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Adathoda vasica - an Intelligent Fabricator of Gold Nanoparticles

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ABSTRACT

Leaf extract of Adathoda vasica a tropical shrub was used as reducing agent to convert gold ions into monodispersed gold nano particles. Parameters like pH, temperature, concentration of reactants viz. Gold salt and leaf extract was standardized to produce gold nano particles of 10 -20 nm size. The best parameters obtained were high temperature (80 and 100ºC), pH 6 and 50 ppm of aurochlorate. The characterization was based on observations made using UV-Vis spectroscopy, XRD and HRTEM. The reducing agents for synthesis of gold nano particles were speculated to be nitrate reductase and glutathione.

Keywords: Gold nanoparticle**,** *Adhatoda vasica*, Biosynthesis, Nitrate reductase, Glutathione.

INTRODUCTION

Nature has developed variety of processes for the biosynthesis of nano-scaled inorganic materials which are cardinal additions to the development of relatively new and largely un-charted area of research based on the biosynthesis of nanomaterials [1]. Bio-synthesis of metal nanoparticles, exploiting biological systems as an efficient sink has grabbed exceptional attention due to their novel optical [2], chemical [3], photoelectrochemical [4] and electronic [5] properties. Plants respond to heavy metal toxicity in a variety of different ways. Such responses include immobilization, exclusion, chelation and compartmentalization of the metal ions, and the expression of more general stress response mechanisms such as ethylene and stress proteins. These mechanisms have been reviewed comprehensively [6] for plants exposed to Cd, the heavy metal for which there have been arguably the greatest number and most wide-ranging studies over many decades. Understanding the molecular and genetic basis for these mechanisms will be an important aspect of developing plants as agents for the phytoremediation of contaminated sites. Marine algae were explored for their potential for synthesis of GNPs by Oza and co-workers. They used *Sargassum wightii* [7] for bio-fabrication of GNPs. They also studied the impact of ionic strength of the surrounding medium on synthesis of gold nanoparticles. Pandey and co-workers explored the reducing potential of *A.racemosus* [8], *M. charantia* [9] for catalyzing the formation of extremely stable gold nanoparticles.The GNPs were extremely stable than chemically synthesized gold nanoparticles. Such stable GNPs can be used as an ideal vessel for ferrying therapeutic moieties inside the living system. Marine algae were also explored for their potential for synthesis of GNPs. Oza et al used *Sargassum wightii* [7] for bio-fabrication of GNPs. They also studied the impact of pH of the surrounding medium on synthesis of gold nanoparticles. A detailed account of living system used for synthesis of plethora of metal nanoparticles can be understood by referring author's exhaustive review [10]

Adhatoda vasica commonly known as Adulsa or Bakash is a small evergreen tree. The leaf extract of this plant was used for biosynthesis of gold nano particles for the present work.

In the present work, the selection of plants species is done on the basis of information available in the literature about the chemical content and with some initial experimental observation e.g. the content of ideal reducing agents such as Citric acid, Ascorbic acids and Flavonoids. The emphasis is also given on the presence of reducing agents such as reductases and dehydrogenases and extracellular electron shuttlers. This is the first report of synthesis, parametric optimization and understanding the mechanism of synthesis using *Adhatoda vasica.*

MATERIALS AND METHODS

Materials: Fresh *Adathoda vasica* leaves were collected from the wild growing plants in Ambernath, as and when required. Gold aurochlorate and silver nitrate were procured from Sigma Aldrich, USA. The experiments were performed using double distilled water. The glasswares were washed with aqua regia to remove the traces of metal contaminant. The pipettes were pre-calibrated before using for the measurements. In order to record the temperature, local made thermocouple was used.

Preparation of aqueous extract of plants: 10 gram of freshly collected leaves of *A. vasica* was crushed in 50 ml of distilled water. The extract obtained was centrifuged at 10,000 rpm for 15 minutes. The supernatant was used as reducing agent for synthesis of gold and silver nanoparticles. In order to retain the activity of the enzymes and other factors such as glutathione and Phytochelatins, the extract was made in ice box.

