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Antitumor activity of biosynthesized silver nano particles from leaves of *Momordica charantia* against MCF-7 cell line

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Abstract: Nano materials are utilized in the pharmaceutical field which can be developed into novel agents with less side effects and cost effective process. Biosynthesized silver nanoparticle from *Momordica charantia*leaves aqueous extract and Invitro study of anticancer activity against Breast cancer cell line MCF-7 showed significant activity. The MTT assay methods were followed for evaluating the cell viability and cytotoxicity for both plant extract and plant-silver nanoparticle. The subjected cells were drawn for the DNA fragmentation assay and the DNA bands were visualized in GEL-Documentation and the DNA ladder of 1KB used as Marker for reference. The Cell toxicity of 63.26% on plant extract and 67.16% on plant –silver naoparticles synthesized activity were recorded against MCF-7 cell line and DNA bands were sheered and recorded at 1.5 % for plant extract and 2.5% of plant –silver naoparticles. The FT-IR analysis of silver nano particle from plant extract showed presence of alkynes, alkene and amine functional group and SEM-EDX analysis showed the particle size of 96.3nm and presence of Cl, Ag, O, Na, Mg, Si and Al. The functional development of novel compounds from silver nano particle from biosynthesized process has good potential than other characteristic drugs used.
Key words: Silver nano particle, Biosynthesis, MTT, Fragmentation, Cancer.

Introduction:

Nano materials are of reduced physical, chemical and biological materials which can be used to develop novel agents can be used over resistant pathogens and act as anticancer agents[1].The sustainability in the initiatives of using green chemistry to develop and focus issues in multi disciplinary fields of research by significantly cost effective methods to produce nanomaterials which are used in multiple applications including biological and biomedical fields which is reduced in toxicity and depends based on the size, shape, composition and uniformal chemistry to prolong its lifespan of Nanoparticles as it is important to develop stabilizing agents and mechanism which can be produced to biological environment[2].

The physicochemical properties for nanoparticles synthesis include photochemical reduction, laser ablation and electrochemistry which are expensive. There are different methods have been developed to synthesis of nanoparticles by physical, chemical and biological[3].The biological or green synthesis has received complete attention in area of research and Nanoparticle synthesis by using living organism, cellular extracts, and plant based products, bacteria and fungi. And it could be as simple, viable and non-toxic forms compared to other methods of synthesis [4].Silver nanoparticles are studied and applied in variant fields of catalysis, photonics, biosensing, diagnostic and antimicrobial agents[5] and promoted to heal wounds and possess anticancerous activity [6].

The presence of secondary metabolities in plants makes to redox reaction and can be exploited for biosynthesis of nanoparticles. Theses metabolities poses active biocompounds which are stable and can investigate themechanism followed by possible medical value and treatment[7]. The research groups around the world have successfully demonstrated the efficacy of silver nanoparticles having potential cytotoxicity against cancer cells[8] and antiangiogenic property in micro vascular endothelial cells[9].

Naturally produced plant materials could be beneficial to develop as an agent with target based medication which is possible by nanoformulations and properties regarding plans and its constituents were analyzed and information should be gathered from medicinal practioners[10].

Cancer is a disease which is accumulated in a range of 10-12 million new cases, 5-7 million deaths by its multiple forms[11]. The leading forms of cancer treatment were ineffective which has lent to develop newer forms of methods to achieve medical formulations[12]. Though the various cytotoxic agents used in treatment of breast cancer like doxorubicin, cisplatin, bleomycin and many other has temporary relief and its effects are uncertain, for that it is necessary to develop novel treating molecule and its mechanism[13].

The reactive oxygen species (ROS) in the cell is to produce apoptosis and generation of ROS in cells by oxidative stress and treating those free radicals which is produced during cellular metabolism by inhibiting the JNK pathway leads to mitochondrial- dependent apoptosis and includes cytotoxicity by oxidative cell damage[14].The ROS has role in DNA damage and Caspase enzyme is a important molecule in apoptosis by cleaving of inhibitory and translocation of caspase activated DNAse to nucleus results in DNA fragmentation[15]. Because the major compounds of phenolic and flavonoid possess antioxidant molecule which can reduce oxidative stress and lipid peroxidation of the cells leads to either cell damage to form malignant cells[16].

Momordica charantia(Bitter guard) is a flowering plant in the family of Cucurbitaccae has simple leaves with 5-7 palmately lobed tendrils branched or unbranched. Fruits are ovoid, spindle shaped and ridged with seeds are white in appearance before ripening and red after it, the outer skin is edible and both vegetable and fruit is used as raw vegetable. It is used as agents to anthelmintic, antibacterial, antidiabetic, antimicrobial, antioxidant, antitumor, hypoglycemic and cytotoxic[17]. Among various nanoparticles, silver nanoparticle has a wide area of applications in multiple fields and has been taken for the present study by synthesizing nanoparticles using leaves of *Momordica charantia*and treating against cancer cell line.

