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Ecliptaalba extract mediated synthesis of silver nanoparticles and its cytotoxic effect on Hep-2 Cell line

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ABSTRACT
Nanotechnology because of its wide applicability emerges out as an important technology of mankind. Metal nanoparticles have long been synthesized by wet chemical techniques which may be toxic. The recent technology of using plant extract is a green synthesis, and a single step environmentally friendly process for production of nanoparticles.The biomolecules present in the aqueous extract of the whole plant Ecliptaalba (Asteraceae) is responsible for the reduction of silver nitrate to silver nanoparticles and also act as capping agent. Silver nitrate (10^{-3} M) was used with aqueous extract to produce silver nanoparticles. It has very good activity than its macro counterparts and reported to contain antibacterial effect. The surface Plasmon resonance (SPR) caused color change measured using UV-Visible spectrophotometer. The size and shape of the silver nanoparticles was rod shaped of large size E.albamediated AgNPs (microwave exposed) found from Scanning Electron Microscope analysis (SEM). Fourier Transform Infrared spectroscopy (FTIR) was carried out to determine the presence of biomolecules in them. Its cytotoxic effect been studied on HEp-2 (Larynx) cancerous cell line and normal VERO cell line, MTT assay was done to test its optimal concentration and efficacy which gives valuable information for the use of silver nanoparticles for future cancer therapy.

Keywords: Nanotechnology; Eclipta alba; Silver Nanoparticles; Anti-cancer activity; Cytotoxicity; Metal nanoparticles

I. INTRODUCTION

Nanotechnology is an emerging technology that attracts researchers from various fields like physics, chemistry, electrical engineering and material sciences, and now in life sciences especially in biomedical application and biotechnology. It involves the creation and utilization of chemical, physical, and biological systems that exhibit structural characteristics from single atoms or molecules to submicron scales, along with the incorporation of these nanostructures into more extensive systems (Rao and Cheetham, 2011).The main advantage of nanotechnology is the ability to utilize the special properties that materials possess when they have nanoscale dimensions (1-100nm). These properties can include physical, chemical, electrical, or biological characteristics that are not available when the material is in the bulk state. Further, the ability to engineer these particles on such a small scale allows them to interact in special ways with biological systems, as they are roughly the size of many native proteins.

Metallic nanoparticles, also known as metal nanoparticles, have emerged as a novel concept in the realm of nanoparticles over the past few years. Noble metals such as gold, silver, and platinum, known for their positive health effects, are employed in the synthesis of nanoparticles and are referred to as metallic nanoparticles (Bhattacharya and Mukherjee, 2008).Currently, there is a significant emphasis among researchers on the study of metal nanoparticles, nanostructures, and the synthesis of nanomaterials.This interest stems from their remarkable properties, which have proven beneficial in various applications such as catalysis(Narayanan and El-Sayed, 2004), the preparation of composite materials like polymers(Moura et al., 2017), disease diagnosis and treatment (Banerjee et al., 2017), advancements in sensor technology(Shaikh et al., 2016), and the labeling of optoelectronic recorded media(Gracias et al., 2000).

There are many reports about the synthesis of nanoparticles using different plant extracts as reducing agents (Jacob et al.,2013;Vinmathi and Packia Jacob, 2015;Jacobet al., 2017). Our study deals with plant extract mediated synthesis of

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AgNPs. The plant was chosen and they have very good medicinal property such as anticancerous, antibacterial, antihepatotoxic etc(Uddin et al., 2010). Several studies been conducted to prove efficacy of plant extract. We extend the process by green synthesis using plant extract. The nanoparticles synthesized were tested for its cytotoxic activity against HEp 2 (Larynx cancerous) cell line. It should be non-toxic to normal cells so a comparative study was done to prove the efficacy. The synthesized nanoparticles were expected to kill the cancerous cell. This process can be used for large scale and can be synthesized with minimum cost and maintenance. The nanoparticles also remain stable for longer time.

II. MATERIALS AND METHODS

2.1.Collection of plant material

The plant sample Ecliptaalba was collected from the farms of Arakkonam district, it was cleaned thoroughly with tap water. The collected plant material was identified by Botanist, Central Research Institute of Siddha, Chennai and the voucher specimen was preserved in herbarium. The plant materials were thoroughly washed with water several times. The whole plant was used for obtaining the extract

2.2.Procedure

The plant parts were chopped into pieces and 25 g of this plant material was weighed.100 ml of distilled water were added. To this, the weighed plant material was added. The mixture was boiled at 90°c for about 20 minutes. It was allowed to cool in room temperature. Filtered with Whatman no: 40 filter paper 3 times. The filtrate was taken and kept in individual bottle. The extract were stored in refrigerator for future purpose.

2.3.Synthesis of nanoparticles

50ml of 1mM (10^{-3} M) silver nitrate solution mixed with 50 ml of plant extract. Then it was exposed to various conditions like microwave, dark and sunlight exposure. After exposure the mixture has to be centrifuged at 10000rpm for 10minutes. The pellet was obtained by drying in hot air oven. Supernatant was stored in refrigerator. The pellet was used for analysis.

2.4. Microwave exposure

The mixture of equal proportion of plant extract and silver nitrate solution was exposed to microwaves at 90°c. It was done at 30sec intervals to check the formation of color change due to surface plasmon resonance and time taken for the appearance was noted. The near infra red region in closed environment avoids interference from other sources so that reaction will be specific.

