Anti Cancer Activity of Biosynthesized Silver Nanoparticles using Vitis vinifera L against HT-29 Human Colon Carcinoma Cells through ROS Mediated Apoptosis

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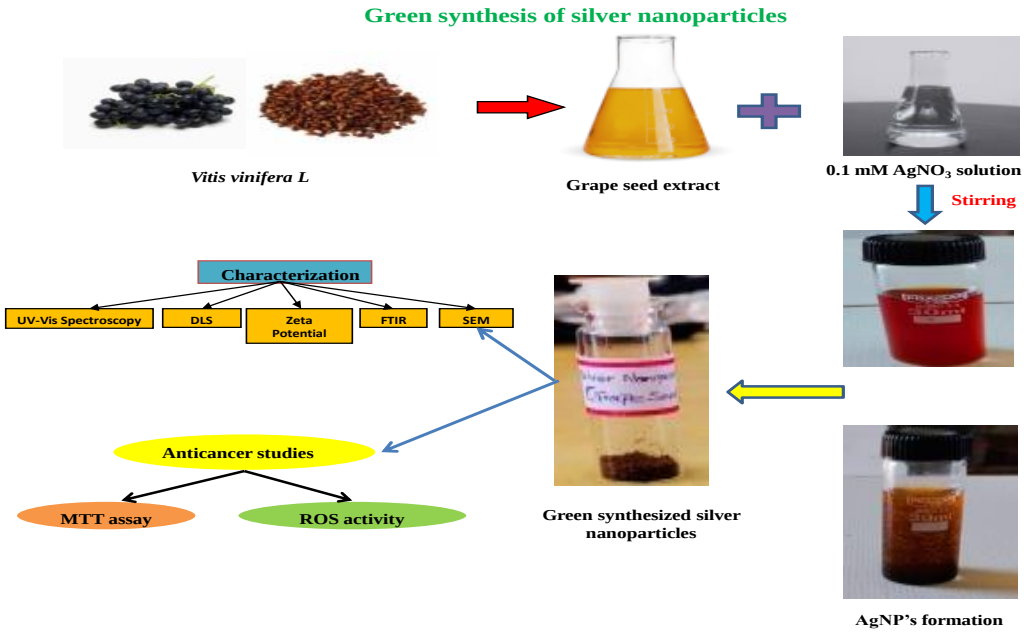
GRAPHICAL ABSTRACT



ABSTRACT:
The biosynthesis of silver nanoparticles, or AgNP's has garnered a lot of attention in the realm of nanotechnology because to its antimicrobial and medicinal qualities. In the current research context, it is expected that the green synthesis of metal nanoparticles will be a more economically and environmentally viable solution. We are presenting the synthesis, characterisation, and anticancer potential of AgNP's derived from the seed extract of Vitis vinifera as part of this endeavor. The synthesized AgNPs have been evaluated by tools including UV-Vis spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Zeta potential, and Dynamic Light Scattering (DLS). With an IC50 value of 8.05 μg/ml, the anticancer activity of the AgNP,s proved notable cytotoxicity towards HT-29 colon cancer cells. Additionally, the increased formation of ROS indicated pro-oxidant activity.

Keywords: Green Synthesis, Silver Nanoparticles, *Vitis vinifera L,* Anticancer Activity, HT-29 Colon Cancer Cells, Reactive Oxygen Species.

1. INTRODUCTION

Nanotechnology has emerged as a pioneering scientific discipline with diverse applications across multiple sectors, including biomedicine, agriculture, electronics, mechanics, optics, pharmaceutics, sensors, energy, cosmetics, and textiles[1]. This broad applicability has led to the development of innovative fields like nanobiotechnology[2]. The extensive growth of the field has sparked increased scientific interest in developing eco-friendly nanoparticles (NPs) from various plant sources. The primary rationale for synthesizing metallic nanoparticles from distinct plant components is that simplicity, cost-effectiveness, sustainability, and environmental friendliness of these processes[3]. Among the various types of nanoparticles, metal and metal oxide nanoparticles (NPs) are considered particularly effective[1].