Experimental:

Collection of plant:

The plant leaves were collected from the locals of chengalpet region of Kanchipuram District, Tamil Nadu, India with the help of local villagers and washed with running tap water to remove dust materials.

Preparation of leaf extract

The leaves of *Momordica charantia* were rinsed with distilled water and shade dried in room temperature for 3 weeks and then 150g of dried leaves were powdered using kitchen blender. 5g of the powdered leaf was dissolved in 100ml of distilled water in 250ml of conical flask .The mixture was allowed to boil for 20mins at 55-60^{0}C.After that the leaf extract was filtered through Whatman filter paper No. 1 and stored at 4°C.Then the solution was used as stock solution.

Preparation of 1mM silver nitrate solution:

Analytical grade silver nitrate ($AgNO_3$) was purchased from Krishna scientific chemical suppliers. A 1mM stock solution of $AgNO_3$ in distilled water was prepared according to the following calculations

The molecular weight of $AgNO_3$:
[Ag-107.87, N-14, O-16] = 107.87 + 14 + (16 × 3) = 169.87
Therefore, Molar mass of $AgNO_3$ = 169.87 g

$$\text{Molarity} = \frac{\text{Molecular weight x Required Molarity x Required volume}}{1000}$$

0.169g of silver nitrate was added in 1000ml of distilled water to prepare 1Mm silver nitrate solution.

Synthesis of silver nanoparticle

The 10ml of *Momordica charantia* leaf extract was added to 90 ml of 1mM aqueous of $AgNO_3$ for reduction of silver nitrate in to silver ions. Aqueous mixture was incubating in direct sunlight condition. After the sunlight exposure color changes from yellowish to dark brown color due to the reduction of silver nitrate into silver ions. The color changes indicate the formation of silver nanoparticles.

Characterization methods for synthesis of silver nanoparticle:

UV-Vis spectroscopy

UV-Vis spectroscopy can be comprehended as absorption spectroscopy in the spectral region of ultra-violet and visible spectra. It uses light in visible and near-UV range to promote outer electrons to higher energy levels. The UV-Vis spectra have broad features that are of limited use for sample identification but are very useful for quantitative measurements. The concentration of analytes in solution can be determined by measuring the absorbance at specific wavelength and applying the Beer-Lambert Law, the UV-Vis range spans the range of human visual acuity and to observe the optical property of biosynthesized silver nanoparticles, samples were periodically analyzed for UV–Vis spectroscopic studies at room temperature at 200-700nm range and it is useful to characterize the absorption, transmission, and reflectivity of a variety of technologically important materials, such as pigments, coatings etc.

Scanning electron microscopy and Energy dispersive x-ray spectroscopy

Scanning electron microscopy (SEM) is a technique that uses electrons instead of light to form an output image and allowed researchers to examine a much larger variety of specimens. It has a large depth of field, which allows more of a specimen to be in focus at one time. The SEM also has much higher resolution closely spaced specimens can be magnified at much higher levels.Energy Dispersive X-Ray Spectroscopy (EDX) is a technique that provides the elemental curve as output. This analytical technique is generally used in conjunction with the Scanning Electron Microscopy (SEM). EDX technique primarily detects the X-rays emitted from the sample during the process of bombardment by an electron beam for characterizing the elemental composition of the sample of interest. Quantitative results can be obtained from the relative x-ray counts at the characteristic energy levels for the sample constituents. Some typical applications include alloy identification, foreign material analysis, coating composition analysis etc. EDX helped to verify the presence of silver in the sample and its percentage as well.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performedfor characterizing size and shape of biosynthesizedsilver nanoparticles. The sample was firstsonicated (Vibronics VS 80) for 15 min. A drop ofthis solution was loaded on carbon-coated coppergrids, and solvent was allowed to evaporate underInfrared light for 30 min. TEM measurements wereperformed on Philips model CM 200 instrumentoperated at an accelerating voltage at 200 kV.

X-ray diffraction (XRD)

X-ray diffraction (XRD) measurements of film of thebiologically synthesized silver nanoparticles solutioncast onto glass slides were done on a εMMAdiffractometer operating at a voltage of 40 kV and current of 20 mA with Cu K (α) radiation of1.54187 nm wavelength. The scanning as done inthe region of 2h from 20°C to 80°C at 0.02°/min and thetime constant was 2s.

Fourier Transform infrared spectroscopy

For Fourier transform infrared spectroscopy (FTIR)measurements, the reaction mixture was centrifugedat 15,000 rpm for 15 min after complete reduction ofAgNO3 by the *Momordica charantia*leaf extract to separate Agnanoparticles from biomass or other bioorganiccompounds which may interfere in analyzing molecule–AgNPs interaction. The Ag nanoparticles pelletobtained after centrifugation were re-dispersed inwater and washed (centrifugation and re-dispersion)with distilled water for three times. Finally, thesamples were dried and grinded with KBr pellets andanalyzed on a Nicolet IR 200 (Thermo electron corp)model.