2.5. Incubation in dark

Mixture was incubated in dark room for 24 hrs at room temperature. The mixture was observed at various time intervals to check the appearance of dark brownish color.

2.6. Sunlight exposure

The mixture was taken in a glass beaker and temperature of 33°c was recorded in Chennai Metrological Centre. It was directly exposed to sunlight and was mixed continuously to maintain homogeneity. It was carried till the appearance of dark brownish color was observed.

2.7. Characterization of nanoparticles

2.7.1. UV-Visspectroscopy

The UV-Visible (UV-Vis) spectrophotometric data were obtained by the use of UV Plus (Motras Scientific) Double Beam Spectrophotometer. The aqueous samples of rGO, Chitosan and rGO-Chitosan conjugate were taken as UV-Vis analytes, and deionized as the reference. The absorption spectra were recorded.

2.7.2.Fourier Transform-Infrared Spectroscopy (FT-IR)

Third-generation infrared spectrometers, often referred to as FTIR spectrometers, are employed to retain the fundamental absorption characteristics of diverse compounds for the purpose of identifying the functional groups within compounds (Khan et al., 2018). The Fourier-transform infrared (FTIR) spectra of isolated compounds were analyzed using a FTIR spectrophotometer (Jasco FT/IR-6600typeA). FT-IR spectroscopy can be broadly classified into two main categories: functional group analysis, which encompasses wavenumbers ranging from 4000 cm^{-1} to 1600 cm^{-1}, and fingerprint analysis, which pertains to wavenumbers below 1600 cm^{-1}.

2.7.3. Scanning Electron Microscopy

The appearance and content of the produced ZnO nanoparticles were studied using field emission scanning electron microscopy (FE-SEM, JEOL-JSM 6500F, Japan).

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2.8. Cytotoxicity and anticancer activity

2.8.1. MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was obtained from Invitrogen, USA. All other fine chemicals were obtained from Sigma, Aldrich.

2.8.2. Cell culture

HEp2and VERO cells obtained from NCCS (National Centre for Cell Science, Pune) were cultured in Rose-well Park Memorial Institute (RPMI) medium, supplemented with 10% fetal bovine serum, penicillin/streptomycin (250 U/mL), gentamycin (100 µg/mL) and amphotericin B (1 mg/mL) obtained from Sigma Chemicals, MO, USA. All cell cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2. Cells were allowed to grow to confluence over 24 h before use.

2.8.3. Cell growth inhibition studies by MTT assay

Cell viability was measured with the conventional MTT reduction assay (Mosmann, 1983). Briefly, HT29 cells were seeded at a density of 5×10^3 cells/well in 96-well plates for 24 h, in 200 µL of RPMI with 10% FBS. Then culture supernatant was removed and RPMI containing various concentrations (3.906–500 µg/mL) ofZinc nanoparticleswere added and incubated for 48 h. After treatment, the cells were incubated with MTT (10 µL, 5 mg/mL) at 37°C for 4 h and then with DMSO at room temperature for 1 h. The plates were read at 595 nm on a scanning multi-well spectrophotometer.

$$\text{Cell viability (\%)} = \left[\frac{\text{Mean OD}}{\text{Control OD}}\right] \times 100$$

III. RESULTS

3.1 Synthesis of silver nanoparticles

Plant extract been exploited for the green synthesis of silver nanoparticles. The formations of the nanoparticles were confirmed by the color change observed from light green to dark brownish color. This may be due to the surface Plasmon vibrations which correlates with previous works carried out in this method.

Exposure Conditions	E.albamediated AgNPS	Yield /100ml
Microwave	65 seconds	250mg
Dark	Incubated -24 hrs	145 mg
Sunlight	7 minutes	32 mg

Table.1. Time taken for appearance of dark brownish color

The color change in microwave is due to the microwaves radiation, which may avoid interference from other sources help to reduce the silver nitrate into silver nanoparticles and the plant extract help in microwave assisted production. The above result (Table.1) showed that microwave gave maximum yield within 65 sec with 250mg yield for E.alba. But in the case of dark incubation yield was observed after 24 hrs which compared to the sunlight treatment, the yield in dark incubation was sizable.

3.2 UV-Vis spectroscopy analysis

The UV-Vis spectroscopy analysis was done to confirm the presence of silver nanoparticles. The appearance of dark brownish color was due to excitations of surface plasmon vibrations. The analysis was done by scanning the samples between the range of 200 -800 nm which gave the λ max value at 400nm. This was the characteristic feature of silver which gave maximum absorbance at 400nm and confirmed the presence of silver nanoparticles. In the exposure conditions followed microwave exposed gave maximum absorbance. In the case of E.alba – AgNPs microwave treatment a peculiar peak at 600 nm was observed which indicated that there may be rod shaped particles formed (Henglein, A., 1993).