Because of their submicroscopic size, nanoparticles have a variety of material properties and can be used practically in a variety of fields, such as drug delivery, engineering, nanomedicine, natural indemnity, and catalysis. They can also be used to treat disorders of the skin, liver problems, cardiovascular diseases (CVD), melanoma, and many other conditions[4]. Therefore, drugs enabled by nanotechnology may enhance their bioavailability and efficacy[5]. Nanoparticles can treat cancer by destroying all malignant cells[6], although they are less effective. Clinical trials using approved nanomedicines to treat cancer were approved by the Food and Drug Administration (FDA) in 2015[7].

One of the most common diseases, in 2018, 9.6 million people died from cancer worldwide; in low- and middle-income nations, 70% of these fatalities took place[8]. The main methods used to manage and treat cancer like chemotherapy, hormone therapy, radiation therapy and surgery. Various medicinal herbs with cytotoxic and anti-cancer properties have already been discovered [9]. The biological activity of plants has been attributed to polyphenols, including terpenes, alkaloids, flavonoids, and phenolic acids[10–12]. It has been known that certain triterpenoids, such as ursolic acid, boswellic acids, fomitellic acids, oleanolic acid, and pomolic acid, exhibit cytotoxic properties[13].There have also been reports of flavonoids with anticancer potential, such as quercetin, kaempferol, myricetin, and rutin[11].

The use of nanotechnology to fight cancer has opened up new research opportunities for interdisciplinary teams from chemistry, biology, engineering and medicine to diagnose, detect treat the disease[14]. The Food and Drug Administration (FDA-USA) accepts recently authorized the clinical use of nano-based anti-cancer medications[15].

Grapes (*Vitis vinifera L.)*[16–17] are a popular fruit around the world. They are a plentiful source of biologically active compounds that possess considerable antiinflammatory, anti-bacterial, hepatoprotective, and cardio protective activities[18-21].

This paper provides an explanation of nanoparticles, their varieties, and their uses across a range of fields, with an emphasis on the environmentally friendly production of silver nanoparticles from *Vitis vinifera L* seed extract and their anticancer efficacy.

