



Research Article

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Evaluation of *in vitro* antioxidant and anti-inflammatory property exhibited by silver nanoparticles stabilized by *Adathoda vasica*

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ABSTRACT

Adathoda vasica is native to India and is commonly used in a broad variety of medicinal applications. *A.vasica* is a traditional medicinal plant widely used in India, but there are few reports in the literature of studies on its chemical compositions and biological properties. In this study, the antioxidant and anti-inflammatory activities of the plant were evaluated and the total phenolic compounds were determined. The antioxidant activity of the extracts was measured using hydroxyl radical-scavenging activity, hydrogen peroxide radical scavenging assay and ferric reducing antioxidant power assay. The anti-inflammatory activity was evaluated using inhibition of albumin denaturation. Our findings suggest that silver nanoparticles stabilized by *A.vasica* possess potential antioxidant and anti-inflammatory compounds, which could be tested as drug candidates against oxidative and inflammation-related pathological processes in medicinal chemistry studies.

Key words: *Adathoda vasica*, antioxidant, inflammation, silver nano particles

INTRODUCTION

Inflammation and oxidative stress play an important role in various diseases. Inflammation is an immunological defense mechanism elicited in response to mechanical injuries, burns, microbial infections, allergens and other noxious stimulus [1-5]. Use of anti-inflammatory agents may therefore, be helpful in the treatment of inflammatory disorders [6]. Non-steroidal anti-inflammatory drugs (NSAID) or steroidal anti-inflammatory drugs (SAID) are commonly used to treat different inflammatory diseases. The adverse effects of the currently available anti-inflammatory drugs however, pose a major problem in their clinical use therefore; naturally, originated agents with very little side effects are desirable to substitute chemical therapeutics [7,8]. Medicinal plants continue to provide humanity with new remedies. It is therefore important to explore medicinal plants for their safety, quality, toxicity, appropriate amount of plant materials to use, and efficacy. Natural products of plants possess several biological activities including antioxidant and anti-inflammatory activity [9,10]. Medicinal plants are rich in active phytochemical compounds with various biological activities. Antioxidants play an important role in neutralizing free radical species which are produced as end or byproducts of normal biochemical reactions in normal system [11]. High amounts of free radical molecules cause oxidative stress in cells which result in damaging essential macromolecules including DNA, lipids, and proteins. The damage of macromolecules leads to inflammation and many degenerative conditions. Reactive oxygen species are also reported as carcinogenic and mutagenic agents [12]. Signs of inflammation are swollen joints, joint pain, stiffness, and loss of joint functions. Nonsteroidal anti-inflammatory drug (NSAIDs) such as ibuprofen and naproxen are anti-inflammatory drugs currently used for treatment of inflammation. These drugs are known to cause severe side effects in the body such as heart attacks and stroke [13].

EXPERIMENTAL SECTION

Chosen plant

Leaves of *Adathoda vasica* were collected from Vayallur road sides at Tiruchirappalli District, Tamilnadu, India and the plant was authenticated by the Botany department of St. Joseph's College and the voucher specimen number is (001). The plant chosen for the study had been washed, macerated and lyophilized. About 500g of all the three plant leaves yielded 33g powder respectively. The procedure was repeated to collect the needed quantity.

Preparation of Silver Nanoparticles from plant extract

Adathoda vasica leaves were washed to remove dust and impurities and shade-dried for a week. The leaves were dried at 37°C for two days in an incubator to remove any moisture left. They were then ground to fine powder. The powder was added in deionised water to form a suspension. Suspension was thoroughly mixed using a magnetic stirrer at 25 rpm for 10 mins, followed by incubation in shaker incubator for 30 min at 37°C. Then the mixture was centrifuged and filtered using 2.5 µm membrane filter [qualitative filter paper – Hi-media] paper. After addition of leaf extract to the silver nitrate solution, instant color change in the reaction mixture was observed from nearly colorless to yellowish brown. The color change occurs due to the reduction of silver ions to elemental form.

