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FORMULATION AND *IN VITRO* CYTOTOXICITY TESTING OF BIOSYNTHESIZED SILVER NANOPARTICLE GEL OF *AEGLE MARMELOS* IN HUMAN MELANOMA CELLS SK-MEL-2

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ABSTRACT

Silver nanoparticles (AgNPs) have gained received great interest in cancer nanomedicine owing to its wide range of biomedical applications. The present study aimed to investigate the anticancer activity of AgNPs gel formulated with *Aegle marmelos* leaf extract against human melanoma cell lines SK-MEL-2. Biogenic synthesis of AgNPs was carried out using *A. marmelos* leaves extract acting as a reducing agent. Biosynthesized nanoparticles were characterized by a UV-spectroscopic technique, demonstrating a peak at 421 nm. Four batches of AgNP gel (A-D) were formulated using varying concentrations of carbopol 934 as a gelling agent and potential anticancer activity was evaluated against human melanoma cell lines SK-MEL-2 by Sulforhodamine B (SRB) assay. Among the four batches, AgNP gel A exhibited the highest percent control growth of 53.5% at 40 μg/mL concentration, which is comparable to that shown by positive control ADR. AgNP gel A also exhibited significant anti-proliferative activity with LC_{50} value of 79.6 μg/mL, which is greater than that of ADR (LC_{50} =50.5). Both the GI_{50} and TGI values for AgNP gel A were found to be <10 μg/mL, which is similar to that of positive control ADR. Thus, it can be concluded that AgNP gel formulated from *A. marmelos* can be a potential anticancer agent for skin cancer.

Keywords: Silver nanoparticles, *Aegle marmelos,* cytotoxicity, sulforhodamine B assay, anticancer, SK-MEL-2 cell lines

INTRODUCTION

Skin cancer, including both melanoma and non-melanoma, constitutes a small but significant proportion of the population. Basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma are the most frequent primary skin cancers. In India, SCC is reported to be the most prevalent skin cancer **[1]**. It is a not common malignancy but its rate of occurrence is increasing due to increased exposure to ultra-violet (UV) radiation. According to GLOBOCAN 2018, the worldwide cancer burden has risen to 18.1 million cases and 9.6 million cancer deaths **[2]**. Presently, chemotherapy, radiation therapy, and surgical excision remain the mainstay for the treatment of skin cancer. Chemotherapy has significant side effects; moreover, it has relatively low success rates due to the development of multi-drug resistance. Owing to these limitations, there is a need for the development of alternative novel, non-invasive therapy which will be safe and effective against melanoma.

Cancer nanomedicine is new and rapidly evolving field merging nanotechnology and biomedical sciences. It is gaining widespread attention after several reports demonstrated the success of nanodrugs in cancer treatment **[3-5]**. In recent years, silver nanoparticles (AgNPs) have been extensively applied ina wide variety of fields such as catalysis, pharmaceutics, electronics and biosensing due to the unique physicochemical properties of silver at the nanoscale **[6, 7]**. Notably, AgNPs have also received great attention because of their antimicrobial and anticancer activities **[8]**. Several methods have been described for the synthesis of AgNPs, such as chemical reduction, microemulsions, photochemical reduction, etc. **[9]**. Among these techniques, biogenic synthesis involving reduction of the metal precursor (silver nitrate) using plant extracts as reducing agents has received the most attention. The method is preferred as it is environment friendly, sustainable and allows synergy between the metal precursor and reducing agent **[10]**. Various plant materials like *Cassia angustifolia* **[11]**, *Stevia rebaudiana* **[12]**, *Andrographis echioides* **[13]**, etc. have been exploited for the synthesis of AgNPs.

Aegle marmelos, commonly known as Bilva or Sriphal or Baelis indigenous to India for over 5000 years. The leaves, root, bark, fruit and seed of *A.marmelos* are of great value in the folk systems and the traditional ayurvedic system of medicine in India. *A.marmelos* has demonstrated anticancer activity in various pre-clinical studies. *A. marmelos* leaf extracts were effective in inhibiting the growth of leukemic K562, T-lymphoid Jurkat, B-lymphoid Raji, erythroleukemic HEL, melanoma Colo38, and breast cancer cell lines MCF7 and MDA-MB-231[14]. Also, *A. marmelos* leaf extract has shown antineoplastic effects on the Ehrlich ascites carcinoma in Swiss albino mice **[15]**. Through experiments, it is observed that phytochemicals such as cineole, lupeol, citral, eugenoland d-limonene present in *A. marmelos* possess antineoplastic effects **[16]**. Moreover, leaves of *A.marmelos* are also reported to possess antioxidant activity **[17-19]**. Thus, this plant could be an attractive option as a reducing agent in the biogenic synthesis of AgNPs.

