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BIOFABRICATION OF SILVER SULPHIDE NANOPARTICLES FROM CINNAMOMUM TAMALA LEAVES: A NEXT GENERATION ANTI-INFLAMMATORY AGENT

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ABSTRACT

Objectives: Inflammation is a body response to fight against various infections, toxins and injuries. The inflammation could be acute or chronic. A few factors such as age, obesity, diet, low sex hormones, sleep disorders, cardiovascular disease and cancer are the root cause of inflammation. The objective of the present study was to biosynthesize nanoparticles with potential anti-inflammatory activity against standard acetylsalicylic acid (aspirin drug). **Materials and Methods:** Silver sulphide nanoparticles were fabricated using the sol-gel method by mixing of bay leaf extract in silver nitrate solution. The synthesised Ag 2 SNPs were then later characterised using UV-Visible spectroscopy, FT-IR, SEM, TEM and XRD. Further proceed for their valuation of anti-inflammatory activity through protein- denaturation, protease inhibition assay and heat induced haemolysis method. **Result:** The various bio physical characterization proved that the particle size was below 100nm and of spherical shape. FTIR spectra analysis showed the presence of various biomolecules, which have a very significant role in capping and stabilising silver sulphide nanoparticles. The synthesised Ag^2 S NPs showed anti- inflammatory activity against standard drug acetylsalicylic acid. Using protein denaturation, protease inhibition assay and heat induced haemolysis method. **Conclusion:** The biosynthesised silver sulphide nano particles could be used in several biomedical applications. The proposed synthesis is cost effective, eco-friendly and promising candidate for formulation of various topical applications like formulation of ointment, gel etc.

KEYWORDS: Bio fabrication, Nanostructure, Spectroscopic characterization, Anti – inflammatory activity.

1. INTRODUCTION

Recently special attention has been emphasized on synthesise of metallic nanoparticles because of their unique properties from their bulk metals.[1] These metallic nanoparticles have various size dependent optical properties.[2,3,4] Due to anti-fungal, anti-bacterial, anti-oxidant and anti-inflammatory properties nano structured silver is an excellent choice in formation of various biomedical apparatus.[5,6,7] Nano silver shows strong antimicrobial activity.[8] Nano silver and their compounds shows strong antibacterial spectrum.[9,10] Due to their strong antimicrobial activity nano silver have vast biomedical application like wound dressing material[11,12], catheters, biodegradable fibres[13] and toys.[14] The synthesise of silver sulphide nanoparticles involves three distinct methods. The first method involves the application of strong reducing agent.[15,16] The second method involves high energy radiation techniques.[17,18,19] The third method involves the slow heating of silver salt solution in presence of weak reducing agent.[20,21,22] Reported methods involves the use of strong reducing agent and non-aqueous solvents[23,24,25] which are cancer producing reagents. So, it is necessary to develop green approach to reduce the hazardous effects of metallic nanoparticles. Literature reported that the applications of various plant parts for bio fabrication of silver sulphide nanoparticles.[26,27] Indian bay leaves are considered as ayurvedic medicine which are used in the formulation of various herbal drugs like tablet, gel, ointments as an analgesic, anti-inflammatory[28], antioxidant.[29] Hence, the synthesiser of silver sulphide nanoparticles using Indian bay leaves is cost effective, eco-friendly, biodegradable and has many biomedical applications. Indian bay leaves extract with silver salt solution and thio- semicarbazide hydrochloride solutions results in synthesise of silver sulphide nanoparticles in less than 12 hours due to the functionalisation of various functional groups with metallic silver ions. The biosynthesised silver sulphide nanoparticles showed potent analgesic and anti-inflammatory activities. According to our research bio fabricated silver sulphide nanoparticles could be considered as an alternative approach towards the preparation of various biomedical equipment and formulation of drugs.

2. MATERIAL AND METHODS

Silver nitrate, thio- semi carbazide hydrochloride was procured from Merck, India Pvt. Limited. Indian bay leaves were obtained from medicinal garden of ITM University Gwalior.

2.1 Preparation of Indian bay leaves extract

The dried leaves of Cinnamomum Tamala tree are called Tej patta and used as a spice in

ayurvedic medicine formulation. The leaves of Cinnamomum Tamala contain a volatile oil which contain phytochemical constituent such as monoterpenes and sesquiterpenes. These leaves also contain Eugenol essential oil. It is responsible for the anti-bacterial and antifungal activities of Indian bay leaves. So, it can also be used in human yeast infections. About 50 gm dried leaves of Cinnamomum tamala tree were ground to yield coarse material. 50 gm of dry leaves were boiled with 100 ml double distilled water for ½ hour. After cooling, the aqueous extract of leaves was filtered using Whatman filter paper no. 1 and stored the aqueous extract of leaves at 4^0c for further use.

2.2 Green fabrication of silver sulphide nanoparticles

0.01M aqueous solution of silver salt and thio-semicarbazide hydrochlorides were taken in a round bottom flask. The medium of solution was alkaline and maintained P^H the solution at 12. then leaf extract of Cinnamomum tamala was added dropwise in different ratios (1:1, 1:2, 1:3) and further stirred the solution for another 30 min. at 60^0c. A black coloured precipitate was obtained which was centrifuged at 4000 rpm for 20 min. and washed the precipitate with double ionised distilled water. During drying process, complete conversion of silver sulphide nanoparticles was taken place.