Biosynthesis of the Gold Nanoparticles: Clear leaf extract was used for the biosynthesis of gold nano particles. The parameters and their variables considered for biosynthesis were,
(a) pH 3, 4, 6, 8, 10 and inherent pH of plant extract (b) *Concentration of the aurochlorate* – after few initial trials it was decided to keep it 50 ppm and (c) 4, 30, 60 and 100^0C temperature.

A stock solution of 50,000 ppm aurochlorate was prepared and diluted as per the pre-requisite of the experiment. The concentration of the gold salt in the plant extract was 50ppm was diluted to make the total volume 10 ml using double distilled water. In a boiling solution of the plant extract, gold salt was immediately added to make the concentration 50ppm. After addition of the gold salt, the solution was agitated till the colour becomes wine red.

Characterization of Biosynthesized Gold Nanoparticles: The characterization methods used included:

UV- Vis Spectroscopy - of the gold nanoparticles was recorded using dual beam spectroscopy Lambda 25 Perkin Elmer, USA. High quality quartz cuvette (Perkin Elmer optics, USA) was used as a vessel to record the spectra.

High Resolution Transmission Electron Micrographic Analysis- was done to elucidate the morphology of the gold nanoparticles using Carl Zeiss Microimaging, GmbH, Germany. Sample was ultrasonicated for 15 minutes and then coated on ultraclean carbon coated copper grid for analysis. The SAED pattern of the gold nanoparticles indicates presence of crystalline gold nanoparticles as deciphered using the diffraction pattern using X-rays.

X-Ray diffraction studies (XRD)- To peep into the crystallinity and the lattice properties of the gold nanoparticles, XRD (PAnalytical, Philips PW 1830, The Netherlands) operating at 40 kV and a current of 30 mA with Cu Kα radiation (λ = 1.5404 Å) was used. The colloidal suspension containing metal nanoparticles was dried on a small glass slab.
Nitrite Reductase [NiR EC 1.6.6.4] Assay - For extraction of Nitrite Reductase from leaves of *A. vasica*, 100 mg plant material was homogenized with Tris-HCl buffer (pH 8.0) and then centrifuged at 0^0C at 2000 rpm for 15 min. The supernatant was used as enzyme source.

Nitrite Reductase activity was measured by Vega and Cardenas method [11] with few variations. The reactions were conducted in uncapped test tubes to maintain aerobic condition. 0.3ml of 0,5M Tris HCl buffer (pH 8,0) + 0.2 ml of 20 mM $NaNO_2$ + 0.2 ml of 5.0 M Ferredoxin + 0.1 ml diluted enzyme and 0.3 ml of Sodium dithionite solution (freshly prepared was taken in a test tube and incubated in dark for 10 min at 30^0C. During incubation some amount of $NaNO_2$ gets converted to NH_3 with catalytic action of nitrite reductase and Ferredoxin. After incubation the reaction was stopped by vigorously shaking the test tube on cyclomixer until dithionite was completely oxidized and the dye became colorless. Then 1ml of diazo coupling reagent (1% Sulphanilamide in 3 ml HCl and 0.02% N-(1-naphthyl) ethylenediamine hydrochloride) was added to 3 ml reaction mixture and diluted 10 folds to detect the remaining NO_2. After 30 min of incubation in dark at 30^0C for development of color; O.D. was recorded at 540 nm. The result was calculated against the standard graph of nitrite.

FTIR – spectra were taken to assess the involvement of possible capping proteins (Glutathione) using Brucker Instruments.

RESULTS AND DISCUSSION

Impact of different pH on formation of gold nano particles at 30 and 100^0C are presented in table -1, which shows that pH 6 has yielded the best results at both the tried temperatures. Therefore, further trials were done using pH 6 and a range of variable temperatures (Table-2)