Antitumor activity:

The anticancer activity was performed on the breast cancer cell line (MCF-7) which is procured form NCCS, Pune, India and maintained in specific medium for its growth and carried MTT assay for plant extract and Silver nanoparticle of the plant extract.

The MTT assay[18] is based on the ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product. Cells were maintained in DMEM medium, supplemented with 10% Fetal Bovine Serum, at 37^{o}C in humidified atmosphere with 5% CO_2.The cells were plated in 96 well flat bottom tissue culture plates at a density approximately1.2 X 10^4 cells/well and allowed to attach overnight at 37^{o}C. The medium was then discarded and cells were incubated with different concentrations of the extracts for 24 hours. After the incubation, medium was discarded and 100µl fresh medium was added with 10µl of MTT (5mg/ml). After 4 hours, the medium was discarded and 100µl of DMSO was added to dissolve the formazan crystals. Then, the absorbance was read at 570nm in a microtitre plate reader. Cyclophosphamide was used as a positive control.

Cell survival was calculated by the following formula:
Viability % = (Test OD/ Control OD) X 100
Cytotoxicity % = 100 – Viability%

DNA fragmentation assay:

The experiment was characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of roughly 50 base pairs (bp) and multiples of (100, 150 etc.). This effect can be used to detect apoptosis, for example via the DNA laddering assay.

In 24 flat-wells plate, incubate 2x10^5 cancer cells (triplicate wells of 10^5 per well) with different samples (GS, CS and quercetin IC 50 range concentrations) (10^5 target cells per well). Add fresh DMEM medium and allow for 24 hour incubation, collect the cell sample in 1.5 ml eppendorf tube, spin down, resuspend with 0.5 ml PBS in 1.5 ml eppendorf tubes, and add 55µl of lysis buffer (40 ml of 0.5 M EDTA 5 ml of 1 M TrisCl buffer pH 8.0 5 ml of 100% Triton X-100 50 ml of H2O) for 20 min on ice (4°C). Centrifuge the eppendorf tubes in cold at 12,000 g for 30 minutes. Transfer the samples to new 1.5 ml eppendorf tubes and then extract the supernatant with 1:1 mixture of phenol: chloroform (gentle agitation for 5 min followed by centrifugation) and precipitate in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. Spin down, decant, and resuspend the precipitates in 30ul of deionized water-RNase solution (0.4ml water + 5ul of RNase) and 5ul of loading buffer for 30 minutes at 37°C. Also insert 2ul of DNA ladder (marker) on the outer lanes. Run the 1.2% gel at 5V for 5min before increasing to 100V. After the dye front reach ¾ of the gel, observe the image of DNA shearing in 312nm UV illuminator[19].

Results:

Synthesis of Nanoparticles:

The aqueous plant extract was prepared and preceded to synthesis of silver nano particles under controlled conditions. The plant extract color change from initial color to brown color shows the conversion and synthesis of nanoparticles by redox reaction and subjected characterization studies (Figure 1).

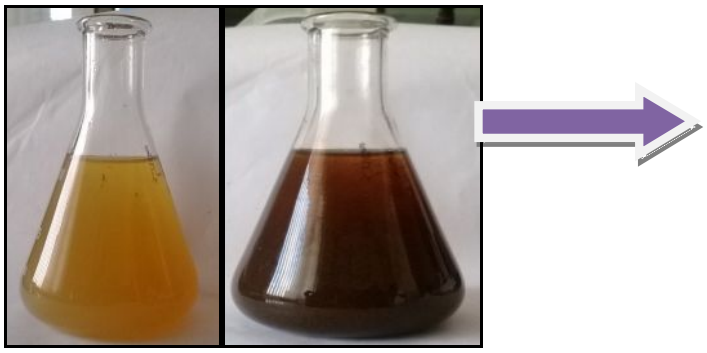

Figure 1: a)Before synthesis of AgNPsb)After synthesis of AgNPs

UV-Vis spectroscopy analysis:

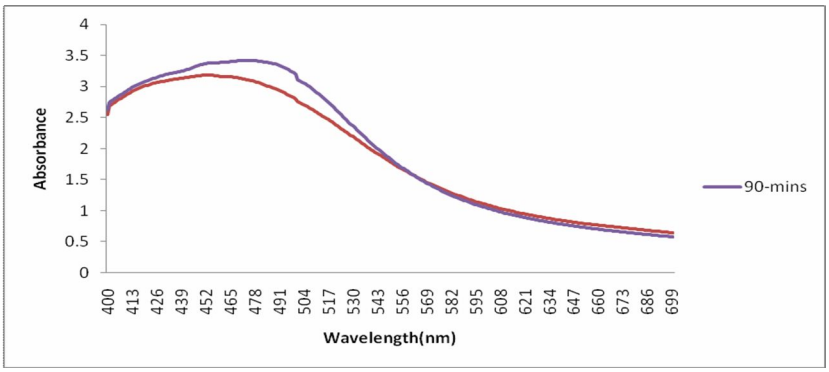

Figure 2: UV spectrophotometer analysis of synthesized nano particle.