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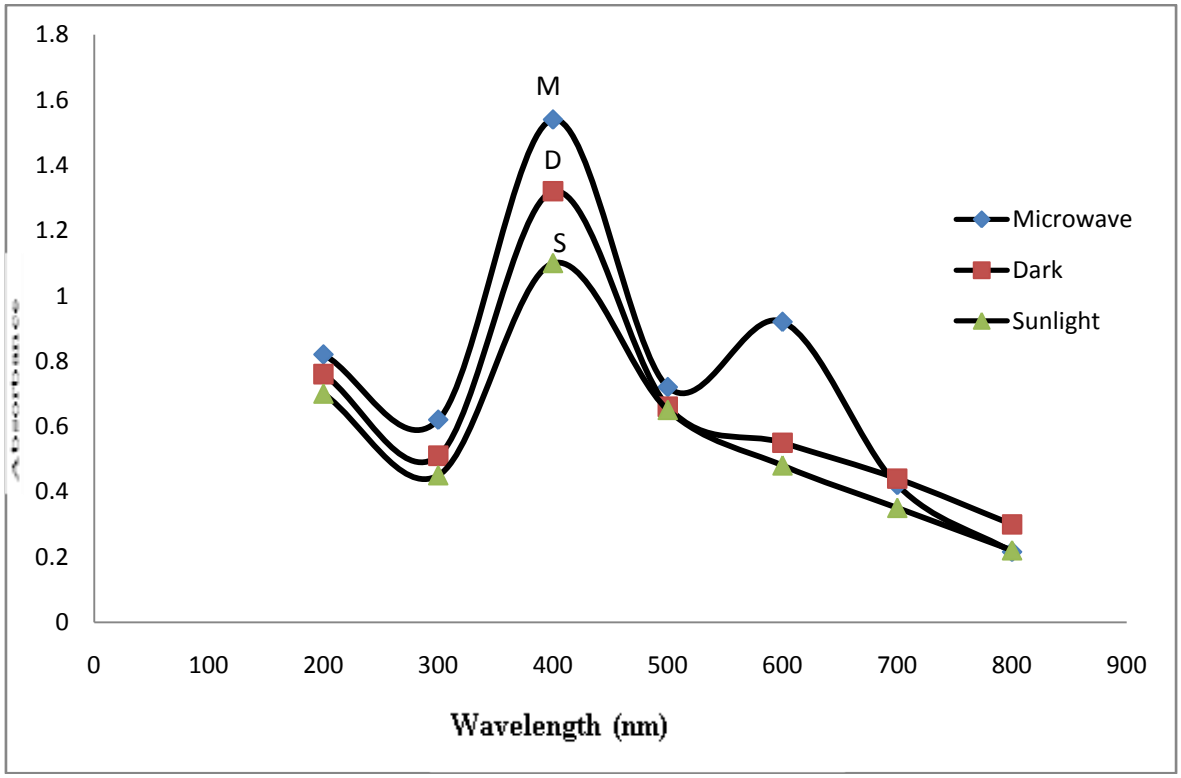

Figure 1: UV-Vis spectra of E.alba extract mediated AgNPs

3.3 Scanning Electron Microscopy Analysis (SEM)

The sizes and shape of the nanoparticles were obtained by the High Resolution Field Emission Electron Microscope analysis. The samples which have been exposed to microwave and incubated in dark been analysed and its size and shape been determined. Its been correlated with UV-Vis absorption spectra analysis.E.albamediated AgNPs exposed to microwaves (Fig 2) obtained rod shaped nanoparticles of length ranging between 1500 -1700 nm with an average of 1600nm and thickness 400 nm. Whereas the particles incubated in dark (Fig 3) obtained spherical nanoparticles in the range of 45 – 95 nm with mean diameter of 72 nm.

Figure 2 :SEM image of AgNPs synthesized by E.albaextract – microwave exposure

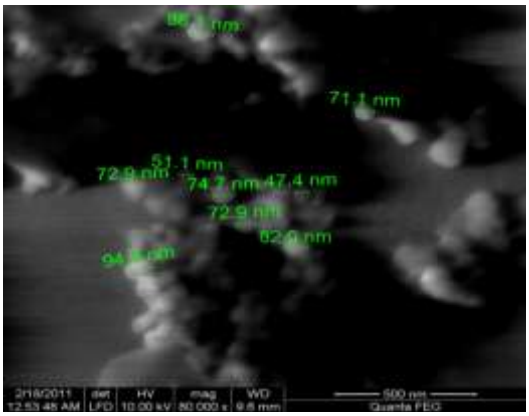

Figure 3 : SEM image of AgNPs synthesized by E.albaextract – dark incubation

3.4 Fourier Transform Infra red Spectroscopy analysis (FTIR)

The presence of biomolecules can be very well determined with FTIR analysis and it's been correlated with standard IR fingerprint data. The broad band in the region 3100 – 3400 cm^{-1} is due to O-H stretching vibrational frequency which can be occurred due to (Fig 12) hydroxyl group of phenol, alcohols. The intense band at 1640 cm^{-1} due to C=C stretching of aromatic ring which present in

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terpenoids(Fig 13 and 14) play a major role in reduction of metal ions. It may be also due to C=O ketones which present in flavonoids. The presence of NO_2 stretching was also found in the silver nitrate analysis. Peaks at 1020 – 1220 cm^{-1} implies C-N stretching vibration of aliphatic amides. Many medium to weak bands between 1200-1300 cm^{-1} is due to the binding of molecule to the surface of AgNPs through COOH carboxylate group. This originated from the OH bending vibrational mode from alcohol or phenol functional group or from C-O stretching and OH bending vibrations from COOH. These above bonds commonly present in protein indicate the presence of protein as a ligand for reducing silver nitrate to AgNPsand thereby increase the stability of nanoparticles. These reducing agents are usually present as large fraction in plant extract