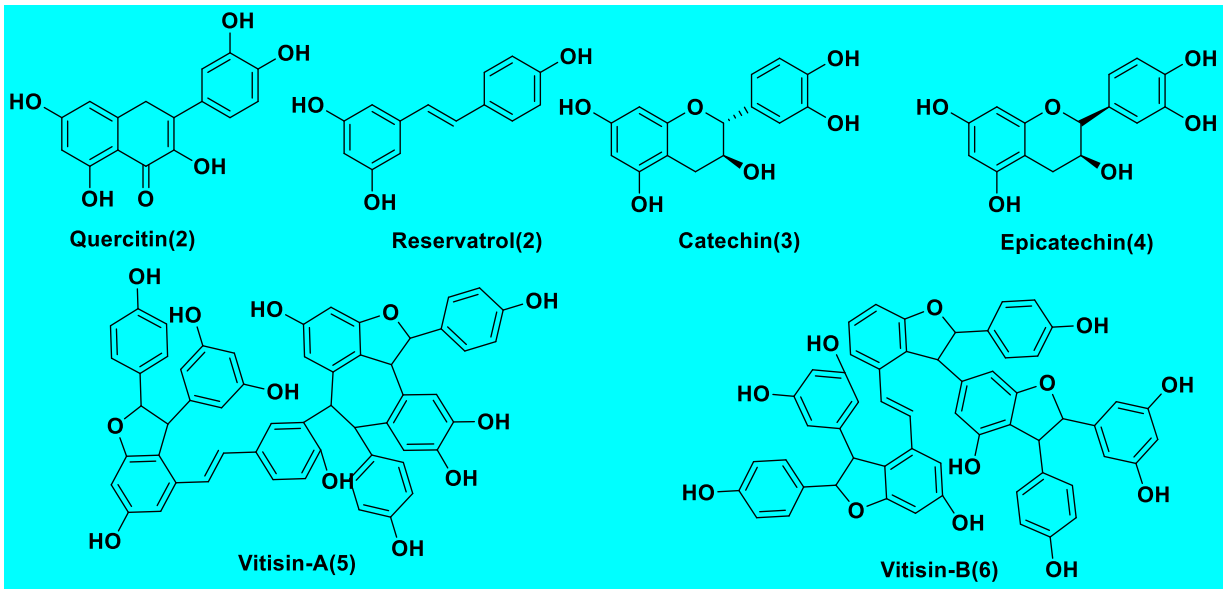


Figure.1: Phytochemical Constituents of *vitis vinifera L.*

2. MATERIALS AND METHODS:

2.1. Material Required:

Silver nanoparticles (AgNP's) were synthesized from ethanolic extract of seeds from *Vitis vinifera L* using silver nitrate ($AgNO_3$) as precursor for nanoparticle formation. Nanoparticles were thoroughly characterized using Zeta potential, SEM, FTIR, DLS, and UV-visible spectroscopy. AgNP's were biologically evaluated for their potential applications in various fields including biomedicine and environmental science.

2.2. Experimental Methods:

2.2.1. Source of fruit material:

We procured relatively fresh black grapes (*Vitis vinifera L*) from the local market in Kurnool, Andhra Pradesh, India. For identification and authentication, it was sent to Prof. Dr. Madhavasetty of the Department of Botany at SV University in Thirupathi, Andhra Pradesh, India. The specimen was stored in our laboratory for future reference and was given the voucher number 4213.

Figure 2: Fruit Sample

2.2.2. Preparation of Grape Seed Extract:

Fresh black grapes *(Vitis vinifera L)* were washed, crushed, and their seeds were dried and powdered. Defatting was carried out using petroleum ether followed by extraction with ethanol. The resulting extract was stored for future use.

2.2.3. Phytochemical Analysis:

The grape seed extract on phytochemical screening was found to have alkaloids, glycosides, carbohydrates, phenolic compounds, flavonoids, proteins, amino acids, and fixed oils. *Vitis vinifera L*. seed extract's phytochemical components were identified qualitatively using accepted methods previously detailed elsewhere.[22-23]. Phytochemicals tested included alkaloids, glycosides, carbohydrates, phenolic compounds, flavonoids, proteins, amino acids, and fixed oils.

2.2.4. Synthesis of silver nanoparticles (AgNP's):

A 0.1 mM $AgNO_3$ solution (mL) was combined with grape seed extract (mL) for the synthesis of silver nanoparticles. Toward formation of synthesizing nanoparticles, the mixture was heated to 40°C for 30 min and then allows stirring at ambient temperature. The finished product was centrifuged at 3000 rpm for 15 min in order to extract the AgNPs. The pellets were subsequently washed with distilled water and allow to air dry.

2.2.5. In Vitro Anticancer Studies:

2.2.5.1 MTT Assay: Cells in 96-well plates are exposed to various concentrations of the test substances (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml). MTT solution was added and incubated for four hours following 48 hours of incubation at 37°C with 5% CO_2. After DMSO was used to dissolve the resulting formazan crystals, absorbance was measured at 570 nm using a microplate reader. Graph Pad Prism Version 5.1 was used to calculate the IC_{50} values.

2.2.5.2. Measurement of Intracellular ROS Generation: In this study, intracellular reactive oxygen species (ROS) levels were measured using HT-29 colon cancer cell. The cells were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with low glucose and fetal bovine serum (FBS), along with an antibiotic-antimycotic solution to maintain cell viability. For the ROS assay, cells was seeded in 24-well flat-bottom micro plates containing coverslips and incubated overnight at 37°C in a CO_2 incubator to allow for adherence and growth. Following overnight incubation, the cells had been treated with 200 µl/mL each of the respective test compounds for 8 hours.

After treatment, cells were exposed to H_2DCFDA (D399), a non-fluorescent probe that permeates cells and is oxidized by ROS to form fluorescent dichlorofluorescein (DCF). The cells were subsequently fixed for 30 minutes with 4% paraformaldehyde after being washed with phosphate-buffered saline (PBS). The fixed coverslips were then examined under a fluorescent microscope to quantify intracellular ROS levels, providing insights into the oxidative stress response induced by the test compounds in HT-29 cells. This methodological approach allowed for the assessment of potential antioxidant or pro-oxidant properties of the compounds under investigation, contributing to the characterization of their biological activity relevant to oxidative stress pathways in cancer cells.