Table – 1: Impact of pH and Temperature on the Biosynthesis of gold nano particles using 50 ppm Aurochlorate and *Adathoda vasica leaf extract*

pH	Temperature	
	30^0C	**100^0C**
2	Change in color in 24 h Very Weak UV-Vis peak at 550nm XRD - Crystalline structure	Change in color in < 5 sec Good peak at 535 nm XRD Crystalline structure
4	Change in color in 24 h Weak UV-Vis peak at 550 XRD Crystalline structure	Change in color in < 5 sec Intense Peak at 550 nm XRD Crystalline structure
6	Change in color in 24 h Intense Peak at 550 nm XRD Crystalline structure TEM Both iso & anisotropic gold nanoparticles could be seen	Change in color in < 5 sec Intense Peak at 550 nm XRD Crystalline structure TEM Both iso & anisotropic gold nanoparticles could be seen
8	Change in color in 24 h Peak of medium intensity at 550 nm XRD Crystalline structure	Change in color in < 5 sec Good peak at 535 nm XRD Crystalline structure
10	Change in color in 24 h Weakest peak at 550 nm XRD Crystalline structure	Change in color in < 5 sec Weak broad peak at 530 nm XRD Crystalline structure
Inherent pH of plant extract 8.2	Change in color in 24 h Intense peak at 550 nm XRD Crystalline structure	Change in color in < 5 sec Good peak at 535 nm XRD Crystalline structure

Table – 2: Impact of different temperatures on the Biosynthesis of gold nano particles using 50 ppm Aurochlorate and *Adathoda vasica* leaf extract at pH 6.

Temperature	Observations			
	Visual	**UV-Vis Peak**	**XRD**	**TEM**
4 ºC	Change in color in > 24 h	Broad peak at 545 nm	Crystalline	Both isotropic & anisotropic gold nanoparticles
RT (28 ± 2ºC)	Change in color in < 24 h	Broad peak at 545 nm	Crystalline	
60 ºC	Change in color within 10 min	Broad peak at 545 nm	Crystalline	
80 ºC	Change in color in <5 sec	Intense peak at 545 nm	Crystalline	
100 ºC	Change in color in <5 sec	Intense peak at 545 nm	Crystalline	Both isotropic & anisotropic gold nanoparticles

Visual observation: When experiment was conducted at 4, RT (Room Temperature) and 30ºC Table 1 & 2); at all the pH it took 24 h to exhibit change in color. But at higher temperatures of 60, 80 and 100ºC reaction was very fast and change in color took place immediately. At 100^0 C immediately after the addition of leaf extract to gold aurochlorate; a change in color from pale yellow to blue, ruby red or pink was observed (see insets of fig.1&2) The change in color was due to is a property which emerges due to a size dependent phenomenon called quantum confinement. This property becomes dominant when, the De-Broglie wavelength of the valence electrons becomes equal to or less than the size of the particle. Due to this phenomenon the freely mobile electrons are caged in gold nanoparticles and exhibit a characteristic collective coherent oscillation of plasmon resonance giving rise to surface plasmon resonance (SPR). The consequence of SPR is a sharp peak observed between 500-600nm [12]. It can be said that A. *vasica* is an excellent sink for bio-fabrication of the gold nanoparticles due to its controlled reducing power as well as presence of capping proteins. This is speculated after observing the SPR of gold nanoparticles at different reaction conditions. Nanoparticles formed were extremely stable at physiological temperature which makes it an ideal candidate for drug delivery.

UV-Vis Spectroscopic studies: Optimum intensity of UV-Vis spectra peak or SPR band cantered between 500 – 600 nm (Fig 1 & 2) occurred at pH 6 for all the tried temperatures. The SPR band cantered between 500 – 600 nm indicate the formation of 30 to 50 nm gold nanoparticles [13]. However, the intensity of peak decreased at pH above and below 6.

At 30°C, gold nanoparticles synthesised at pH 6, 8 and inherent pH; exhibited an intense peak at 556 and a weak broad peak at 770nm (fig.1a). Presence of two humps indicates the formation of non-spherical gold nanoparticles owing to transverse and longitudinal surface plasmon resonance at 556 and 770nm respectively [14]. Appearance of

the peak in near infra red spectrum may be either because of the (i) agglomeration of the nano particle that may be due to time or due to the impact of dielectrics of the medium and/or electrical double layers around the gold nanospheres in the solution [15] or (ii) formation of non-spherical gold nanoparticles with increasing aspect ratio with time. The combination of both the above phenomenon is also possible

Gold nanoparticles synthesised at pH 2 and 10 exhibited agglomeration as seen in the inset of fig. 1a; which can be seen in the form of broadening of the area under the peak. As mentioned above the agglomeration may be due to the dielectric constant of the medium or destabilisation of electrical double layer around the gold nanoparticles due to unfavourable ionic strength of the medium.