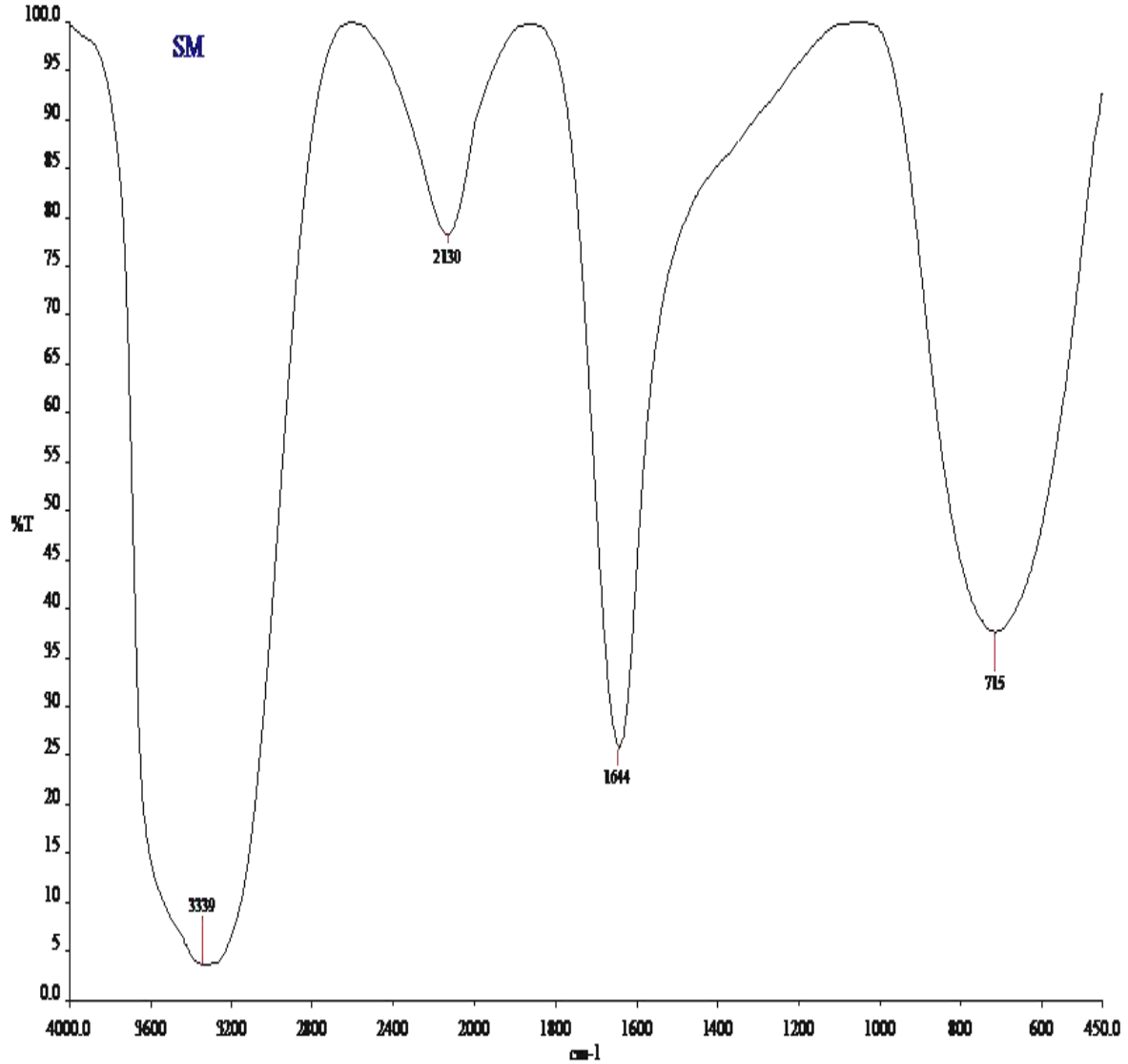

Figure 4: FTIR spectra of Silver Nitrate ($AgNO_3$)

