Research Article

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IN VITRO ANTI- INFLAMMATORY STUDIES ON SILVER NANOPARTICLES SYNTHESIZED FROM *CENTRATHERUM PUNCTATUM* CASS.

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ABSTRACT

This paper presents an empirical analysis on the use of aqueous extract of *Centratherum punctatum* Cass (Asteraceae) for the production of silver nanoparticles (AgNPs) from aqueous silver nitrate. Phytochemical analysis of the extract revealed the presence of essential components such as flavonoids, alkaloids, carbohydrates, tannins and vitamin C, some of which serve as efficient reducing and capping agents for the reduction of silver nitrate to silver nanoparticles. The AgNPs synthesized were characterized by UV -Vis spectroscopy, FT-IR, XPS, SEM, TEM, Zetasizer and TG-DSC analyzer. In the present study, attempts have also been made to investigate the anti-inflammatory and anti-oxidant activity of the synthesized AgNPs. Anti-oxidant activity was assessed using DPPH method. Anti-inflammatory potential was evaluated through *in vitro* inhibition of protein denaturation, protease activity and improved membrane stabilization property.

Keywords: *Centratherum punctatum* Cass., nanoparticles, anti-oxidant, anti-inflammatory, SEM and TEM.

INTRODUCTION

Herbs and herbal extracts have been used to treat various ailments since ages. Their derivatives have attracted tremendous attention therapeutically and are promising as remedies to treat diseases of diversified origin. Herbs especially have fallen into limelight, anticipating their replacement with sophisticated drugs. More than 50% of modern drugs existing in clinical use today are derived from plants[1].Metal nanoparticles have proved to be of significance due to their lesser volume to surface area ratio along with their catalytic, optical, electrical and magnetic characteristics[2], that are extensively used owing to their anti-microbial properties[3]. Silver nanoparticles contribute even more, due to their advantages as anti-oxidants and anti-inflammatory agents[4-7]. Moreover, they are highly conductive, chemically stable and highly economical[8]. The plant extract was used for the preparation of silver nanoparticles owing to its least toxicity and lesser need for elaborate purification as compared to the chemical methods. The present work essentially deals with increasing therapeutic efficacy of the selected drug in its nanoparticle form.

The plant *Centratherum punctatum* Cass. (family Asteraceae) is a common perennial herb bearing strigose leaves, cauline with acute apex, serrate margin and terminal inflorescence. The plant contains sesquiterpene lactones, flavones, glycosides and phenolic compounds. Among these, flavonoids and phenolic compounds play a vital role in the reduction of silver nitrate to silver nanoparticles. This drug besides being anti-inflammatory is also known for its anti-tumorous, anti-depressant, anti-hypertensive, and anti-microbial properties[9]. As the selected plant source *Centratherum punctatum* Cass. is enriched with therapeutic potential as mentioned above, attempts have been made empirically, on green synthesis of AgNPs using this plant.

MATERIALS AND METHODS

Fresh plants of *Centratherum punctatum* Cass. were collected from the Herbal Garden of SASTRA University, Thanjavur, Tamil Nadu during the month of March,2014. Identification was carried out using Flora of Gamble[10] and authenticated with the help of Herbarium specimen deposited at Royal Botanical Garden, Kew (Voucher specimen number K000373089).

Preparation of the Extract

Fresh leaves of *Centratherum punctatum* Cass. were identified in the department of CARISM, SASTRA University. The leaves after a Double Distilled (DD) water wash, were cut into pieces, dipped in 500 ml of distilled water for approximately an hour. The condensed liquid after separation was analyzed for its major chemical constituents and further used for the synthesis of nanoparticles.

Reagents Used

Major reagents used were DPPH and silver nitrate procured from Sigma Aldrich. KBr pellets were purchased from Merck, and other reagents used in chemical analysis were of AR grade.

Synthesis of Nanoparticles

20 ml of the plant extract was mixed with 80 ml of 3mM of previously prepared silver nitrate solution. Color change from yellow to reddish brown indicated the formation of silver nanoparticles. AgNPs thus obtained were purified by repeated centrifugation at 5000 rpm for 10 minutes. The pellet was

collected and dried. Proteins and vitamin C were estimated in AgNPs[11].pH of the solution was also determined.

Preliminary Phytochemical Screening
Preliminary phytochemical screening was carried for the synthesized nanoparticles[12].

