



**International Journal of Biology, Pharmacy  
and Allied Sciences (IJBPAS)**

*'A Bridge Between Laboratory and Reader'*

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**GLYCYRRHIZA GLABRA MEDIATED SILVER NANOPARTICLES:  
SYNTHESIS, CHARACTERIZATION, STANDARDIZATION, AND  
CARDIOPROTECTIVE BIO EVALUATION**

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Received 16<sup>th</sup> Jan. 2024; Revised 20<sup>th</sup> Feb. 2024; Accepted 24<sup>th</sup> July 2024; Available online 1<sup>st</sup> May 2025

<https://doi.org/10.31032/IJBPAS/2025/14.5.9021>

**ABSTRACT**

SARS-CoV-2 exacerbated the risk of cardiovascular diseases (CVDs), threatening world health. Novel drug delivery systems (NDDS) incorporating herbal medicines have the potential to boost CVD therapy's efficacy. The key intent of this study was to utilize aqueous root extract of *Glycyrrhiza glabra* Linn as a green reducing and capping agent for the silver nanoparticles (AgNPs) fabrication, followed by their characterization, standardization, and assessment for cardioprotective activity. Stable AgNPs formation was achieved by mixing the aqueous root extract with 1mM silver nitrate (AgNO<sub>3</sub>) solution. SEM, TEM, and DLS showed spherical GGNPs with an average size of 20–50nm. The 430nm surface plasmon band was characteristic. EDAX (41.69% weight) and ICP-AES (324ug/ml) verified silver content. The synthesized AgNPs were standardized by validated HPLC method for the content of glycyrrhizic acid as the marker compound was found to be 2.44±0.105mg. In-vitro release study suggested GGNPs follows the diffusion and degradation mixed release pattern for the sustain and controlled release. Synthesized AgNPs were tested for cardio-protection utilizing the CAM assay. GGNPs concentration raised the number of branching arteries in chicken choriollontoic membrane by 33 ± 2 at 1mg/ml, while

100mg/ml GG extract concentration increased it by  $38 \pm 2$ . This shows GG NPs are more pro-angiogenic. In H9C2 cell line toxicity studies, GG NPs had no toxicity after 24 hours. The aforementioned experimental data on metal nanomedicine derived from herbal sources offers valuable insights into the scientific foundation for the development of novel treatment therapies in cardiovascular diseases (CVDs).

**Keywords:** *Glycyrrhiza glabra* Linn, green synthesis, silver-nanoparticles, Cardioprotective, pro angiogenesis

## 1. INTRODUCTION

CVDs stand as the primary contributing factor of the death among a significant portion of the global population. Pre-existing CVDs have been identified as contributing to worsened outcomes and heightened mortality risk in individuals with COVID-19. Moreover, COVID-19 itself has been shown to potentially induce cardiovascular complications. The interplay between drugs and diseases in COVID-19 cases, culminating in the development of CVDs, is becoming an increasingly serious concern. Numerous therapeutic approaches have been devised for the management of CVDs. Nevertheless, these treatment modalities may be associated with substantial negative impacts on the body. Nowadays, the preference for herbal drugs over conventional and synthetic medications has witnessed a notable surge [1, 2]. Incorporation of the herbal extracts into the novel drug delivery systems have some added advantages, such as dependable platform for regulated and targeted drug delivery to cure

the diseased condition. Nanoparticles have garnered a great fascination in medicine due to their distinct physicochemical properties that enhance biological functions. Additionally, modifying the properties of nanoparticles can decrease the side effects of drugs and enable targeted drug delivery. Therefore, synthesizing herbal nanoparticles holds great potential for the treatment of CVDs [3, 4].

Among the metal nanoparticles, AgNPs were commonly utilized used because of their broad spectrum activities [5, 6]. *Glycyrrhiza glabra* Linn. (Licorice), a traditional Indian herb, contains glycyrrhizic acid, a triterpene saponin glycoside, as its primary bioactive phytoconstituents. It has been shown to reduce oxidative damage in ischemia-reperfusion injuries [7, 8]. Numerous studies have also demonstrated licorice's anti-inflammatory, antitumor, antimicrobial, and cardioprotective effects [9, 10]. The present study will establish a scientific data for use and emphasis on the

synthesis of the AgNPs containing the extracts of roots of *G glabra*. Their standardization with respect to the marker compound glycyrrhizic acid and the bio evaluation for the cardio protection activity, this research underscores the potential of utilizing readily available traditional herb as a beneficial resource for cardio protection.

