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Green synthesis, characterization and anticancer activity of fungal asparaginase from *Aspergillus terreus* conjugated on MgO-ZnO nanocomposite against liver cancer cells

Baskar G*, Abinesh AR, Sheik Mohammed Raja A, Keerthana K, Supriya A & Pravin R

Department of Biotechnology, St. Joseph's College of Engineering, Chennai - 600 119, Tamil Nadu, India

L-Asparaginase exists as an effective chemotherapeutic drug for various cancer cell types. However, the L-asparaginase enzyme in its free state is unstable and has reduced half-life period with numerous side effects. Here, as a green synthesis approach, we have tried a nanocarrier to reduce its side effects and thereby the environmental pollution and toxicity. We used leaf extract of *Mesosphaerum suaveolens* (L.) Kuntze, commonly called Pignut, Wild spikenard or Horsehound, to produce a Mg-ZnO nanocomposite, which has antitumor activity by arresting the cell cycle of tumour cells. To the synthesized nanocomposite, extracted L-asparaginase enzyme from *Aspergillus terreus* was impregnated. The presence of ZnO helps to preserve the biological activity of immobilized L-asparaginase along with MgO making the nanocarrier biocompatible and biodegradable. This helps in increasing the stability and anticancer property of the L-asparaginase. The enzyme-impregnated nanobiocomposite was characterized using UV-Vis spectroscopy which ensured the existence of the Asp-Mg-ZnO in the nanobiocomposite. The presence of functional group and the crystallinity nature of the nanobiocomposite were studied using FT-IR and XRD, respectively. The morphological structure and elemental composition of the nanobiocomposite were determined using the SEM-EDAX. The anticancer activity of the synthesized Asp-Mg-ZnO nanobiocomposite was determined using the MTT assay, which helped to determine the viability and proliferation capacity of the cancer cells.

Keywords: Anticancer, Antitumor, Chemotherapy, Conjugation, Horsehound, *Mesosphaerum suaveolens*, Pignut, Wild spikenard

Nanomaterials developed in the field of nanobiotechnology offer a wide range of applications in biomedical and industrial uses. Green synthesis of nanoparticles has proven to reduce the pollution and decrease toxicity[1]. The *Mesosphaerum suaveolens* (L.) Kuntze, commonly called Pignut, Wild spikenard or Horsehound, belongs to the Lamiaceae family, which has ethnomedical value and is applied in various medical and biological applications[2,3]. The *M. suaveolens* contains essential phytochemicals such as polyphenols, phenolic acid, alkaloids[4], flavonoids[5] and essential oil which can be used as a source for nanoparticle synthesis, as they are potential reducing agents. The binding of biological drug molecules to various nanomaterials has been employed to increase the bioavailability of the drugs[6]. Due to their biological characteristics such as their ability to be antimicrobial and anticancer, nanoparticles are employed in a wide range of processes[7].

Cancer cells change constantly, making it difficult to find a solution. American Cancer Society reprotdthat the Liver cancer incidences is 3 times higher in men than in women. The liver cancer was found stabilized in men with gae of 50 years and older, and decreased in youngsters in United States based on the cases reported during 2015 to 2019[8,9].

The application of nanoparticles has helped in the process of finding a potential drug to combat cancer[10]. Each kind of cancer requires a different treatment plan, hence a precise cancer diagnosis is crucial for the right kind of treatment. Surgery, radiation, chemotherapy and systemic therapy are frequently used in treatment. Among these, chemotherapy is widely deployed, in which L-asparaginase is a promising chemotherapy drug. An amino hydrolase called L-asparaginase breaks L-asparagine amino acid into aspartic acid and ammonia. Aspartic acid from the above reaction can be converted to L-asparagine in normal cells due to the presence of the L-asparagine synthetase gene; however, neoplastic cells cannot recycle aspartic acid into asparagine amino acid due to mutation in theL-asparagine synthetase gene[11,12]. This ensures the amino acid degradation that is essential during cell proliferation, which ultimately retards cancerous

*Correspondence:
E-Mail: basg2004@gmail.com

cell growth. Due to this property, the enzyme L-asparaginase can be used in chemotherapy treatments.