UV -Vis Spectra Analysis
Reduction of pure silver ions was confirmed by measuring the UV-Vis spectrum of the reaction mixture against distilled water as blank. Spectral analysis was carried out using double beam Perkin Elmer spectrophotometer with a resolution of 1nm and the observed spectrum was recorded from 370 nm to 800 nm.

FT-IR Analysis
PerkinElmer spectrometer FT-IRSPECTRUM ONE in the range of 4000–400cm^{-1} at a Resolution of 4cm^{-1} was used. The sample was mixed with KBr procured from Merck chemicals. Thin sample pellet was prepared employing the Hydraulic Pellet Press and subjected to FT-IR analysis.

TG-DSC Analysis
Thermo gravimetric differential scanning calorimeter analysis was carried out using TG-DSC (SDT Q600 V20.9 model, TA instruments, USA). 1.444 mg of the sample was taken in an alumina cup holder and heated up to 1000^0C at the rate of 10^0C/min in nitrogen atmosphere.

X-Ray Photoelectron Spectroscopy Analysis
X-Ray photoelectron spectroscopic analysis of the synthesized AgNPs was done using K-Alpha instrument (XPS K-Alpha surface analysis, Thermo fisher scientific, UK). 5mg of the dried sample was taken in the stub and packed tightly until uniform surface was obtained and placed in the sample arm. Analysis was carried out at the binding energy of 0 to 1350 eV.

Elemental Analysis by SEM and Energy Dispersive Analysis of X-rays (EDAX)
The samples were mounted on a brass stub and sputter coated with gold, further introduced into the specimen chamber of the cold field emission scanning electron microscope (SEM - JSM-6701F, JEOL, Japan) under ultra-high vacuum for EDAX analysis.

TEM Analysis
Morphology and size analysis of the silver nanoparticles was carried out using the FieldEmission Transmission Electron Microscope (FE-TEM), JEOL (Model 2701F).

Particle Size Measurement
Particle sizing experiments were carried out employing Laser Diffractometry using Zeta Sizer nano-series (Malvern).

***In vitro* Antioxidant Studies**
Determination of antioxidant activity (Scavenging Activity of DPPH Radical)
Traditional plant sources offer a wide range of natural antioxidants and there is not much work regarding the scientific validation of anti-oxidant plants and their complexes. The *in vitro* methods provide easy and useful indications of anti-oxidant activities[13]. In the present work extract of the selected plant and its NP-complex, was subjected to DPPH free radical scavenging assay as described by the method[14].This study will help in suggesting an enhanced anti- oxidant from biological sources for the betterment of human health care.

***In vitro* Anti-inflammatory Studies**
Inhibition of Protein Denaturation
The reaction starts with addition of 5% aqueous bovine serum albumin to various concentrations of plant extract (50, 100, 250, 500 and 1000 µg/ml), with pH maintained at 6.3 using 1N HCl. Each reaction mixture tube was incubated for 20 min at 37 ^{0}C, followed by heating at 57 ^{0}C for 3min and cooling at room temperature.
PBS (pH 6.3) was added to each tube resulting in the formation of turbid solution measured spectrophotometrically at λ 660 nm. The percentage inhibition of protein denaturation was calculated as follows:

Percent inhibition = 100- (O.D. of test – O.D. of product control) / O.D. of Control) x 100

Acetyl salicylic acid (250 µg/ml) was used as a positive control.

Effect on Membrane Stabilization
Hypotonic saline (0.25% NaCl) was mixed with 0.15 M phosphate buffer (pH 7.4) and 1 mL of various concentrations of test solution (50, 100, 250, 500 and 1000 µg/ml). Reaction was initiated with the addition of 10% human RBC to the above solution. Control tests contained isotonic saline in place of test solution, whereas product control was short of red blood cells. This reaction mixture was incubated at 56^{0}C for 30 min. Each tube was then cooled and centrifuged at 1500 rpm for 10 min followed by measurement of the resultant supernatant spectrophotometrically at λ 560 nm.

Membrane stabilizing activity was calculated as follows:

Percent Stabilization = 100- (O.D. of test – O.D. of product control) / O.D. of Control x 100

Acetyl salicylic acid (250 µg/ml) was used as a positive control.

Proteinase Inhibitory Activity
The reaction mixture contained 0.06 mg trypsin, 25 mM Tris-HCl buffer (pH 7.4) and test extracts (50, 100, 250, 500 and 1000 µg/mL). This was then incubated for 5 min at 37^{0}C followed by the addition of 0.8% (w/v) casein and incubated for an additional 20 min. Reaction was terminated by the addition of 70% (v/v) perchloric acid. This was centrifuged and the supernatant was read spectrophotometrically at 280 nm against buffer as blank. Percentage of inhibition was calculated as follows:

% Inhibition = 100- (O.D. of test – O.D. of product control) / O.D. of Control x 100

Acetyl salicylic acid (250 µg/ml) was used as a positive control.