Characterization of the synthesized AgNPs

UV- visible spectroscopy analysis [Perkin Elmer lambda 35 model]

The preliminary characterization of the silver nanoparticles was carried out using UV-visible spectroscopy. Noble metals, such as silver [Ag] and gold [Au] nanoparticles exhibit unique and tunable optical properties because of their Surface Plasmon Resonance [SPR], depending upon shape, size and size distribution of the nanoparticles. The reduction of silver ions was monitored by measuring the UV-visible spectra of the solutions after diluting a small aliquot [0.2 mL] of the aqueous component. UV-visible spectroscopy analysis has been done using a Perkin-Elmer Lambda-35 spectrophotometer operated at a resolution of 1 nm as a function of reaction time. The nanoparticles solution was diluted to 20 times with deionized water to avoid errors due to high optical density of the solution. Deionised water was used as a blank.

FR-IR Analysis

Perkin-Elmer spectrometer FTIR Spectrum ONE in the range 4000–400 cm⁻¹ at a resolution of 4 cm⁻¹ was used. The deposited residues in the sample were dried and grinded with KBr to obtain pellet. Thin sample disc was prepared by pressing with the disc preparing machine and placed in Fourier Transform Infrared [FTIR] for the analysis of the nanoparticles.

XRD Analysis

The silver nanoparticle solution thus obtained was purified by repeated centrifugation at 5000 rpm for 20 min followed by re-dispersion of the pellet of silver nanoparticles into 10 ml of deionized water. After freeze drying of the purified silver particles, the structure and composition were analyzed by XRD and SEM. The dried mixture of silver nanoparticles was collected for the determination of the formation of Ag nanoparticles by an X'Pert Pro x-ray diffractometer (PAN analytical BV, The Netherlands) operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in a θ- 2 θ configuration.

EDX Measurement

EDS makes use of the X-ray spectrum emitted by a solid sample bombarded with a focused beam of electrons to obtain a localized chemical analysis. All elements from atomic number 4 (Be) to 92 (U) can be detected in principle, though not all instruments are equipped for 'light' elements (Z < 10). Qualitative analysis involves the identification of the lines in the spectrum and is fairly straightforward owing to the simplicity of X-ray spectra. Quantitative analysis (determination of the concentrations of the elements present) entails measuring line intensities for each element in the sample and for the same elements in calibration Standards of known composition.

X-Ray Diffraction analysis [XRD]

Crystalline AgNPs were determined by X-ray diffraction analysis. The biosynthesized AgNPs were laid onto the glass substrates on a Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30 mA with CuKα radiation. These results were used to predict the average particle size of nanoparticles with the help of JCPDS data.

Determination of cellular toxicity using sheep erythrocytes [Xian-guo and Ursula 1994]

The method described by Xian-guo and Ursula [14] was employed to study cellular toxicity.

Evaluation of *in vitro* antioxidant activity assays

Hydrogen peroxide radical scavenging assay

The ability of AVE extract to scavenge hydrogen peroxide was determined by following the standard procedure [15]. Hydrogen peroxide 10 mM solution was prepared in the phosphate buffer saline of 0.1 M, pH 7.4. 1 ml (0.25 mg) of the extract was rapidly mixed with 2 ml of hydrogen peroxide solution. The absorbance was measured at 230 nm in the UV spectrophotometer against a blank (without hydrogen peroxide) after 10 min of incubation at 37°C. The percentage of scavenging of hydrogen peroxide was calculated using the following formula

$$\% \text{ scavenging (H}_2\text{O}_2) = (A_0 - A_1 / A_0) \times 100$$

A₀ - absorbance of control A₁ Absorbance of sample)

Total phenolic content

Phenolic contents of crude extracts were estimated by the method of Taga *et al.*, [16]. Briefly 100 µl of aliquot sample was mixed with 2.0 ml of 2% Na₂CO₃ and allowed to stand for 2 min at room temperature. After incubation, 100 µl of 50% Folin Ciocalteu's phenol reagent was added and the reaction mixture was mixed thoroughly and allowed to stand for 30 min at room temperature in the dark. Absorbance of all the sample solutions was measured at 720 nm using spectrophotometer (Phenolic content are expressed as Gallic acid equivalent per gram).