In the present study, silver nanoparticles were biosynthesized using *A.marmelos* leaf extract as a reducing agent. The gel formulation of biosynthesized AgNPs was evaluated for its potential anticancer activity against human melanoma cell lines SK-MEL-2 by sulforhodamine B assay (SRB).

MATERIAL AND METHODS

Material

Leaves of *Aegle marmelos* were obtained from Dr. Babasaheb Ambedkar Marathwada University Aurangabad. Methanol, petroleum ether and chloroform were used for extraction. All the chemicals were obtained from Dipa Chemicals, Aurangabad. All the chemicals and reagents used were of analytical grade. Human melanoma cell lines SK-MEL-2 were obtained from Advanced Centre for Treatment, Research and Education in Cancer, Mumbai.

Methods

Extraction of *Aeglemarmelos* Leaves

Soxhlet extraction method was used to extract active constituents from the leaves of *Aegle marmelos*. Leaves were extracted with petroleum ether for about 12hto remove fatty material and chlorophyll. Marc was collected and extracted similarly using methanol.

Biogenic Synthesis of Silver Nanoparticles (AgNP)

The biogenic synthesis of AgNPs using *A. marmelos* leaf extract is pictographically depicted in **Figure 1**. The dried extract of *A. marmelos* (0.2 g) was added to 50 ml de-ionized water and stirred on a magnetic stirrer (EQUIP-TRONICS EQ770) at room temperature for 1h. Clear leaf extract was then added in a drop-wise manner to 0.001M solution of $AgNO_3$ (50 mL). The solution was shaken on the rotary orbital shaker (HMG INDIA P-1451) at 200 rpm for 48 hrs and incubated at 30°C in dark condition. The progress of the reduction reaction was regularly monitored by observing a colour change. During the reduction process formation of the AgNPs is indicated by a change in the colour of the reaction solution from colourless to dark brown which can be visually observed. The development of brown colour in the reaction mixture indicated the synthesis of

AgNPs **(Figure 1)**. The reduction of Ag^{+} ions was confirmed by using UV-Vis spectrophotometer(Shimadzu UV-1700, Kyoto, Japan) at an interval of 24 h. AgNPs were recovered from solution by centrifugation at 4000 rpm for 30 min, washed with water and dried.

Formulation of Silver Nanoparticle (AgNPs) Gel

Four batches of silver nanoparticle gel (Batch A-D) were formulated using varying concentrations of carbopol 934 as a gelling agent **(Table 1)**. Triethanolamine was added to the colloidal suspension of the AgNPs to raise the pH of the system.