The UV-VIS spectrometer analysis consists of plot which is recorded the scanning limits employed for nano particle synthesis and dielectric constant of solution helps to create uniform size of the particle with no evidence for aggregation or variance until the 410-490 nm during reaction period (Figure 2) and consists of silver nano particle which is confirmed by SEM and TEM analysis.

Scanning electron microscopy and Energy dispersive x-ray spectroscopy

Scanning electron microscopy provided further insight into the morphology and size details of the silver nanoparticles. The SEM image showing the high density silver nanoparticles synthesized by the *M.charantia* extract further confirmed the development of silver nanostructures and EDAX analysis at size of approximately 91.63nm(Figure 3).

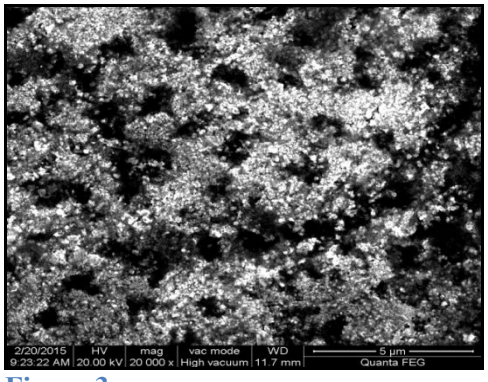

Figure 3a

Fig 3a)SEM image with 5µm

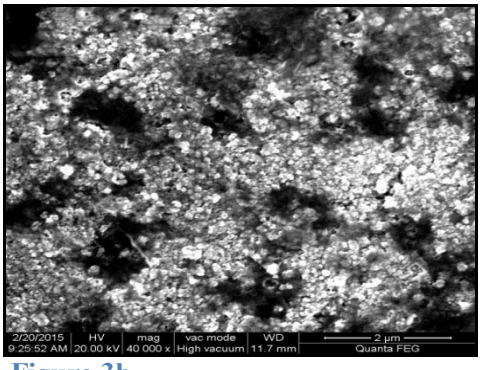

Figure 3b

Fig 3b) SEM image with 2µm

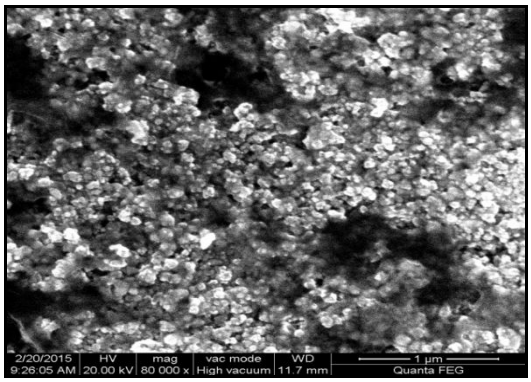

Figure3c

Fig 3c) SEM image with 1µm

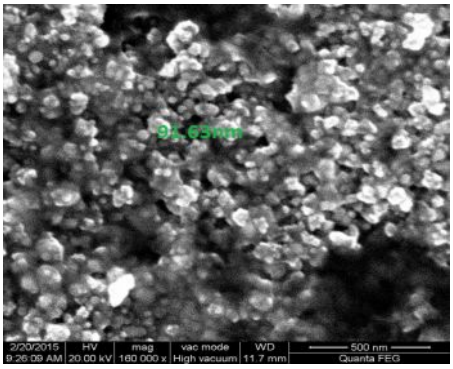

Figure 3d

Fig 3d) SEM image with 500nm

Figure 3:SEM images with different scales

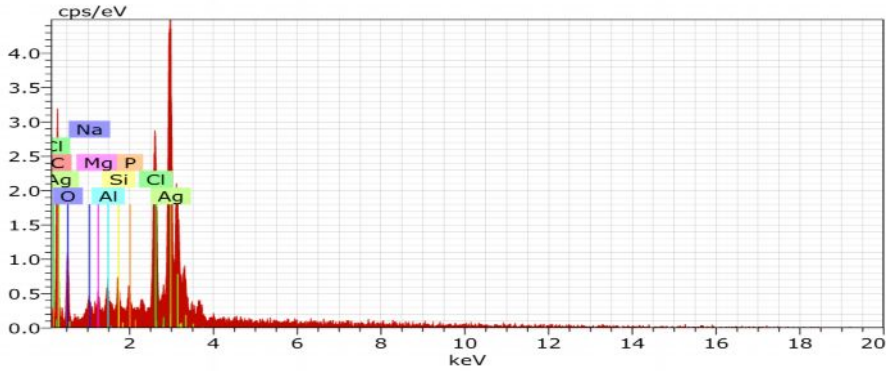

Figure 4: EDAX analysis of synthesized nano particle.