At 80 and 100°C, The nanoparticles synthesised at all the pH except pH 10 showed sharp peak at 556 nm. The sharpness of the peak and vivid ruby red color of the gold nanoparticles [15] at pH 6 shows the formation of highly stable and monodisperse gold nanoparticles. pH 2, 4, 8 and inherent exhibited broadening of the peaks.

The SPR of the gold nanoparticles was centered at 563nm as seen in fig.2b. The pH values other than 6, exhibited agglomeration of the gold nanoparticles in the solution. This may be due to absence of the stabilising proteins called capping proteins or the enzymes involved in the reduction or both. There was no formation of the gold nanoparticles at pH 10, which was in stark contrast with all the other cases studied earlier.

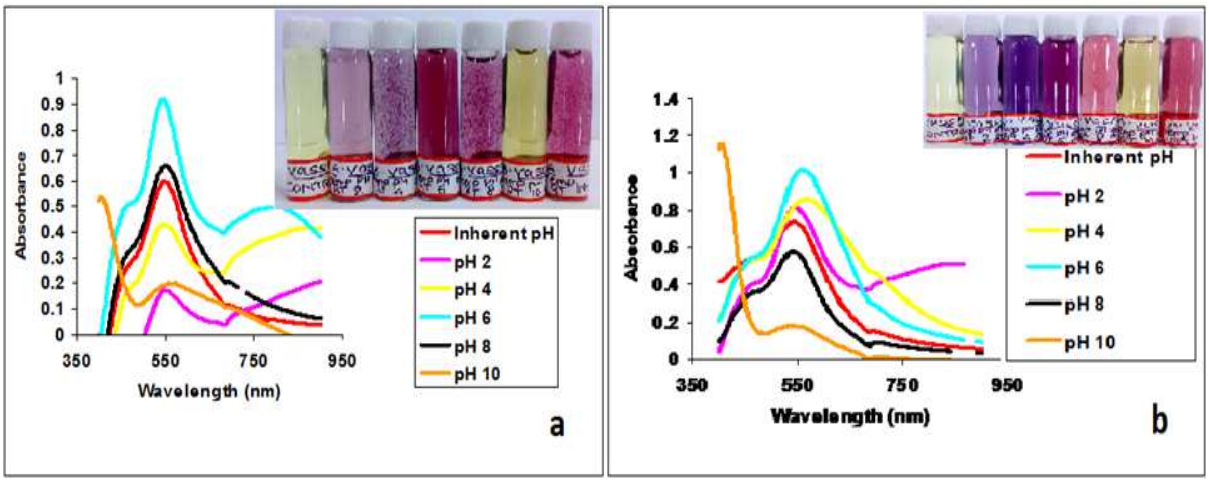

Figure-1: Impact of temperature and pH on bio-fabrication of gold nanoparticles using *A. vasica*, as shown by UV-Vis Spectroscopy (a) impact of 30°C & different pH values (b) impact of 100°C & different pH values. The inset shows the change in SPR with respect to different parameter

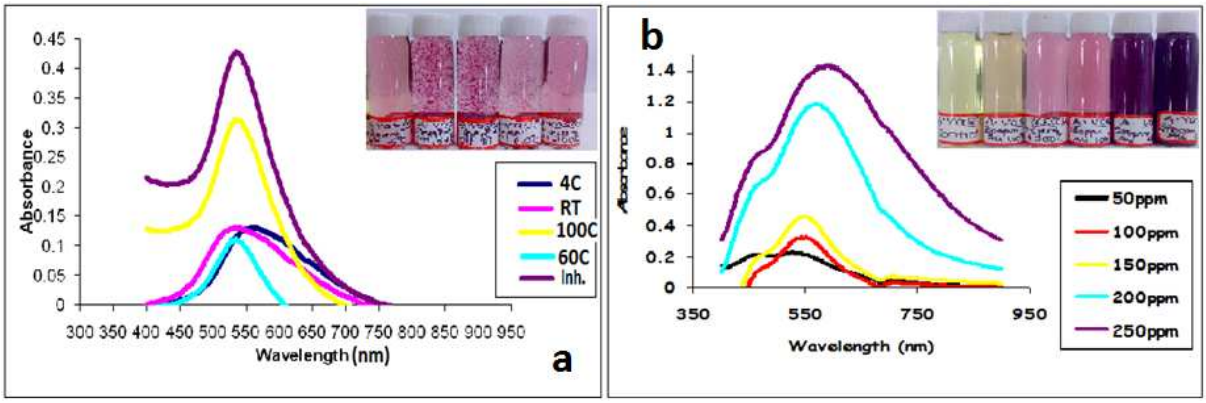

Figure- 2: UV-Vis spectra of gold nano particles biosynthesized at pH 6 using leaf extract of *A. vasica* at (a) different temperatures and (b) different concentrations of aurochlorate

