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Invitro antioxidant and antidiabetic assay of Methanol stem extract and synthesized Silver nanoparticles of *Cissus quadrangularisL*. stem

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ABSTRACT

Cissus quadrangularis is a succulent plant belonging to the family Vitaceae and is native to tropical regions like Asia, Africa, and the Arabian Peninsula. It has been in use in almost all the traditional systems of medicine, especially in Ayurveda. The Cissusquadrangularis stem-mediated silver nanoparticles are also being studied for their potential pharmaceutical benefits. The secondary metabolites in both the methanol stem extract and synthesized silver nanoparticles contribute to the antioxidant and antidiabetic properties of the plant. The present study provided a comparative quantitative analysis of alkaloid, tannin, and phenolic content of the methanol stem extract and synthesized silver nanoparticles. Furthermore, the free-radical scavenging and blood sugar-lowering effects were evaluated employing standard protocols. The results revealed the total alkaloid and phenolic content was higher in the synthesized AgNPs and tannin content was higher in the methanolic stem extract. The glucose utilization was found to be more efficient in the Cissusquadrangularis stem-mediated AgNPs, whereas the methanol extract was shown to have more antiradical properties.

Keywords: *Cissusquadrangularis, ,antioxidant, antidiabetic, Silver nanoparticle, Antiradical, Glucose utilization.*

1. Introduction

Medicinal plants are the cornerstone of almost any ancient medical system in the world.Indian civilization is considered the best vault or safe house for information on medicinal plants and their usage.*Cissusquadrangularis* is a succulent medicinal plant found growing in the tropical and sub-tropical regions of the world.The development of plant extract-based green synthesis has been aided by the unquestionable need for a dependable, safe, and ecologically pure method of producing nanoparticles [1].

The biotransformation process is brought about by the functional groups attached to the biomolecules. Among all the metallic nanoparticles, silver nanoparticles have gained momentum due to their wide spectrum of biological significance. Silver's bactericidal properties are well-established and have a lengthy historical background. When compared to its macroscopic equivalent, silver exhibits comparatively significantly greater antibacterial activity in its nano form [2].Ionic silver metal is bio-reduced into nanoparticles by a variety of significant secondary metabolites obtained from plant extracts, including sugars, alkaloids, phenolic acids, flavonoids, and terpenoids[3].Researchers now have more avenues to explore in their quest to understand the compounds and mechanism of action causing these therapeutic benefits thanks to the AgNPs extract [4].

It has been demonstrated that silver nanoparticles have superior antioxidant and anticancer qualities, and they may be used to create new medicinal medicines[5]. Moreover, the therapeutic potential of biomolecules present in the plant extract is enhanced after its reduction into nanoparticles. The current study aims to compare the antidiabetic and antioxidant potential of the methanolic plant extract of *Cissusquadrangularis* stem.

2. Materials and Methods

2.1 Identification of the Plant

Cissusquadrangularis L. was procured from a nearby nursery located in Hyderabad. Prof. P. Kamalakar of Department of Botany, Osmania University, Hyderabad verified the plant's authenticity, and the university lodged a voucher specimen of the plant for future use.

2.2 Preparation of methanolic extract

The stem of the plant was initiallyrinsed with running tap water, followed by distilled water to get rid of surface contaminants.Following that, the stem is stretched out and let to dry at room temperature in the shade until it is completely dehydrated. The shade dry process required about two to three weeks since the plant is xeric in its natural habitat. Using an electric blender, the plant material is ground into a fine powder after drying. Using a rotatory magnetic stirrer, 100 grams of plant powder were macerated in 100 milliliters of methanol for a duration of 72 hours. Next, it was filtered using Whatman filter paper No. 42. The crude methanolic extract of the plant *Cissusquadrangularis* L. was left behind after the solvent was evaporated from the collected filtrate in the rota-evaporator.

