In vitro hepatoprotective activity of *Azima tetracantha* leaf extract and silver nanoparticle in hepatocytes

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ABSTRACT

Hepatocytes have become valuable tools to evaluate the possible protective effect of drugs in the recent past. The techniques for high yield hepatocytes are made it as useful model. The study was aimed to synthesize the nanoparticles and evaluate the In vitro hepatoprotective activity of Azima tetracantha leaf extract and Silver nanoparticle (100, 200 and 300μg/ml) through CCl$_4$ induced toxicity in hepatocytes. Silver nanoparticles were effectively synthesized from aqueous leaf extract of Azima tetracantha under pH and temperature-dependent condition. 1mM Silver nitrate. All the variables tested as Protein, ALP, GOT and GPT recorded a significant Alteration observed in CCl$_4$ exposed rats. However treatment with Silver nanoparticle restored the level to near normal was observed than Azima tetracantha leaf treated groups. Nanoparticles were characterized using UV–Vis absorption spectroscopy and SEM analysis showed the average particle size of 100nm as well as revealed their cubic structure. The potential hepatoprotective activity of Silver nanoparticleis due to radical scavenging property of Silver ion.

Keywords: Hepatocytes, Silver nanoparticles, *Azima tetracantha, In vitro* hepatoprotective
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INTRODUCTION

Nanomaterials are part of a commercial revolution that has resulted in an explosion of hundreds of new products due to their diverse physico-chemical properties, enabling their usage in a wide range of innovative applications [1]. To avoid the use of toxic organic solvents and severe reaction conditions (temperature, pressure, and long refluxing time) for the preparation of nanomaterials, researchers recently have been exploring the possibilities of preparing nanomaterials in aqueous medium with the help of stabilizing or capping agents [2]. Nanoparticles can be synthesized using various approaches including chemical, physical, and biological methods. Although chemical method of synthesis requires short period of time for synthesis of large quantity of nanoparticles, this method requires capping agents for size stabilization of the nanoparticles. Chemicals used for nanoparticles synthesis and stabilization are toxic and lead to non-ecofriendly byproducts. The need for environmental non-toxic synthetic protocols for nanoparticles synthesis leads to the developing interest in biological approaches which are free from the use of toxic chemicals as byproducts. Thus, there is an increasing demand for ''green nanotechnology'' [3].

Silver nanoparticles are being extensively synthesized using many different biological sources including fungi, bacteria and plants [4]. Among them the plant mediated nanoparticles synthesis is getting more popular because of

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the high reactivity of plant extract and easy availability of plant materials. This method of nanoparticles synthesis involves no toxic chemicals and termed as green chemistry procedure. The main phytochemicals responsible for the synthesis of nanoparticles are terpenoids, flavones, ketones, aldehydes amides etc. In continuation of the efforts for synthesizing Silver   nanoparticle, here we report a facile, green and one pot synthesis using the leaf extract of *Azima tetracantha .* The biosynthetic route for nanoparticles has not yet been extended for the synthesis of Silver nanoparticles and its evaluation of *in vitro* hepaoprotective activity.

MATERIALS AND METHODS

SYNTHESIS OF AG NANOPARTICLES USING AZIMA TETRACANTHA EXTRACT
For the Ag nanoparticles synthesis, 5 ml of *Azima tetracantha* extract was added to 45 ml of 1 mM aqueous $AgNO_3$ solution in a 250 ml Erlenmeyer flask. The flask was then incubated in the dark at 4hrs (to minimize the photo activation of silver nitrate), at room temperature. A control setup was also maintained without *Azima tetracantha* extract. The Ag nanoparticle solution thus obtained was purified by repeated centrifugation at 8,000 rpm for 15 min followed by re-dispersion of the pellet in de-ionized water. Then the Ag nanoparticles were freeze dried using SEM analysis [5].

CHARACTERISATION OF SILVER NANOPARTICLES
Silver nanoparticles are characrterized by UV-Vis schimadzu 1600 spectrophotometer. The bioreduction is monitored in the UV absorption spectrometer from 300 to 700 nm range. Then the solution was centrifuged at 18,000 rpm for 30 min at room temperature to precipitate the nanoparticles . The resulting pellet is dissolved in deionized water and filtered through whatman filter paper No: 42. An aliquot of this filtrate containing silver nanoparticles are used for Fourier transmission Infrared spectroscopy (FTIR).