RESULTS AND DISCUSSION

When the leaf extract of *Centratherum punctatum* Cass. was mixed with $AgNO_3$ solution, brownish golden color of aqueous extract changed to pale yellow color immediately within 10 min, indicating the formation of silver nanoparticles (Figure 1).

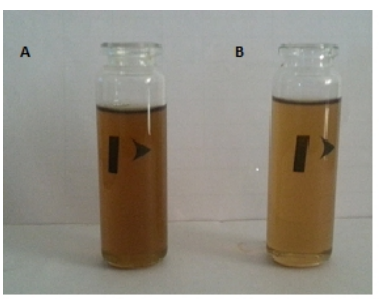


Figure 1: (A) Plant extract and (B) Synthesized Ag-NPs

Phytochemical screening of the *Centratherum punctatum* Cass. extract showed the presence of alkaloids, tannins, flavonoids, carbohydrates and vitamin C (Table 1).

Table 1: Preliminary phytochemical screening for plant extract and synthesized NPs.

Name of the Constituents	Plant Extract	AgNPs
Alkaloids	+	+
Flavonoids	+	+
Carbohydrates	+	+
Tannins	+	+
Vitamin C	+	+
Sterols	+	-

The reduction of pure silver ions was confirmed by UV Visible Spectra where the maximum absorbance was seen at 475 nm (Figure 2).

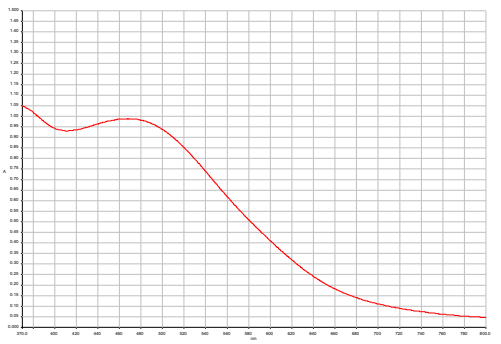


Figure 2: UV-Vis spectra of synthesized Ag-NPs

Strong IR bands were observed at 3408, 2923, 2307,1646,1383.7,1019 and 534 cm^{-1} (Figure 3). Bands that appeared at 3408 and 2923 cm^{-1}correspond to O-H stretching and aliphatic C-H stretching. The bands at 2307 and 1646.9 cm^{-1} correspond to CO2 and C=C and primary amine (N-H stretching). IR bands at 1383.7 and 1019 cm^{-1} may be ascribed to –C-O and C-O-C stretching modes respectively. Hence the phytoconstituents containing these functional groups such as phenols, terpene lactones, flavonoids and alkaloids present in the leaf extract of the plant *Centratherum punctatum* Cass. may be responsible for the chemical reactions involved in the synthesis of silver nanoparticles. The preliminary phytochemical screening of the extract used for silver nanoparticle synthesis also confirmed the presence of tannins, sterols, terpenes, carbohydrates, quinones and alkaloids.

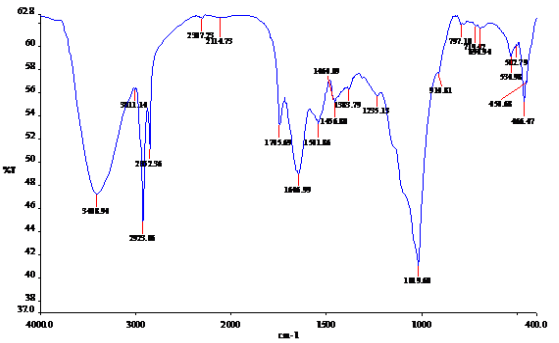


Figure 3: FT-IR spectrum of *Centratherum punctatum* Cass. extract

The XPS analysis (Figure 4) of synthesized AgNP's revealed the presence of Ag3d, C1s and O1s. The core level Ag3d signal was observed at the binding energies of 367.32 eV and 373.32 eV. In this 367.32 eV represents $Ag3d_{3/2}$ and 373.32 eV represents $Ag3d_{5/2}$ signals respectively. The doublet of Ag3d has the slitting level of 6 eV which suggested the presence of metallic silver. The core level O1s XPS signals at 532.27 indicates the presence of hydroxyl group and its attribution to the formation of AgNP's. There is core level C1s XPS signals observed at 284.78 eV, 286.10 eV and 288.18 eV after fitting of the spectrum. The peak at 284.78 eV indicates C-C signal along with the other peaks at 286.10 eV and 288.18 eV represent C-O and C=O signals respectively[15].