The peak observed in different ranges showing the principal structures of Cl, C, Ag, O, Na, Mg, Si and Al which including synthesized nanoparticles of silver and plant constituents which is reduced along with Naoparticles (Figure 4)

Transmission electron microscopy

The transmission electron spectrometer resulted in outer surface of the synthesized nanoparticle images and exhibiting its resolution in multiple nm(Figure 5)

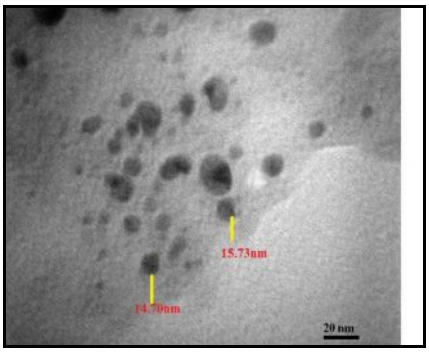

Figure 5a

Fig 5a) TEM image with 20nm.

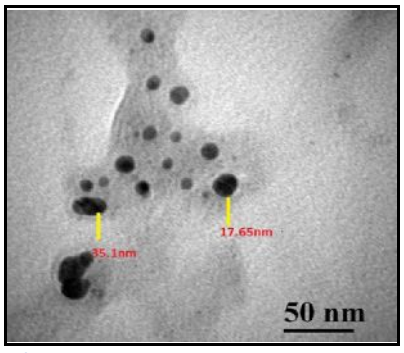

Figure 5b

Fig 5b) TEM image with 50nm

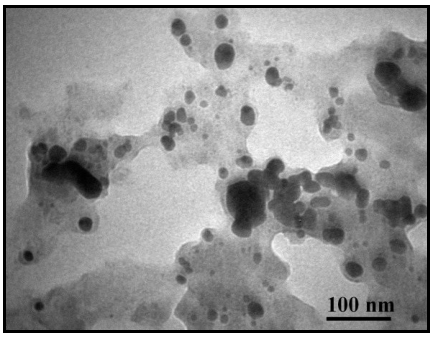

Figure 5c

Fig 5c) TEM image with 100nm

Figure 5: TEM images with different scales

X-ray diffraction analysis

The XRD pattern of synthesized Silvernanoparticle using *Momordica charantia* extract were recorded and typical XRD pattern is shown in The diffraction peaks are indexed as(111), (200), (220), (311) planes ofa pure face centered crystalline (fcc) structureof silver. Crystallite size of AgNPs asestimated from the FWHM of different peaksusing the Scherer's formula and diffractionlines observed at 2θ angle. Apart from these peaks, therecorded XRD pattern shows additional unassigned peaks. This may be due to theformation of the crystalline bio-organic compounds/metalloproteins that are present in *Momordica charantia* (Figure 6).

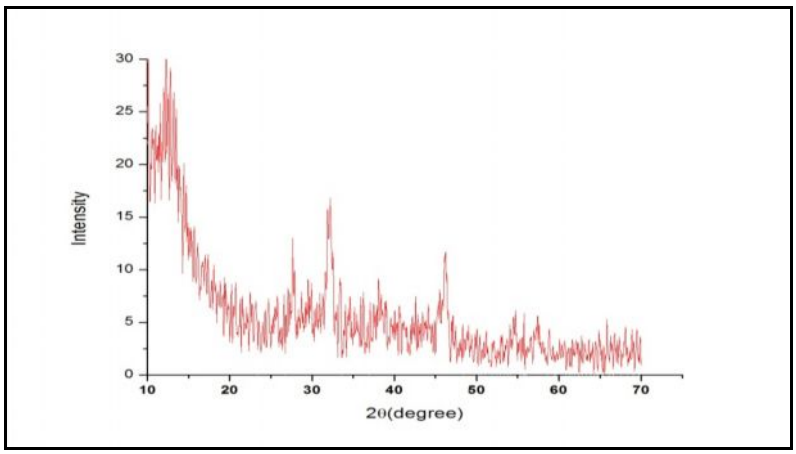

Figure 6: XRD analysis.