SEM ANALYSIS OF SILVER NANOPARTICLES
Scanning electron microscopic (SEM) analysis was done using VEGA3 TESCAN machine. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid. Extra solution was removed using a blotting paper and then the films on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

IN VITRO HEPATOPROTECTIVE ACTIVITY
The *in vitro* hepatoprotective activity was carried out by the method of [6]. The liver was excised and weighed in a tared beaker of cold calcium-free Locke's solution. Sufficient solution was removed to give a ratio of 1 g of liver to 10 ml of final suspension. The liver and solution were then transferred to a homogenizer tube, and the liver broken up by pressing down with a loose-fitting lucite pestle. This was followed by twenty even up and down strokes by hand. Shreds of connective tissue containing many cells remained after this treatment, but they were readily removed by straining through bolting silk. Experience has shown that further homogenization to release more whole cells. The isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10% newborn calf serum, antibiotics, dexamethasone and bovine insulin. The cell suspension was incubated at 37 ^{o}C for 30 min in a humidified incubator under 5% CO_2. After incubation of 24 hrs, the hepatocytes were exposed to the fresh medium containing CCl_4 (1%) along with different Concentrations of *Azima tetracantha* leaf extract and AgNPs (100, 200 and 300μg/ml). Group I serves as normal, group II served as control as CCl_4 treated, group III to V served as different concentrations of *Azima tetracantha* leaf extract and AgNPs (100, 200 and 300μg/ml). After 60 minutes of CCl_4 treatment, the liver markers were determined.

BIOCHEMICAL PARAMETERS
The glutamate oxaloacetae transaminase (GOT) was estimated by the method of [7]. The glutamate pyruvate transaminase (GPT) was estimated by the method of [7]. The serum alkaline phosphatase (ALP) activity was estimated by the method of [8]. Protein was estimated by the method of [9].

RESULTS AND DISCUSSION

Table 1 shows the phytochemical constituents of the aqueous extracts of *Azima tetracantha* medicinal plants. Our results revealed that the presence of the active phytochemical constituents like Flavonoids, Steroids, Terpenoids, Polyphenol, Saponin, Carbohydrate, Amino acids, Anthroquinone, & Glycoside. Moreover, in current research it

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was reported that the flavonoids and terpenoids present in these plant leaf extracts are the surface active molecules stabilizing the nanoparticles [10]. The time of addition of extract into 1mM $AgNO_3$ solution was considered as the starting point of the reaction. It is well known that silver nanoparticles exhibit a yellowish-brown colour in solution due to excitation of Surface Plasmon Resonance (SPR) vibrations which in turn is due to the presence of free electrons [11]

Table 1. Qualitative Phytochemical screening of *Azima tetracantha*

Test	Result
Tannin	-
Phlobatannins	-
Saponin	+
Flavonoids	+
Steroids	++
Terpenoids	+
Triterpenoids	-
Alkaloids	-
Carbohydrate	+
Amino acid	++
Anthroquinone	+
Polyphenol	++
Glycoside	++

(+) Presence, (++) highly Presence;   (-) Absence

SYNTHESIS OF SILVER NANOPARTICLES
The green synthesis of silver nanoparticles through plant extracts were carried out. Silver nitrate is used as reducing agent as silver has distinctive properties such as good conductivity, catalytic and chemical stability. Applications of such eco-friendly nanoparticles in bactericidal, wound healing and other medical and electronic applications, makes this method potentially exciting for the large-scale synthesis of other inorganic materials (nanomaterials). The aqueous silver ions when exposed to herbal extracts were reduced in solution, there by leading to the formation of silver hydrosol. The time duration of change in colour varies from plant to plant. The phytochemicals present in the *Azima tetracantha* extract were considered responsible for the reduction of silver ions. It is well known that silver nanoparticles exhibit yellowish - brown colour in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles The appearances of brown colour (**Figure** 1) in the reaction vessels suggest the formation of silver nanoparticles (SNPs) [12].

