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Pharmacological Properties of *Cissus quadrangularis* Loaded Silver Nanoparticles: An *In-vitro* Study

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Abstract

Plant extracts traditionally used in ancient medical systems such as Ayurveda and Unani for thousands of years is now being revisited with interests turning towards utilizing them as biologically synthesized nanoparticles with better delivery. *Cissus quadrangularis* Linn. is one such important medicinal plant belonging to the family Vitaceae. In India, *C. quadrangularis* stems are widely used as medicine as well as in traditional cuisine owing to the presence of several bioactive compounds in it. In the present study, parts of *C. quadrangularis* plant were collected and ethanolic (Soxhlet and cold centrifuge) extracts were made from fresh and dried samples. Green synthesis of silver nanoparticles with extracted *C. quadrangularis* was carried out and its efficacy was checked. Phytochemical properties of *C. quadrangularis* extract was evaluated and various pharmacological properties such as anti-microbial, anti-oxidant, anti-inflammatory and anti-cancerous properties of both *C. quadrangularis* extracts and *C. quadrangularis* loaded sliver nanoparticles were assessed and compared. Overall, the green synthesized *C. quadrangularis* loaded silver nanoparticles had positive pharmacological properties, which were demonstrated *in-vitro* through this study.

Keywords: *Cissus quandrangularis*; Silver nanoparticles; Green synthesis

Introduction

Cissus quadrangularis Linn. is a perennial plant belonging to Vitaceae (grape) family. It is commonly known as Veldt-grape, Devil's backbone and adamant creeper, and in Indian regional languages known as Asthisamhari (Sanskrit), Hadjod (Hindi) and Pirandai (Tamil). *C. quadrangularis* is a tendril climbing shrub with stout, fleshy quadrangular stems that is found throughout warmer regions of India and Ceylon and other south east Asian nations [1,2]. The roots and stems are traditionally used for healing of fracture of the bones, hence the name Hadjod. The stem is given internally and also applied topically over broken bones and used in complaints related to the spine and back [3]. The plant has been well documented in Ayurveda for treatment of osteoarthritis, rheumatoid arthritis, osteoporosis as well as gout, syphilis, venereal diseases, piles, leucorrhoea [4,5].

Phytochemical studies of *C. quadrangularis* using different solvent extracts has demonstrated the presence of high amount of ascorbic acid, carotene, phytosterol substances, fatty acid, fatty acid ester, alcoholic compounds and hydrocarbons, and existence of n-hexadecanoic acid, ethan-1,1-diethoxy, 9,12,15-octadecatrienoic acid-methyl ester and calcium, making it ideal for many pharmacological applications [6-9].

Nanotechnology as an interdisciplinary tool has gained its popularity in recent times in medical fields. Nano-materials are prepared between the ranges of 1-100 nm. These nano-materials or nanoparticles demonstrate unique properties with change in the surface area. The production of the nanoparticles can be done by both physical and chemical methods. Green synthesis of nanoparticles from various plant sources and micro-organisms are carried out in large numbers which are not harmful for the environment and are also cost effective in nature[10,11]. The present study aims to synthesise *Cissus quadrangularis* loaded silver nanoparticles and assess *in-vitro* its various pharmacological applications.

Materials and Methods

Plant collection and processing

Cissus quadrangularis Linn. is distributed widely in south India owing to climatic conditions suitable for its growth. Plant specimen was collected from Chennai City, and was authenticated in the Plant Biology and Plant Biotechnology Department, Women's Christian College, Chennai. The stem parts collected were divided them into two batches: One batch was used immediately for fresh extraction and another was left under shade for drying for two weeks, until completely dried & then finely powdered. Both the fresh and dried samples were extracted using ethanol (analytical grade) as solvent by Soxhlet method and cold centrifugation.

10.0 g of each of the samples (fresh and dried) were ground with 25 mL of ethanol in a table-top blender. The pulp from each of the sample was then filtered and the residue and the filtrate obtained were separated into two equal portions. The residue was air-dried to remove alcohol. One portion of the air-dried residue was placed in the extraction thimble; one portion of the filtrate was placed in the extraction flask, which was made up to 250 mL with ethanol. The extraction process was carried out for 5 hours using Soxhlet apparatus.