## 2. MATERIAL AND METHODS

### 2.1 Materials

The roots of *G glabra* were procured from the Dawa bazar, Mumbai, India. Dr. Harshad M Pandit. Former head and adjunct professor of botany, Guru Nanak Khalsa college Mumbai, identified and certified the plant sample (sample #: rbs p 01222522). Silver nitrate ( $\text{AgNO}_3$ , 99.9%) was acquired from Sigma Aldrich.

### 2.2 Preparation and evaluation of aqueous root extract

Approximately 20g of powdered root material was extracted with 100mL of distilled water. The mixture was refluxed for 3 hours at  $60^\circ\text{C}$ . The extract was filtered through Whatman filter paper and dried at  $45^\circ\text{C}$ . In vitro quantitative phytochemical assays were conducted to quantitatively estimate the antioxidant efficacy of the plant extract [11, 12].

### 2.3 Synthesis of *G glabra* mediated silver nanoparticles (GGNPs)

2.0 mL of GG extract (1000ppm) was added to 18ml aqueous  $\text{AgNO}_3$  (1mM) solution kept in beaker at room temperature with constant stirring at 500rpm for 3 hrs. After 15 mins, the solution started turning dark yellow to dark brown and then grey black after 3 hrs, demonstrating the formation of AgNPs. The reduction of silver ions was supervised periodically by the UV-visible spectrophotometry [13]. The resulting colloidal solution of GGNPs was lyophilized and kept in a cold, dry and dark area till their further characterization and use.

### 2.4 Characterization of GGNPs

The observed transition in hue from yellow to gray-black is indicative of a reduction of silver ion facilitated by the root extract, hence signifying the synthesis of GGNPs. This transformation was further confirmed Surface plasma resonance (SPR) vibrations using UV-Visible spectrophotometer (Jasco V-730) between 200nm to 800nm. Malvern zetasizer was used to analyze the particle size, zeta potential and Polydispersity index of GGNPs. The Shimadzu FTIR spectrophotometer was used to analyze the spectra of purified GGNPs and GG extract in the range of  $450\text{--}4000\text{ cm}^{-1}$ , with a resolution of  $4\text{ cm}^{-1}$ . X ray diffraction (XRD) data was acquired at room temperature using nickel-filtered  $\text{Cu K}\alpha$  radiations at 40kv voltage, and

7° to 70° C (2θ) range. Dried AgNPs were placed on carbon grid of Scanning electron microscope (SEM) to evaluate size and shape at 6000X and 12000X, while Transmission electron microscopy (TEM) sample was scanned at 1,50,000X–3,00,000X to acquire a structurally clear picture. The sample was placed on a carbon grid and subjected to low vacuum (10–130 pa) with a voltage of 20 keV for Energy Dispersive X-ray Analysis (EDAX). At first, the picture was scanned at a magnification of 3000X. A specific area was then identified and examined using EDAX to verify the existence and measurement of silver metal. The quantitative determination of silver concentration at the ppm level was conducted using inductively coupled plasma atomic emission spectroscopy (ICP-AES). A calibration plot for known silver was created using concentrations of 0.1, 1, 10, and 100 ppm at 328.06 nm. The silver concentration was measured three times and given as mean (µg/ml) with standard deviation.

### **2.5 Invitro release study of the GG extract and GGNPs**

Franz diffusion cell drug release investigations were done in- vitro for both GG extract and GGNPs separately. GG extract and GGNPs were first placed on dialysis membrane of two Franz diffusion cells [14]. Dissolution medium of simulated stomach

fluid (pH 1.5) and intestinal fluid (pH 7) were used to examine how pH affects release kinetics. With UV spectrophotometry at 254nm for GG extract and 430nm for AgNPs, cumulative drug release was computed.

### **2.6 Application of mathematical models in release kinetics of GGNPs**

The data collected from the Invitro release research under varied pH conditions was further analyzed using multiple mathematical models, including Zero-order, First-order, Higuchi, and Korsmeyer-Peppas models. The best fit model that matches the drug release pattern was determined using graphical presentations, correlation coefficient (R<sup>2</sup>), and release exponent (n) [15].

### **2.7 Stability studies of GGNPs**

Stability studies of the prepared GGNPs were determined by storing optimized formulation at 30 ± 2°C/65 ± 5%RH and 40 ± 2°C/75 ± 5%RH in stability chambers for 6months. The samples were examined after a time period of 1, 15, 30days followed by 2nd, 3rd, and 6th months for their drug content and particle size distribution [16, 17].