The formation of colour depends on time duration . The *Azima tetracantha*   leaf was responsible for the reduction of Silver   ions. It is well known that Silver   nanoparticles exhibit brown colour in aqueous solution due to excitation of surface plasmon vibrations in Silver   nanoparticles. This colour formation supported by [13] studies. The appearances of brown colour in the reaction vessels suggest the formation of Silver   nanoparticles (AgNPs).
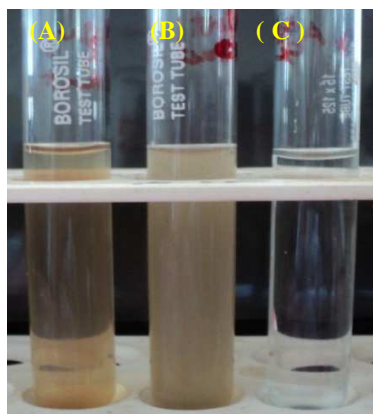

Figure 1: (A) AgNPs, (B) *Azima tetracantha* leaf extract and (C) $AgNO_3$

It is generally recognized that UV–Vis Spectroscopy could be used to examine size- and shape-controlled nanoparticles in aqueous suspensions. **Figure** 2 shows the UV-Vis spectra recorded from the reaction medium after

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5 hours. Absorption spectra of silver nanoparticles formed in the reaction media has absorbance peak at 436 nm, broadening of peak indicated that the particles are polydispersed.
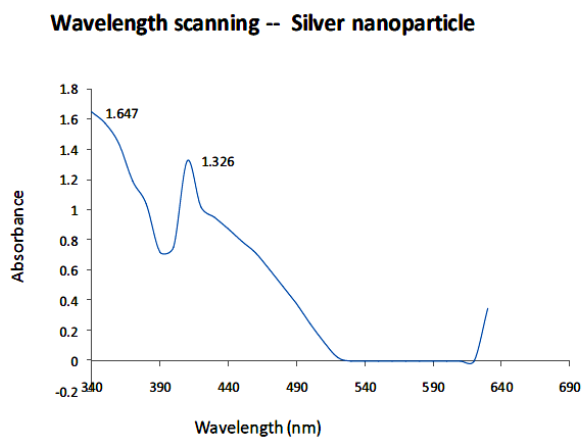

Figure 2: UV-Vis absorption spectrum of Silver nanoparticles after 5 hrs

FTIR is an important tool which enables us to understand the involvement of functional groups in the interactions between metal particles and biomolecules. In the present work, FTIR spectra are used in the identification of biomolecules responsible for capping and stabilizing the silver nanoparticles. FTIR spectrum of *Azima tetracantha* extract shows bands at 696, 1124, 1401, 1566, 1637, 2086 and 3410. The FTIR spectra of the *Azima tetracantha* is given in the **Figure**.3 which show the presence of silver nanoparticles, peak at 3410cm-1 which are assigned as –OH stretching in alcohols and phenolic compounds [14].The band appeared at about 1637 cm-1can be assigned for aromatic rings. The strong broad band appearing at 3410 cm-1can be associated to the stretching vibrations of alcoholic and phenolic O–H. At 1124cm-1a peak is observed that could be for plant ascribed to multiplet C=O group.
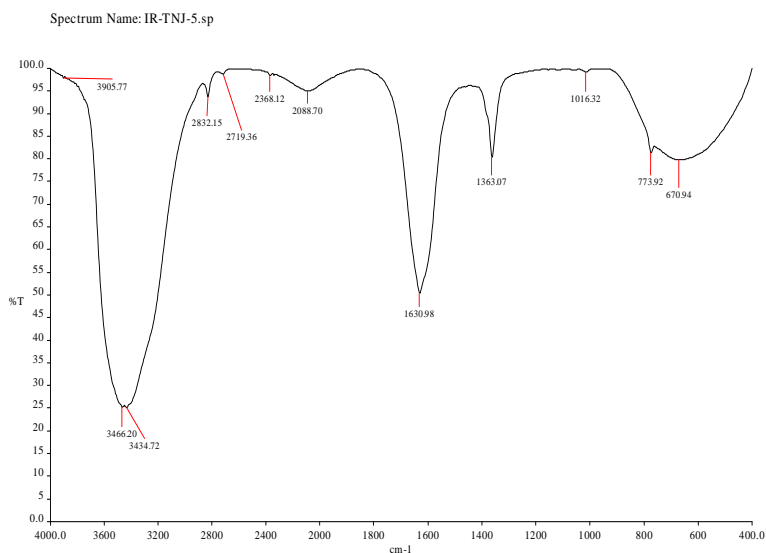

Figure 3: FTIR analysis of silver nanoparticles synthesized by treating 1mM aqueous AgNO3 solution with *Azima tetracantha* extract