For cold extraction, both the fresh and dried samples (1 g each) were soaked in 25 mL of ethanol for 48 hours, with periodical shaking. Cold centrifugation was then carried out, at 5000 rpm for 20 min and the supernatant obtained was collected.

Both the extracts were dried on a Laboratory Rocker to concentrate the samples. The residual powder obtained was weighed and re-dissolved in ethanol to get a final concentration 1 mg/mL, and stored in airtight containers under refrigerated condition for further analysis.

Phytochemical analysis

The extracts were subjected to qualitative analysis to check for the presence of Alkaloids, Carbohydrates, Cardiac glycosides, Flavonoids,

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Phenols, Saponins, Tannins, Terpenoids, Quinones, Coumarins and Proteins using standard protocol described by Prabhavathi et al. [12].

Biosynthesis of *C. quadrangularis* loaded silver nanoparticles

10 mL of *C. quadrangularis* extract was mixed with 90 mL of 1 mm aqueous silver nitrate solution for reduction into Ag^+ ions and kept in magnetic stirrer for 1 hour at room temperature and further in dark until colour change was observed. Formation of *C. quadrangularis* loaded silver nanoparticles was noted in 25 min by colour change in the solution from pale green to honey brown indicating the reduction reaction which was further confirmed by UV-visible spectroscopy and Scanning Electron Microscopy.

In-vitro pharmacological assays

The following assays were carried out to check the pharmacological application of the biosynthesized *C. quadrangularis* loaded silver nanoparticles:

Antimicrobial assay: Antimicrobial assay of *C. quadrangularis* loaded silver nanoparticles was carried out using Well Diffusion method [13]. Mueller Hinton agar medium was used for bacterial strains and Potato dextrose agar medium was used for fungal strains. The media were poured into sterilized petri-plates. Wells of 8 mm diameter were made through the surface of sterile media using cork borer. Pure bacterial isolates of *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhi* and pure fungal isolates of *Candida albicans, Candida krusei, Candida tropicalis,* obtained from King's Institute, Chennai, India, were used for this study. Varying concentrations (40 µL, 60 µL, 80 µL, and 100 µL) of the *C. quadrangularis* loaded silver nanoparticle were placed in wells made and sterile distilled water was used as control. The plates were incubated at 37˚C for 24 hour. The inhibition diameters of the zones were measured, after incubation period.

Assessment of *in-vitro* anti-inflammatory activity:

Inhibition of albumin denaturation: The percentage inhibition of protein denaturation was calculated by method described by Sakat et al. [14] by measuring absorbance at 660 nm (Shimadzu, UV-1280), using formula given below:

$$\% \text{ INHIBITION} = (Abs_{Control} - Abs_{Sample}) * 100 / Abs_{Control}$$

Heat induced hemolysis: The anti-inflammatory activity was determined on the basis of membrane stability [14], which was measured at 560 nm (Shimadzu, UV-1280) and calculated as below:

$$\text{Percentage membrane stability activity} = (Abs_{Control} - Abs_{Sample}) * 100 / Abs_{Control}$$

Assessment of *in-vitro* anti-coagulant activity: Anticoagulant activity was determined through measurement of Prothrombin Time (PT) as described by Ramya et al. [15]; pure platelet plasma obtained from a healthy volunteer was divided into three groups:

Negative Control- (GROUP I) having 0.2 mL of plasma+0.1 mL of 0.9% saline+0.3 mL of 25 mL $CaCl_2$

Positive Control- (GROUP II) having 0.2 mL plasma+0.1 mL of 50 mg/g EDTA+0.3 mL $CaCl_2$

Test sample 1- (GROUP III) having 0.2 mL plasma+200 µL *C. quadrangularis* extract+0.3 mL $CaCl_2$

Test sample 2- (GROUP IV) having 0.2 mL plasma+200 µL *C. quadrangularis* loaded silver nanoparticle+0 .3 mL $CaCl_2$.