2.3 Synthesis of silver nanoparticles

2.3.1 Preparation of Plant Extract

To get rid of surface contaminants and debris, 10g of fresh *Cissusquadrangularis* L. stem was cut and thoroughly washed under running water. It was thereafter thoroughly cleaned and sterilized using absolute alcohol. Following a brief heating period of around 20 minutes, the stem was chopped into small

pieces and put in a beaker filled with 100 milliliters of distilled water. Using No. 1 Whatman filter paper, a filtrate is obtained from the plant extract after it has reached room temperature. Refrigirate the contents for further use below 4 degrees Celsius.

2.3.2 Preparation of Silver Nanoparticles

The synthesis of silver nanoparticles was carried out using a 0.1 Molar standard aqueous solution of silver nitrate ($AgNO_3$) that was prepared by dissolving 17 gm of silver nitrate in 1 litre of deionized water. Subsequently, 5 ml of plant extract of the *Cissusquadrangularis* was added to 45 ml of standard 0.1M $AgNO_3$ solution in a 100 ml Erlenmeyer flask. The entire procedure was carried out at room temperature to assist in the formation of silver nanoparticles, and the change in color from white to dark brown could be regarded as a positive indicator for the formation of silver nanoparticles.

70% alcohol is used to wash the solution after it has been centrifuged at 10,000 rpm to extract the nanoparticles from the reaction medium.

2.4 Quantitative Analysis

2.4.1.Test for total alkaloid content

The spectrophotometric method was used to estimate the total alkaloid content in the methanolic and AgNPs extract of the *Cissusquadrangularis* stem [6]. 2 mL of extract was diluted in 2N hydrochloric acid and filtered through a Whatman filter paper. 1ml of this solution was taken to a separatory funnel and rinsed with 10ml of chloroform (3 times). With 0.1 N NaOH, the pH of this solution was adjusted to neutral. This solution was stirred with 5 ml of BCG (Bromocresol Green) solution and 5 ml of phosphate buffer. The mixture was vigorously agitated, and the complex produced was extracted with 1, 2, 3, and 4 mL $CHCl_3$. The extracts were assembled in a 10-ml volumetric flask and made up to the volume with chloroform.

Preparation of Standard Solution: The Atropine was used as a standard for plotting the standard atropine calibration graph. Prepare 0.4, 0.6, 0.8, 1.0 and 1.2 ml of atropine solutions and transfer them into a different separating funnel. Stir in 5 mL of phosphate buffers with pH 4.7 and 5 mL of BCG (Bromocresol Green) solution, followed by 1, 2, 3, and 4 mL of chloroform.The extracts were transferred in a 10 ml volumetric flask and diluted with chloroform to adjust the volume.

At 470 nm, measure the absorbance of the standard solutions and extracts to that of theblank solution made using the same procedure except for the addition of atropine.

2.4.2. Test for total tannin content

The total tannin content in the samples was estimated using the Folin-Ciocalteu technique [7]. To a 10mL volumetric flask, 0.1 ml of the extract is added. To the extract, add 0.5 ml Folin reagent, 7.5 ml deionized water, and 1 ml of 35% Na_2Co_3. The mixture was then diluted with 10 ml of distilled water, well agitated, and allowed to stand at room temperature for 30 minutes.

Preparation of Standard Solution: The tannic acid was used as a standard for the plotting of standard tannic acid calibration graph. Prepare 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of tannic acid solutions and add 0.5 ml Folin reagent, 7.5 ml deionized water and 1 ml of 35% Na_2Co_3. After that, the mixture was diluted with 10 ml of distilled water, well agitated, and left to stay at room temperature for 30 minutes.

At 770 nm, evaluate the absorbance of the standard solutions and extracts to that of the blank solution obtained using the same method except with tannic acid addition.

2.4.3. Test for total phenolic content

The total phenolic content was estimated by the Folin-Ciocalteu method as described by Singleton &Rossi (1965) [8]. Dissolve the AgNPs extract and methanolic extract of the *Cissusquadrangularis* stem while maintaining 10mg/ml concentration. Take 1ml of the test sample and add 5ml of Folin-Ciocalteu reagent(diluted ten folds) and 4ml of 25% Sodium bicarbonate andallow the mixture to incubate for 30 min at 20 degrees Celsius temperature.