Silver nanoparticles are being extensively synthesized using many different biological sources including fungi, bacteria and plants [4]. Among them the plant mediated nanoparticles synthesis is getting more popular because of the high reactivity of plant extract and easy availability of plant materials. This method of nanoparticles synthesis

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involves no toxic chemicals and termed as green chemistry procedure. In this present study, *Azima tetracantha* extract was used for the synthesis of silver nanoparticles. The aqueous $AgNO_3$ solution turned to brown colour in 30 min with the addition of *Azima tetracantha* extract, indicating the formation of AgNPs in the reaction solution probably as a result of the excitation of surface plasmon resonance (SPR) bands [15]. The control tubes (AgNO3) showed no change in colour when incubated in a similar condition.

SEM analysis was carried out to understand the topology and the size of the Ag-NPs, which showed the synthesis of higher density polydispersed spherical Ag-NPs of various sizes. The SEM image showing the high density silver nanoparticles synthesized by the *Azima tetracantha* extract further confirmed the development of silver nanostructures. Most of the nanoparticles aggregated and only a few of them were scattered, as observed under SEM. The SEM analysis showed the particle size between 10-60nm as well the cubic, face-centred cubic structure of the nanoparticles (**Figure** 4).
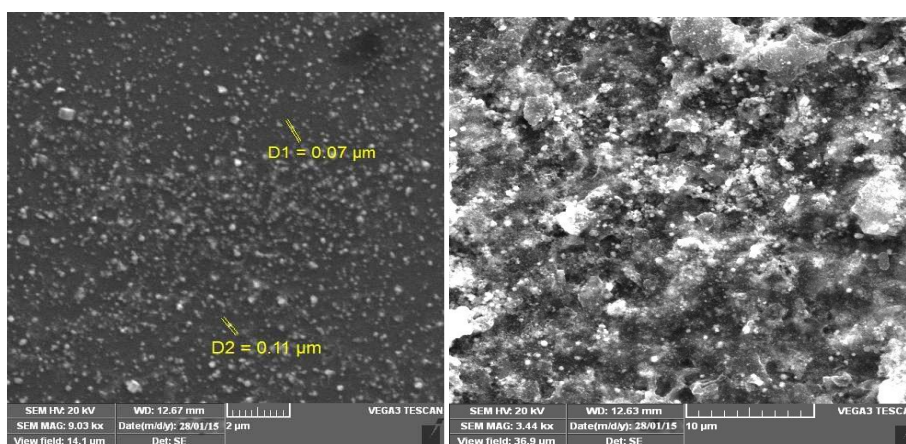

Figure 4: High resolution scanning electron microscopic (SEM) image of silver nanoparticles (AgNPs). Polydispersed (Cluster) AgNPs ranged between 70–110 nm

IN VITRO HEPATOPROTECTIVE ACTIVITY AZIMA TETRACANTHA AND AGNPS ON CCL₄ INDUCED OXIDATIVE STRESS IN HEPATOCYTES

The present study was carried out to evaluate the *In vitro* hepatoprotective activity of *Azima tetracantha* and AgNPs on CCl_4 induced hepatotoxicity. The observations made on different groups of experimental and control animals were compared as follows.

Table 2 represents the % of viability of control and experimental hepatocytes. Group II CCl_4 induced oxidative stress showed a significant decreased in the % of cell viability when compared to Group I. Group III, IV and V CCl_4 induced hepatotoxicity group treated with *Azima tetracantha* and AgNPs (100, 200 and 300μg/ml) significantly increased in % of cell viability when compared to group I. The cell viability is concentration dependent. The % of viability increased with increased concentration. AgNPs treated group increased the cell viability than *Azima tetracantha* extract treated group.

Table 2 shows the % of viability

Treatment groups	% of viability	
	Azima tetracantha	AgNPs
Group I Normal	100	100
Group II Control	37.43	43.21
Group III 100μg /ml	64.87*	78.45*
Group IV 200μg /ml	73.56*	87.43*
Group V 300μg /ml	86.57*	93.35*

Values were expressed as mean ± SD for triplicate in each group.
*Significantly different from Group II (p < 0.05)

Table 3 and Fig 5 represents the GPT activity of control and experimental hepatocytes. Group II CCl_4 induced oxidative stress showed a significant increased in the activity of GPT when compared to Group I. Group III, IV and V CCl_4 induced hepatotoxicity group treated with *Azima tetracantha* and AgNPs (100, 200 and 300μg/ml) significantly restored in the activity of GPT when compared to group I. The activity of GPT is concentration dependent. The activity of GPT increased with increased concentration. AgNPs treated group decreased GPT activity than *Azima tetracantha* extract treated group.