2.3 Ultraviolet spectroscopy

UV-visible spectroscopy was performed using UV-visible spectrophotometer Perkin- Elmer UV /V is Lambda 25 (wavelength range 200- 800nm) in Sophisticated instrumentation laboratory, ITM University, Gwalior.

2.4 Fourier- Transform spectroscopy

This spectroscopy helps to identify the presence of various functional groups in aqueous extract of Cinnamomum tamala leaves. This spectroscopy was performed using Perkin-Elmer FT-IR spectrophotometer in sophisticated instrumentation laboratory, ITM University, Gwalior in the range of 400-800 cm-1.

2.5 Fluorescence spectroscopy

This technique is widely used to obtain fluorescence spectrum for dried and powdered silver sulphide nanoparticles using Perkin-Elmer LS-55 Fluorescence spectrometer in Sophisticated instrumentation laboratory, ITM University, Gwalior.

2.6 X -ray diffraction

x-ray diffractometer was designed for obtaining the ultimate quality diffraction data, combined with ease of use and flexibility to quickly switen to different applications. XRD of samples was performed in the range of 20-80 at 40kv and 15 milliampere with a divergence slit of 10 mm in 20/ continuous scanning mode.

2.7 EDX study

This study determines the elemental composition of a sample. The EDX study samples were performed using JEOL-JSM 6360 equipped with an energy dispersive analyser.

2.8 Transmission Electron Microscopy (TEM)

Suspension of bio fabricated silver sulphide nanoparticles in double distilled water was used for TEM studies at an increasing voltage of 200 KV. The results was clearly visualized using Olympus Siv ewer from CIF, Jiwaji University, Gwalior.

2.9 Scanning Electron Microscopy (SEM)

SEM is a surface imaging technique used for measurement of particle size and distribution of nano particles.[32,33,34,35] SEM images of silver sulphide nano particles were taken from Hitachi –PU 5.0 KV 7.8 mm × 120 K, LA30 (U) (SAIF) Punjab university, Chandigarh.

2.10. Anti-inflammatory activity

2.10.1 Inhibition of Protein Denaturation

This activity was evaluated by biosynthesized silver sulphide nanoparticles and aqueous extract of Cinnamomum tamala leaves by protocol of Mizushima et al.[36] In this method 500ml of 1% BSA was added to mixture of leaf extract and bio fabricated Ag 2 S NPS. Heat the mixture at 50˚c for 30 min. After cooling the mixture, absorbance was taken at 660nm with the help of UV-visible spectrophotometer. In this method acetyl salicylic acid was used as a positive control. The experiment was performed in triplicates and percentage inhibition of protein denaturation activity was calculated by the following formula:

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

Where,

A_1=Absorbance of aqueous leaf extract

A_2 =Absorbance of mixture of leaf extract and biosynthesised silver sulphide nano particles Ag_2SNPs

Ao= Absorbance of positive control (Aspirin)

2.10.2 Protease inhibition assay

This assay was evaluated by the method of sakat et al 37. In this assay, mixture 100ml of BSA and 100ml of leaf extract and bio fabricated AgSNPs was incubated at room temperature for 15 min. then add 250 ml of trypsin solution for inhibition of protease and centrifuge the mixture. After centrifugation supernatant was collected and taken absorbance of mixture at 210 nm with the help of UV visible spectrophotometer. The experiment was performed in the triplicates and the percentage protease inhibition was calculated by the following formula:

$$\% \text{ Protease inhibition} = \left\{\frac{100-\{A1-A2\}}{Ao} \times 100\right\}$$

Where,

A_1=Absorbance of aqueous leaf extract

A_2 =Absorbance of mixture of leaf extract and biosynthesised silver sulphide nano particles Ag_2SNPs

Ao= Absorbance of positive control (Aspirin).

2.10.3 Heat induced haemolysis assay

This assay was performed by using a standard protocol 38, 39. 5 ml healthy human blood sample was centrifuged at 3000rpm for 15min. cells were washed with saline solution (Ph 7.2) then equal volume of leaf extract, bio fabricated Ag2SNPs 100% RBC suspension was centrifuged and kept on a water bath for 20 min. the absorbance of the sample was monitored at 520 nm with the help of UV-VISIBLE spectrophotometer at time interval of 10 minute. In this assay, acetyl salicylic acid was taken as standard.

3. RESULTS AND DISCUSSION

This research work reports the bio fabrication of silver sulphide nanoparticles using Cinnamomum tamala leaf extract which was free from any impurity and does not require continuous stirring of reaction mixture. The appearance of black coloured particles indicates the formation of crystalline silver sulphide nanoparticle.

3.1 Phytochemical screening of Cinnamomum tamala leaf aqueous extract

In order to understand the presence of various phytochemical constituent in aqueous extract of Cinnamomum tamala leaves, the extract was subjected to qualitative phytochemical

screening. For this, some specific functional group tests were performed to check the availability biomolecules like flavonoids, alkaloids, glycosides, steroids etc.

Table 1: The qualitative estimation of phytoconstituents present in Cinnamomum tamala leaves 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.	Phytosterols and	+
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 Cinnamomum tamala 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]

Figure 1: Biosynthesised Silver sulphide nano particles from Cinnamomum tamala leaf extract.