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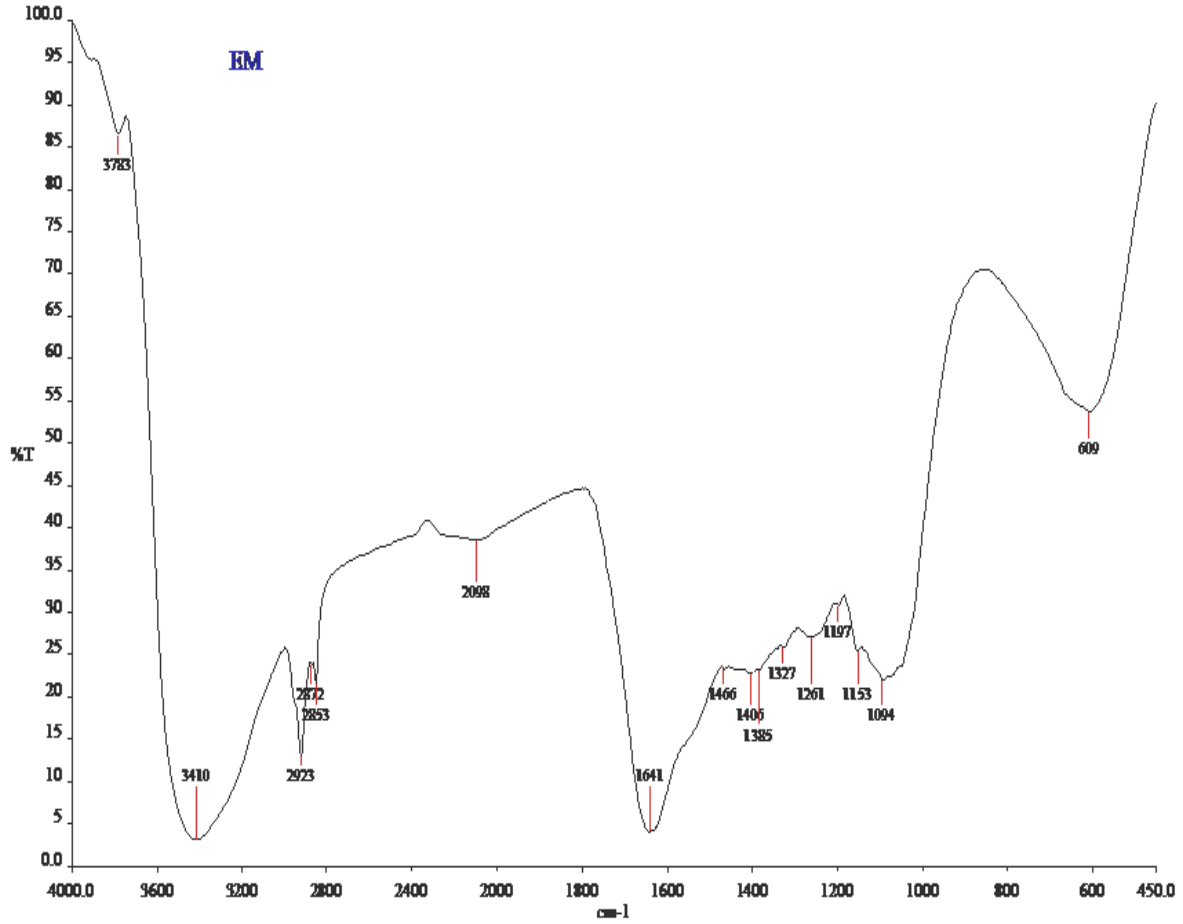

Figure 5: FTIR spectra of E.albamediated AgNPs (microwave exposed)

3.5 Cytotoxic assay (MTT)

The plant extract mediated nanoparticles was tested for its cytotoxic effect using MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) on HEp-2 cell line and VERO cell line. Drugs of varying concentration in cell lines were tested after 24 hrs of incubation at 37°c in 5% CO2. Two cell lines were used HEp-2 Larynx cancerous cell line to test the optimal drug concentration for 50% cell death LD 50 and VERO cell line to prove that the drug is non-toxic to normal cell line.

S.no	Concentration (µg/ml)	Dilutions	Absorbance at 595 nm		EM cell viability%	SN cell viability%
			EM	SN		
1	500	Neat	0.09	0.25	14.28	35.21
2	250	1:1	0.10	0.35	15.87	49.29
3	125	1:2	0.15	0.39	23.80	54.92
4	62.5	1:4	0.22	0.45	34.92	63.38
5	31.25	1:8	0.29	0.54	39.68	76.05
6	15.625	1:16	0.32	0.59	50.79	83.09
7	7.8125	1:32	0.48	0.65	76.19	91.54
8	3.906	1:64	0.59	0.67	93.65	94.36
9	Cell control	-	0.63	0.71	100	100

Table 2:Invitro cytotoxicity effect ofE.alba mediated AgNPs (microwave exposed) and silver nitrate on HEp-2 cancerous cell line

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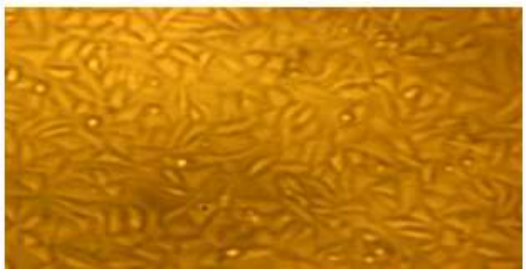
S.no	Concentration (µg/ml)	Dilutions	Absorbance at 595 nm		EM cell viability%	SN cell viability%
			EM	SN		
1	500	Neat	0.32	0.20	46.37	27.03
2	250	1:1	0.37	0.27	53.62	36.49
3	125	1:2	0.44	0.33	63.76	44.59
4	62.5	1:4	0.54	0.45	78.26	60.81
5	31.25	1:8	0.59	0.50	85.50	67.57
6	15.625	1:16	0.61	0.52	88.40	70.27
7	7.8125	1:32	0.63	0.56	91.30	75.68
8	3.906	1:64	0.64	0.60	92.75	81.08
9	Cell control	-	0.69	0.74	100	100

Table3: Invitro cytotoxicity effect ofE.alba mediated AgNPs (microwave exposed) and silver nitrate on VERO cell line