Preparation of Standard Solution: The gallic acid is used as a standard for plotting the standard Gallic acid calibration graph. Gallic acid stock solution is prepared by dissolving 10mg of Gallic acid in 100ml of methanol. This stock solution was used to make various dilutions of standard Gallic acid. Prepare 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml concentrations of Gallic acid solutions and add 5ml of Folin-Ciocalteu reagent (diluted ten folds) and 4ml of 25% Sodiumbicarbonate and allow the mixture to incubate for 30 min at 20 degrees Celsius temperature.

At 765 nm, evaluate the absorbance of the standard solutions and extracts to that of the blank solution obtained using the same method except with Gallic acid addition. The total phenolic content was calculated using the formula:

C = C1 x V/m

Here,

C = Total phenolic content in mg/ml

C1=Concentration of Gallic acid established from the calibration curve

V = Volume of the extract in ml

m = Weight of the plant extract in grams

2.5 Antioxidant assay

The DPPH (1,1-diphenyl-2-picryl hydrazyl) method of analysising the free radical scavenging activity was employed to assess the antioxidant activity in the methanolic and AgNPsof *Cissusquadrangularis* stem extract [9]. This method involves reducing DPPH in methanol in the presence of a hydrogen-donating antioxidant, resulting in the formation of non-radicals from DPPH- H and a color shift from purple to yellow.

This test employed various concentrations of test compounds (10,25, 50, 75, and 100 µg/ml) in 1 ml of DPPH methanol solution (0.2 mM). The O.D. value must be set to 0.8 for the fresh DPPH of 0.2 mM. Add DPPH if the O.D. is less than 0.8, and methanol if it is higher than 0.8. The blend was incubated for 30 minutes after being thoroughly mixed.

The optical density of the solution was measured at 517 nm using a Hitachi 2010 spectrophotometer. Using the formula below, the percentage inhibition of antioxidant activitywas calculated, and the test sample results were compared to the ascorbic acid which is regarded as a positive control (Vitamin C).

Percentage of inhibition of DPPH = ((Control OD – Test OD) / Control OD) X 100

2.6 Antidiabetic Activity

The HepG2 cells were allowed to grow in DMEM with 4.5 g/L D-glucose and 10% heat-inactivated FBS at 37°C in a 5% CO2 condition. The cells were then trypsinized and counted using the tryphan blue test prior to being planted into 96-well plates

with 5000 cells per well and six blank wells and left to grow for three days.

Post seeding for three days, the extracts were added in triplicates at concentrations of 5,10, 25, 50 and 100 µl. After 48 hours of incubation, the spent culture media was drained and refilled with a 25-litre incubation buffer (DMEM diluted with PBS, 0.1 percent BSA, and 8 mm glucose), which was incubated for yet another 3 hours at 37 degrees Celsius. 0.1µ g/ml. The metformin was considered as the positive control, whereas the negative control (untreated) cells were treated with just incubation buffer without extract.

Following incubation, 10 µl of the incubation liquid was withdrawn from each well andplaced in a fresh 96-well plate containing 200 µl of glucose-oxidase reagent to assess the concentration of glucose in the medium. After 15 minutes of incubation at 37 degrees Celsius,the absorbance of the samples was noted at 492 nm with a Multiscan plate reader. The difference between the cell-free and cell-containing wells was found to calculate the quantity of glucose utilized. The percent of glucose utilization was estimated in comparison to untreated controls.