3.3 UV-Visible spectroscopic analysis

This is a very useful and reliable technique for the primary identification of formation nanoparticles 42. For this UV- Visible absorption maxima exhibit in the range of 200-800nm. The results obtained from spectra showed approximately at 285nm and 310 nm for 1:1 ratio, 280nm for 1:2 ratio and 285 nm for 1:3 nm radio indicating the formation of silver sulphide nanoparticle. A broad absorption peak at 290nm was aroused due to the surface plasmon resonance absorption band along with free electronic vibrations of silver sulphide nanoparticles in a resonance with a light wave.

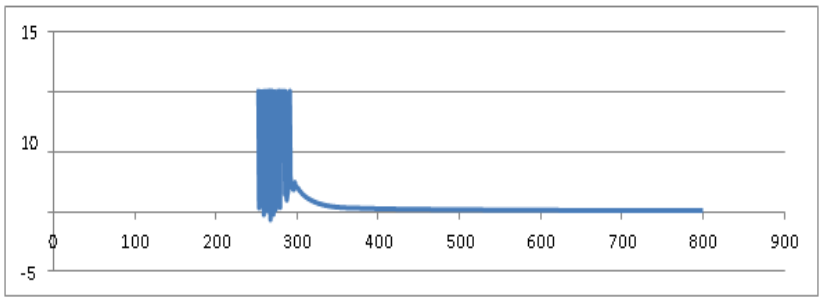

Figure 2 UV-Visible spectra of biosynthesised silver sulphide nano particles (1:1 ratio).

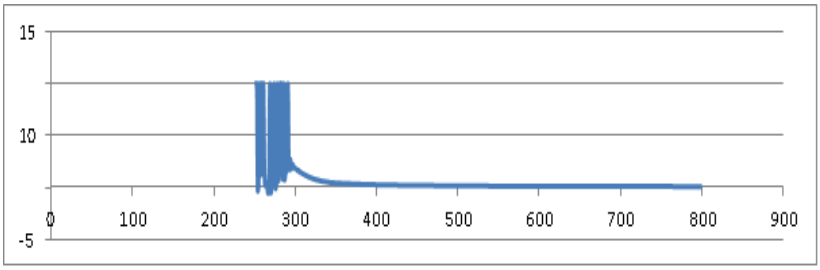

Fig. 3: UV-Visible spectra of biosynthesised silver sulphide nano particles (1:2ratio).

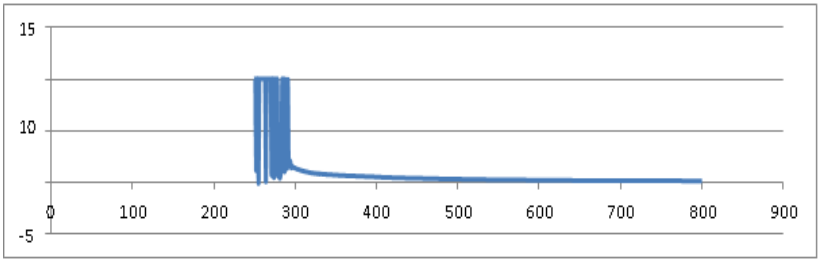

Fig. 4: UV-Visible spectra of biosynthesised silver sulphide nano particles (1:3 ratio).

3.4 Fourier transform spectroscopic analysis

This is a very reliable and accurate spectroscopic method to characterized to presence of functionally active sites of the leaf extract of Cinnamomum tamala which are responsible for capping ability to stabilized the bio fabricated Ag2SNPs.

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

Serial No.	1:1 ratio	1:2 ratio	1:3ratio
1.	3827	3787	3864
2.	3786	2916	2918
3.	2917	2848	2179
4.	2075	2074	1768
5.	1605	1607	1558
6.	1515	1517	1358
7.	1384	**1384**	1020
8.	1075	1019	573
9.	830	616	374
10.	361	358	

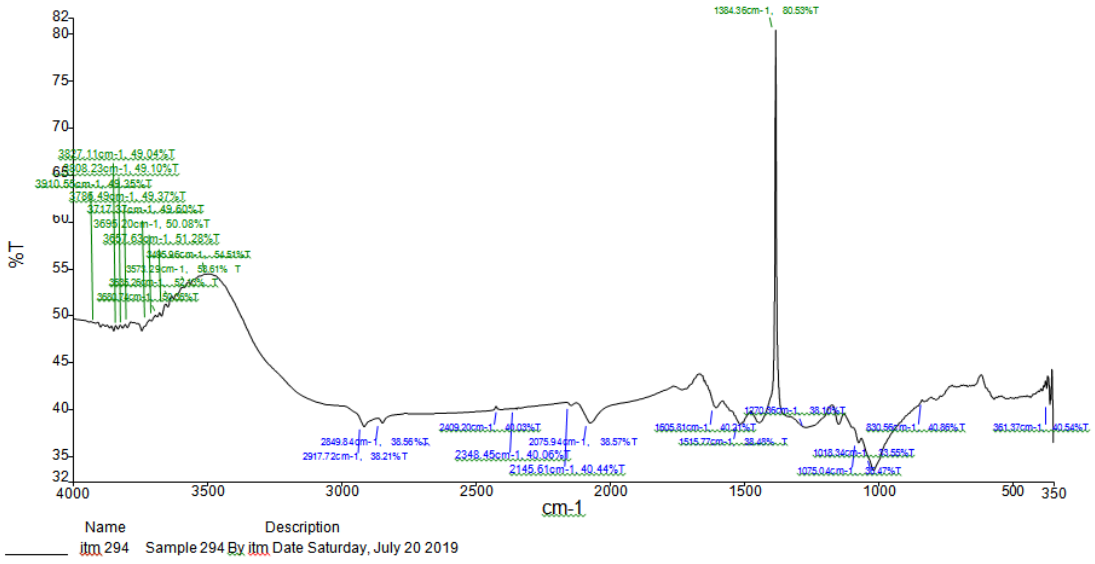

Fig. 5: FT-IR spectra of biosynthesised silver sulphide nano particles (1:1 ratio).