Total antioxidant activity

Total antioxidant activity was measured following the method of Prieto *et al.*, [17]. To 7.45 ml of sulphuric acid (0.6 mM solution), 0.9942 g of sodium sulphate (28 mM solution) and 1.2359 g of ammonium molybdate (4 mM solution) were mixed together in 250 ml distilled water and labeled as Total Antioxidant Capacity (TAC) reagent. 300 µl of extract was dissolved in 3 ml of TAC reagent. Blank was maintained with distilled water replacing the TAC reagent. Absorbance of all sample mixtures was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Hydroxyl radical-scavenging activity

Hydroxyl radical scavenging activity of extract was measured according to the method of Halliwell *et al.* [18]. One milliliter of the final reaction solution consisted of aliquots (500 µL) of various concentrations of the extract, 1 mM FeCl₃, 1 mM EDTA, 20 mM H₂O₂, 1 mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 h at 37 °C, and further heated in a boiling water bath for 15 min after addition of 1 mL of 2.8% (w/v) trichloroacetic acid and 1 mL of 1% (w/w) 2thiobarbituric acid. The color development was measured of 532 nm against a blank containing phosphate buffer.

Ferric reducing antioxidant power assay

Reducing power of different crude extract was determined by the method of Oyaizu [19]. Briefly, 1.0 ml of different solvent extract containing different concentration of samples were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferric cyanide (1%). Reaction mixture was kept in a water bath at 50°C for 20 min. After incubation, 2.5 ml of Trichloroacetic acid (10% of TCA) was added and centrifuged at 650 rpm for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml of FeCl₃ (0.1%). Absorbance of all the solution was measured at 700 nm. Ferric reducing antioxidant Power is expressed as the number of equivalents of ascorbic acid.

Assessment of *in vitro* anti-inflammatory activity

Inhibition of albumin denaturation

The anti-inflammatory activity of AVE was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima [20] and Sakat [21], followed with minor modifications. The reaction mixture was consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37 °C for 20 min and then heated to 51 °C for 20 min, after cooling the samples the turbidity was measured at 660nm. (UV-Visible Spectrophotometer Model 371, Elico India Ltd) The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows:

$$\text{Percentage inhibition} = (\text{Abs Control} - \text{Abs Sample}) \times 100 / \text{Abs control}$$

RESULTS

Characterization of silver nano particles of *A.vasica*

From this study, it has been found that the leaf extract of *Adathoda vasica*, a traditional medicinal plant has the potential to reduce silver nitrate ions to silver nanoparticles. The light brown color of leaf extract of *A.vasica* was changed to yellowish brown after 10 mins of incubation due to the excitation of surface plasmon vibrations in silver nanoparticles. The intensity of the color increased with the incubation time. Nanoparticles size can also be determined by the changes in the color of the reaction solution.

UV-visible spectroscopy analysis

The corresponding UV-Vis absorption spectra for the synthesis of silver nanoparticles using *A.vasica* at 5% concentration for various temperatures [30 °C, 60 °C, 90 °C and 95 °C] are shown in Fig 1. The leaf extract of *A.vasica* [control] shows no evidence of absorption peak in the range in 200 to 900 nm. AgNPs at various temperatures indicated an absorbance peak at 30 °C [448 nm], 60 °C [437 nm], 90 °C [444 nm] and at 95 °C [445 nm] respectively. The spectrum with bands in this range has been associated with the surface plasmon resonance of nano-sized silver metal, confirming the occurrence of silver nanoparticles in the solution after exposure to UV light.