Although L-asparaginase is an effective chemotherapeutic drug, the quality must be improved for better results due to its inadequate stability and nonspecificity[13]. It can be improved by conjugating the enzyme with nanoparticles. This research aims to synthesize a new L-asparaginase carrier — a nanocomposite to improve the activity and enhancing the enzyme's half-life and stability[14]. Accordingly, zinc nanoparticles (ZnO) exhibit anticancer properties and high resistance to the microbiome. The improved electrostatic properties of zinc oxide allow it to have various charges that ensure stability in both acidic and basic environments. As cancer cells contain a large concentration of a negative charge phospholipid bilayer, this feature can be utilized to conjugate therapeutic chemicals and internalize nanoparticles to cancer cells. The conjugation of therapeutic agents with ZnO makes it more stable and resourceful[15,16].

Due to distinctive physicochemical characteristics, such as exceptional thermodynamic stability and biodegradability, magnesium oxide (MgO) is an ecologically safe, commercially feasible, and important nanoparticle. Additionally, its ability to induce and elevate ROS production in cancerous cells makes it a potential therapeutic nanocarrier. Furthermore, because of their biocompatibility, MgO nanoparticles are used in the biomedical engineering sector for the development of antitumour therapeutic agents, tissue regeneration, implant coatings, bioimaging and enhancement of the wound healing phenomenon[17]. In the current work, in order to enhance the biological activity and bioavailability of L-asparaginase and increase the potency of the drug, we conjugated the fungal L-asparaginase to a nanocomposite. The produced Asp-Mg-ZnO nanobiocomposite was characterized using SEM, XRD, UV-Vis, EDX and FTIR analysis. The synthesized nanobiocomposite was tested for its anticancer activity against liver cancer cell lines (HepG2).

Materials and Methods

Chemicals used

The chemicals used for the research work such as sodium nitrate ($NaNO_3$), magnesium chloride ($MgCl_2$), ferrous sulfate ($FeSO_4$), dipotassium hydrogen phosphate (K_2HPO_4), zinc chloride ($ZnCl_2$), copper sulfate ($CuSO_4$), sodium nitrate ($NaNO_3$), sodium hydroxide (NaOH), magnesium sulfate ($MgSO_4$), potassium chloride (KCl) and zinc sulfate ($ZnSO_4$) were purchased from LOBA chemicals, Mumbai, India. All the chemicals used were of analytical grade and with 99% purity. The *Aspergillus terreus*fungal strain MTCC 1782 used for the synthesis of L-asparaginase enzyme was obtained from the CSIR-Institute of Microbial Technology, Chandigarh, India.

L-asparaginase enzyme production

Czapek-Dox agar slant was used to culture *A. terreus,* afungal organism. To prepare the fungal asparaginase enzyme, the prepared culture was transferred to a liquid medium, a modified form of Czapek-Dox medium using a saline solution[18]. The composition of the liquid medium was 1% L-asparagine, 0.20% glucose, 1% sodium nitrate, 0.0010% zinc sulfate, 0.052% potassium chloride, 0.152% di-potassium hydrogen phosphate, 2% L-proline, 0.001% copper sulfate, 0.001% ferrous sulfate and 0.052% magnesium sulfate. Finally, to ensure the optimal growth, the pH was altered to 6.2. With 120 rpm at 32ºC in an orbital shaker, the culture was aerobically agitated for 72 h. After this, the crude L-asparaginase enzyme was obtained by filtering the culture using Whatman filter paper and finally stored at 4 °C for further use[19].

Preparation of Mesosphaerumsuaveolens leaf extract

The *M. suaveolens* plant leaves were collected from local areas of Chennai, Tamil Nadu India. The collected leaves were washed with distilled water and homogenized using a motor and pestle. 100 g of the homogenized leaves were mixed with 200 mL double distilled water and kept at 80ºC for 10 min. The solution was then cooled and then filtered with a Buchner funnel to obtain the plant extract and the extract was stored at 4°C for further use[20].

Green synthesis of Mg-ZnO nanocomposite

The 25 ml of 25 mM $ZnCl_2$ solution was added to 50 mL of *M. suaveolens* leaf extract in the glass beaker and vigorously mixed for 10 min. About 25 mL of 25 mM $MgCl_2$ was slowly added to the above mixture and then kept in a 90ºC water bath to initiate nucleation. The solution containing precipitated nano-composite was centrifuged at 15,000 rpm for 30 min. The collected nanocomposite was washed with distilled water and ethanol to remove impurities and dried to collect a powdered form of nanocomposite[20].