In this research, levels of intracellular reactive oxygen species (ROS) were assessed using HT-29 colon cancer cells. The cells were grown in Dulbecco's Modified Eagle Media (DMEM) that was supplemented with low glucose and fetal bovine serum (FBS), along with an antibiotic-antimycotic solution to ensure cell viability. For the ROS assay, the cells were placed in 24-well flat-bottom microplates with coverslips and incubated overnight at 37°C in a CO2 incubator to facilitate adherence and growth. After the overnight incubation, the cells were treated with 200 µl/mL of each respective test compound for duration of 8 hours. Following the treatment, the cells were treated with H2DCFDA (D399), a non-fluorescent probe that enters cells and is oxidized by ROS, resulting in the formation of fluorescent dichlorofluorescein (DCF). The cells were then fixed for 30 minutes using 4% paraformaldehyde after being rinsed with phosphate-buffered saline (PBS). The fixed coverslips were subsequently analyzed using a fluorescent microscope to measure intracellular ROS levels, offering insights into the oxidative stress response triggered by the test compounds in HT-29 cells. This methodological strategy enabled the evaluation of potential antioxidant or pro-oxidant characteristics of the compounds being studied, aiding in the characterization of their biological activity related to oxidative stress pathways in cancer cells.

3. RESULTS AND DISCUSSION

The ethanolic extract of *Vitis vinifera L*. contained phenolics, flavonoids, glycosides, alkaloids, tannins, and terpenoids, according to the qualitative examination of phytochemicals.

3.1. Visible Observation:

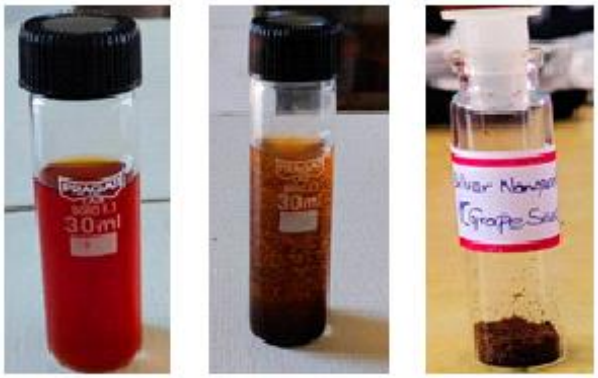


Figure. 3: Colour change of *Vitis vinifera* seed extract before after addition of $AgNO_3$.

3.2. Characterization of GSE Silver nanoparticles (AgNp's)

3.2.1. UV-Vis Spectroscopy:

The color changes observed upon exposure to plant extracts indicated a reduction of silver ions into silver nanoparticles. The phenomenon of Surface Plasmon Resonance (SPR) is the cause of the color shift. About 440 nm for grape seed extract, the distinctively sharp bands of silver nanoparticles were visible (Fig: 4).

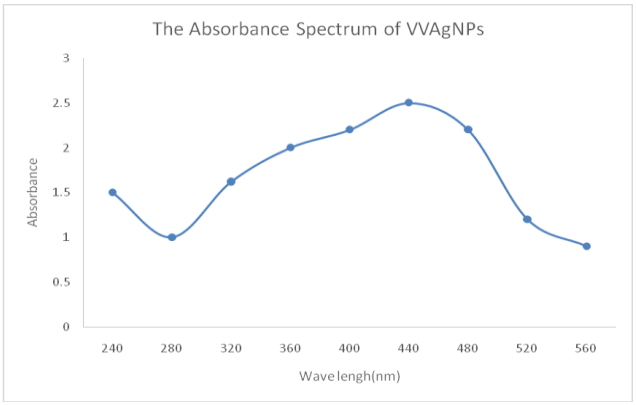


Figure.4: The absorbance spectrum of GSE silver nanoparticles Showing maximum absorbance near 440 nm.