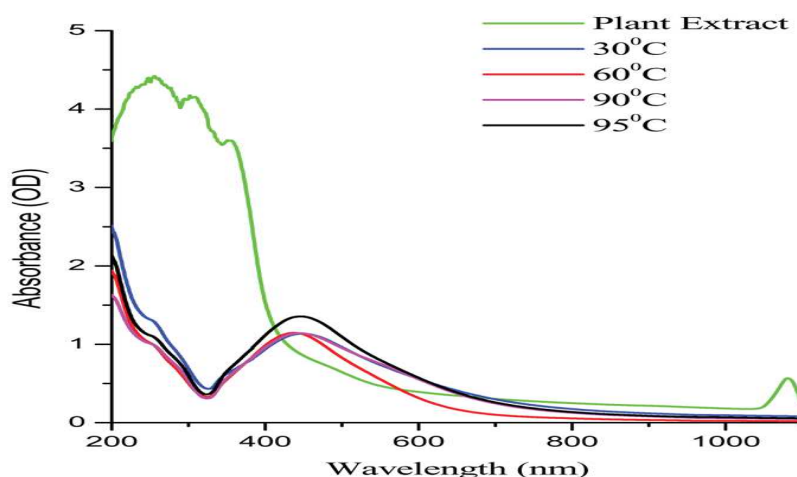


Figure 1: UV-Vis spectroscopy analysis of AgNPs synthesized using *A.vasica*

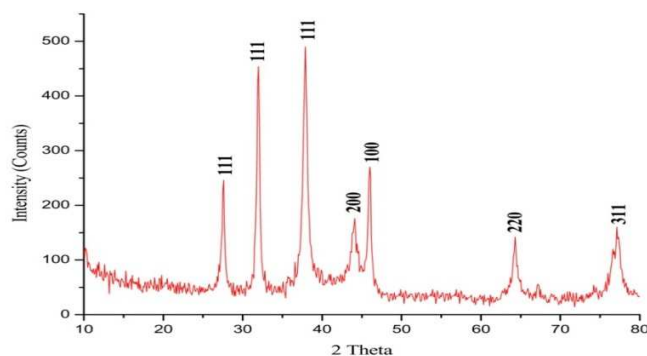
FT-IR [Fourier Transform Infra Red spectroscopy] analysis

FT-IR [Fourier Transform Infra Red spectroscopy] analysis an infrared spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material. Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, infrared spectroscopy can result in a positive identification [qualitative analysis] of every different kind of material.

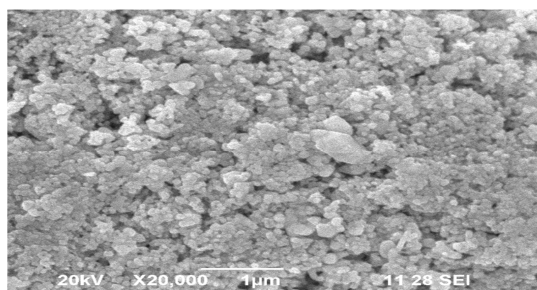
The FTIR spectrum of silver nanoparticles synthesized using *A.vasica* at various temperatures is shown in Fig 2. The band around 3433 cm^{-1} denotes O-H stretches corresponding to amines and alcohols and phenols, 1563 cm^{-1} indicates to $\alpha\text{-CH}_2$ bending corresponding to aldehydes and ketones. The peak at 1641 cm^{-1} denotes to NH_2 corresponding to amines and primary amide bonds related to carboxylic acids and their derivatives, 1355 cm^{-1} denotes O-H bonding corresponding to alcohols and phenols, 678 cm^{-1} denotes C-H deformation corresponding to alkynes. A peak at 2096 cm^{-1} indicates that the Silver atoms reduced [22].

Figure 2: FTIR spectroscopy analysis of AgNPs synthesized using *A. vasica***X-ray diffraction analysis [XRD]**

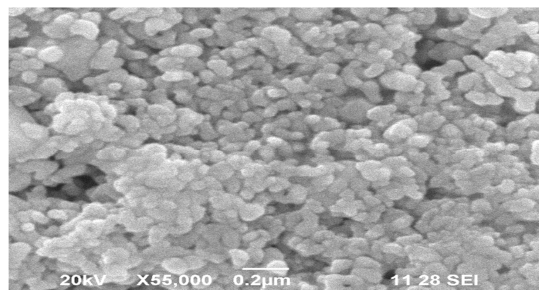
XRD pattern of biosynthesized AgNPs [Figure 3] shows four intense peaks in the spectrum values ranging from 10 to 80 XRD spectra of pure crystalline silver structures have been published by the Joint Committee on Powder Diffraction Standards [File no.04-0783]. A comparison of our XRD spectrum with the standard confirmed that the silver particles formed in our experiments were in the form of amorphous and crystalline phases coexist. The crystalline peaks at 2θ values of 27.6° , 32° , 37.9° , 44° , 46° , 64.3° , 77° can be indexed as 1 1 1, 1 1 1, 1 1 1, 2 0 0, 1 0 0, 2 2 0, 3 1 1 planes of fcc silver.