Table 3 Effect of *Azima tetracantha* and AgNPs on GPT (IU/L) activity

S.NO	Concentrations	*Azima Tetracantha*	AgNPs
1.	Group I Normal	27.97 ± 1.95	27.97 ± 1.95
2.	Group II Control	81.12 ± 5.67	81.12 ± 5.67
3.	Group III 100μg /ml	32.67 ± 2.28	28.99 ± 2.02
4.	Group IV 200μg /ml	31.68 ± 2.21	29.95 ± 2.09
5.	Group V 300μg /ml	30.12 ± 2.10	28.18 ± 1.97

Values are expressed as Mean ±SD for triplicate
*Significantly different from Group II (p < 0.05)
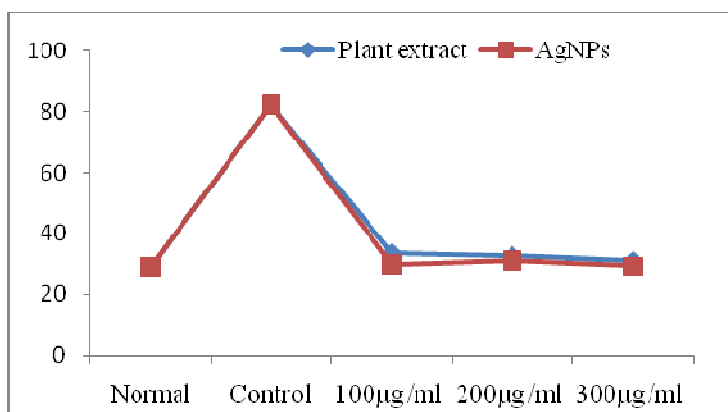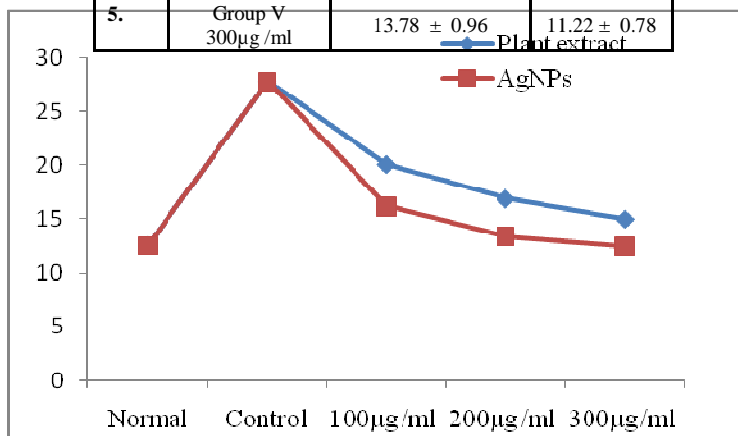*AgNPs = Silver Nanoparticles*

Figure 5: GPT activity of *Azima tetracantha* extract and AgNPs at different concentrations

Table 4 Effect of *Azima tetracantha* and AgNPs on GOT (IU/L) activity

S.NO	Concentrations	*Azima Tetracantha*	AgNPs
1.	Group I Normal	11.33 ± 0.79	11.33 ± 0.79
2.	Group II Control	26.63 ± 1.86	26.63 ± 1.86
3.	Group III 100μg /ml	18.89 ± 1.32	15.18 ± 1.06
4.	Group IV 200μg /ml	15.69 ± 1.09	12.15 ± 0.85
5.	Group V 300μg /ml	13.78 ± 0.96	11.22 ± 0.78

Values are expressed as ... *Mean ±SD for triplicate*
*Significantly different ... *from Group II (p < 0.05)*
AgNPs = Silver ... *Nanoparticles*

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Figure 6: GOT activity of *Azima tetracantha* extract and AgNPs at different concentrations

Table 4 and Fig 6 represent the GOT activity of control and experimental hepatocytes. Group II CCl$_4$ induced oxidative stress showed a significant increased in the activity of GOT when compared to Group I. Group III, IV and V CCl$_4$ induced hepatotoxicity group treated with *Azima tetracantha* and AgNPs (100, 200 and 300μg/ml) significantly restored in the activity of GOT when compared to group I. The activity of GOT is concentration dependent. The activity of GOT increased with increased concentration. AgNPs treated group decreased GOT activity than *Azima tetracantha* extract treated group.