3. Result

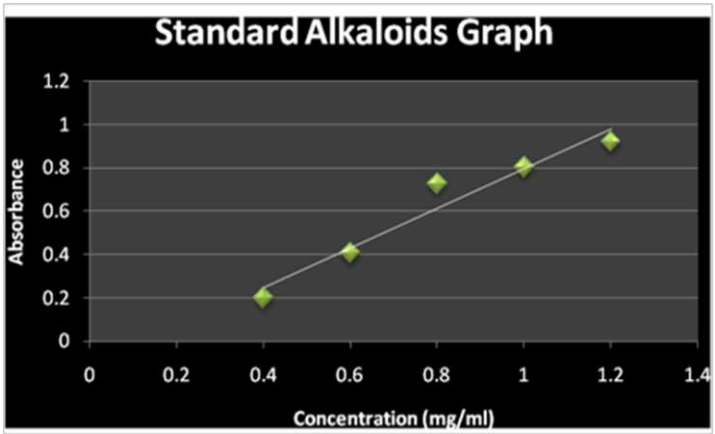
3.1 Quantitative Analysis:

3.1.1 Estimation of Total Alkaloid content in methanolic and AgNPs extract

The total alkaloid content is determined using the Bromocresol green solution with the spectrophotometer. A spectrophotometer is used to detect absorbance at 470nm at varying doses. The methanolic extract has an absorbance of 0.648, and AgNPs have an absorbance of 0.823 at 470 nm, and the concentration is calculated using a calibration graph. This graph illustrates the concentrations of methanolic and AgNPs extracts, with 0.836 and 1.017, respectively. The findings show that AgNPs have a greater concentration than methanolic extract, implying desirable properties that may be used in future studies.

Table:1 Total alkaloid content in the extracts

Tube	Concentration	Absorbance
1.	0.4	0.201
2.	0.6	0.408
3.	0.8	0.728
4.	1.0	0.804
5.	1.2	0.921
Methanolic Extract	0.836	0.648
AgNPs	1.017	0.823



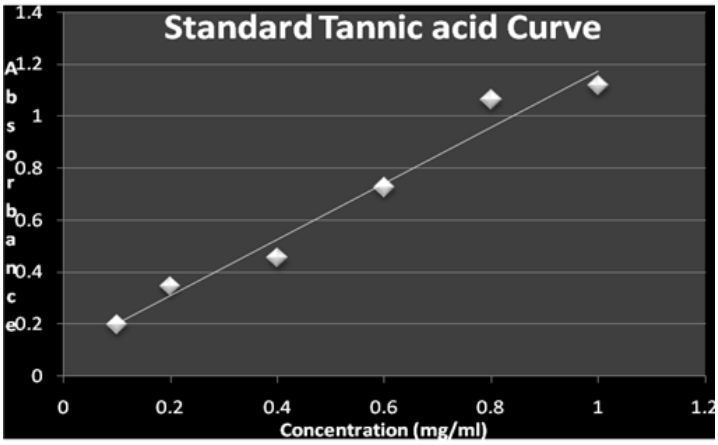
Graph:1 Total alkaloid content

3.1.2 Total tannin content determination in methanolic and AgNPs extracts

A folinciocolteau reagent technique employing sodium carbonate and distilled water is adopted to determine tannins. The tannic acid is used as a standard in the protocol.A spectrophotometer is used to measure the absorbance of standards, methanolic, and AgNPs extracts at 700 nm, and the absorbance of the standards, methanolic, and AgNPs extract changes with the concentration. The methanolic extract has an absorbance of 0.466, while the AgNPs extract has an absorbance of 0.427. To determine the concentration of the extract and the standard, a calibration graph is plotted against it. The concentration of the methanolic extract and AgNPs is estimated to be 0.34977 and 0.31472, respectively.