All four test tubes were incubated at room temperature and were tilted at an angle of 45˚C for every 30 seconds upto one hour to observe clotting and measure the clotting time, referred to as Prothrombin Time (PT).

Assessment of *in-vitro* antioxidant activity: Antioxidant property was evaluated through DPPH radical scavenging activity described by Brand-Williams et al. [16]. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) is a stable free radical with an odd electron, which is a violet solution in ethanol. In the presence of a free radical scavanging antioxidant, it is reduced leading to decolourization (yellow). The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants, spectrophotometrically. The ability of the *C. quadrangularis* loaded silver nanoparticles to scavenge the DPPH radical was calculated using the formula given below, inhibition curve versus concentration was plotted, and the concentration of each sample required to reduce DPPH radical by 50% was extrapolated.

$$\% \text{ INHIBITION} = (Abs_{Control} - Abs_{Sample}) * 100 / Abs_{Control}$$

Where, Ascorbic acid was used as control and absorbance readings were taken at 517 nm (Shimadzu, UV-1280).

Assessment of *In-vitro* cytotoxicity and *in-vitro* anti-cancer activities: MTT assay was used to determine both cytotoxicity and anti-cancer activities of *C. quadrangularis* extracts and *C. quadrangularis* loaded silver nanoparticles. Vero cell lines were used to determine the cytotoxic activity and HeLa cells and A549 Lung cancer cells were used to determine the anticancer activity of the plant extract and the biosynthesized nanoparticles. Previously grown and maintained cells were trypsinized and were seeded in 96-well plates, with 5×10^3 cells/100 µL and incubated for 24 hours at 37˚C. The cells were then treated with *C. quadrangularis* extracts and *C. quadrangularis* loaded silver nanoparticles and incubated for another 24 hours at 37˚C in a 5% CO_2 atmosphere.

The %cell viability of the three different cells was calculated using the formula mentioned below [17-19].

%Cell Viability =(A570 of treated cells/A570 of control cells)*100

Results and Discussion

Phytochemical analysis

Phytochemical analysis of ethanolic extracts from fresh and dried stems of *Cissus quadrangularis* demonstrated the presence of alkaloids, carbohydrates, phytosterols, phenols, tannins, terpenoids, flavonoids, saponins, cardiac glycosides, quinones, coumarins and proteins, consistent with the previous studies [6,7,12].

Characterization of *C. quadrangularis* loaded silver nanoparticles

The formation of *C. quadrangularis* loaded silver nanoparticles was noted through color change from pale green to honey brown color (Figure 1) in both the extracts within 25 min of keeping after stirring over a magnetic stirrer for one hour. This indicated the successful reduction of silver ions (Ag^+) to silver (Ag) by the phytochemicals present in the *C. quadrangularis* extract.

UV-visible spectroscopy: Formation of nanoparticles was monitored by measuring the UV-Visible spectrum of the reaction mixture and the absorbance was recorded at 200-800 nm using UV-Vis spectrophotometer (Shimadzu, UV-1280). The UV–Vis spectra of the reaction mixture of *Cissus quadrangularis* stem extracts, fresh and dried, with 1 mM silver nitrate solution showed clear absorption peak

Figure 1(a): Fresh and dry ethanolic extracts of *Cissus quadrangularis*.

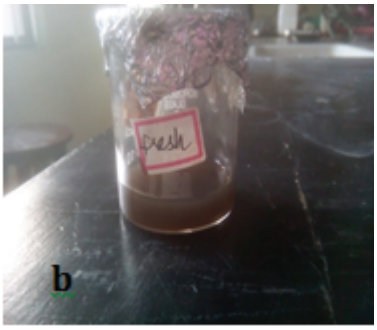

Figure 1(b): *C. quadrangularis* extract (fresh) in silver nitrate solution after 25 min of incubation showing honey brown coloration.

Figure 1(c): *C quadrangularis* extract (dry) in silver nitrate solution after 25 min of incubation showing honey brown coloration.

at 417-413 nm (Table 1) indicating the presence of silver nanoparticles synthesized by *C. quadrangularis* extracts; the characteristic peak formation at this wavelength is due to the effect of surface Plasmon resonance of electrons in the reaction mixture and poly-dispersal of the nanoparticles formed. The absence of such a peak in the extracts at same wavelength was also noted. The results obtained were consistent with previous study by Renugadevi et al. [20].