3.2.2. DLS and Zeta Potential studies:

Dynamic light scattering (Photon Correlation Spectroscopy) revealed that AgNP's have an average size of 499.6 nm. The poly dispersity index (PDI) of grape seed silver nanoparticles is 0.844. The synthesized grape seed silver nanoparticles exhibited a zeta potential of -37.3 mV, indicating stability at room temperature. (Fig: 5)

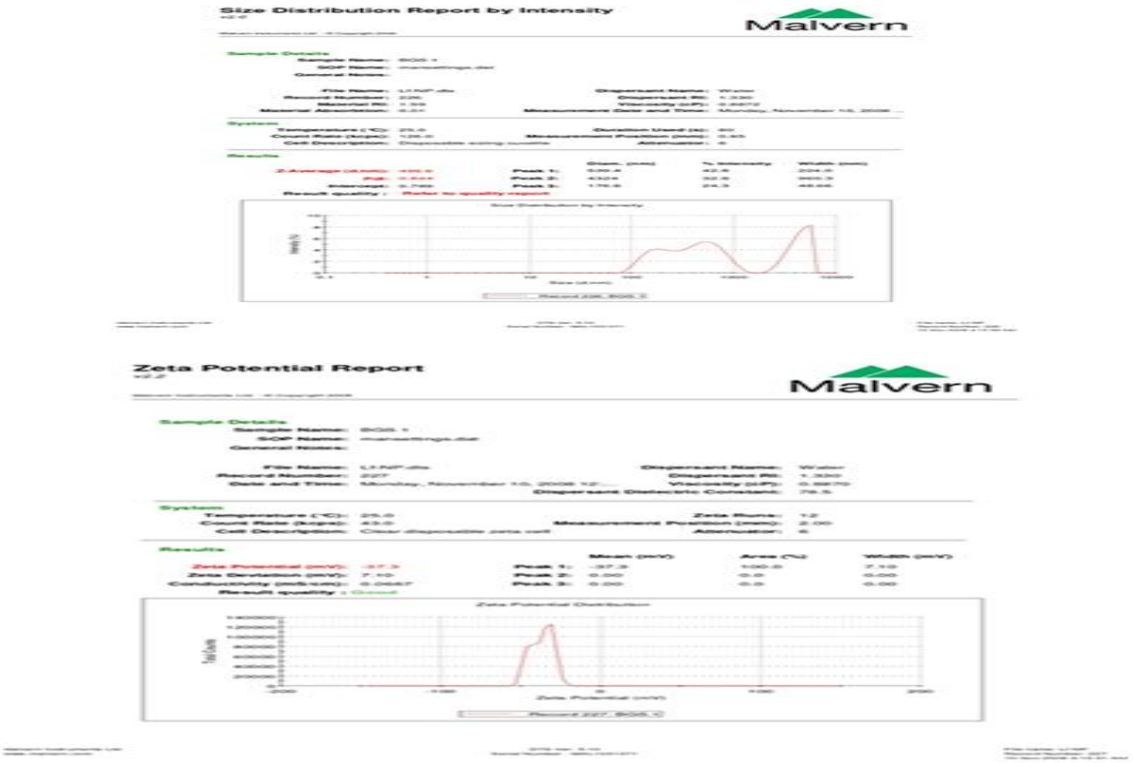


Figure 5: DLS & Zeta Potential of GSE-AgNPs

3.2.3. FTIR Analysis:

The main objective of FTIR analysis of AgNP's is to detect the reduction of silver ions and to identify the molecules that are used as coating and stabilizing agents. FTIR analysis spectra revealed peaks at 3349 (OH), 2923(Ar C-H), 3009, 2853(Ar C-H), 2076(C=O, NCS), 1743 (C=O) and 1608 cm^{-1} (Olefins).

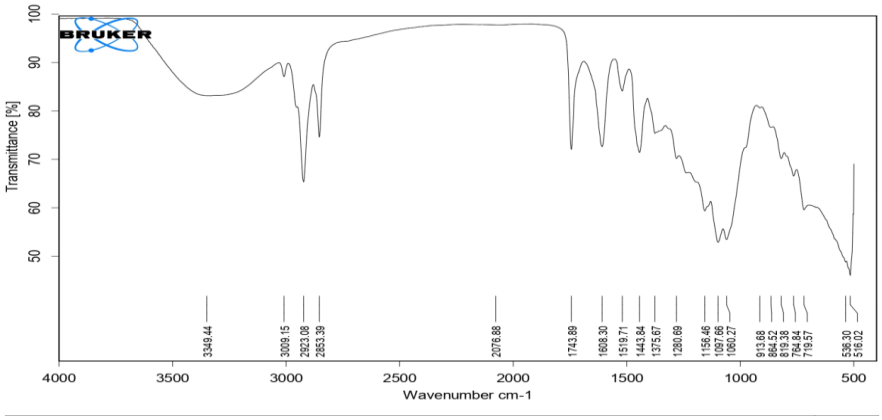


Figure.6: FTIR spectra of GSE-AgNP's

3.2.4. Scanning Electron Microscopy:

The concept of scanning electron microscopy is to provide the surface morphology of particles.