### **2.8 Standardization of GG extract and GGNPs**

The standardization of GG extract and GGNPs for the content of Glycyrrhizic acid a biomarker was done using suitable optimized

and validated HPLC method. The HPLC system (Shimadzu Liquid Chromatograph Mass Spectrometer-8060NX™), consisting of LC-20AT pump, UV detector (Shimadzu SPS-20A) and ACE Generix C18 (5) 250 x 4.6 mm column with compatible guard column was used [18-20].

## 2.9 In-vitro evaluation of cardioprotective activity

### 2.9.1 Trypan blue size exclusion assay

A suspension of 200µl of H9C2 rat cardiomyocyte cells was transferred in a 96-well plate, without any test agents and allowed the cells to grow for 24 hours. Following that, GG extract (100, 200, 300, 400, 500 mg/ml) and GGNPs (2, 4, 6, 8, 10 mg/ml) were separately added to the system. The plates were nurtured at 37°C in 5% CO<sub>2</sub> for 24 hours. The plates were taken from the incubator and washed once with DPBS. A 50 microliter infusion of 0.4% Trypan Blue was added at room temperature for 5 minutes and washed three times with 1x sterile DPBS. The cells were examined using under an inverted biological microscope, and their cellular count was determined. The live or dead cells were counted manually and calculated the % of cell viability [21].

### 2.9.2 Chicken chorio-allantoic membrane (CAM) assay

12 days old fertilized poultry eggs underwent a cleaning process using 70% ethanol and were thereafter kept at a constant humidity of 37°C. During the treatment session, a tiny aperture was created at the slender extremity. The window was sealed using clear tape and then subjected to incubation once again. Following a 24-hour incubation period, a tiny window was accessed in the shell, and a sterile gel foam piece measuring 3mm x 3mm x 1mm was placed on the membrane. Control group received sterile PBS (pH-7.0) as the vehicle, while the experimental group was exposed to different concentrations of GG extracts (100, 200 mg/ml) and GGNPs (1, 2 mg/ml). The eggs were reintroduced into the incubator and subjected to undisturbed incubation for a duration of 24 hours. Later the photos of each egg treated with CAM were acquired and subjected to analysis for the presence of blood vessels. The spatial distribution of vessel branch points inside a square region equivalent to the area of each sponge was quantified, and the results were examined for each treatment group. The angiogenesis index obtained is calculated as the average of the number of new branch points seen in each set of data [22, 23].

## 3 RESULTS AND DISCUSSION

### 3.1 Extraction and quantitative phytochemical evaluation

Extraction Yield was found to be 3gm. The results of quantitative evaluation were reported in **Table 1**, indicates that the extract contains a high concentration of antioxidant phytochemicals. These phytoconstituents contribute to the production of GGNPs by serving as green reducing and capping agents.

### 3.2 Synthesis of GGNPs

Fabrication of AgNPs was verified by the colour change in the colloidal solution from

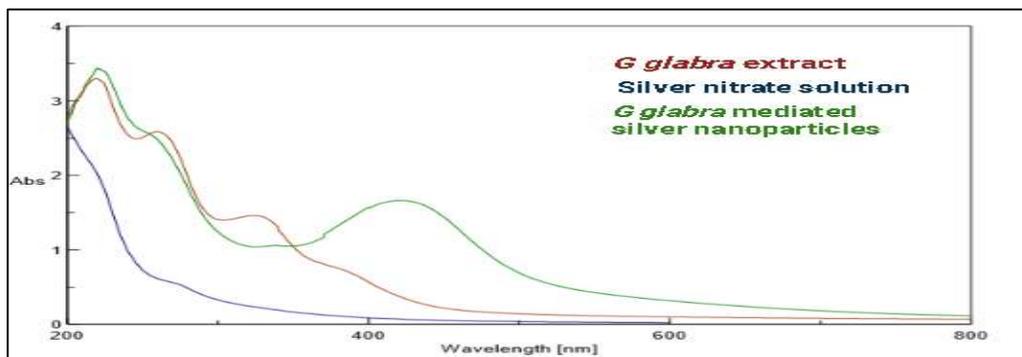
light brown to dark brown and then grayish black, indicating the reduction of  $\text{Ag}^+$  to  $\text{Ag}^0$  in solution as shown in **Figure 1A**. The UV- spectrum obtained had the surface plasmon resonance (SRP) within the absorption band range of 430 to 436 nm, as seen in **Figure 1B**.