Figure 3 : XRD analysis of AgNPs synthesized using *A. vasica***Scanning electron microscope [SEM] analysis**

SEM image shown in Figure 4, [X20,000 magnification] and 12 [X30,000 magnification] confirms that the synthesized AgNPs are irregular and spherical in shape and in mono dispersed manner with average diameter range of 50 – 60 nm.



1 μm



0.2 μm

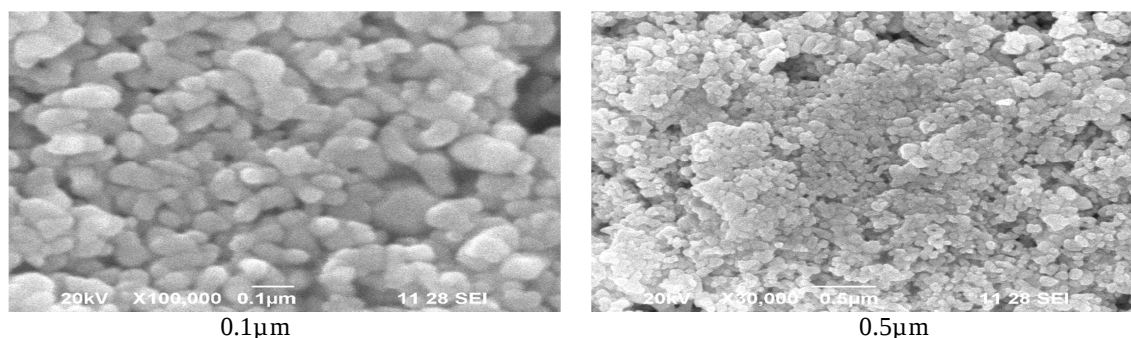


Fig 4: SEM image of AgNPs synthesized from the plant extract

The morphology and size of the green synthesized AgNPs were studied by SEM. Fig. 4 shows the morphology of nanoparticles with a different size range. The green synthesis AgNPs strongly recommend that Ag was the major element which has an optimal absorption in the range due to the surface Plasmon resonance.

Determination of cellular toxicity using human erythrocytes

The compound showed no cellular toxicity as no hemolysis was observed.

Scavenging activity of hydrogen peroxide

The scavenging activity of hydrogen peroxide content of extract *Adathoda* were determined and the results are presented in Fig.5 & table 1. Without the hydrogen peroxide is used as a reference value. Among the different concentrations used, 50μl (13%) shows lowest anti-oxidant activity and 125μl (45%) of concentration shows the highest anti-oxidant activity. OD values were taken at 230nm.