SEM analysis: Scanning Electron Microscopy (SEM) images provide information about the morphology and average size of the biosynthesized silver nanoparticles. The silver nanoparticles synthesized using *C. quadrangularis* extracts (Figure 2) were found to be spherical in shape and were distributed evenly. The diameter of synthesized nanoparticles was in the range 10-30 nm, with an average of 20 nm.

Anti-microbial activity: The *C. quadrangularis* loaded silver nanoparticles (*C. quadrangularis* SNP) showed good antibacterial activity against the tested organisms *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae* and *Salmonella typhi* (Figure 3 and Table 2). Biosynthesised nanoparticles showed better zones than the pure extracts. Dried *C. quadrangularis* extract was marginally better in zones of inhibition than the fresh *C. quadrangularis* extract against the organisms screened. Both the biosynthesised nanoparticles and the extracts did not show any appreciative zones against the three *Candida* spp. screened in this study.

In-vitro anti-inflammatory activity:

Inhibition of Albumin denaturation: The anti-inflammatory activity of the extracts and the *C. quadrangularis* loaded silver nanoparticles (*C. quadrangularis* SNP) was checked by their ability to resist heat induced protein (albumin) denaturation. Maximum inhibition of 61% was noted in 16 µL concentration of both fresh and dried extract of the plant, while a higher percentage of 78.46% was noted in 16 µL of *C. quadrangularis*

Compound	Wavelength (nm)	Absorbance
C. quadrangularis loaded silver nanoparticle (Fresh)	417.00	0.356
C. quadrangularis loaded silver nanoparticle (Dried)	413.00	0.989
Fresh stem extract	224.00	3.986
Dry stem extract	220.00	3.999

Table 1: Absorbance peak of *C. quadrangularis* SNP and *C. quadrangularis* extracts at different wavelengths.

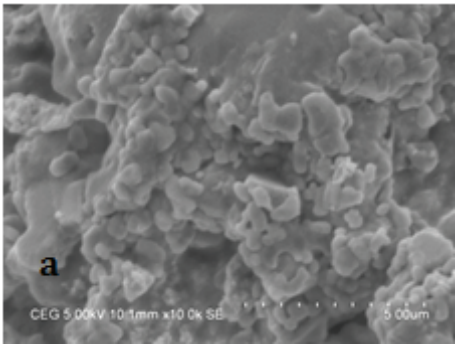

Figure 2(a): SEM image of *C. quadrangularis* loaded silver nanoparticle (Fresh).

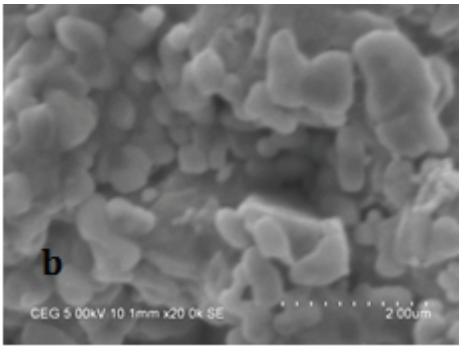

Figure 2(b): SEM image of *C. quadrangularis* loaded silver nanoparticle (Dried).