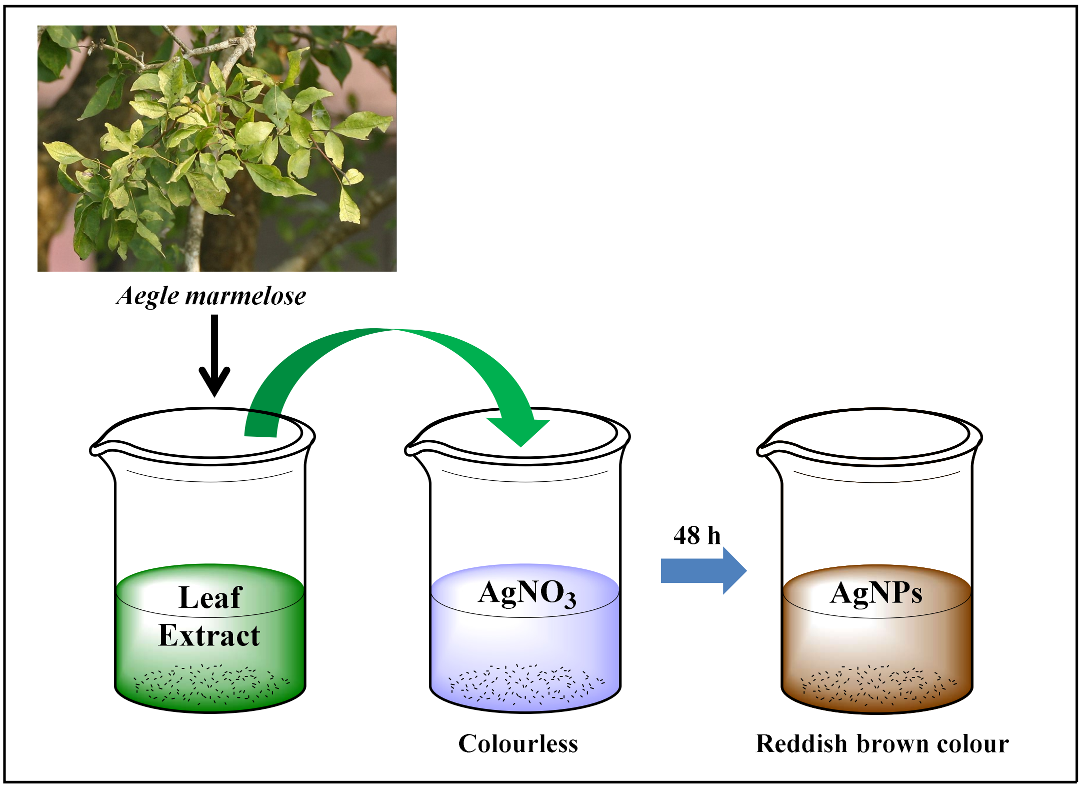

Figure 1: Biogenic synthesis of silver nanoparticles (AgNPs) from *Aegle marmelos* leaf extract

Table 1: Gel formulations of silver nanoparticles (Batch A-D)

Batch	Concentration of carbopol (%)
A	0.5
B	1.0
C	1.5
D	2.0

***In-vitro* anticancer activity using SRB Assay**

Cytotoxic effect of formulated silver nanoparticle gel was evaluated by SRB assay on human melanoma cell lines SK-MEL-2. The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0×10^{5} cells/ml using a medium containing 10% newborn sheep serum. 0.1 mL of the diluted cell suspension was added to each well of the 96 well microliter plate (1×10^4 cells/well).After cell inoculation, the microtiter plate was incubated at 37°C, 5% CO_2, for 24 h before the addition of test compounds. After 24 hours, when a monolayer was formed, the supernatant was discarded. AgNP gels of all four batches (A-D) were added to the cells in the microtiter plate in increasing concentrations (10, 20, 40, 80 μg/ml). The microtiter plate was incubated at 37°C, 5% CO_2, for 72 h and microscopic examination was carried out every 24 h. After 72 h, an assay was terminated by the addition of cold trichloroacetic acid (TCA). Cells were fixed *in situ* by the gentle addition of 25μL of 50% (w/v) TCA and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air-dried.0.1%SRB solution (100 μL) was added to each well and the plate was incubated for 30 min at room temperature. After staining, unbound SRB was recovered and residual SRB was removed by washing four times with 1% acetic acid and plates were air-dried. Bound SRB was subsequently solubilized with 10 mM tris base and absorbance was measured at a wavelength of 540 nM. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells ×100 **(Equation 1)**.

$$\%\ \text{Growth} = \frac{\text{Average absorbance of test}}{\text{Average absorbance of control}} \times 100$$

…..Equation 1

The results obtained in the cytotoxicity testing were expressed in three parameters i.e. LC_{50}, GI_{50}, and Total Growth Inhibition (TGI). The LC_{50} value is the concentration of drug causing 50% cell lethality. The GI_{50} value is the concentration of drug which causes 50% inhibition in the growth of cells. The TGI value is the concentration of a drug that leads to total inhibition of cell growth. Along with the experimental samples, a positive control drug doxorubicin (ADR) was used in this study. It is an anti-cancer agent used for chemotherapy. Experiment was performed in triplicates and average percent control growth was reported.

RESULT AND DISCUSSION

The present study reports the biogenic synthesis of AgNPs using *Aegle marmelos* leaf extract, their characterization by UV-spectroscopic technique and *in-vitro* evaluation of anticancer activity against human melanoma cell lines SK-MEL-2 by SRB assay.

Characterization of AgNPs

The leaf broth of *Aegle marmelos* plant showed the colour change of the medium to dark brown-black when treated with

0.001M silver nitrate and incubated overnight in dark. The reduction of Ag^+ was confirmed by UV-visible spectroscopic technique and sharp bands of silver nanoparticles were observed around 421 nm, confirming with absorbance maxima reported in the literature **[20]**.