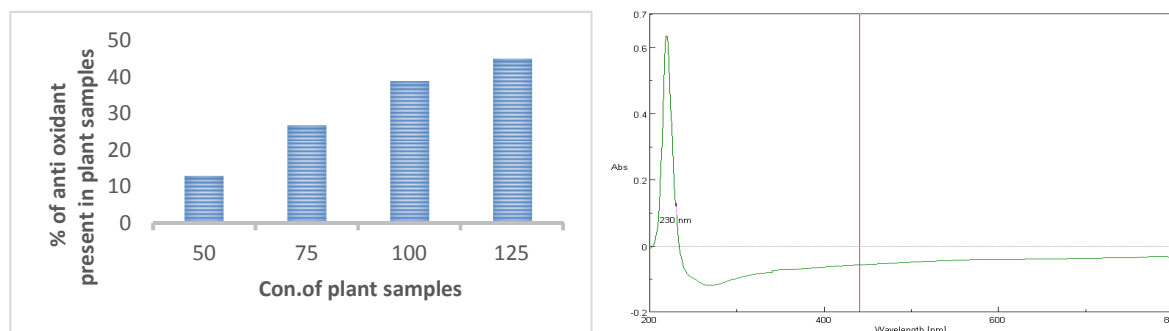


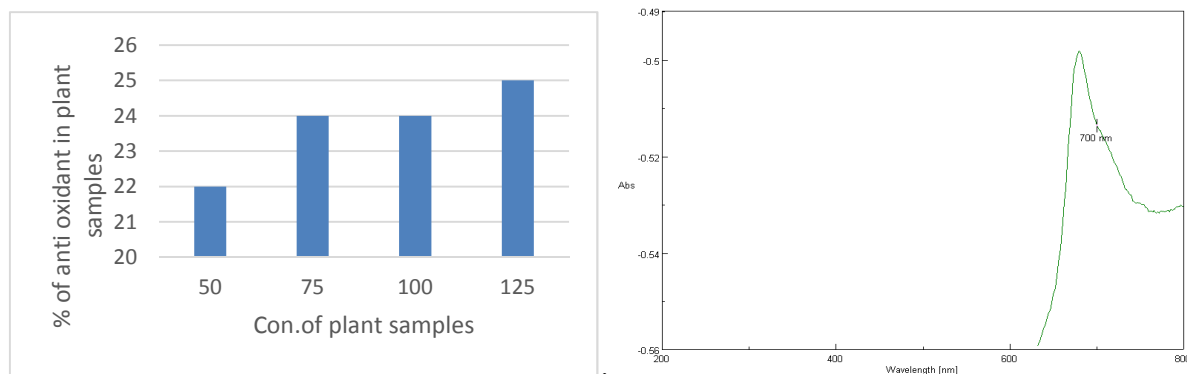
Fig. 5: Scavenging activity of hydrogen peroxide of *A.vasica*

Table 1: Scavenging activity of hydrogen peroxide of *A.vasica*

S.NO	CON.OF PLANT SAMPLE	PERCENTAGE OF ANTI OXIDANT ACTIVITY
1	50μl	13%
2	75μl	27%
3	100μl	39%
4	125μl	45%

Ferric reducing antioxidant assay

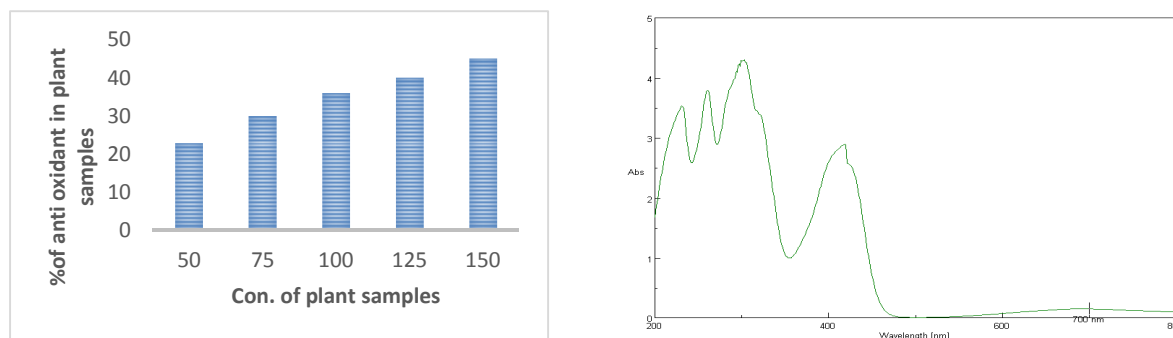
The Ferric reducing antioxidant content of extract *Adathoda* were determined and the results are presented in Fig.6 & table 2. Ascorbic acid is used as a reference value. Among different concentrations, 125μl (25%) shows highest anti-oxidant activity and 50μl (22%) shows the lowest anti-oxidant activity. OD values were taken at 700nm.

Fig 6: Ferric reducing antioxidant assay of *A.vasica*Table 2: Ferric reducing antioxidant assay of *A.vasica*