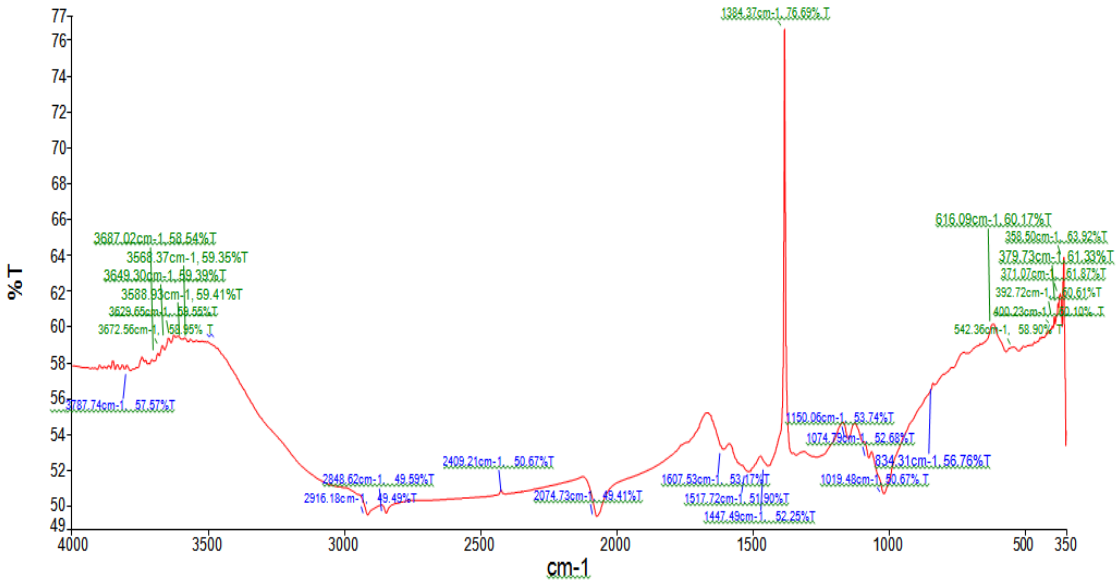

Fig. 6 FT-IR spectra of biosynthesised silver sulphide nano particles (1:2 ratio).

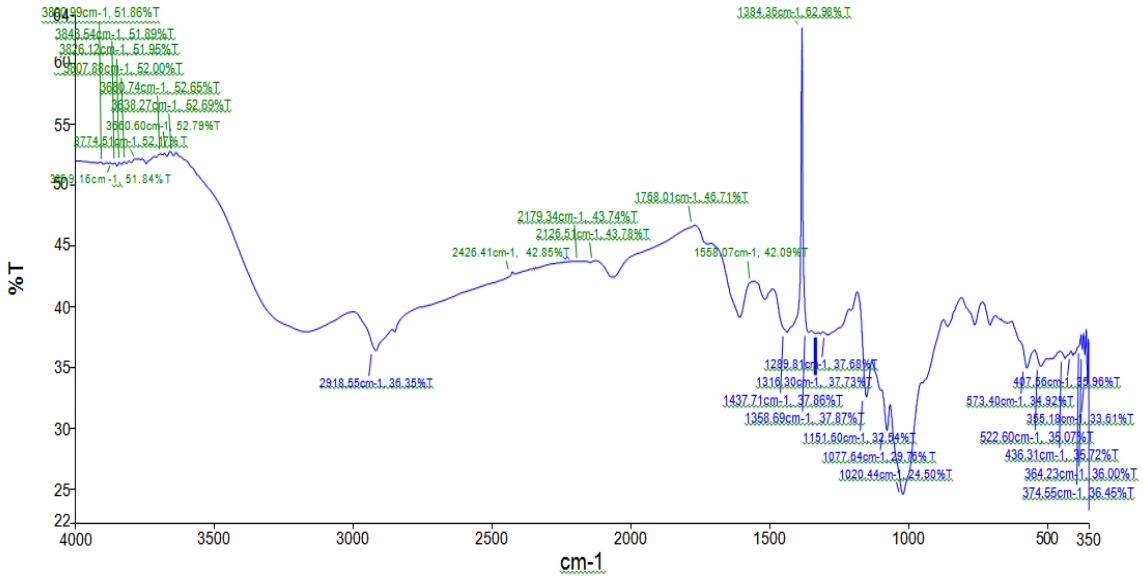

Fig. 7 FT-IR spectra of biosynthesised silver sulphide nano particles (1:3 ratio).