Evaluation of anticancer activity by SRB assay

SRB assay is routinely used for cytotoxicity determination, based on the measurement of live cell protein content. SRB is a fluorescent dye and under mild acidic conditions, it binds to protein basic amino acid residues of trichloroacetic acid (TCA)–fixed cells. It can be quantitatively extracted from cells and solubilized by weak bases such as tris base (tris(hydroxymethyl) aminomethane) for measurement of absorbance.

The standard protocol for anticancer activity testing uses ADR (doxorubicin) as a reference standard. *In-vitro* anticancer activity of four batches of AgNP gel (A-D) was evaluated in comparison with pure *A. marmelos* leaf extract (E) and positive control doxorubicin (ADR) against human melanoma cell lines SK-MEL-2 **(Table 2)**. Among all batches of AGNPs gel (A-D), AgNPs batch A exhibited highest activity against S K-MEL-2 cell lines, indicating -53.5% control growth at 40μg/mL concentration, which is slightly lower than % control growth of -63.1% shown by ADR at the same concentration. Batches B-D did not exhibit comparable results to that of ADR. The pure extract of *A.marmelos* (E) was also noted to exhibit low activity with giving lowest % control growth of 106% at 10μg/mL concentration. The % control growth is graphically represented in **Figure 2**.

The LC_{50}, TGI, and GI_{50} values obtained for each test compound are summarized in Table 3.The GI_{50} value and the TGI value for AgNP gel A was found to be <10 μg/mL, which was similar to that of positive control ADR. SK-MEL-2 cell proliferation was significantly inhibited by AgNP gel A with a LC_{50} value of 79.6μg/mL, which is greater than that of ADR (LC_{50}=50.5). Percentage control growth is shown with photo plates for SK-MEL-2 cell line **(Figure 3)**.

Table 2: Percentage control growth of Human Melanoma Cell Lines SK-MEL-2after treatment with AgNP gel (A-D), *A. marmelos* pure leaf extract (E) and positive control doxorubicin (ADR)

Human Melanoma Cell Lines SK-MEL-2																
% Control Growth and Test compound concentrations (ug/mL)																
Test Sample	Experiment 1				Experiment 2				Experiment 3				Average			
	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
A	24.8	-16.8	-55.1	-32.4	13	-18.3	-55.4	-42	-8.4	-15.4	-50	-40	9.8	-16.8	-53.5	-38.1
B	104.6	25.1	41	10.8	78.9	38.4	32.1	20.2	62.1	24.8	32.9	24.2	81.9	29.5	35.4	18.4
C	91.3	42.9	43.1	38.9	76.5	30	33.1	28.9	40.2	33	30.8	28.4	69.4	35.3	35.7	32.1
D	111.8	137.9	206.3	212.4	115.2	129.4	182.6	209.5	108.1	135.3	182.9	187.9	111.7	134.2	190.6	203.2
E	106.3	103.5	119.1	130.7	107.5	114.5	160	138.7	104.1	99.8	109.9	135	106	106.6	129.7	134.8
ADR	-69.9	-57.8	-55.1	-21.9	-61.6	-66.3	-64.7	-34.1	-66.3	-68	-69.4	-41.0	-65.9	-64	-63.1	-32.4

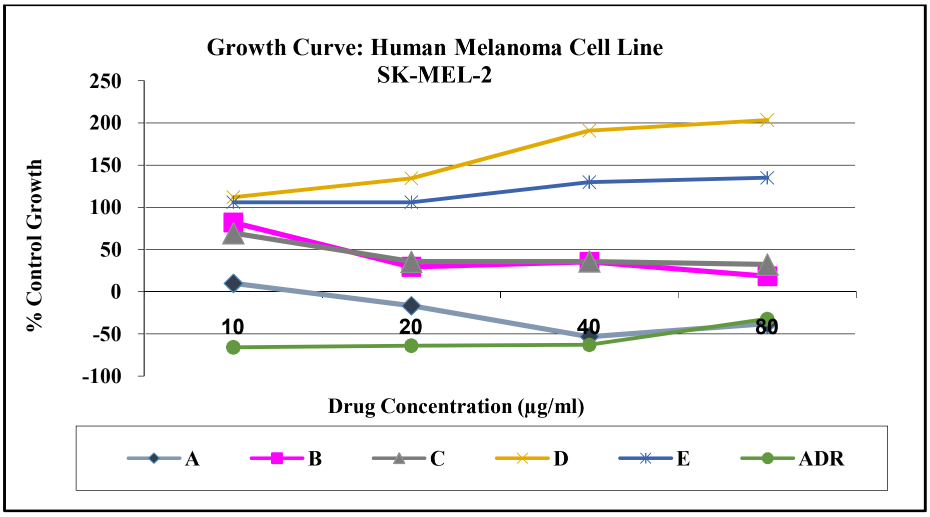

Figure 2: Comparative % control growth of AgNPs gel (batch A-D), *A. marmelos* pure leaf extract (E) and positive control doxorubicin (ADR)