The cytotoxic effect of silver nanoparticles synthesized using E.albaextractswere determined using HEp-2 cell lines by MTT-assay. Significant cytotoxic effect (85 %) was observed at 500 µg/ml concentration of AgNPs produced using E.alba extracts (Fig 6) and the LD 50 was observed at 15.625µg/ml. Whereas, silver nitrate (AgNO3) showed 65% cell death at 500 µg/ml concentration (Fig 7) and the LD 50 was observed at 250 µg/ml concentration. In the noncancerous cells (VERO cell) maximum cell death of 54% was observed in E.alba mediated AgNPs (Fig 8) at 500 µg/ml concentration .In the case of silver nitrate at 500µg/ml 83% cell death was observed and LD 50 was observed at 62.5µg/ml. This kind of killing effect was identified due to the generation of ROS in the gliomacells(Stella et al, 2009).ROS typically include the superoxide radical, hydrogen peroxide and the hydroxyl radical, which cause damage to cellular components such as lipids, DNA and proteins and eventually lead to death (Xia et al,2006) The toxicity of silver nanoparticles against rapidly dividing HEp-2 cells raises exciting opportunities for their potential use as anti-cancer agents. Since AgNPs are cytotoxic to normal cell line (VERO cells) at higher concentrations, careful usage of AgNPs at reduced concentration may be used as an efficient anti-cancerous agent. Further studies are needed to clarify the possible anticancer application of these nanoparticles on human use and also to analyze the mechanisms behind the effects observed.



a) Normal HEp-2 cell line

b) High conc. 500µg/ml

c) LD 50(15.625 µg/ml)

Figure 6: Invitro cytotoxic effect of E.albamediatedAgNPs (microwave exposed) at various conc. On HEp-2 cell line

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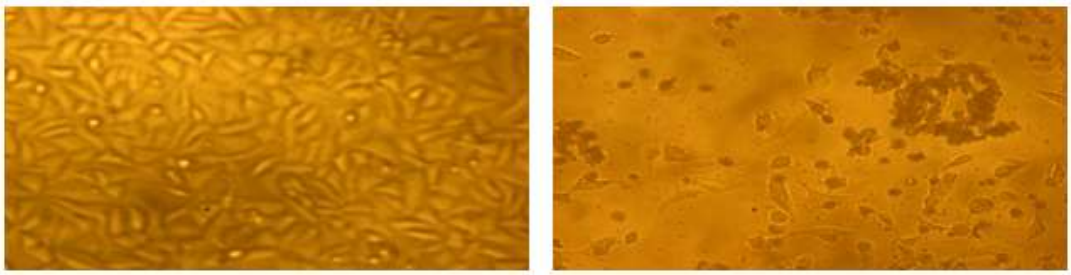
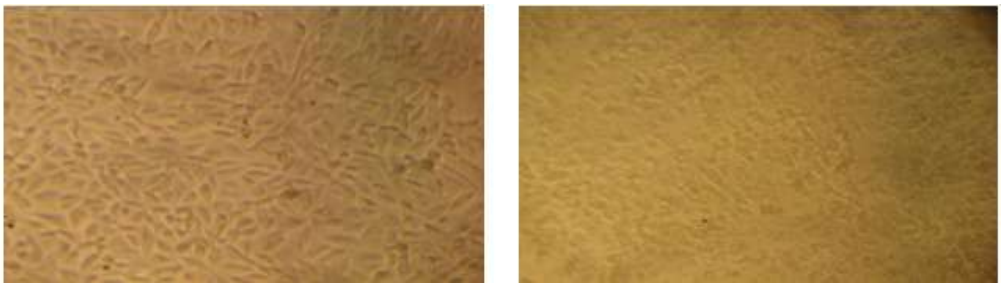


a) Normal HEp-2 cell line b) High conc. 500µg/ml

c)LD 50 (250 µg/ml)

Figure 7: Invitro cytotoxic effect of silver nitrate at various conc. on HEp-2 cell line



a) Normal VERO cell line b) High conc. 500µg/ml

c)LD 50 (125 µg/ml)

Figure 8: Invitro cytotoxic effect of E.albamediatedAgNPs (microwave exposed) at various conc. on VERO cell line

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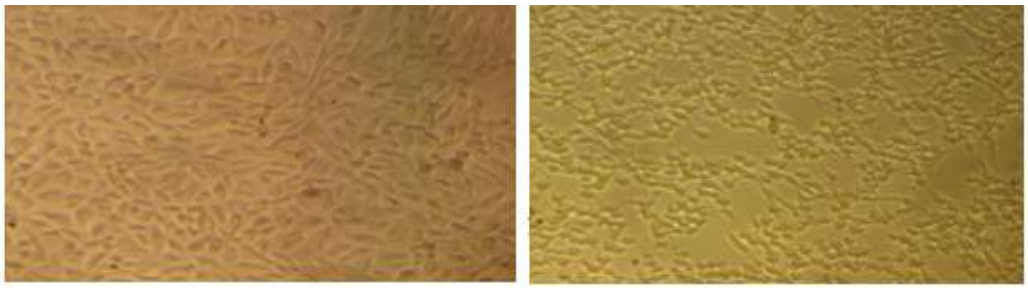

a) Normal VERO cell line b) High conc. 500µg/ml

c)LD 50 (62.5 µg/ml)

Figure 9: Invitro cytotoxic effect at increasing conc. of silver nitrate on VERO cell line