The UV-Vis spectra shown in fig. 2, explains the impact of various temperatures (4, 28 ± 2, 60 &100°C) at pH 6 and 50ppm of gold salt on biosynthesis of gold nanoparticles. A sharp and narrow absorption at 553nm at 4°C indicates the formation of highly stable spherical gold nanoparticles in the solution. It can also be speculated from clear pink color of the gold nanoparticles is due to SPR, as shown in the inset. At 100°C, however, agglomeration of the

nanoparticles was observed after 2 days. Broadening of the area under the peak as well as minor red shift at 100°C supports the above observation.

High Resolution Transmission Electron (HRTEM) microscopic studies: A typical HRTEM image is presented in Fig 3, shows gold nano particles of a maximum of 20 nm size. Both anisotropic and isotropic nano particles could be seen in the micrograph. However, nano particles synthesized at 100°C, were found to considerably spherical. HRTEM clearly shows the lattice fringe of gold nanoparticles (Fig3b). The microscopic observation is in agreement with the UV-Vis spectroscopic studies.

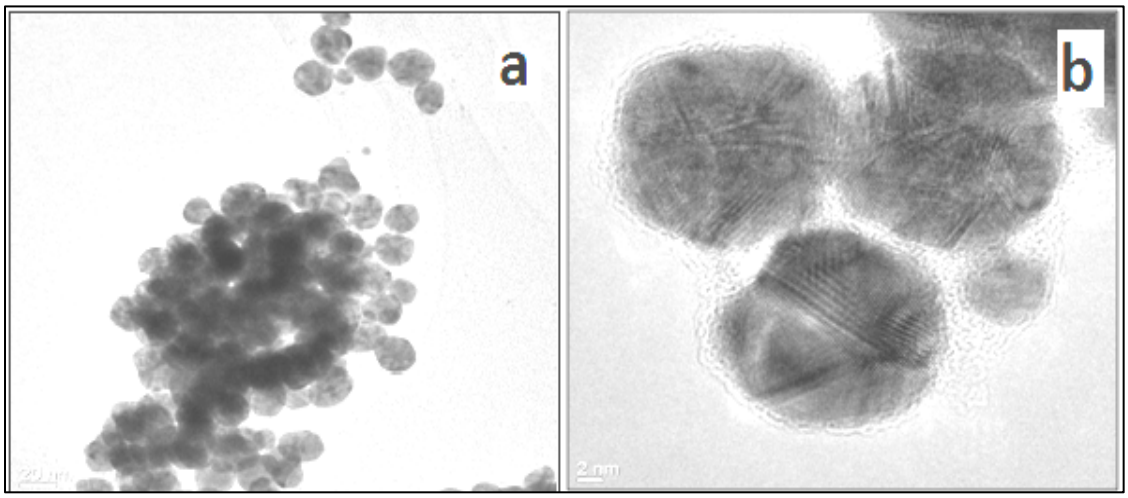

Figure- 3: HRTEM showing the impact of temperature and pH on biosynthesis of gold nanoparticles using *A. vasica* (a) 30°C at pH 6 (b) 100°C at same pH