Table 5 Effect of *Azima tetracantha* and AgNPs on Protein content (g/dl)

S.NO	Concentrations	*Azima Tetracantha*	AgNPs
1.	Group I Normal	7.31 ± 0.51	7.31 ± 0.51
2.	Group II Control	4.85 ± 0.33	4.85 ± 0.33
3.	Group III 100μg /ml	5.46 ± 0.38	6.38 ± 0.44
4.	Group IV 200μg /ml	6.38 ± 0.44	6.96 ± 0.48
5.	Group V 300μg /ml	6.81 ± 0.47	7.57 ± 0.52

Values are expressed as Mean ±SD for triplicate
*Significantly different from Group II (p < 0.05)
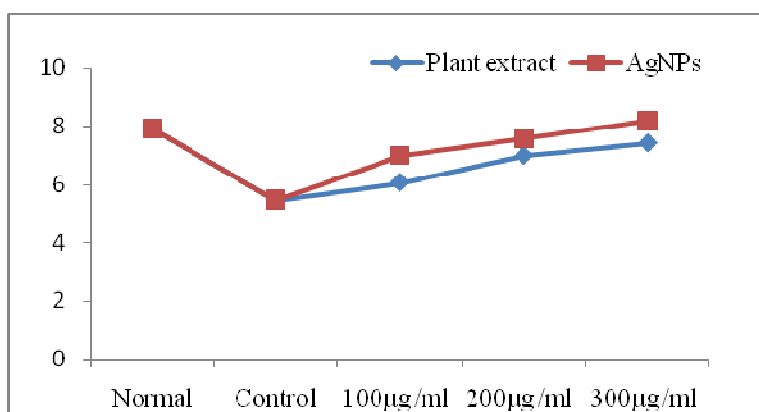*AgNPs = Silver Nanoparticles*

Figure 7: Protein content of *Azima tetracantha* extract and AgNPs at different concentrations

Table 5 and Fig 7 represents the protein content of control and experimental hepatocytes. Group II CCl$_4$ induced oxidative stress showed a significant decreased in protein content when compared to Group I. Group III, IV and V

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CCl_4 induced hepatotoxicity group treated with *Azima tetracantha* and AgNPs (100, 200 and 300μg/ml) significantly restored in protein content when compared to group I. The activity of ALP is concentration dependent. The activity of ALP increased with increased concentration. AgNPs treated group increased protein content than *Azima tetracantha* extract treated group.

Table 6 and Fig 8 represents the ALP activity of control and experimental hepatocytes. Group II CCl_4 induced oxidative stress showed a significant increased in the activity of ALP when compared to Group I. Group III, IV and V CCl_4 induced hepatotoxicity group treated with *Azima tetracantha* and AgNPs (100, 200 and 300μg/ml) significantly restored in the activity of ALP when compared to group I. The activity of ALP is concentration dependent. The activity of ALP increased with increased concentration. AgNPs treated group decreased ALP activity than *Azima tetracantha* extract treated group.

Table 6 Effect of *Azima tetracantha* and AgNPs on ALP (IU/L) activity

S.NO	Concentrations	*Azima Tetracantha*	AgNPs
1.	Group I Normal	14.21 ± 1.07	14.21 ± 1.07
2.	Group II Control	24.21 ± 1.69	24.21 ± 1.69
3.	Group III 100μg /ml	20.55 ± 1.43	16.5 ± 1.15
4.	Group IV 200μg /ml	18.38 ± 1.28	14.55 ± 1.01
5.	Group V 300μg /ml	15.21 ± 1.06	12.5 ± 0.75