S.NO	CON.OF PLANT SAMPLES	PERCENTAGE OF ANTI OXIDANT ACTIVITY
1	50µl	22%
2	75µl	24%
3	100µl	24%
4	125µl	25%

Total antioxidant assay

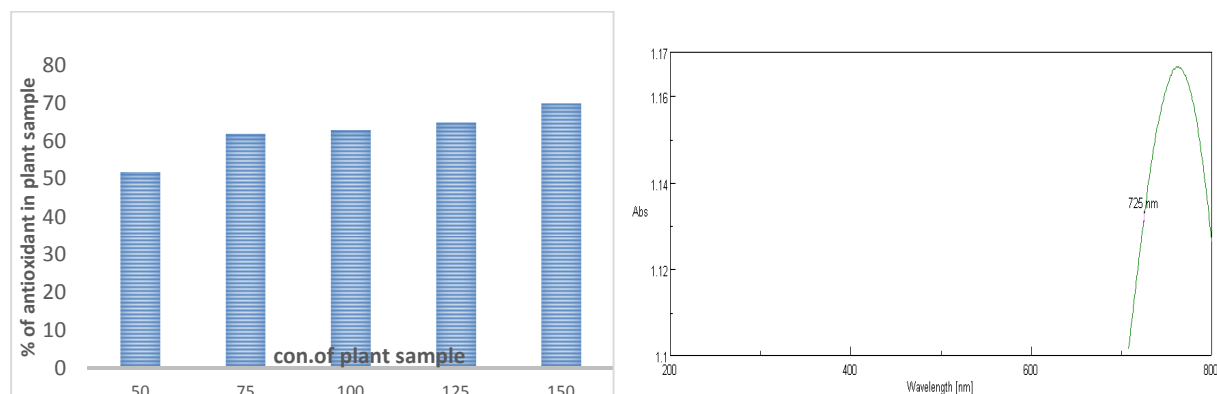
The total antioxidant content of extract *Adathoda* was determined and the results are presented in Fig.7 & table 3. Ascorbic acid is used as a reference value. Among different concentrations used, 150µl (45%) shows highest anti-oxidant activity and 50µl (23%) of concentration shows the lowest anti-oxidant activity. OD values were taken at 695nm.

Fig 7: Total antioxidant assay of *A.vasica*Table 3: Total antioxidant assay of *A.vasica*

S.NO	CON.OF PLANT SAMPLES	PERCENTAGE OF ANTI OXIDANT ACTIVITY
1	50µl	23%
2	75µl	30%
3	100µl	36%
4	125µl	40%
5	150µl	45%

Total Phenolic content

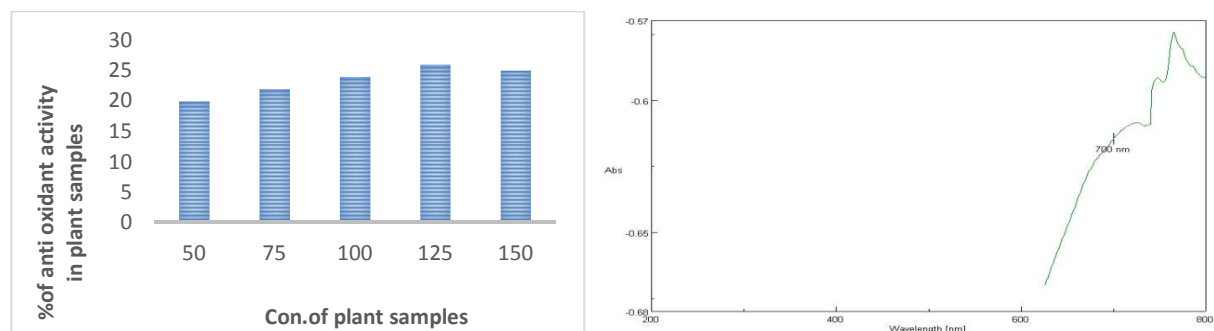
The total phenolic content of extract *Adathoda* were determined and the results are presented in Fig.8 & table 4. Gallic acid is used as a reference value. Among different concentration 150µl (70%) shows highest anti-oxidant activity and 50µl (52%) of concentration shows the lowest anti-oxidant activity. OD values were taken at 720nm.

Fig 8: Total phenolic content of *A.vasica*Table 4: Total phenolic content of *A.vasica*

S.NO	CON.OF PLANT SAMPLES	PERCENTAGE OF ANTI OXIDANT ACTIVITY
1	50 μ l	52%
2	75 μ l	63%
3	100 μ l	62%
4	125 μ l	65%
5	150 μ l	70 %

Hydroxyl radical scavenging assay

The Hydroxyl radical scavenging assay content of extract *Adathoda* was determined and the results are presented in Fig.9 & table 5. Phosphate buffer is used as a reference value. Among different concentrations, 150 μ l (25%) shows highest anti-oxidant activity and 50 μ l (20%) shows the lowest anti-oxidant activity. OD values were taken at 532nm.