3.5 Fluorescence study

Biosynthesized AgSNPs are reported to exhibit visible photoluminescence and their fluorescence spectra are shown in Fig (8,9,10). The optimized Ag 2 S NPs were found to be luminescent with three emissions at 275nm for 1:1 ratio, 280 nm for 1:2 ratio and 290 nm for 1:3 ratio, for an excitation at 350 nm. When Ag 2 SNPs were excited at 300nm, it showed excitation at 350 nm, the excitation of 375 nm 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 Ag2S NPs synthesized using Cinnamomum Tamala leaf extract are also reported to be luminescent with emission band at 340 and 440 nm.[49]

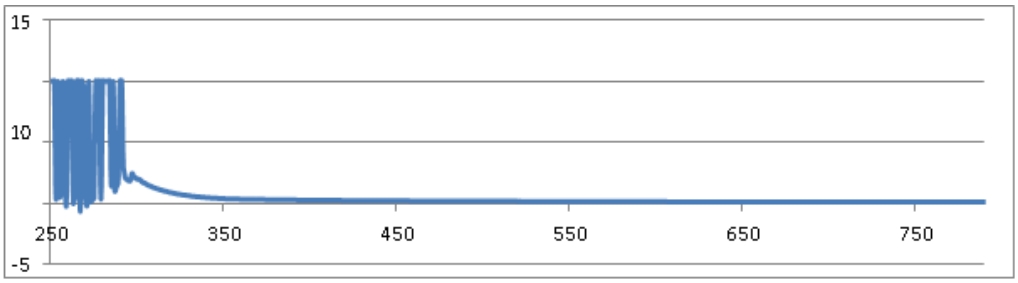

Figure 8: Fluorescence spectra of biosynthesised silver sulphide nano particles 1:1 ratio.

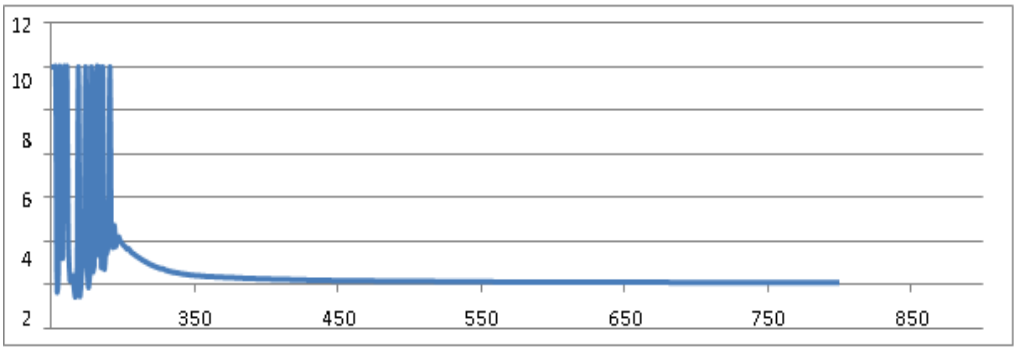

Figure 9: Fluorescence spectra of biosynthesised silver sulphide nano particles 1:2.

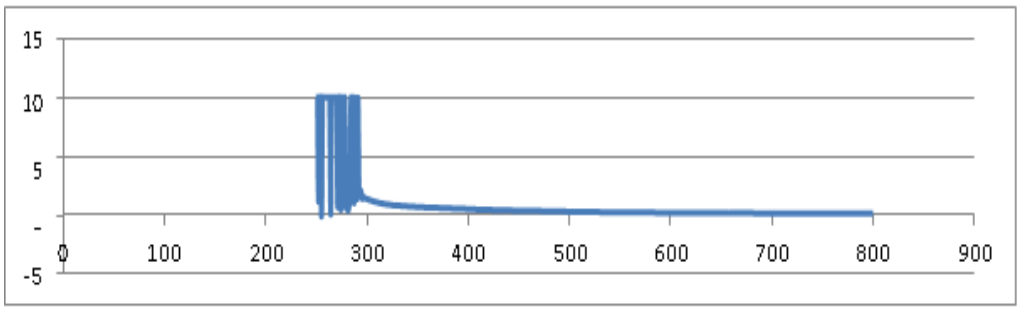

Figure 10: Fluorescence spectra of biosynthesised silver sulphide nano particles 1:3.

3.6 Powder X-Ray diffraction analysis

X-Ray diffraction is a technique which has been used for both molecular and crystal structures, qualitative identification of compounds, quantitative study of chemicals which are responsible for degree of crystallinity, isomorphous substitution and the particle size of nanoparticles. This technique is also used for the characterization of organic and inorganic crystalline compounds.

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.,

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

Where,

D = Crystalline size

λ= wavelength of X- Rays

β= Full width at half maximum of the diffraction peak

θ= 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.[58]

Table 3 –X-Ray diffraction peaks (2θ values) of various ratios of biosynthesised silver sulphide nanoparticles.

