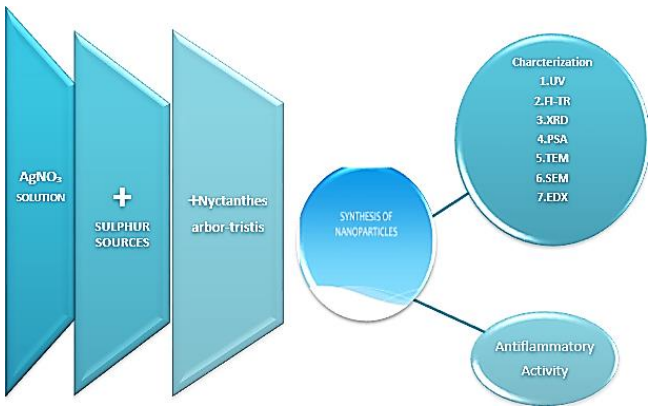
7. Footnotes

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

8. Graphical Method



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