Table:2 Total tannin content in the extracts

Tube	Concentration	Absorbance
1.	0.1	0.198
2.	0.2	0.346
3.	0.4	0.456
4.	0.6	0.727
5.	0.8	1.064
6.	1.0	1.119
Methanolic Extract	0.34977	0.466
AgNPs	0.31472	0.427



Graph:2 Total tannin content

3.1.3 Estimation of total phenolic content in both methanolic and AgNPs extract

The total content was estimated using the folinciocolteau reagent technique, and absorbance was measured at 765nm using sodium carbonate, folin reagent, and gallic acid as a standard. A spectrophotometer at 765 nm indicated that the methanolic-extracts and AgNPs have absorbances of 0.36 and 0.71, respectively. Plotting a calibration curve with y=mx+b yields the concentration and total phenolic content. The methanolic extract has a concentration of 0.388, whereas the AgNPs extract has a concentration of 0690. Total phenolic content was determined using the concentration, and the standard equation graph was y=1.077x+0.055, with an R2 of 0.931.For all concentrations, a formula yields standard equation values that reflect 0.3093 in methanol and 0.5903 in AgNPs.

$C = C1 X V/m$

C: Total phenolic content in mg/g

C1: Concentration of gallic acid established from the calibration curve

V: Volume of extract in ml

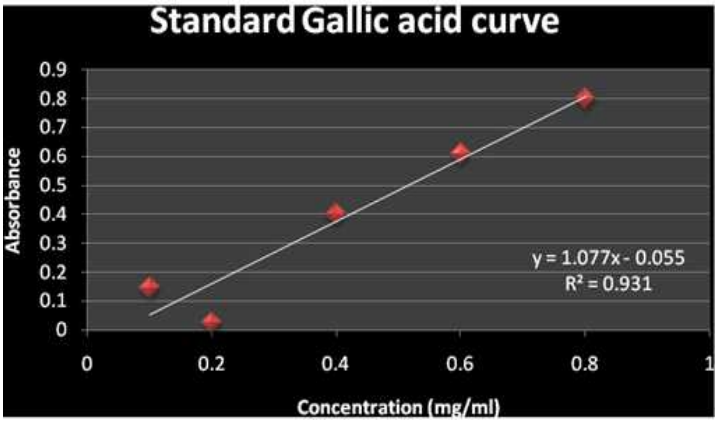
m: weight of the plant extract in gms

Standard equation formula

Y = mx – b
X = y – b / m
Where: y = concentration
m = 1.077
b = 0.055
Methanolic Extract:
X = (0.388180 – 0.055) / 1.077
X = 0.3093

AgNPs Extract:
X = (0.5903– 0.055) / 1.077

X = 0.5903



Graph:3 Total phenolic content

Table:3 Total tannin content in the extracts

Tube	Concentration	Absorbance	SEC
1.	0.1	0.147	0.04
2.	0.2	0.026	0.13
3.	0.4	0.4	0.32
4.	0.6	0.61	0.51
5.	0.8	0.801	0.69
Methanolic Extract	0.388180	0.36	0.3093
AgNPs	0.690814	0.71	0.5903

3.2. Antioxidant activity

The antioxidant activity was studied using the DPPH approach (1,1-diphenyl-2-picryl hydrazyl radical), which is based on the formation of non-radical from DPPH- H during the reduction of methanol DPPH in methanol solution in the presence of a hydrogen-donating antioxidant. The DPPH molecule, which contains a stable free radical, has long been used to evaluate anti-oxidants' potential to eliminate free radicals. The ascorbic acid is considered the standard in the assay. The table readings indicate that the ascorbic acid's antioxidant potential is higher than the two test samples. As the concentration of the standard and the test samples increases, the % inhibition also increases. The IC50 (efficient concentration value) parameter is utilized to analyze the DPPH method's results and is defined as the substrate concentration that reduces DPPH activity by 50%. (colour).As a result, the antioxidant activity is inversely proportional to the IC50 value. The ascorbic acid standard exhibited 50.87 percent inhibition activity and an IC 50 value of -3.30 at a 10 µg/ml concentration. On the contrary, the methanolic extract of *C. quadrangularis* shows 7.85 % inhibition and an IC50 value of -0.07 at the same concentration. With a percent inhibition of 1.24 and an IC50 value of 2.17, the *C. quadrangularis*AgNPs appear to have the least antioxidant capacity at the concentration of 10µg/ml.