SEM images revealed the production of AgNP's from grape seed extract in spherical shape as shown in Fig.7.

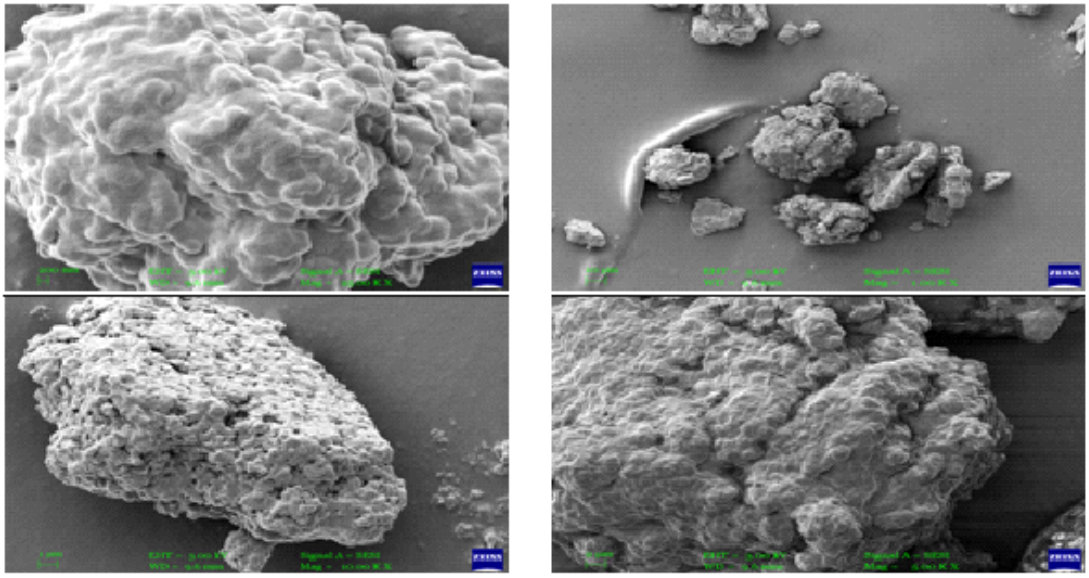


Figure.7: SEM studies of GSE-AgNPs

3.3. Cytotoxic studies against HT-29 Colon Cancer cell line by MTT assay:

The MTT assay was used to assess the cytotoxicity capability of VV AgNP's against the HT-29 colon carcinoma cell line. A significant increase in the % of cytotoxic value of the VV AgNP's treated cells was noted when compared to the standard. The IC_{50} for cytotoxicity was found to be the standard was 3.35 µg/ml and cells treated with VV AgNP's were 8.05

µg/ml being the most potent inhibitor. VV AgNP's demonstrated good cytotoxicity in HT-29 cell lines at concentrations of 50 µg/ml and 100 µg/ml, moderate cytotoxicity at 25 µg/ml, slight cytotoxicity at 6.25 µg/ml and 12.5 µg/ml, and no discernible cytotoxicity in HT-29 colon cancer cell lines exposed to 3.125 µg/ml VV AgNP's. However, when the concentration of VV AgNP's was raised to 100 µg/ml, there was a notable drop in cellular relative viability. The findings showed that VV AgNP's were cytotoxic to the examined colon cancer cell lines (HT-29) at greater concentrations and in a dose-dependent manner.