Table 3: LC50, TGI and GI50 values of human melanoma cell lines (SK-MEL-2), after treatment with AgNP gel (A-D), *A. marmelos* pure leaf extract (E) and positive control doxorubicin (ADR)

Drug concentrations (µg/ml) calculated from graph			
Batch	LC_{50}	TGI	GI_{50}
A	79.6	<10	<10
B	NE	>80	24.3
C	NE	>80	19.1
D	NE	NE	NE
E	NE	NE	NE
ADR	50.5	<10	<10

*NE = Non-evaluable data, experiments need to be repeated, test values highlighted in yellow indicate activity

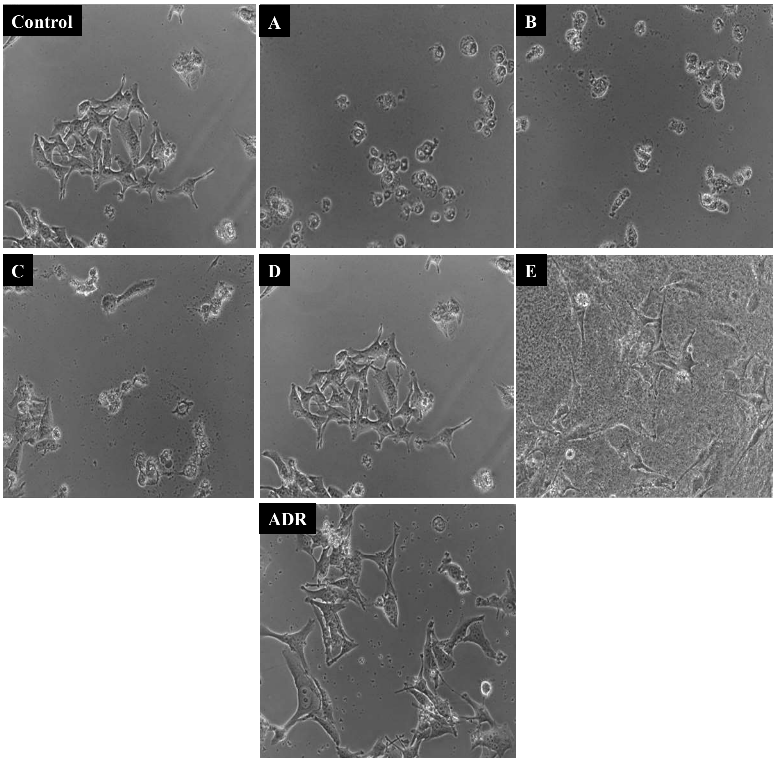

Figure 3: Effect of test compounds as observed on human melanoma cell lines as observed under inverted microscope: Control (SK-MEL-2 before treatment), A-D (AgNPs gel), E (*A. marmelos* pure leaf extract) and ADR (positive control doxorubicin)

CONCLUSION

The present study reported a novel gel formulation of AgNPs biosynthesized from leaf extract of *A. marmelos*. Biogenic synthesis of AgNPs was carried out using *A. marmelos* leaves extract acting as reducing agent and reduction of silver ions was confirmed by UV-spectroscopic technique. Four batches of AgNP gel (A-D) were formulated using varying concentrations of carbopol 934 as a gelling agent and their potential anticancer activity was evaluated against human melanoma cell lines SK-MEL-2 by SRB assay. Among the four batches, AgNP gel A exhibited the highest percent control growth of 53.5% at 40 µg/mL concentration, which is comparable to that shown by positive control ADR. LC_{50} was found to be 79.6 µg/mL, demonstrating greater anti-proliferative activity than that of ADR (LC_{50} =50.5). Both the GI_{50} and TGI values for AgNP gel A were found to be <10 µg/mL, which is similar to that of positive control ADR. Thus, the optimum concentration of carbopol for gel formulated was found to be 0.5% carbopol. Formulation of AgNPs biosynthesized from *A. marmelos* may serve as potential alternative for conventional chemotherapy for skin cancer.

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