Table:4 Calculation of % inhibition and IC50 from DPPH assay

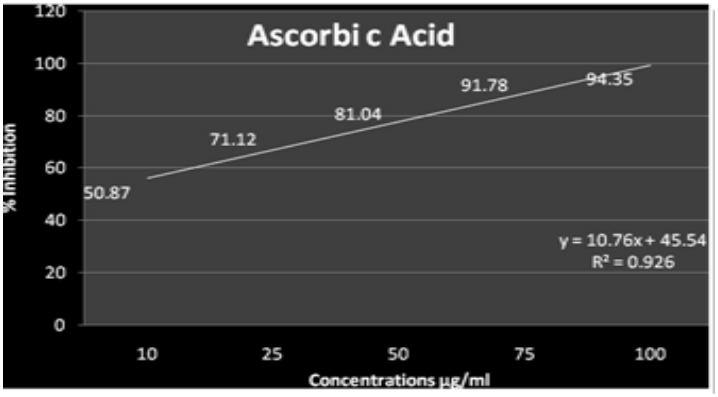
Test Compound	Concentration (µg/ml)	Control	OD	% Inhibition	SD	IC50
Ascorbic acid	10	0.549	0.27	50.87	0.146	-3.30
	25	0.549	0.158	71.12	0.682	-1.91
	50	0.549	0.104	81.04	0.623	0.41
	75	0.549	0.045	91.78	0.829	2.74
	100	0.549	0.031	94.35	1.105	5.06

Table:5 Calculation of % inhibition and IC 50 value from DPPH assay of methanolic extract of Cissusquadrangularis

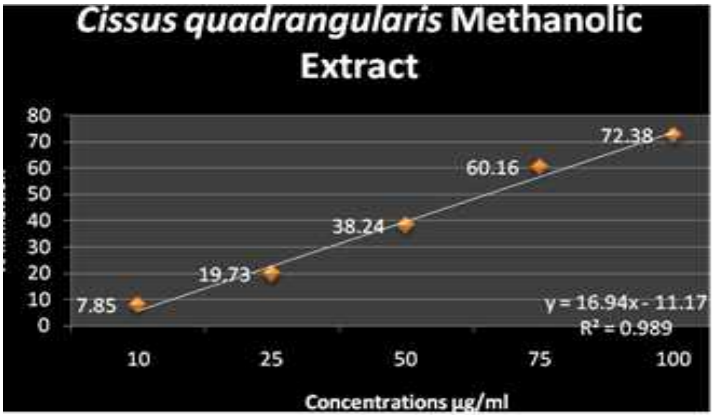
Test Compound	Concentration (µg/ml)	Control	OD	% Inhibition	SD	IC50
Methanolic extract of *C.quadrangularis*	10	0.549	0.507	7.85	0.117	-0.07
	25	0.549	0.44	19.73	0.248	0.82
	50	0.549	0.34	38.24	0.364	2.29
	75	0.549	0.213	60.16	0.387	3.77
	100	0.549	0.152	72.38	0.621	5.24

Table:6Calculation of % inhibition and IC 50 value from DPPH assay of AgNPs extract of Cissusquadrangularis

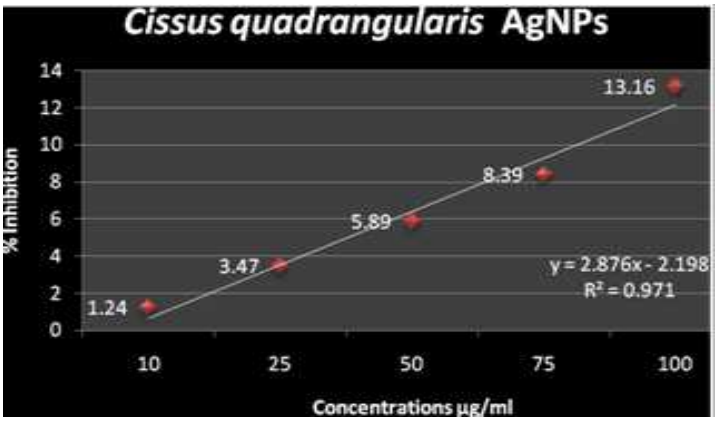
Test Compound	Concentration (µg/ml)	Control	OD	% Inhibition	SD	IC50
AgNPs of *C.quadrangularis*	10	0.549	0.54	1.24	0.086	2.17
	25	0.549	0.531	3.47	0.137	7.93
	50	0.549	0.517	5.89	0.163	16.62
	75	0.549	0.503	8.39	0.218	25.31
	100	0.549	0.476	13.16	0.275	34.1