Fourier transform infrared spectroscopy analysis:

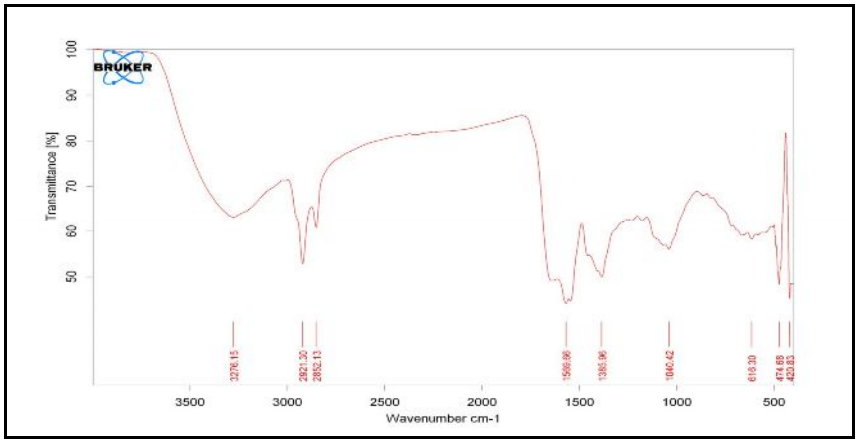

Figure 7: FT-IR analysis of *Momordica charantia.*

The functional group determination through FT-IR analysis has ensured the presence of multiple patterns at 3276.15, 2921.30, 2852.13, 1569.66, 1385.96, 1040.42, 616.30, 474.68 and 420.83 cm-1 of silver nanoparticles of *Momordica charantia*showing the presence of primary amines, alkanes, alkynes and aromatic functional groups (Figure 7).

MTT assay:

The cell viability and toxicity assay were carried out for the plant extract and silver nano particles of*Momordica charantia*were tabulated in table 1.

Table 1: Viability of the MCF-7 Cancer cell line against Plant and Ag nanoparticle extract.

Concentration in µg/ml	Plant leaf extract viability	Silver nanoparticle with plant leaf extract viability
12	90.51	88.01
25	84.36	78.06
50	79.18	69.08
100	36.74	32.84

From the inferred data the plant extract cytotoxicity is 63.26% at the concentration 100 µg/ml of stock concentration (Figure 9) and silver nanoparticle extract showed cytotoxicity of 67.16% at concentration of 100 µg/ml (Figure 10), a fraction higher than the plant extract which can be developed into active substance by studying and characterizing its active principle.

The graphical figure shows the viability of the Plant and silver nanoparticle extract of *Momordica charantia*(Figure 8).

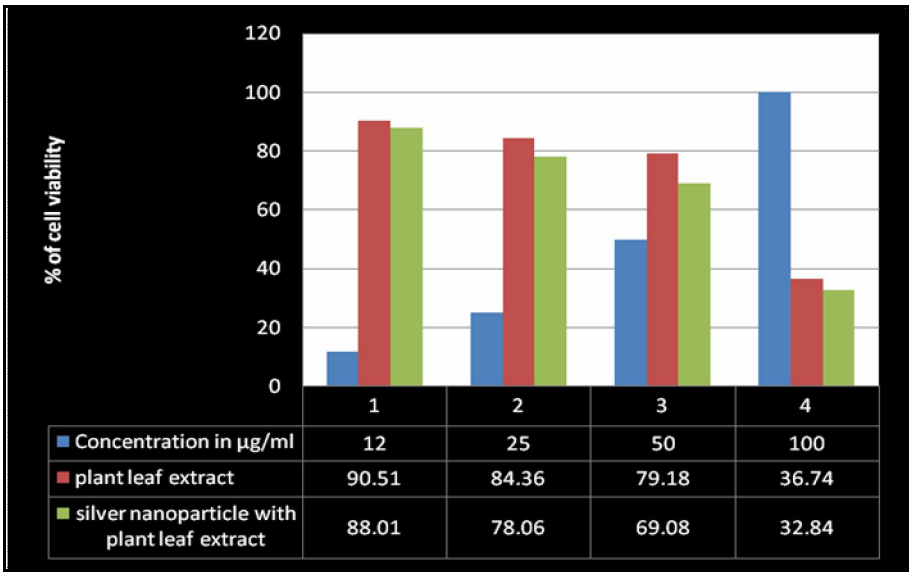

Figure 8: The viability of plant and nanoparticle of the extract.

The figure below represented the activity of Plant and Nanoparticle extract against MCF-7 cell line.