**Table 1: Quantitative phytochemical evaluation of aqueous root extract**

Sr. No	Test	GG Extract	Supernatant left (After centrifugation of colloidal GGNPs)
1.	Total Flavonoid content $\mu\text{g/ml}$ of Quercetin/ $\text{gm}$ of dry extract	$113.59 \pm 5.55$	$8.14 \pm 1.97$
2.	Total antioxidant capacity $\mu\text{g/ml}$ of Ascorbic acid / $\text{gm}$ of dry extract ( $\text{IC}_{50}$ )	$79.41 \pm 5.55$	$431 \pm 27.6$
3.	Total phenolic content $\mu\text{g/ml}$ of Gallic acid / $\text{gm}$ of dry extract	$198.2 \pm 1.25$	$5.55 \pm 3.5$



**Figure 1A: Colour change in the mixture after addition of 1mM  $\text{AgNO}_3$  solution to the GG extract for nanoparticles synthesis**



**Figure 1B: UV- visible spectra of GG extract,  $\text{AgNO}_3$  solution and GGNPs indicating the change in surface plasmon resonance after formation of nanoparticles**

### 3.3 Characterization of GGNPs

The lyophilized GGNPs were utilized for the FTIR analysis to identify the possible phytoconstituents from GG extract that are responsible for the reduction of silver ions and nanoparticle stabilization. The broad FTIR peak imply a phytochemical coating on AgNPs. Phenolic chemicals attach to AgNPs

via –OH stretching vibrations, as seen by the peak at 3327cm<sup>-1</sup>. The amide group at 1643 cm<sup>-1</sup> is caused by amide linkage of proteins that cap AgNPs and stabilize them. Peak shift towards 3400cm<sup>-1</sup>, indicate AgNPs production by reducing plant extract carbonyl groups, including C-H and O-H of acid origin groups [24] (Figure 2, 3).

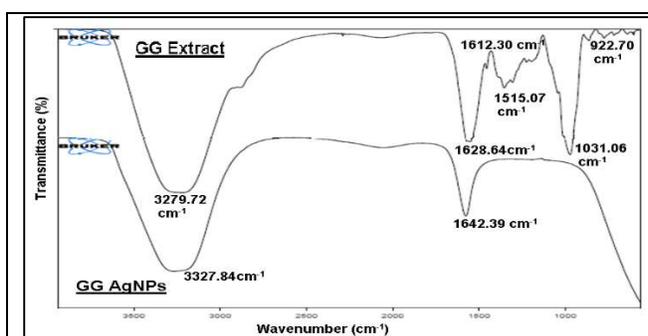


Figure 2: FTIR spectrum of GG extract and GGNPs.

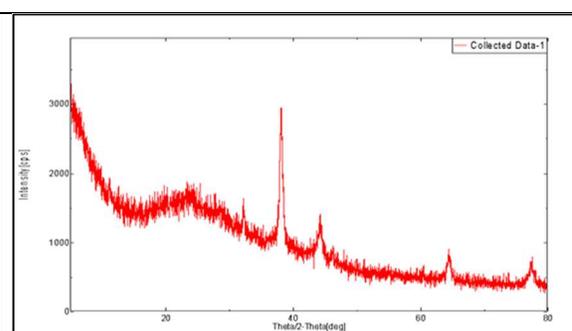


Figure 3: XRD pattern of GGNPs indicating crystalline nature

The XRD analysis of fabricated AgNPs from GG extract showed diffraction peaks at 2-theta= 38.08 °, 44.30 °, 64.49 °, 77.34 °. The X-Ray pattern displays a comprehensive sharp Bragg's peak indicating the crystalline nature of the GGNPs [25]. The average crystallite size was calculated using Scherer's

equation and was found to be  $113 \pm 43.72$ nm. GGNPs have hexagonal, spherical polydispersed particles, according to SEM examination. TEM study accurately measured nanoparticle size and high-resolution clear morphology scans revealed 20-50nm GGNP particle size [26] (Figure 4, 5).