Graph: 4 DPPH assay in Ascorbic acid



Graph:5 DPPH activity in methanolic extract of Cissusquadrangularis



Graph: 6 DPPH activity in AgNPs extract of Cissusquadrangularis

% inhibition = control OD- test OD /control OD *100
IC50 value is calculated from the graph using the slope intercept formula-

y = mx + b
Where,
y = Concentration
m = Slope
b = Intercept
Therefore, the formula to calculate the IC50 value is
IC50 = y – b / m

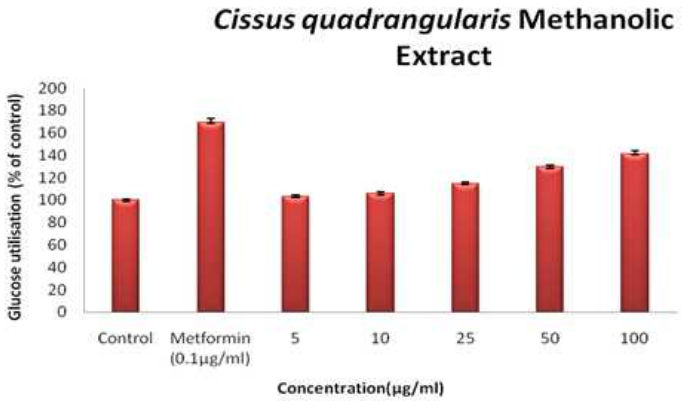
3.3. Antidiabetic activity

The test was carried out on HepG2 cells cultured at 37°C in a 5% CO2 atmosphere in DMEM with 4.5 g/L D-glucose and 10% heat-inactivated FBS.After the cells have been prepared and incubated, the absorbance at 492 nm is measured using a Multiscan plate reader. The amount of glucose utilized was calculated using the difference between the cell-free and cell-containing wells.The percent of glucose utilization was measured in comparison to the untreated controls.

The table gives the OD value, glucose utilization of the control, metformin standard, and the varying concentrations of the test sample or extract. The OD values of the control and the metformin are 0.612 and 1.035, respectively. By using OD parameters and the control, it is found that the metformin utilization value at 0.1 µg concentration is 169.26, which is regarded as the standard. Glucose utilization of the test sample is calculated based on these OD values and Glucose utilization control.Metformin at 0.1 g has the maximum glucose utilization, whereas methanolic extract and the AgNPsat a 5 g/ml concentration have glucose uptake of 103.35 and 106.41, respectively. It is evident from the graphs that with the rise in the concentrations of the extracts, the rate of glucose utilization was also enhanced.

Table :7 In vitro Anti-diabetic activity of Cissus quadrangularis Methanolic extract

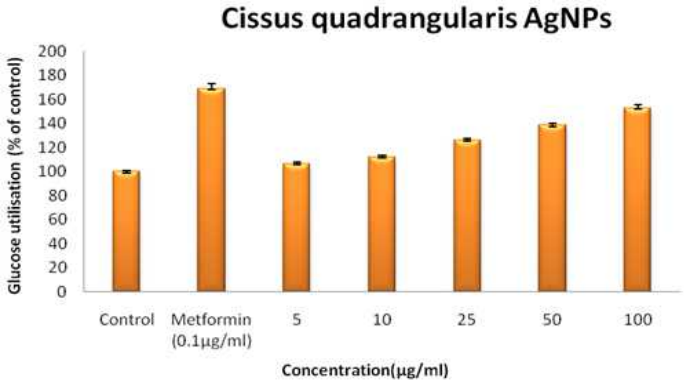
Extract (µg/ml)	OD	Glucose Utilization(% Control
Control	0.612	100±1.18
Metformin (0.1µg/ml)	1.035	169.26±3.89
5	0.632	103.35±1.15
10	0.646	105.61±1.48
25	0.703	114.94±1.37
50	0.792	129.47±1.64
100	0.866	141.53±2.36