Values are expressed as Mean ±SD for triplicate
Significantly different from Group II (p < 0.05)
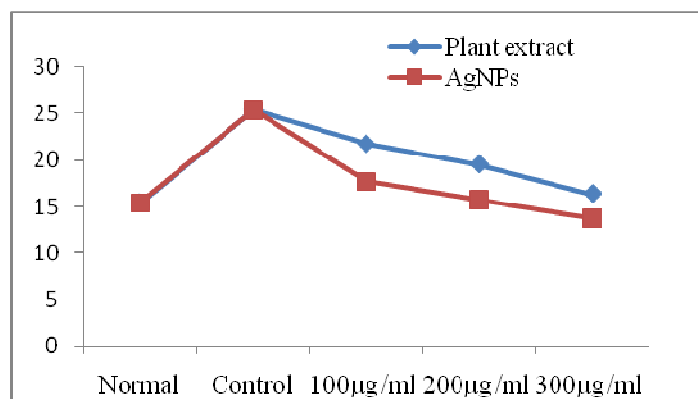*AgNPs = Silver Nanoparticles*

Figure 8 ALP activity of *Azima tetracantha* extract and AgNPs at different concentrations

The present study was performed to assess the hepatoprotective activity in hepatocytes against carbon tetrachloride induced acute hepatic injury. CCl_4 induced hepatic injury is commonly used as an experimental method for the study of hepatoprotective effect of drugs. The hepatotoxicity induced by CCl_4 is due to its metabolite $CCl_3^{\bullet}$, a free radical that binds to lipoprotein and leads to peroxidation of lipids of the endoplasmic reticulum [16]. The ability of a hepatoprotective drug to reduce the injurious effects, or to preserve the normal hepatic physiological mechanisms which have been disturbed by a hepatotoxin, is an index of its protective effects. Although transaminase enzymes levels are not a direct measure of hepatic injury, they show the status of the liver. The lowering of marker liver enzymes is a definite indication of hepatoprotective action of the drug. The transaminase enzymes GOT and GPT levels are reliable markers of liver function [17].

Hepatocytes have become valuable tools to evaluate the possible protective effect of drugs in the recent past. The techniques for high yield hepatocytes are made it as useful model [6]. In the assessment of liver damage by carbon tetrachloride, the determination of enzyme activities such as aspartate aminotransferase (GOT) and alanine aminotransferase (GPT) is largely used. Activities of GOT, GPT, Protein and alkaline phosphatase (ALP) are the

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most frequently utilized indicators of hepatocellular injury. Necrosis or membrane damage releases the enzymes into circulation; and therefore, they can be measured in hepatocyte. GPT is more specific to the liver, and is thus a better parameter for detecting liver injury. Elevated levels of hepatocyte enzymes are indicative of cellular leakage and loss of functional integrity of the cell membrane in the liver [18]. The mechanism by which alkaline phosphatase reaches the circulation is uncertain; leakage from the bile canaliculi into hepatic sinusoids may result from leaky tight junctions and the other hypothesis is that the damaged liver fails to excrete alkaline phosphatase made in the bone, intestine and the liver [19]. Total protein level, on other hand, are related to the function of hepatic cells i.e they reveal the functional status of the hepatic cells. Decreased levels of total protein are indicative of the failure of the biosynthetic function of the hepatocyte [20].

In the present study, the CCl_4 treated hepatocyte showed a significant elevation (Table- 2, 3, and 5) in the activities of GPT, GOT and alkaline phosphatase activity, while significantly decreasing the levels of total protein (Table – 4) as compared to the control hepatocyte, thereby indicating oxidative damage. Co-treatment with *Azima tetracantha* and AgNPs at doses of 100, 200 and 300μg/ml, significantly prevented the rise in the levels of the marker enzymes and as well as it significantly prevented the decrease in the total protein. The diminished rise of hepatocyte enzymes, together with the diminished fall in the levels of total protein in the *Azima tetracantha*   and AgNPs (Table-3) treated groups, is a clear manifestation of the hepatoprotective effect of the *Azima   tetracantha* . The results of the present study agreement with [6, 21] studies. Among the *Azima tetracantha*   leaf extract and AgNPs treatments, AgNPs possess significant hepatoprotective activity than leaf extract.

The present study concluded that the bio-reduction of Silver ions through. *Azima   tetracantha*   leaf extract and testing for their hepatoprotective activity. The aqueous Silver   ions exposed to the extract, the synthesis of Silver nanoparticles were confirmed by the of formation of brown colour. These environmentally benign Silver nanoparticles were further confirmed by using SEM. The SEM analysis showed the particle size around 100nm as well the polydispersed spherical nanoparticles. The result obtained from this study supports hepatoprotective activity of Silver nanoparticle in hepatocytes.

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