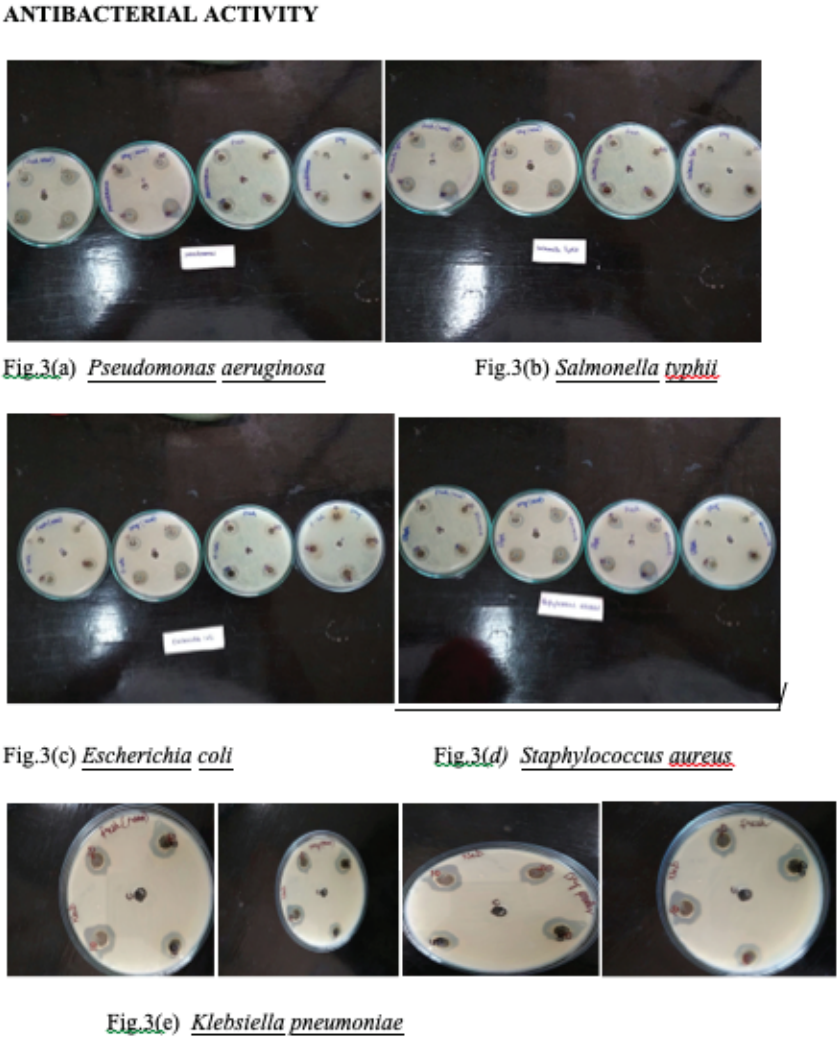

Figure 3: Antibacterial activity of *C. quadrangularis* loaded silver nanoparticles at varying concentrations (40 µL, 60 µL, 80 µL, 100 µL) against different organisms screened.

SNP, indicating that the anti-inflammatory effect was better when *C. quadrangularis* extracts were loaded in silver nanoparticles (Table 3).

***Membrane stabilization*:** *C. quadrangularis* stem extracts and *C. quadrangularis* SNP were effective in inhibiting heat induced hemolysis of RBC membrane at different concentrations, with the *C. quadrangularis* SNP showing better percentage of inhibition of 92.3% against 86% value of *C. quadrangularis* extracts at highest concentration tested. Aspirin was used as standard, which showed the maximum inhibition of 87% at a concentration of 250 µg/mL.

***In-vitro* anticoagulant activity:** The coagulation activity was prolonged by both *C. quadrangularis* stem extracts as well as *C. quadrangularis* SNP by indicating a prolonged Prothrombin Time (PT) of more than 10 min when compared to the control, indicating that *C. quadrangularis* extract had good anticoagulant activity. Phytochemical compounds present in the *C. quadrangularis* stem extracts can be attributed to the prolonged PT thereby demonstrating anticoagulant activity.

***In-vitro* antioxidant activity:** *In-vitro* antioxidant activity was assessed by DPPH scavenging assay method. The percentage inhibition obtained for *C. quadrangularis* extracts and *C. quadrangularis* SNP have been presented in (Table 4). *C. quadrangularis* SNP showed better antioxidant potential at various concentrations. From the graph (Figure 4) it was evident that the IC_{50} value of *C. quadrangularis* SNP was at a lower concentration of 9.26 µL than *C. quadrangularis* extracts (21.74 µL), indicating that *C. quadrangularis* has better antioxidant properties at lower concentration when loaded in silver nanoparticles.