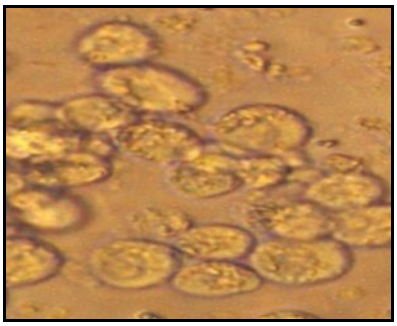

Figure 9a
Toxicity-12µg/ml

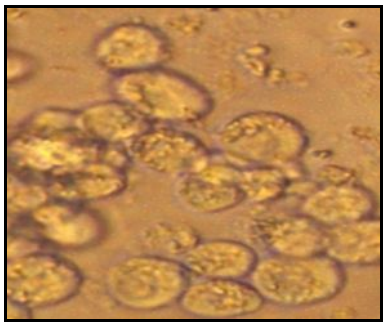

Figure 9b
Toxicity-25µg/ml

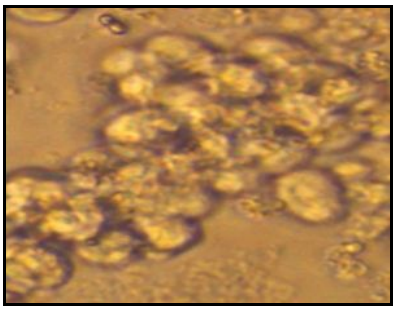

Figure 9c
Toxicity-50µg/ml

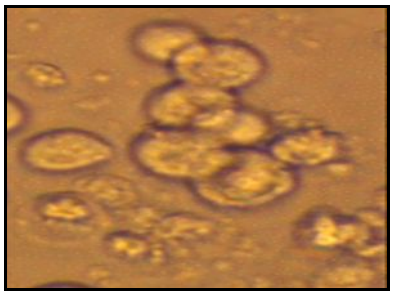

Figure 9d
Toxicity-100µg/ml

Figure 9 a, b, c,d).Cytotoxicity of plant leaf extract against MCF-7 cell line by MTT cell viability assay.

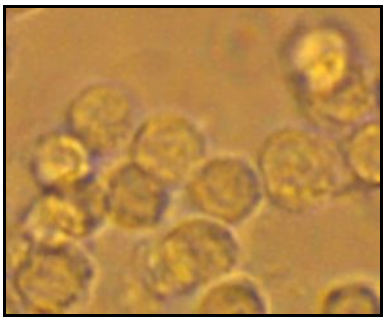

Figure 10a
Toxicity-12µg/ml

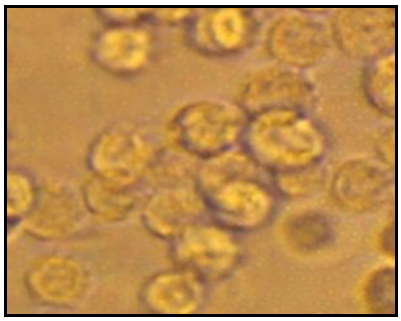

Figure 10b
Toxicity-25µg/ml

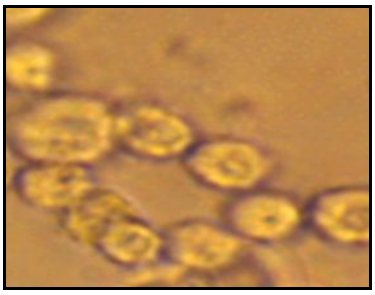

Figure 10c
Toxicity-50µg/ml

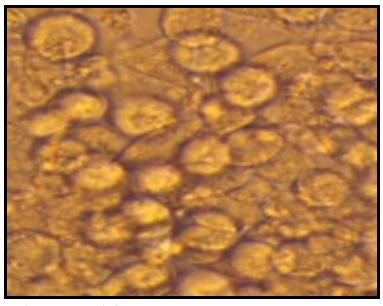

Figure 10d
Toxicity-100µg/ml

Figure 10 a, b, c,d).Cytotoxicity of synthesized silver nanoparticle with plant leaf extract againstMCF-7 cell line by MTT cell viability assay.

DNA fragmentation assay:

The excretion of the DNA apoptosis is evaluated by band of the DNA obtained from the treated cell against plant extract and Silver nano particle of the *Momordica charantia*is tabulated in table 2, Figure 11 and 12.

Table 2: The DNA fragmentation % of the treated cell line.

% of DNA Damage	Control	Plant extract	NP-Plant extract
	1.1	2.12	2.76

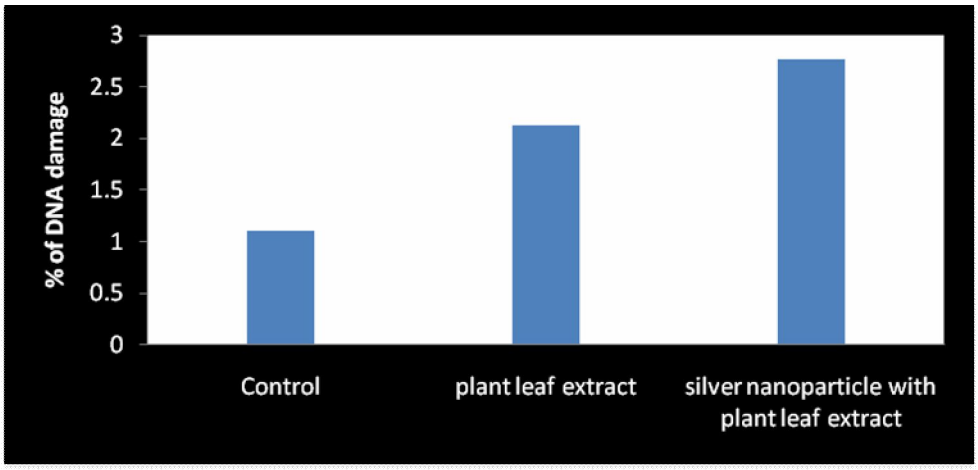

Figure 11: Graphical representation of the DNA fragmentation.