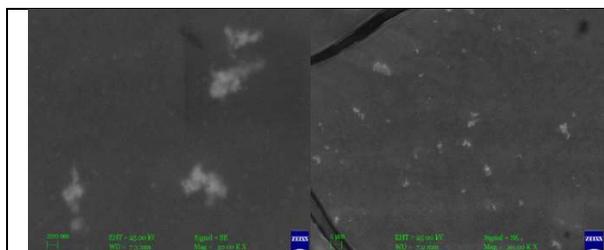


Figure 4: SEM images of GGNPs

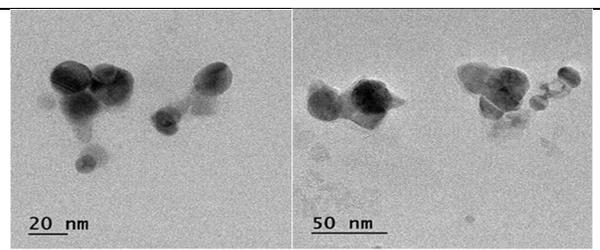
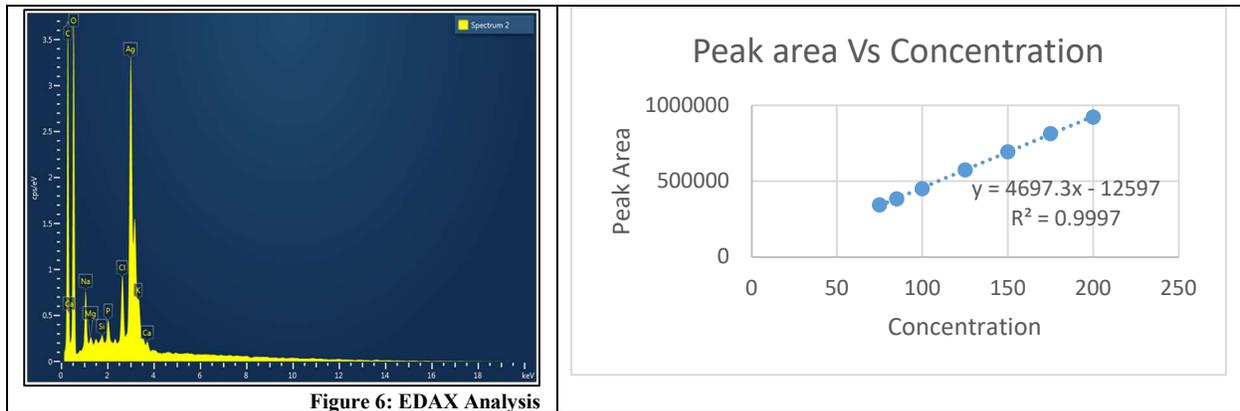


Figure 5: TEM images of GGNPs

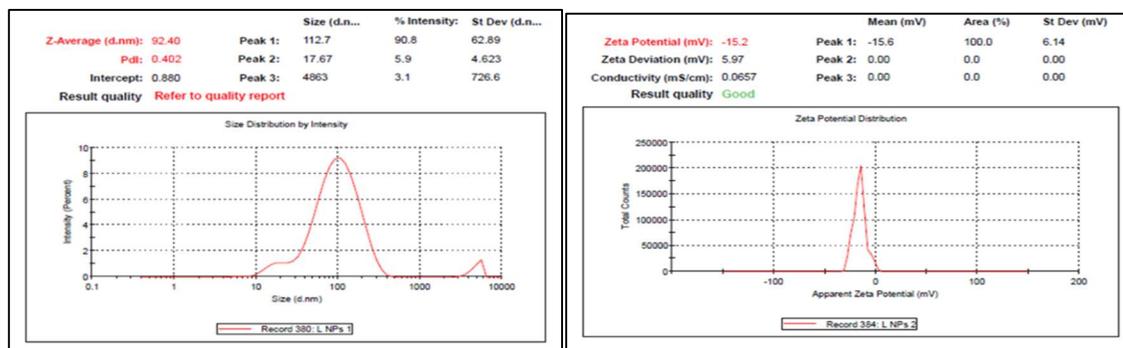
EDAX revealed, Silver (Ag) (51.97%), Oxygen (O) (23.48%), and Carbon (C) (20.63%) absorption peaks were prominent in

GGNPs. GGNP spectra exhibit oxygen and carbon peaks, confirming alkyl chain stabilizers (**Figure 6**).



DLS analysis was done to determine the hydrodynamic average particle size of GGNPs and was found to be 92.40nm with PDI value of 0.402 and zeta potential -15.2, indicating the polydispersed suspension of

GGNPs is more stable [27, 28]. The concentration of AgNPs from GG extract was measured to be 324  $\mu\text{g/ml}$  using ICP-AES (**Figure 7**).



**Figure 7: Zeta potential and DLS analysis of GGNPs**

### 3.4 Invitro release study of the GG extract and GGNPs

In-vitro release was performed at pH 1.5 and 7 respectively for GG extract and GGNPs

separately for various time span such as 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 24h (**Figure 8A, B**).