***In-vitro* cytotoxicity and anti-cancer activity:** MTT assay, a colorimetric assay for measuring the activity of cellular enzymes that reduce the yellow tetrazolium dye MTT to its insoluble purple colored formazan crystals, was used to assess the cell viability. Cells from Vero cell line (normal cells) were used for cytotoxicity assessment while HeLa cells (cervical cancer cell line) and A549 cells (Lung cancer cell line) were used to assess the anti-cancer activity of *C. quadrangularis* extract and *C. quadrangularis* SNP. To evaluate the inhibition of cell viability of the two cancer cells and compare it with cell viability of normal cells, the cells were treated with different doses of *C. quadrangularis* extracts and *C. quadrangularis* SNP and viability was assessed post-incubation, colorimetrically. Both the samples were least cytotoxic to normal cells (Vero) and displayed anti-cancer properties with decreasing viability in increasing concentrations. *C. quadrangularis* SNP was better in its

ORGANISMS	SAMPLES IN DIFFERENT CONCENTRATIONS			
	10 µL	20 µL	40 µL	80 µL
E. coli Fresh *C. quadrangularis*-SNP	12 mm	14 mm	15 mm	15 mm
Dried *C. quadrangularis*-SNP	14 mm	16 mm	16 mm	18 mm
Fresh *C. quadrangularis* Extract	10 mm	10 mm	12 mm	14 mm
Dried *C. quadrangularis* Extract	10 mm	10 mm	12 mm	14 mm
Staphylococcus aureus Fresh *C. quadrangularis* -SNP	10 mm	12 mm	14 mm	16 mm
Dried *C. quadrangularis* - SNP	12 mm	14 mm	16 mm	18 mm
Fresh *C. quadrangularis* Extract	10 mm	12 mm	14 mm	14 mm
Dried *C. quadrangularis* Extract	8 mm	10 mm	12 mm	14 mm
Klebsiella pneumoniae Fresh *C. quadrangularis* -SNP	13 mm	14 mm	16 mm	16 mm
Dried *C. quadrangularis* - SNP	14 mm	14 mm	16 mm	18 mm
Fresh *C. quadrangularis* Extract	15 mm	18 mm	18 mm	19 mm
Dried *C. quadrangularis* Extract	14 mm	15 mm	16 mm	20 mm
Pseudomonas aeruginosa _ Fresh *C. quadrangularis* -SNP	15 mm	18 mm	18 mm	18 mm
Dried *C. quadrangularis*- SNP	14 mm	14 mm	16 mm	18 mm
Fresh *C. quadrangularis* Extract	10 mm	12 mm	12 mm	14 mm
Dried *C. quadrangularis* Extract	10 mm	12 mm	14 mm	14 mm
Salmonella typhi Fresh *C. quadrangularis* -SNP	14 mm	14 mm	16 mm	16 mm
Dried *C. quadrangularis* - SNP	14 mm	14 mm	16 mm	18 mm
Fresh *C. quadrangularis* Extract	12 mm	12 mm	14 mm	14 mm
Dried *C. quadrangularis* Extract	10 mm	12 mm	14 mm	14 mm

Table 2: Antibacterial activity of *C. quadrangularis* Extracts and *C. quadrangularis* loaded Silver nanoparticles.

Sample tested	Concentration	Percentage of Inhibition (%)	
		Albumin denaturation	Membrane stabilization
Cq fresh extract and Dried Extract	2 µL	50%	78%
	4 µL	53.84%	82%
	8 µL	60%	85.4%
	16 µL	61%	86%
Cq extract loaded silver nanoparticles	2 µL	63.07%	88.5%
	4 µL	68.61%	87.6%
	8 µL	75.38%	89.1%
	16 µL	78.46%	92.3%
Aspirin	250 µg/mL	-	87%

Table 3: Effect of *C. quadrangularis* extracts and *C. quadrangularis* loaded silver nanoparticles on Albumin denaturation and membrane stabilization.