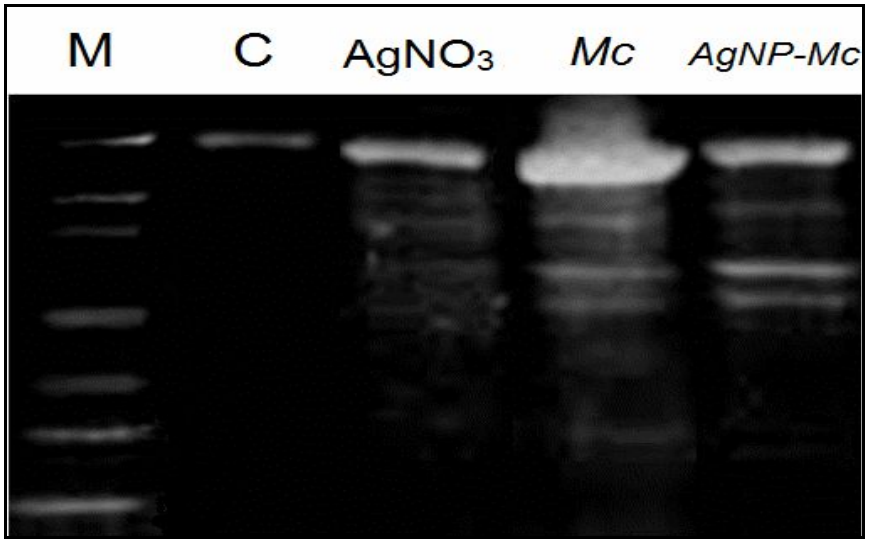

Figure 12: GEL DOCUMENTATION of the DNA bands.

Discussion:

The bio based synthesis of nanoparticle which showed significant activities on cancer studies which can be developed into potential anticancer agent and develop into active pharma molecule.*M.charantia* possess secondary metabolities in various extracts which can be treated against human cancer cell lines and it has activity over significant percentage could develop into active biocompounds which is studied both *Invitro and Invivo*[20].The anticancerous activity of *Momordica charantia* plant possess bioactive compounds which are less toxic to normal cells and affecting more targeted cancer cells which is studied against breast cancer cell which

proliferated by producing cell cycle regulatory genes which promotes apoptosis[21]. Dose dependent cytotoxicity against DLA cells through caspase enzyme were evaluated *Invitro* against silver nano particles which showed activity and it is confirmed by histopathological studies of DLA cells[8].

Silver nanoparticles synthesized from *Catharanthus roseus* leaves aqueous extract posses antiplasmodial activity with the nanoparticle showing 35-55 nm in size and it is recorded against *P.flaciparum*[22]. The methanol extract of *M.charantia* fruit were studied against different cancer cell lines upon that Breast cancer cell line (MCF-7) shows significant activity around 99% of cytotoxicity at 100 µg/ml concentration and aqueous extract showing 42% cytotoxic activity at 100 µg/ml concentration which can be developed into anticancerous agent[23].

Invitro analysis of anticancer potentials of green synthesized nanoparticle from *Citrullus colocynthis* were studied against different cancer cell lines of Colon (HCT-116), Breast (MCF-7), Liver (Hep-G2) and Intestine (CACO-2) and shows significant activity in HCT-116 and Hep-G2 with lesser activity against MCF-7 cell lines[24]. Combined activity of silver nanoparticle and chemotherapeutic agents like Cyclophosphamide, mercaptopurine and busulfan which is used to treat cancer showed significant activity over 75% cytotoxicity when compared to tested separately against THP-1 cancer cell line[25]. Jayachitra *et al.,* 2014 has tested MCF-7 cancer cell line against nanoparticle synthesized from *Cassia fistula* leaf extract and showed significant anticancer activity by dose dependent manner by increasing concentration of Silver nanoparticles[26].

Water extracts of *Momordica charantia*on cell viability and its cellular mechanism of action was studied against six different cancer cell lines on dose dependent manner by MTT assay, it shows significant activity and mechanism of action by caspases 3 and 9 showed increases in levels of free calcium through release of cytochrome – c which resulted in the mitochondrial apoptosis[27].Plant mediated silver nanoparticle synthesis based on phytoconstituents which is tested on Breast cancer cell line (MCF-7) using MTT assay and IC50 values evaluated by dose dependent manner[28].

Conclusion:

The plant M.charantia poses important phytoconstituents and it could add only to beneficiary effect of green synthesizing the nanoparticle from the plant. It could develop into active agent of chemotherapy based on the inferred data and activity over significance of 65%. The potential activity may because of the plant phytoconstituents and synthesizing silver nano particle leads to develop a new variant in the field of anticancer agents and present study adds to the case of registering the potential source of it.

Conflict of Interest:

Authors declare no conflict of interest in this research.

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