Z	1:2	1:3
38.1	38.1	38.1
44.4	44.2	43.5
64.4	64.4	64.4
77.4	77.3	77.3
81.4	81.17	81.3

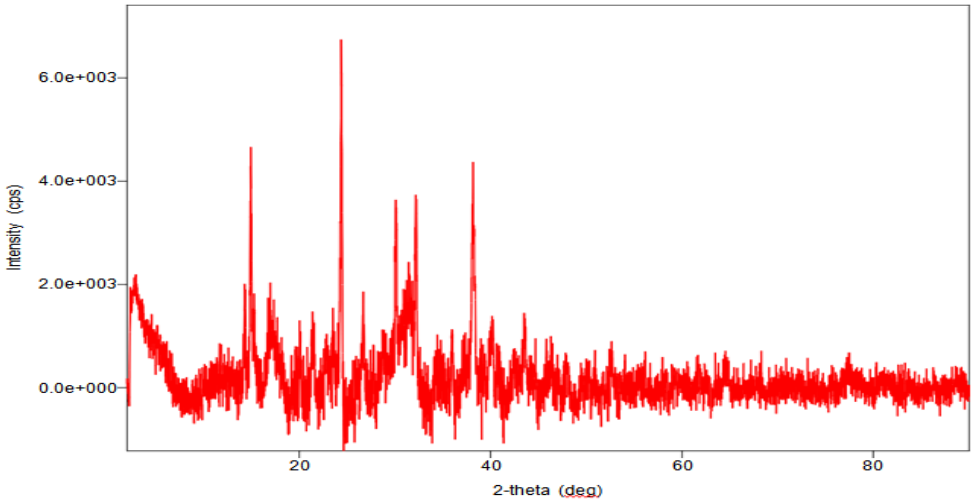

Figure 11: X-Ray diffraction pattern of silver sulphide nano particles (1:1 ratio).

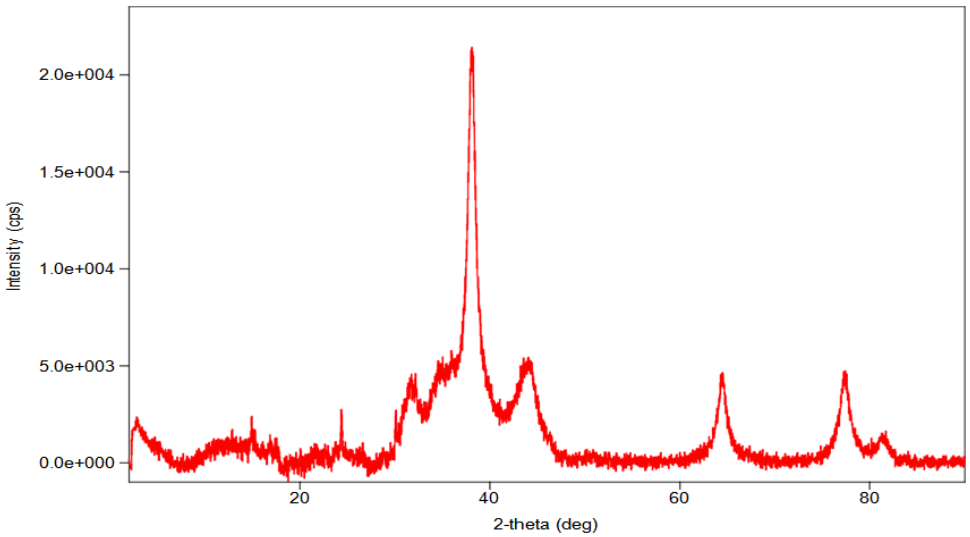

Figure 12: X-Ray diffraction pattern of silver sulphide nano particles (1:2 ratio).

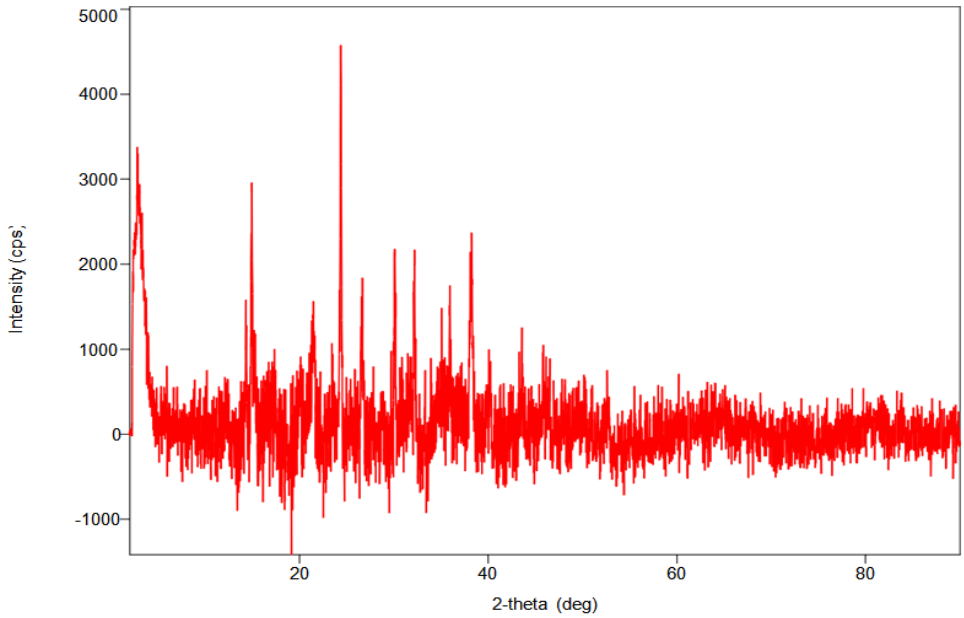

Figure 13: X-Ray diffraction pattern of silver sulphide nano particles (1:3ratio).