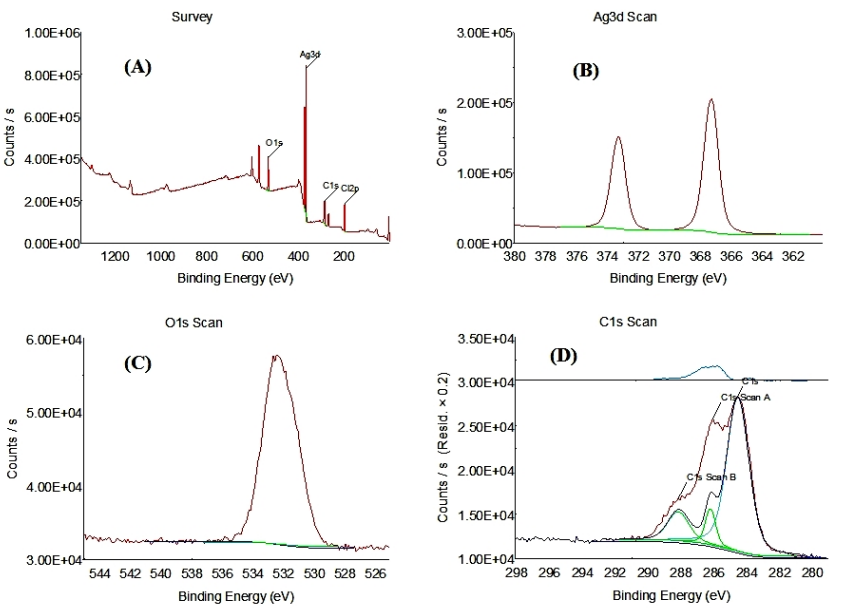


Figure 4: XPS Spectrum of AgNP's (A) represents Survey scan and (B), (C) & (D) represents the respective Ag3d, O1s and C1s narrow scans

SEM images showed aggregation of nanoparticles formed with diameter range between 35 to 40 nm (Figure 5). EDAX further confirmed the significant presence of silver along with carbon and oxygen (Figure 6).

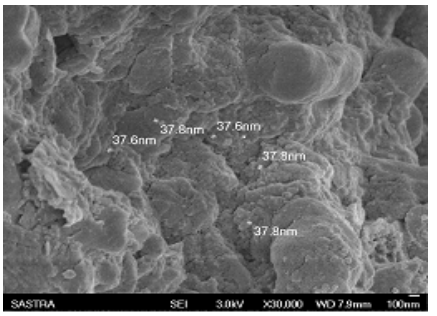


Figure 5: SEM images of synthesized Ag-NPs in colloidal condition at different nanometric scales

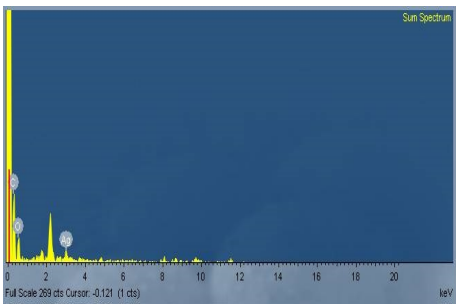


Figure 6: EDAX spectra of synthesized Ag-NPs

The Thermo Gravimetric Curve indicated a weight loss of 46.75% which could be attributed to the loss of organic content. Differential scanning calorimetric graph indicated a peak at 442.16^{0}C which indicated the loss of organic content. The peak at 962.56^{0}C corresponds to the melting point of silver (Figure 7).