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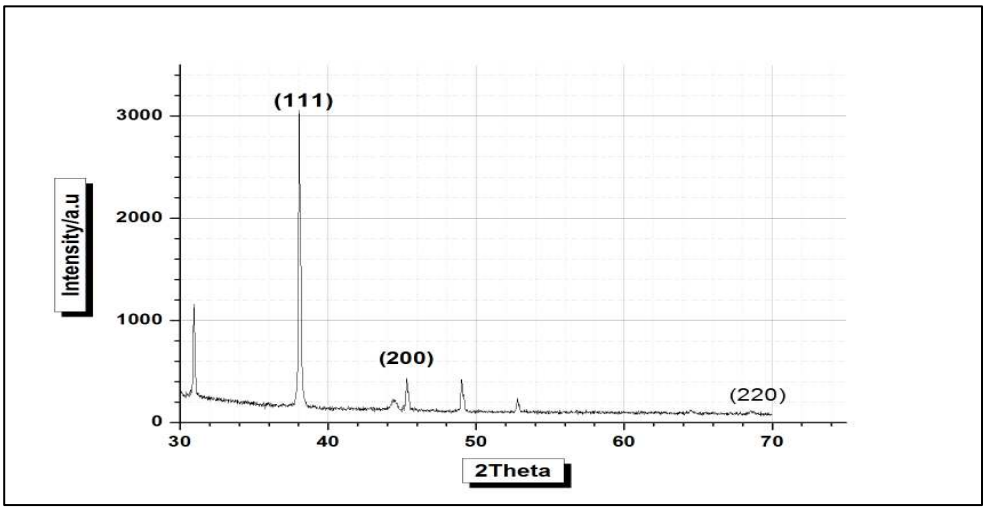

Figure- 4: XRD pattern of gold nano particles synthesized using leaf extract of *A. vasica*, showing typical Bragg reflections for gold nanoparticles

Table 3 – An assay of presence of Nitrate Reductase (in leaf extract of A. vasica) in reactants involved in synthesis of gold nano particles; showing total disappearance of NR activity when aurochlorate was added to the reactant mixture

NR activity in Plant extract µmole/min/gram of plant tissue	NR activity after boiling the plant extract µmole/min/gram of plant tissue	NR activity after biosynthesis of gold nanoparticle µmole/min/gram of plant tissue
0.8293	0.6679	Nil

X-ray diffraction studies: The confirmation of formation of elemental gold nanoparticles is provided by X-ray diffraction (XRD) analysis of the thin film prepared by coating the gold nanoparticle solution on Si (111) substrate. Typical Bragg reflection pattern is shown in fig. 4.21. The prominent peaks due to (111), (200), (220) and (311) Bragg reflection at 2 θ = 38°, 45°, 67° and 78° respectively, were the only characteristic observed corresponding to bulk fcc gold [16].

As it can be seen in the table – 3, there was a substantial decrease in the nitrate reductase activity in solutions (plant extracts) having gold nanoparticles as compared to nitrate reductase activity in plant extracts without gold nanoparticles, thus suggesting the involvement of NR in synthesizing gold nanoparticles.