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 (AgNO3+TSC+Leaf extract)	Average particle size in nm
1.	1:1	0.5732 nm
2.	1:2	0.1312 nm
3.	1:3	0.5974 nm

3.7 TEM

TEM images of nanoparticles provides the information of morphology and particles size of nanoparticles. Figures exhibits that the particles were spherical in shape. The particle size of all 3 ratios were ranging from 1.48 nm to 6.69 nm. The TEM images shows that nano particles are not combined but are separated by equal interspace between the particles, which was confirmed by microscopy visualising under the higher resolution. The TEM images explains that the nano particles are bounded with the phytochemicals present in the leaf extract.

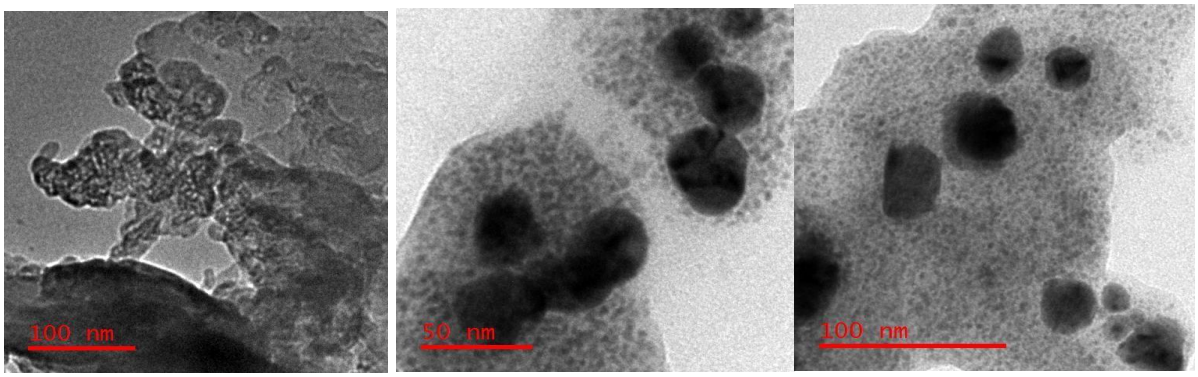

Figure 14: TEM Images of biosynthesised silver sulphide nano particles(1:1,1:2 , & 1:3 ratio).

3.8 SEM

SEM monographs in figures 15 explains well dispersed, versatile and spherical shape of biosynthesised silver sulphide nano particles. With the size range of 1.48-6.69 nm leaf extract added to the mixture of silver nitrate and thio- semicarbazide hydrochloride solutions., does not change the shape of nano particles but it increases the size of nano particles mostly in higher concentrations. The nano particles were assembled into a very open and quasi–linear structure than a dense closely packed assembly.

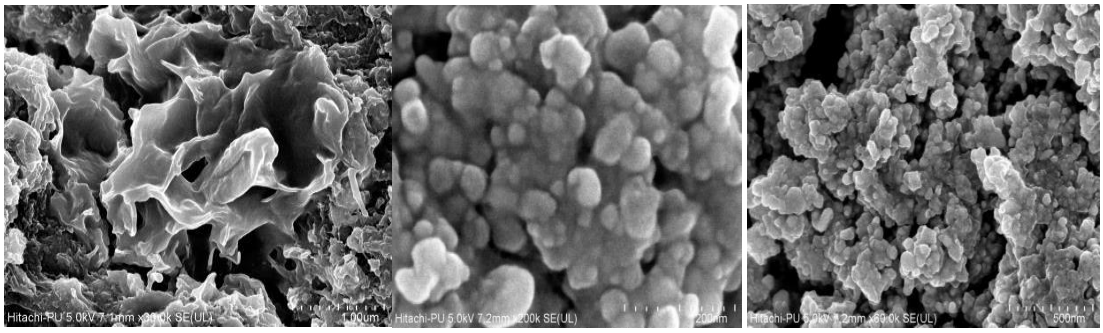

Figure 15: SEM Images of biosynthesised silver sulphide nano particles ((1:1, 1:2 & 1:3 ratio).

3.9 EDX

Fig.16 shows the EDX spectrum of the biosynthesised silver sulphide nanoparticles. Silver signal comes from the Ag2S nanoparticles and the atomic percentage of silver is 5.75%. Except for A, there were also some other peak. The atomic percentages of Carbon(C), Oxygen (O) and sulphur are 28.42%, 26.22% and 2.10% respectively. The weight composition of silver is 5.75% and the atomic composition is then calculated as 1.29% respectively. The signal from the EDX studies confirms the presence of silver. It indicates that the reduction of silver nitrate and thiosemicarbazide hydrochloride solution usinf leaf extract. The other impurities is found such as carbon, oxygen, sulphur was identified because of the interation with the leaf extract during bioprocessing.