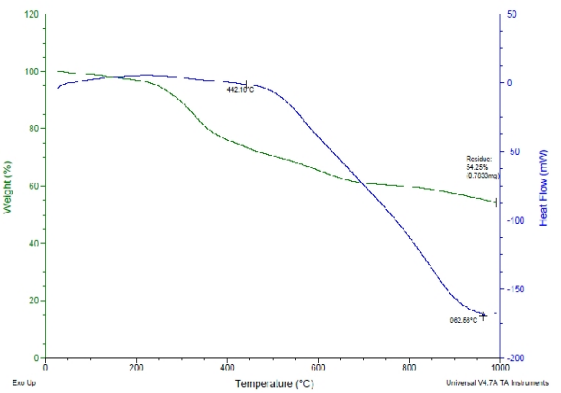
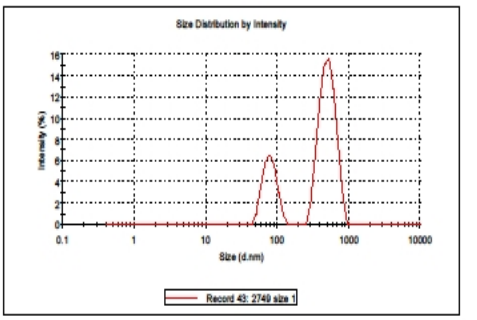


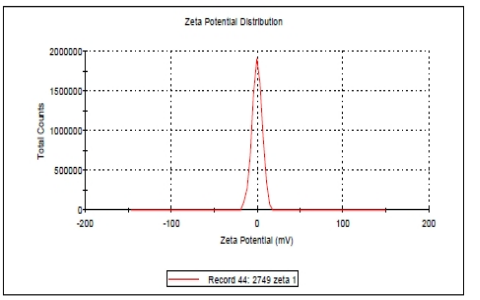
Figure 7: TGA graph showing melting point and the weight loss pattern of synthesized Ag-NPs

The size of the silver nanoparticles dispersed in water (Dielectric constant of the dispersant is 78.5) was determined to be 357.7 (Figure 8.a), at the temperature 25^{0}C. The zeta potential and zeta deviation were determined to be -0.115 mV and 5.82 mV respectively (Figure 8.a) with conductivity was shown to be 9.04e-4 mS/cm. Diameters of the particles vary between 515 nm to 80 nm (Figure 8.b) The Hydrodynamic radius is calculated using the Stokes Einstein equation.

a.



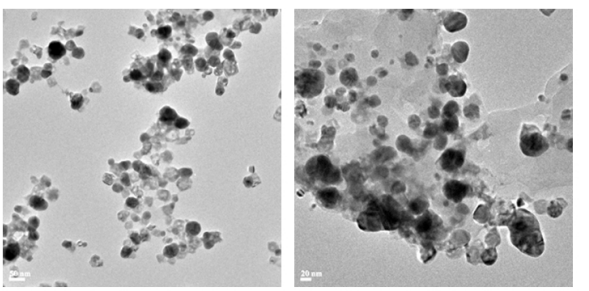
b.



b

Figure 8: Zeta analysis of synthesized Ag-NPs. a. Size and b. zeta potential

TEM analysis showed that the nanoparticles were almost spherical with particle size ranging from 50-100 nm (Figure 9.A, 9.B).



(A) **(B)**

Figure 9: TEM analysis of synthesized Ag-NPs

DPPH Radical Scavenging Activity

DPPH (free radical) scavenging activity is one among the frequently used chemical methods to evaluate free radical scavenging activity of test drug. It is now well established that the potential of natural products in scavenging DPPH free radical scavenging is directly correlated to its antioxidant potential. The decrease in absorption at 517 nm is directly proportional to the formation of DPPH–H (non-radical form)[14]. Figure 10 shows free radical scavenging activity of the NP-*Centratherum punctatum* Cass. extract complex at various concentrations used which proved the potent role of this complex as an antioxidant with an IC_{50} value of 203.6 µg/mL. This also suggested the possible complex formation of silver nanoparticles with various bioactive phytoconstituents present in the plant extract.

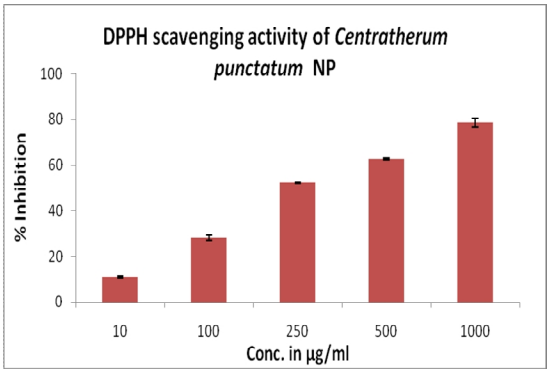


Figure 10" Free radical scavenging activity of synthesized Cp-NPs complex showing an IC50 = 203.6 µg/mL

Anti-Inflammatory Activity

Previous studies[16]hold proof that the cell membrane destabilization particularly lysosomes, denaturation of proteins, in particular blood proteins and activation of proteinases from dying cells are the major causes of arthritis and inflammation. NP-*Centratherum punctatum* Cass. extract complex synthesized was evaluated for its anti-inflammatory potential through *in vitro* assays mainly comprising of inhibition of protein denaturation, RBC membrane stabilization and inhibition of proteinase activity. An IC_{50} of 215.6, 183.0 and 222.5 µg/mL (Figure 11a,b,c) was observed in the inhibition of protein denaturation, RBC membrane stabilization and proteinase assay respectively. The synthesized NP-plant extract complex studied for its anti-inflammatory potential through *in vitro* method suggests that *Centratherum punctatum* Cass. could be a good source for developing green nano medicine for the management of inflammation.