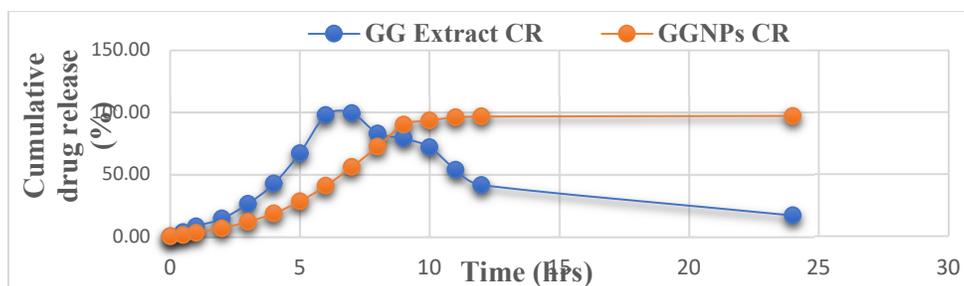


Figure 8A: In vitro release profile of GG extract and GGNPs in simulated stomach fluid (pH 1.5)

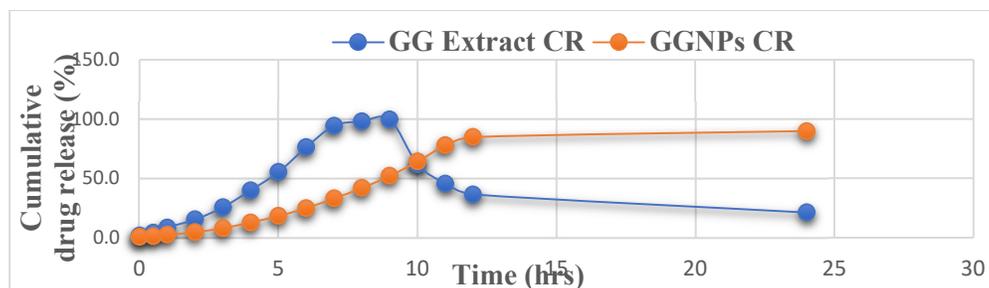


Figure 8B: In vitro release profile of GG extract and GGNPs in simulated intestinal fluid (pH 7)

From the graph, the high release for GG extract and GGNPs was found at pH 1.5.  $96.99 \pm 0.66\%$  nanoparticles were released at pH 1.5 after 24hr and  $89.8 \pm 0.58\%$  nanoparticles were released at pH 7 after 24hrs. From this evidence, it was confirmed that the drug release in GGNPs was sustain and prolonged. In case of GG extract in both the conditions, extract exhibited the highest release of  $99.27 \pm 1.2\%$  at pH 1.5 within first 7hrs and  $99.6 \pm 1.2\%$  pH 7 within 8hrs.

### 3.5 Mathematical modelling and drug release kinetics

Fitting In vitro release data into multiple kinetic models allowed mathematical modeling of GGNP release. **Table 2** shows the higher regression coefficient ( $R^2$ ) and release exponent ( $n$ ) parameters analyzed. The study found that GGNPs released via diffusion and erosion, following the Korsmeyer-Peppas hypothesis.

Table 2: Release kinetics and drug release mechanism of GGNPs at different parameters.

Parameters	Zero order	First order	Higuchi model	Korsmeyer-Peppas model		Drug transport mechanism
	$R^2$	$R^2$	$R^2$	$R^2$	$n$	$R^2$
SGF pH 1.5	0.7183	0.5825	0.8156	0.9420	0.4921	Anomalous diffusion
SIF pH 7	0.8273	0.6409	0.8317	0.9192	0.4725	Anomalous diffusion

### 3.6 Stability studies of GGNPs

Stability study of the GGNPs were carried out at the two different environmental conditions,

$30 \pm 2^\circ\text{C}/65 \pm 5\%\text{RH}$  and  $40 \pm 2^\circ\text{C}/75 \pm 5\%\text{RH}$  stored in stability chambers for 6months. It was observed that with the time

bathochromic shift in the spectra indicates that the GGNPs has a large surface tension energy leading to the aggregation process. There was no major alteration in the drug content of the

colloidal GGNPs were seen for the 6 months. The stability of GGNPs to the particle size distribution and absorbance of UV-Vis was given in **Table 3**.