Graph:7In vitro Antidiabetic activity of Cissus quadrangularis Methanolic extract

Table:8 In vitro Anti-diabetic activity of AgNPs of Cissus quadrangularis

Extract (µg/ml)	OD	Glucose Utilization(% Control
Control	0.612	100±1.18
Metformin (0.1µg/ml)	1.035	169.26±3.89
5	0.651	106.41±1.34
10	0.686	112.18±1.63
25	0.773	126.37±1.97
50	0.848	138.66±2.06
100	0.936	152.95±2.14



Graph: 8 In vitro Antidiabetic activity of AgNPs of Cissus quadrangularis

4. Discussion and Conclusion

The plant *Cissusquadrangularis* L. of the family Vitaceae is an important medicinal plant and is a crucial ingredient in almost all types of traditional and indigenous systems of medicine. The green synthesis of nanoparticles from the aqueous extract of the stem was less toxic, and the synthesized nanoparticles were found to be relatively stable. These AgNPs could be effortlessly harvested after production. Plant extracts are particularly promising for the synthesis of AgNPs due to their viability, low cost, and environmental friendliness, which provides a great opportunity to combine green chemistry and nano synthesis [10]. The synthesis of nanoparticles utilizing plants offers various advantages, such as the usage of safer solvents, less use of harmful chemicals, gentler reaction conditions, practicality, and their flexibility in use for medical, surgical, and pharmaceutical applications [11].

Plants use alkaloids as defense molecules, and their toxicity renders them effective against infections and predators [12]. The results revealed that the concentration of alkaloids was higher in the AgNPs compared to the methanolic stem extract. On the other hand, the concentration of tannins was roughly the same in both methanolic and AgNPs extract. Tannins are known to be powerful antioxidants, and they do this by chelating metal ions like Fe (II) and interfering with one of the Fenton reaction's reaction stages, so delaying oxidation [13]. Tannins' anticarcinogenic and antimutagenic properties could be linked to their antioxidative properties, critical in protecting cells from oxidative damage, like lipid peroxidation [14]. The estimation of total phenolic content was done through the standard graph in combination with the standard slope equation. The concentration of phenols in the AgNPs was higher than its concentration in the methanolic extract. Plants produce natural antioxidants in the form of phenolic chemicals, including phenolic acids and tocopherols [15].

Free radicals are essential in various pathological conditions, including tissue damage, inflammation, and neurodegenerative diseases.Antioxidants play a critical function in protecting the human body from free radical damage [16].The free radical scavenging ability tested using the DPPH method, using the ascorbic acid as the standard revealed that the methanolic stem extract exhibitedantioxidant potential lower than the ascorbic acid standard but higher than that of AgNPs.*Cissusquadrangularis* methanol extract has significant antioxidant and free radical scavenging action in vitro and in vivo systems, attributed to beta–carotene [17].

Plants that promote glucose uptake and disposal by muscle, fat, and hepatic cells and those that influence hepatic glycogen metabolism are all insulin sensitizers [18]. *Cissusquadrangularis*, in addition to its numerous other biological functions, has significant antihyperglycemic properties.The MTT assay used to determine the in vitro antidiabetic activity of the chosen extracts against the HepG2 liver cell line at varying doses, with the metformin taken as the standard indicated that methanolic extract and the AgNPs do not have much difference in the % control of glucose utilization. Thus, the plant *Cissusquadrangularis*possess considerable antihyperglycemic potential in the form of methanolic extract and the nanoparticle form.

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