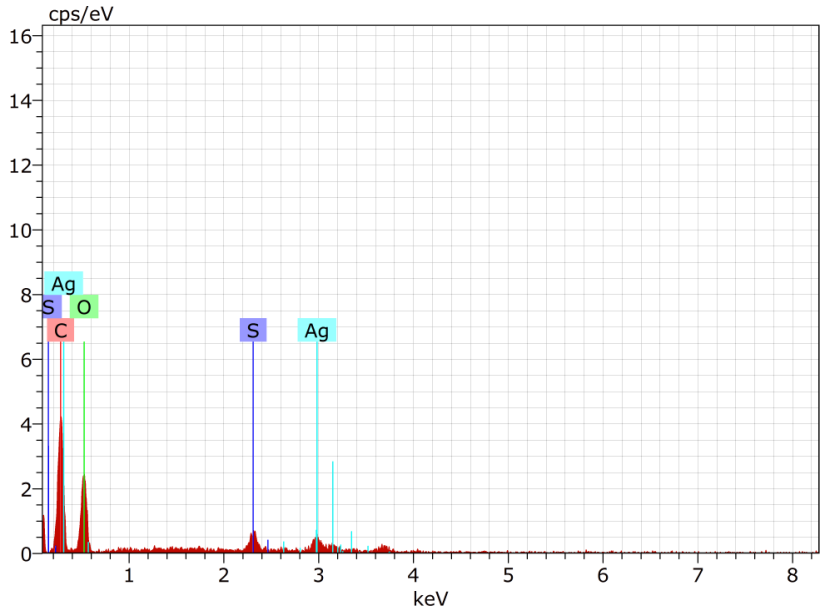

Figure 16: EDX spectra of silver sulphide nano particles which shows the presence of Ag, S, C,O Elements.

Element	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (3 Sigma) [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 tissues which is confirmed by the enhanced production of pro-inflammatory cytokines, the activation of the

immune system, and the release of prostaglandins and chemotactic substances such as complement factors, interleukin –1 (IL –1), TNF – alpha.[63,64,65,66] 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 Cinnamomum Tamala leaf extract were effective in inhibiting induced albumin denaturation. This was observed for crude leaf extract was 95% and biosynthesized Ag2SNPs in different ratios were 92% for 1:1, 96.7% for 1:2, 83.4% for 1:3. And 89% 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	95%	0.01	0.095
2.	Ag $_2$SNPs(1:1)	92%	0.01	0.092
3.	Ag $_2$ SNPs (1:2)	96.76%	0.01	0.096
4.	Ag $_2$ SNPs (1:3)	83.4%	0.01	0.083
5.	Standard	89%	0.01	0.089
6.	Control	No inhibition	—	—

3.10 .2 Inhibition of Anti-proteinase activity

The leaves of Cinnamomum Tamala exhibited significant Anti-proteinase activity. The percentage of inhibition was observed in leaf extract. The standard drug Aspirin showed maximum proteinase inhibitory action 92.8%. The anti-proteinase activity for leaf extract was observed as 95% and for different ratios of AgSNPs were observed as 95.3%, 94.5% and 99.3% respectively.

Table 6: Inhibition of Anti-proteinase activity.

S. No.	Samples	Protection ±SD (%)	P value	Error value
1.	Crude leaf Extract	**95%**	**0.01**	**0.095**
2.	Ag $_2$SNPs (1:1)	**95.3%**	**0.01**	**0.095**
3.	Ag $_2$ SNPs (1:2)	**94.5%**	**0.01**	**0.094**
4.	Ag $_2$ SNPs (1:3)	**99.3%**	**0.01**	**0.099**
5.	Standard drug (Asprin)	**89**	**0.01**	**0.089**
6.	Control	No inhibition	—	—

3.10.3 Invitro Heat – induced haemolysis method

The Invitro anti-inflammatory activity was studied for leaf extract and different ratios of biosynthesized Ag_2S NPs particles. The results are summarised in table 7.

Table 7: Invitro anti-inflammatory activity by heat – induced haemolyses method.

S. No.	Samples	Protection ±SD (%)	P value	Error value
1.	Crude leaf extract	95%	0.01	0.095
2.	1:1 Ag_2SNPs	52%	0.01	0.052
3.	1:2 Ag_2SNPs	62%	0.01	0.062
4.	1:3 Ag_2SNPs	58%	0.01	0.058

4. DISCUSSION

The bio fabrication of silver sulphide nanoparticles is a very unique and cost-effective method. In our study, we reported the various concentration of leaf extract, s silver nitrate and thio-semicarbazide hydrochloride solutions for synthesis of Ag_2 SNPs. Result reveal that surface plasmon resonance absorption band increases with increased concentration of reactants. FT-IR spectra provides valuable information about the presence of potential biomolecules in Cinnamomum tamala leaf extract, which were responsible for the stability of biosynthesised Ag_2S NPs nanoparticles. XRD spectra confirms the cubic phase of nanoparticles corresponding to FCC structure of Ag_2S NPs. From XRD data it has been clear that the Ag_2S NPs were crystalline aggregates, spherical in shape with varied size. Nanoparticles with large size have the tendency to nucleate and grow the twinned particles with their high surface area ratio. Results of in vitro anti- inflammatory activity also revealed that these nano particles are considered as a promising drug candidate for the treatment of arthritis disease.

5. CONCLUSION

Using Indian bay leaves for the bio fabrication of silver sulphide nanoparticles have great medicinal importance due to their effective in vitro anti-inflammatory activity against aspirin drug. This bio fabrications have many benefits like cost, high efficacy and eco-friendly nature of nanoparticles. Silver nanoparticles were characterised from various biophysical technique like UV-VISIBLE, FT-IR, XRD, TEM, SEM.

Biosynthesised silver sulphide nanoparticles were crystalline spherical in shape and property interact with various functional group present in aqueous extract of Indian bay leaves. These

nanoparticles exhibited significant anti-inflammatory activity like protein denaturation, protease inhibition and haemolytic activity of RBCs against a standard drug aspirin at room temperature.

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FOOTNOTES

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