Concentration	Percentage of Inhibition (%)	
	C. quadrangularis fresh and Dried Extract	*C. quadrangularis* extract loaded silver nanoparticles
10 µL	32%	54%
20 µL	46%	78%
40 µL	64%	84%
80 µL	88%	92%
160 µL	94%	98%

Table 4: Antioxidant activity of *C. quadrangularis* extracts and *C. quadrangularis* loaded Silver nanoparticles by DPPH assay.

activity at a lower concentration than *C. quadrangularis* extract. Tables 5(a) and 5(b) represent the percentage viability determined using *C. quadrangularis* extracts and *C. quadrangularis* SNP. Figures 5(a) and 5(b) graphically represent the same. IC_{50} values of *C. quadrangularis* SNP with 50% viability of cancer cells (HeLa and A549) were 40 µL and 73.6 µL respectively, which was at a lower concentration than that of *C. quadrangularis* extract (160 µL), again indicating enhanced activity at lower concentration when synthesized as nanoparticle.

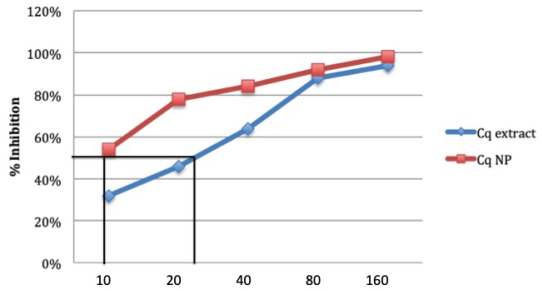

Figure 4: Graph showing IC_{50} value of *C. quadrangularis* NP=9.26 and *C. quadrangularis* extract =12.74.

Concentration	Percentage Viability (%)		
	Vero cells	HeLa cells	A549 cells
Control	100%	100%	100%
10 mL	94%	80%	98%
20 mL	96%	78%	87%
40 mL	96%	70%	72%
80 mL	97%	56%	60%
160 mL	98%	48%	50%

Table 5a: Cytotoxicity and Anti-cancer activity of *C. quadrangularis* extract.

Concentration	Percentage Viability (%)		
	Vero cells	HeLa cells	A549 cells
Control	100%	100%	100%
10 µL	98%	72%	86%
20 µL	98%	64%	74%
40 µL	99%	50%	58%
80 µL	99%	44%	46%
160 µL	99%	32%	40%

Table 5(b): Cytotoxicity and Anti-cancer activity of *C. quadrangularis* loaded silver nanoparticles.

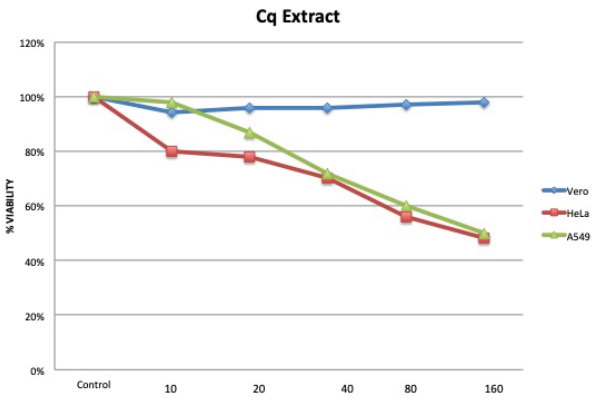

Figure 5(a): Graph showing cytotoxicity and anticancer activity of *C. quadrangularis* extracts on Vero, HeLa and A549 cells.

Figure 5(b): Graph showing cytotoxicity and anticancer activity of *C. quadrangularis* loaded silver nanoparticles on Vero, HeLa and A549 cells.

Conclusion

Biologically synthesized nano-materials are gaining importance in the scientific community with emphasis being laid on their characterization and biomedical application. Plant extracts traditionally used in ancient medical systems such as Ayurveda and Unani are now being revisited with interests turning towards utilizing them as biologically synthesized nanoparticles with better delivery. The present study has demonstrated the various pharmacological properties of *Cissus quandrangularis*, one such popular plant with medical applications. On comparison, the *in-vitro* pharmacological properties displayed by the *C. quadrangularis* loaded silver nanoparticles in this study were better than the *C. quadrangularis* extract. This certainly has potential for exploration in medical application with further *in-vivo* testings.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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