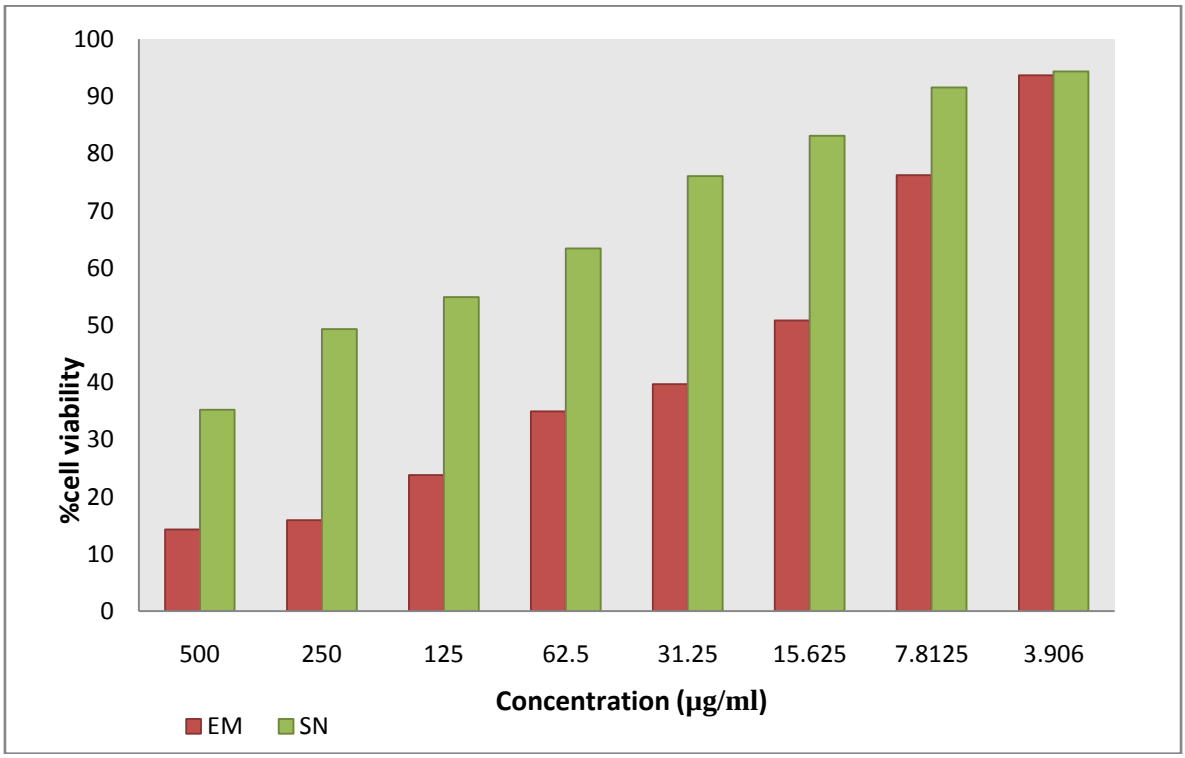

Figure 10: Invitro cytotoxicity effect of E.alba mediated AgNPs (microwave exposed) and silver nitrate on HEp-2 cell line (MTT assay)

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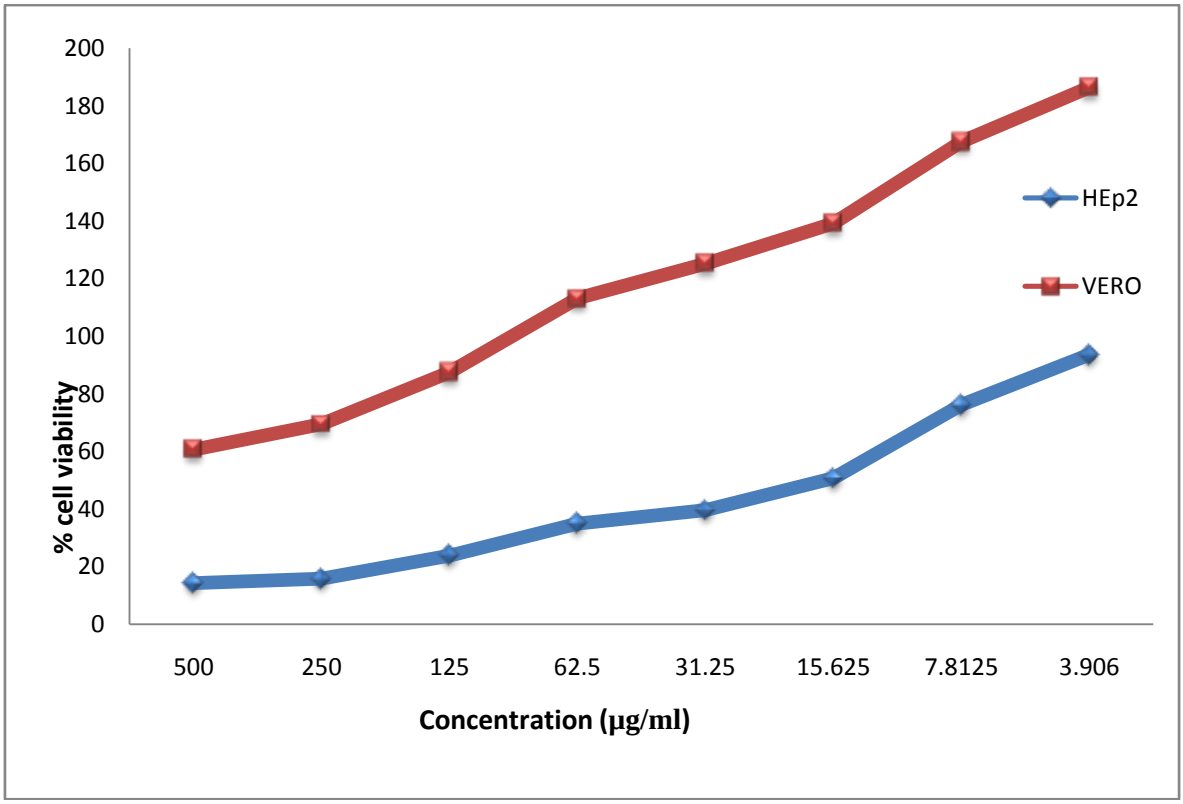