Synthesis of Asp-Mg-ZnO nanobiocomposite

The 0.1 g of synthesized nanocomposite was treated with 0.5 mL of glutaraldehyde solution and kept for incubation at 32ºC for 3 h to functionalize the nanocomposite[21]. After incubation, the above mixture was centrifuged and the pellet was washed consecutively with 50 mM Tris-HCl buffer.The glutaraldehyde-treated nanocomposite was then added to 100 mL of crude asparaginase enzyme and mixed constantly in a magnetic stirrer for 30 min at 250 rpm. After this, the mixture was centrifuged at 15,000 for 30 min to collect the Asp-Mg-ZnOnanobiocomposite. The collected pellets were then lyophilized with freeze dryer at a pressure of 0.150 Kpsi with a temperature of −45°C.

Characterization of Asp-Mg-ZnO nanobiocomposite

The synthesized Asp-Mg-ZnO nanobiocomposite was analyzed using double beam UV-Vis spectrophotometer (Systronics, 2201). The peaks were observed between 300 to 400 nm. Functional group characterization and confirmation of the conjugation of asparaginase on the synthesized nanocomposite was studied by FT-IR analysis (BRUKER RFS 27 MultiRAM). Powder XRD (Enraf Nonius CAD4-MV31) was used to determine the crystalline nature of the nanobiocomposite. The morphology and elemental composition of the nanobiocomposite were characterized using SEM-EDAX analysis.

Anticancer activity of Asp-Mg-ZnO nanobiocomposite

Cancer cell culture

In DMEM with 2 mM glutamine and 10% FBS, HepG2 cell lines were grown in a humid incubator at 37°C with 5% CO_2 and 95% air. The cell culture was continuously passaged and the culture from the fourth to sixth passages was used for the MTT assay. The HepG2 cells were cultured in 96-well tissue plates with 100 µl media and, after 24 h, different concentrations (100, 10 and 1 ng; and 1, 10 and 100 µg) of the lyophilized nanobiocomposite sample were added to the cells. After incubation of cells with the nanobiocomposite for 24 h, MTT reagent was added and incubated for 2-4 h. The reaction was then stopped and the formed crystals of formazan were dissolved with DMSO. The cell viability was found by taking absorbance at 570 nm. On the basis of cell viability, the % cytotoxicity of the nanobiocomposite was calculated using the following formula. Using these values, the graph was plotted and the IC_{50} value was found using Eq. (1).

$$\text{Cytotoxicity, \%} = \frac{(\text{control OD} - \text{Test OD})}{\text{control OD}} \times 100 \quad \ldots (1)$$

Results and Discussion

UV-Vis spectral analysis of Asp-Mg-ZnOnanobiocomposite

The nanobiocomposite was subjected to UV–Visible analysis and peaks were observed between 300 to 800 nm as depicted in Fig. 1A. A peak at 240 nm confirms the presence of the enzyme L-asparaginase. The 320-400 nm shows the presence of Zn and a peak at 500 nm depicts the presence of MgO. Thus, the presence of ZnO, MgO and L-asparaginase in the nanobiocomposite was confirmed using UV-Vis spectral analysis.

FT-IR analysis Asp-Mg-ZnOnanobiocomposite

The presence of various functional groups in the Asp-Mg-ZnO nanocomposite was confirmed using FT-IR analysis[22]. The FT-IR spectra vary from 500 cm^{-1} to 3500 cm^{-1} shown in Fig. 1B. Totally 13 peaks were observed. The presence of ZnO and MgO stretching might be the cause of the peaks at 563 cm^{-1} and 740 cm^{-1}. Carboxylic acid exhibits a medium