**Table 3: The stability of GGNPs.**

Stability	$\lambda_{\max}$ (nm)	Absorbance	Particle size (nm)	Zeta Potential (mV)
<b>30 ± 2°C/65 ± 5%RH</b>				
0 Day	430	1.532	45.4	-10.0
1 <sup>st</sup> Day	430	1.564	45.4	-10.0
15 <sup>th</sup> Day	431	1.571	45.7	-16.2
30 <sup>th</sup> Day	430	1.571	46.7	-17.1
2 <sup>nd</sup> Month	434	1.563	47.8	-17.1
3 <sup>rd</sup> Month	435	1.5598	48.17	-18.1
6 <sup>th</sup> Month	435	1.5501	48.20	-20.1
<b>40 ± 2°C/75 ± 5%RH</b>				
0 Day	430	1.532	45.4	-10.0
1 <sup>st</sup> Day	430	1.598	45.4	-10.0
15 <sup>th</sup> Day	432	1.621	48.2	-17.2
30 <sup>th</sup> Day	435	1.623	49.17	-20.7
2 <sup>nd</sup> Month	435	1.612	49.17	-21.6
3 <sup>rd</sup> Month	438	1.596	50.40	-25.7
6 <sup>th</sup> Month	440	1.456	50.9	-29.1

### 3.7 Standardization of GG extract and GGNPs

The standardization of the GG extract and GGNPs with respect to the Glycyrrhizic acid

was done using an optimized HPLC method conditions as given in **Table 4**. The content of the marker compounds was analyzed by extrapolating from the calibration curve.

**Table 4: Optimized HPLC conditions**

Stationary Phase	ACE Generix C18 (5) 250 x 4.6 mm
Mobile Phase	A. 0.1% aqueous Formic acid solution B. Acetonitrile- 0.1% formic acid
Mode	Gradient (50:50 v/v)
Flow rate	0.9 ml/min
Detection	251 nm
Injection	10 $\mu$ L
Temperature	40° C

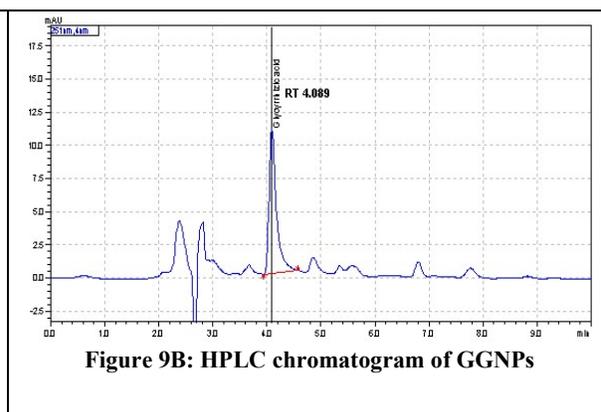
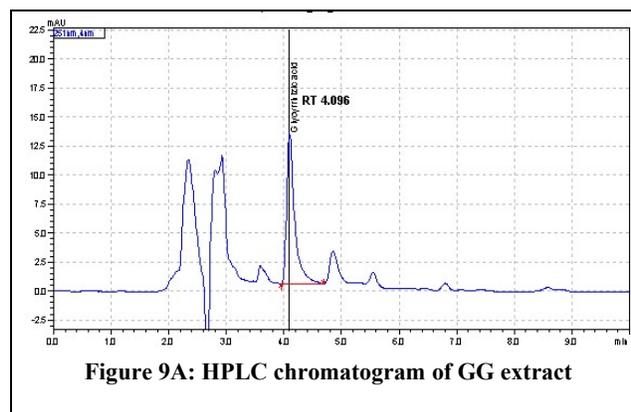


Table 5: Quantitative analysis of Glycyrrhizic acid in GG extract and GGNPs

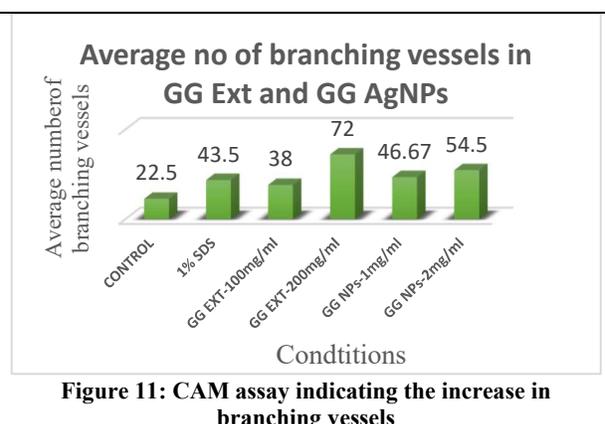
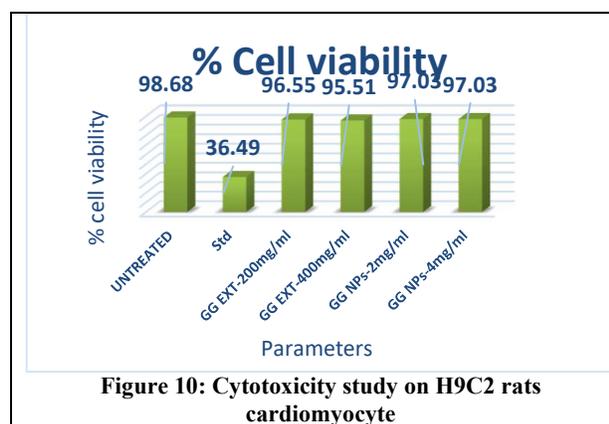
Sr No.	Sample	Concentration (mg) $\pm$ SD	%RSD	Standard deviation	Standard error	Retention time (mins)	Peak area
1	GG Extract (400mg)	5.75 $\pm$ 0.511	1.776	0.511	0.295	4.096 $\pm$ 0	122461 $\pm$ 2938
2	GGNPs (1500mg)	0.89 $\pm$ 0.105	0.430	0.105	0.061	4.089 $\pm$ 0.006	102064 $\pm$ 603.5