Fiure 11: Invitro anticancer effect of E.alba –AgNPs (microwave exposed) - comparison between VERO and HEp-2 cell lines

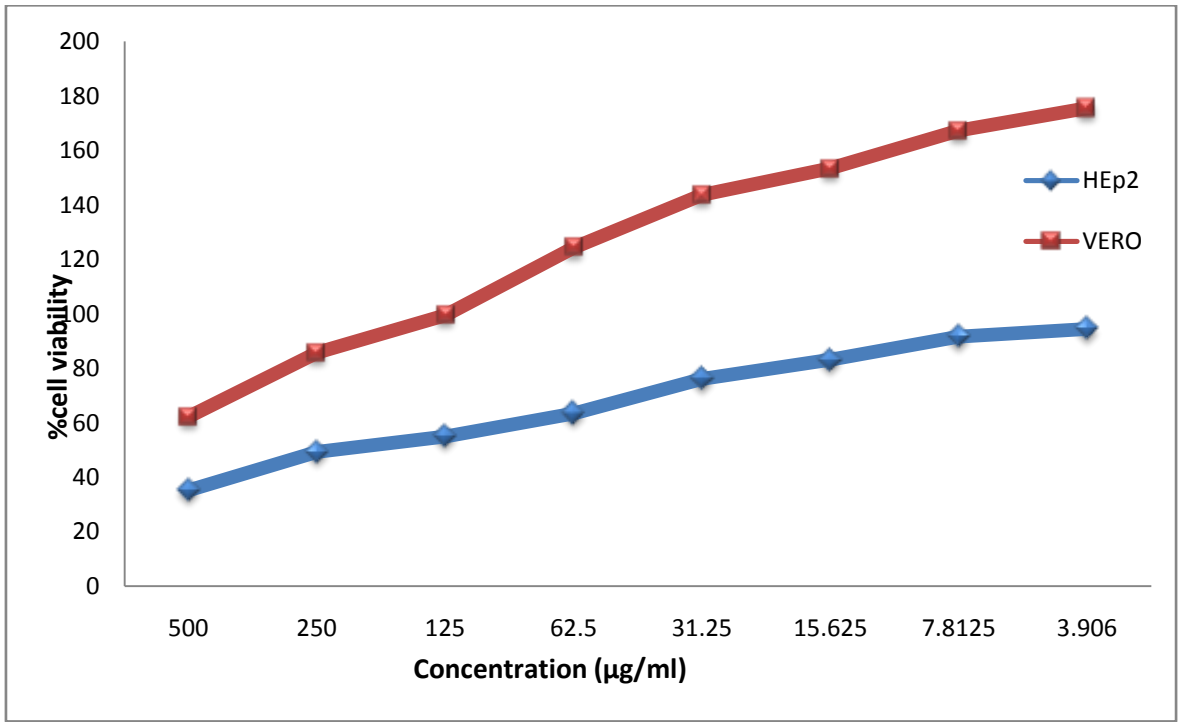

Figure 12: Invitro anticancer effect of silver nitrate- comparison between VERO and HEp-2 cell lines

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IV. CONCLUSION

The plant extract Ecliptaalbamediated synthesis of silver nanoparticles was found to be more effective and the plant itself has got the medicinal property so tapping these resources seems to be ecofriendly and easy to produce these nanoparticles. Bioreduction of Ag^+ ions to silver nanoparticles by the plant extract occur. The plant aqueous extract act as reducing and capping agent of silver nanoparticles. Among several physical conditions microwave treatment gave maximum yield and the colour change occur within 70 seconds at faster rates. Already reports are available on the usage of Nanoparticles for antibacterial activity; hence in our present study we put an innovative step in using these AgNPs for invitro cytotoxic effect on HEp-2 cell line. Several compounds present in these plant extracts acts as a ligand in the reduction process, which would be involved in the stabilization of these AgNPs. E.alba extract mediated AgNPs exposed to microwave was more cytotoxic and LD 50 was at 15.625µg/ml on the cancerous cell line and at the same concentration in normal VERO cell line 90% viable cells were observed. The cytotoxic assay was also carried on normal VERO cell line and it was toxic at very high concentration but at low concentration it was nontoxic to normal cell line. This makes the use of these plant mediated AgNPs for cancerous treatment.Thus, green synthesis seems to be a very effective, innovative step and the nanoparticles produced by this method can be used to treat cancer.

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