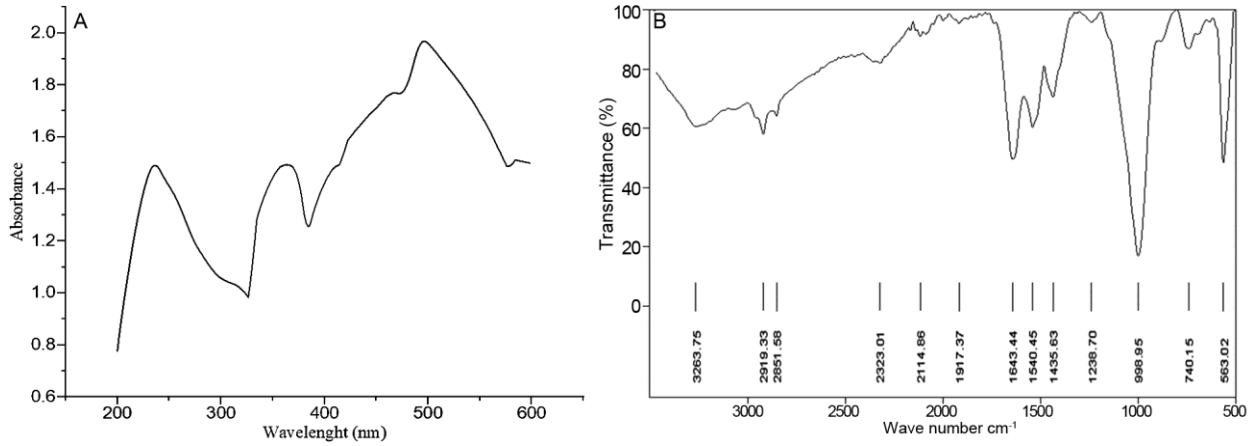

Fig. 1 — (A) UV-Vis spectra; and (B) FT-IR spectra of synthesized Asp-Mg-ZnO nanobiocomposite

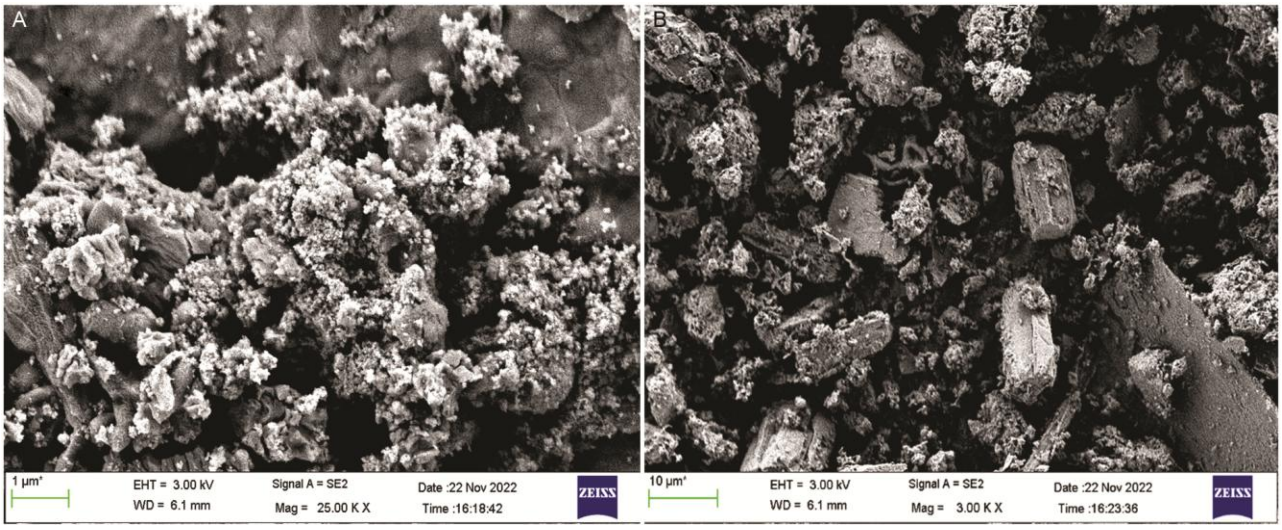

Fig. 2 — SEM image of synthesized Asp-Mg-ZnO nanobiocompositeat the magnifications of (A) 25000 X (B) 3000

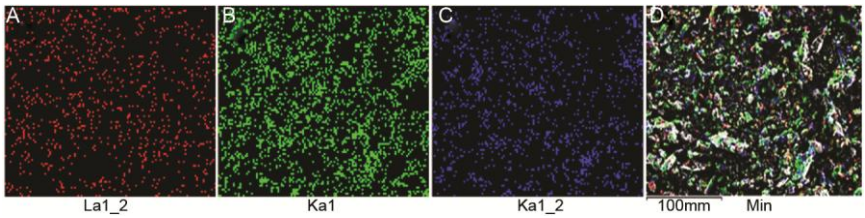

Fig. 3 — Elemental distribution of (A) Zn; (B) O; (C) Mg; and (D) overall in the synthesized Asp-Mg-ZnO nanobiocomposite