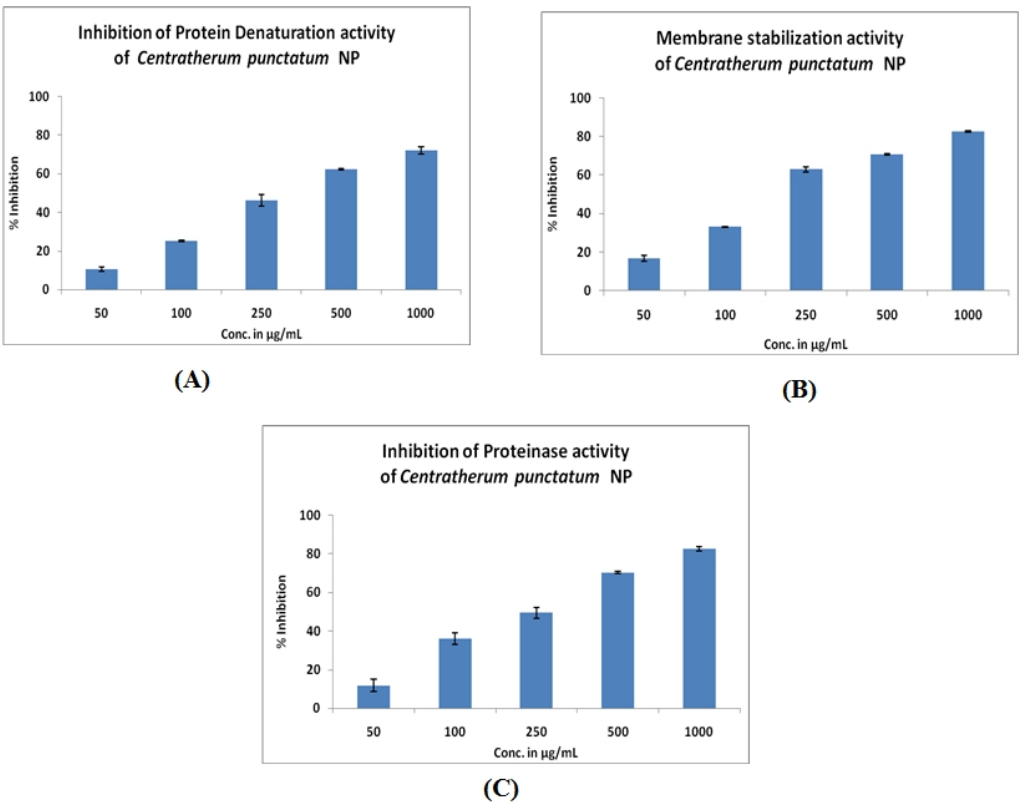


Figure 11: Anti-inflammatory efficacy of synthesized CP-NP complex. a. Inhibition of protein denaturation (IC_{50} = 215.6 µg/mL), b. membrane stabilization (IC_{50} = 183.0 µg/mL) and c. Inhibition of protease activity (IC_{50} = 222.5 µg/mL)

CONCLUSION

The study demonstrates and discusses the synthesis of AgNPs using *Centratherum punctatum* Cass. leaf extract. AgNPs have been prepared by reducing silver ion through ascorbic acid which was dispersed in aqueous extract of *Centratherum punctatum* Cass. The reduction of silver ion peak was observed at 423.75 nm in UV-Vis analysis. The purity and stability of AgNPs were confirmed by TG-DSC and FT-IR analysis. The results of SEM images showed that the average size of the synthesized AgNPs to be 37 nm. EDAX further confirmed the presence of silver nanoparticles. The synthesis of AgNPs using *Centratherum punctatum* Cass. provides a natural, economical and efficient nano form, which could find applications in pharmaceutical industries and that can contribute in providing newer pathways towards the development of nano medicines employing Green synthesis.

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