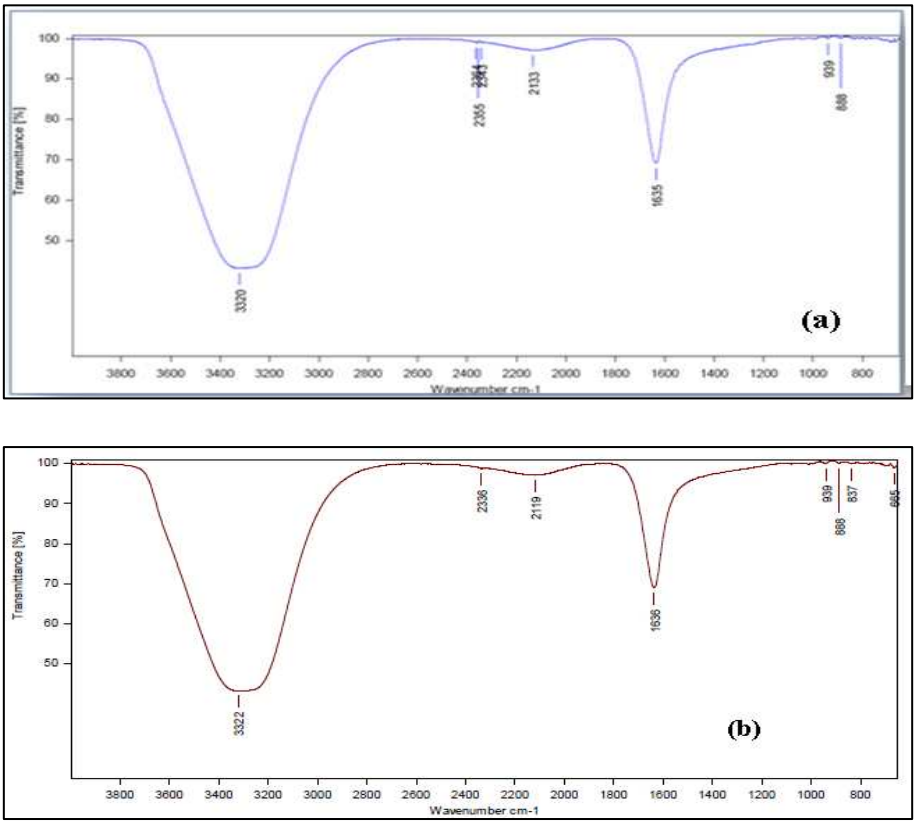

Fig. 5 - FTIR spectra of (a) Glutathione (b) AuNP-encapped with Glutathione

Fourier Transform Infra-Red Spectroscopy (FTIR): To decipher the molecular role of protecting agents which thermodynamically stabilize the gold nanoparticles in the solution FTIR was exploited to understand the functional groups associated with the surface of gold nanoparticles in the solution. The plant extract was ultra filtered in order to rule out the presence of cellular debris and other non specific proteins. According to the comparative FTIR spectra of capping proteins and AuNP- capping proteins conjugate (Fig 5a and b), the S=O stretching at 837 cm^{-1} and 665cm^{-1} indicates that gold nanoparticles has been thiolated by –SH group of amino acids present in capping proteins Glutathione. Moreover, both the spectra show N-H bending at 1636 cm^{-1}. Further, the bands at 939 and 888 correspond to the bending of -NH_2 of amino groups of Glutathione. The capping of AuNPs using thiol ligands desirably protects the surface, decreasing the entropy due to the partial liberation of the solvation shell and exchange reactions happening due to the displacement of carboxylic acid with the thiol group. This exchange reaction is relatively lucid procedure in which we presume that, it proceeds to completion. Owing to the strength of gold-thiol, it is being considered that exchange of thiolated ligands on AuNPs with other thiolated ligands is a more reluctant reaction, but has been widely exploited biologically. As per the previous literatures, the most common proteins associated with gold nanoparticles as capping agents were revealed to be glutathione. Our findings are in partial agreement with presence of glutathione as efficient capping agent. (Fig 5).

The possible mode of interaction between glutathione and gold nano particle is schematically presented in fig-6.

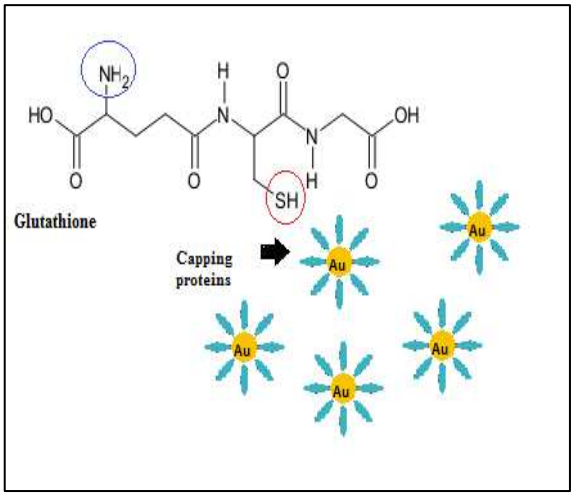

Figure 6: Schematic representation of possible interaction between Glutathione and Aurochlorate. The circle marked in blue and red are the sites where these interactions are studied.

CONCLUSION

Adathoda vasica leaf extract has shown the capability of biosynthesizing gold nano particles from gold salt solution. The optimum conditions for stable gold nanoparticles biosynthesis using *Adathoda vasica* leaf extract, was observed when inherent pH (8.2), 100^{o}C and 100 ppm Aurochlorate was used for synthesis.

Presence of Glutathione in leaf extract of *Adathoda vasica* support the view that they are involved in reducing the gold ions to gold nano particles. It presents a controllable method of tuning the synthesis of desired size and shape of gold nanoparticle. It can be concluded that plants are efficient chiselers for biosynthesis of gold nano particles.

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