Table 4: The IC50 value of compounds (µg/ml)

Sample Codes	**HT-29**	
	Mean	**SD**
VV AgNP's	8.05	1.65
Doxorubicin	3.35	0.16

Table 5: Anti cancer activity of VV AgNP's against HT-29 colon cancer cell lines:

HT-29						
Concentration µg/ml	**VV AgNP's**			**Doxorubicin**		
100	28.92	21.32	33.09	25.31	23.46	23.3
50	31.37	31.86	33.09	27.26	25.98	27.26
25	37.75	38.24	39.71	29.95	27.25	28.39
12.5	42.16	45.83	42.65	30.51	28.93	29.35
6.25	45.83	48.04	47.3	31.78	30.06	32.88
3.125	69.36	69.61	68.14	35.01	34.83	33.57
Negative Control	100					

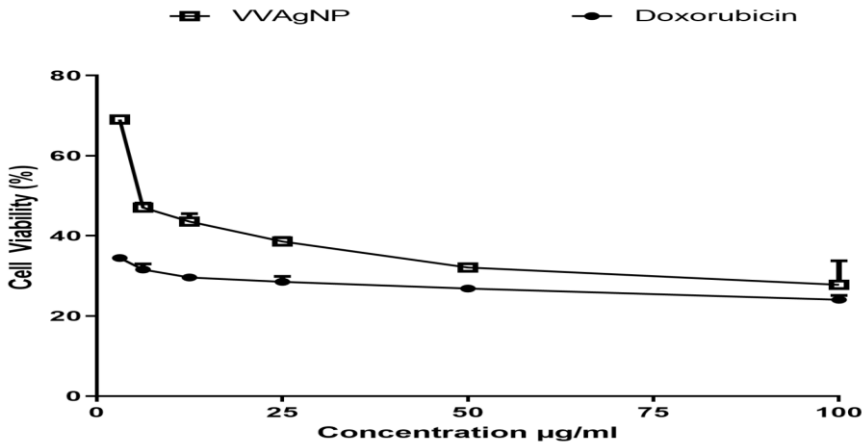


Figure.8: Reduction of HT-29 cell viability in response to VV AgNP's.

3.4. Measurement of intracellular ROS generation:

Reactive oxygen species (ROS) activity in cells is measured by the ROS Assay employing the fluorogenic probe DCFH-DA. Cellular esterases deacetylate this probe, which diffuses into cells and transforms into non-fluorescent DCFH. DCFH undergoes oxidation by ROS to DCF, which produces green fluorescence (excitation: 485 nm, emission: 530 nm).

Table 6: Green fluorescence intensity (%) of GSE AgNP's**.**

Green fluorescence intensity (%)					
SL No	**Sample Code**	**Area**	**Mean**	**Min**	**Max**
1	**NC**	3145728	10.028	0	253
2		3145728	10.962	0	253
3	**VVAgNP's**	3145728	22.656	0	253
4		3145728	25.472	0	253

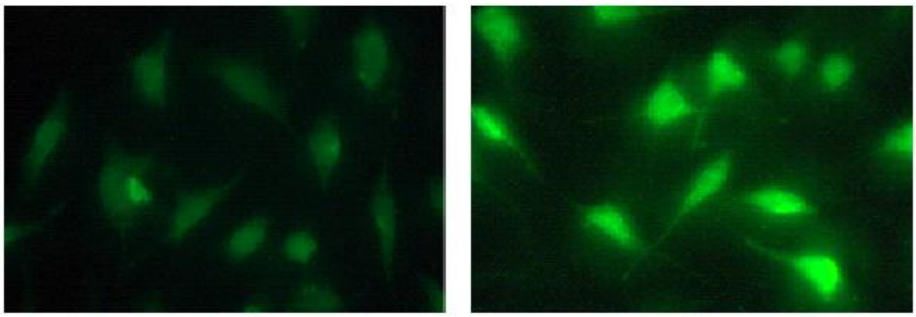


Figure. 9: Green fluorescence intensity of GSE Ag NPs. A) and Normal B) cells of biosynthesized AgNP's against D399 by fluorescent microscope using Dulbecco's Modified Eagle Media.

4. CONCLUSION

In summary, grape fruit extract (Vitis vinifera) was used as a reducing and capping agent in the green synthesis procedures to create VV AgNP's in an easy, economical, and environmentally responsible way. The synthesized Grape Seed Extract of Silver Nanoparticles (GSE AgNPs) has been extensively described and biologically assessed against the HT-29 Colon Cancer cell line using the MTT assay. They showed an IC50 value of 8.05 µg/ml and deserved more research for utilized in biomedical applications.

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CONFLICT OF INTEREST

Authors declare no conflict of interest

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