Fig 9: Hydroxyl radical scavenging assay of *A.vasica*Table 5: Hydroxyl radical scavenging assay of *A.vasica*

S.NO	CONCENTRATION OF PLANT SAMPLES	PERCENTAGE OF ANTIOXIDANT ACTIVITY
1	50 μ l	20%
2	75 μ l	22%
3	100 μ l	24%
4	125 μ l	26%
5	150 μ l	25%

Anti-inflammation

Inhibition of albumin denaturation

Protein Denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammation activity, ability of plant extract to inhibit protein denaturation was studied. Aspirin is used as a reference value. At

different concentrations, the tests were performed and among that 125 μ l (77%) shows the highest anti-inflammation action as compared to 50 μ l (33%). OD values were taken at 660nm as shown in figure 10 & table 6.

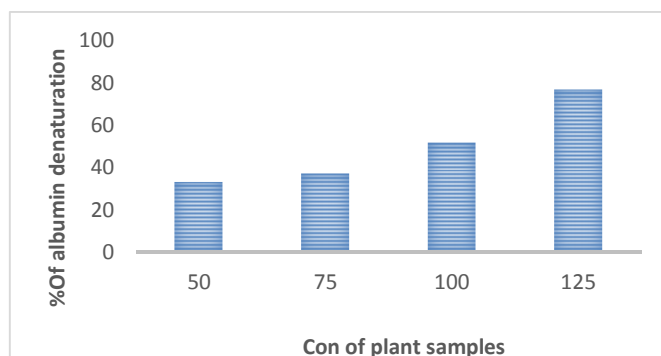


Fig 10: Inhibition of albumin denaturation of *A.vasica*

Table 6: Inhibition of albumin denaturation of *A.vasica*

S.NO	CON.OF PLANT SAMPLES	PERCENTAGE OF ATI OXIDANT ACTIVITY
1	50 μ l	33%
2	75 μ l	37%
3	100 μ l	52 %
4	125 μ l	77%

DISCUSSION

Plant constituents are responsible for both free radicals scavenging and anti-inflammatory activity. Secondary metabolites are responsible for biological activities of plants including terpenoids [23], phenolic compounds (flavonoids, phenolic acids, quinones, coumarins, lignans, stilbenes, tannins), and nitrogen compounds (alkaloids, amines, and betalains) and carotenoids [24]. It is reported that the reactive oxygen species (ROS) like super oxide anion (O_2^-), hydroxyl radicals ($OH^\cdot$), peroxy radical ($ROO^\cdot$) and nitric oxide radical ($NO^\cdot$) easily react with most of the biomolecules including protein, lipoprotein, enzymes, DNA and RNA (Roy, S, 2013). The ample generation of ROS leads to various inflammatory disorders like rheumatoid arthritis, cancer, insulin dependent diabetes mellitus (IDDM) [25-27]. Presently NSAIDs are the most commonly prescribed agents for the management of inflammatory disorders like fever, pain, arthritis, gout etc. Recently, the adverse drug reactions associated with these agents are great matter of concern.

It has been also observed that many plant derived anti-inflammatory compounds possess both anti-inflammatory and free radical scavenging activity. Antioxidants can help in reducing the incidences of inflammation. Multiple antioxidants if consumed in diet, normalized ROS induced release of interleukin-6, thus preventing incidences of lipid peroxidation. Multi antioxidants are also known to reduce inflammatory symptoms in inflammatory joint disease, acute and chronic pancreatitis, and adult respiratory distress syndrome. Thus from the present investigation it can be concluded that antioxidant activity may account for anti-inflammatory activity of AV leaf extract. AV extract showed significant antioxidant and anti-inflammatory activity. The reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid ($HClO$), and free radicals such as the hydroxyl radical ($^\cdot OH$) and the superoxide anion (O_2^-). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules. This species is produced from hydrogen peroxide in metal-catalyzed redox reactions such as the Fenton reaction. These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins. Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms, while damage to proteins causes enzyme inhibition, denaturation and protein degradation. Inflammation is one of the results of free radical damage. When free radical damage is not controlled, you get chronic inflammation. Chronic inflammation is one of the main ways degenerative diseases develop.

CONCLUSION

Our experimental results suggest that silver nanoparticles stabilized by *A. vasica* possess potential antioxidant and anti-inflammatory activity, which may be contributed by the phytochemicals present and could be tested as drug candidates against oxidative and inflammation-related pathological processes in medicinal chemistry studies.

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