### 3.8 Evaluation of cardioprotective activity

#### 3.8.1 Tryphan blue size exclusion assay

The Tryphan blue exclusion study of the GG extract and GGNPs was done to determine the toxicity on H9C2 cell line. The different concentrations of GG extract (100, 200, 300,

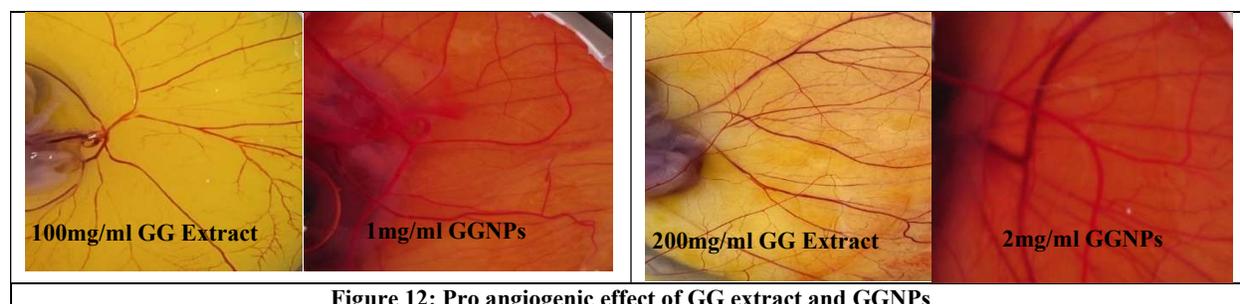
400, 500 mg/ml) and GGNPs (2, 4, 6, 8, 10 mg/ml) were added separately to the cell culture and incubated for 24hrs. The results indicated no toxicity after the 24 hrs of incubation for GG extract as well as GGNPs giving more than 90% of the cell viability.



#### 3.8.2 Chicken chorio-allantoic membrane assay

The GG extract and GGNPs were evaluated to check the pro angiogenesis activity on the fertilized chicken eggs. The results indicated satisfactory increase in the pro angiogenesis index after the incubation of 24 hours of the

drug treatment. % of Angiogenesis index of GG Extract: 100mg/ml = 68.89, 200mg/ml= 220.00 % of Angiogenesis index of GGNPs: 1mg/ml= 46.64, 2mg/ml= 142.22. Suggests the use of GGNPs in the cardio protection by enhancing the regeneration of the damaged cardiomyocytes in case of the CVDs.



#### 4. CONCLUSION

In conclusion, this work clearly offers a low-cost, environmentally benign, and easily repeatable method for synthesizing AgNPs using root extract of *G glabra* as a reducing, stabilizing, and capping agent. Bioactive elements included in *G glabra* root extract include flavonoids, tannins, polyphenols, terpenoids, and alkaloids that are necessary for the synthesis and stabilization of GGNPs. The characterization results using FTIR clearly showed that the capping of these phytoconstituents makes the synthesized GGNPs stable. The spectral results concluded that the produced GGNPs had a spherical form, were negatively charged, moderately polydispersed, stable, and were nanoscale in size. Furthermore, Bio evaluation of the GGNPs revealed the better proangiogenic effect as compared to the GG extract to help regenerate the damaged cardiomyocytes. Thus providing the safety profile and scientific background for the utilization of the AgNPs in the cardioprotective therapeutics.

#### Acknowledgement

The authors acknowledge STIC Kochin, IITB Mumbai, Averin Biotech Hyderabad, Diya labs Mumbai, and Principal. K.M. Kundnani College of pharmacy Mumbai, for extending their instrumental and technical support.

#### Conflict of Interest

The authors state that the research had no commercial or financial conflicts of interest.

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