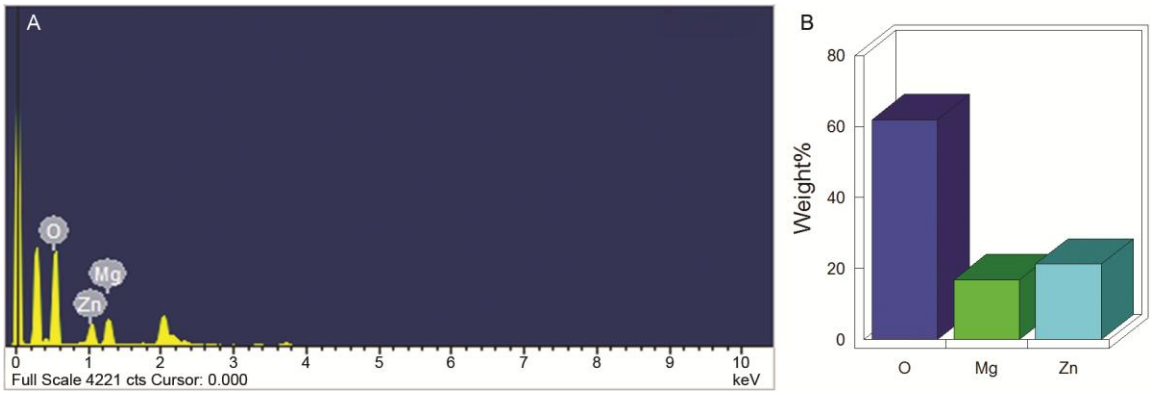

Fig. 4 — (A) EDAX; and (B) XRD analysis of the synthesized Asp-Mg-ZnO nanobiocomposit

bending of O-H at the peak at 1435.63 cm^{-1}, while the nitro compound exhibits a significant N-O stretching in the peak at 1540.45 cm^{-1}. The establishment of an amide bond was observed in the peak at 1643 cm^{-1}. The amide bonds are formed by an imine reaction between the amino group of L-asparaginase and the glutaraldehyde aldehyde group[23]. This confirms the presence of asparaginase immobilized onto the nanocomposite. Peaks at 1917.37 cm^{-1} and 2851.3 cm^{-1}, respectively demonstrate the existence of weak C-H stretching in aromatic compounds and alkene, respectively. Carboxylic acid exhibits substantial O-H stretching at the peak at 3263 cm^{-1}.

SEM-EDAX analysis of Asp-Mg-ZnO nanobiocomposite

The elemental distribution and morphology of the nanobiocomposite were observed by SEM with EDAX analysis. The surface of the Asp-Mg-ZnOnanobiocomposite was found to be heterogeneous, and the particle size was found to be ranging from 40 nm to 60 nm. The clump formation in Fig. 2A was due to immobilized asparaginase. The

EDX analysis determined the composition of ZnO and MgO in the nanobiocomposite. EDAX analysis of the Asp-Mg-ZnO nanobiocomposite showed signals for the presence of Zn, Mg and O. The elemental distribution and the composition of the respective components were found to be Zn – 21.29%, Mg – 16.98%, and O – 61.88% which was inferred from Figs 3 & 4A.

XRD analysis of Asp-Mg-ZnO nanobiocomposite

The crystalline nature of the Asp-Mg-ZnO nanobiocomposite was confirmed with XRD analysis Fig. 4B. It depicts various peak positions at 2-theta angles of 15.08˚, 15.92˚, 16.59˚, 20.98˚, 21.56˚, 30.72˚, 32.05˚ and 33.38˚. Specifically, peaks at 15.08˚, 15.92˚, 16.59˚, 20.98˚ and 21.56˚ represent the presence of Mg in the nanocomposite and the peaks at 30.72˚, 32.05˚ and 33.38˚ signifies the presence of Zn. This confirms the presence of Zn and Mg in the nanocomposite in addition to the SEM/EDX analysis. The miller index for the respective peak was found to be (1,1,0), (1,1,0), (1,1,0), (2,0,0), (2,0,0), (2,1,1), (3,0,0), (3,1,0) and (3,1,0) which concludes the hexagonal wurtzite structure along with a cubic blend of the Asp-Mg-ZnOnanobiocomposite, and it was confirmed with JCPDS (Card no.79-2205). Asp-Mg-ZnO nanobiocomposite average size was estimated using Debye–Scherer's formula ($D = k\lambda / \beta\cos h$), where 'k' is the wavelength of the x-ray used, b is the full width at half maximum of the diffracted peak and h is the Bragg angle, and average size of the nanobiocomposite was estimated to be of 44.6 nm.

MTT assay of Asp-Mg-ZnOnanobiocomposite

After culturing the HepG2 cell line, the anticancer activity of the Asp-Mg-ZnO nanobiocomposite was studied using MTT assay (Fig. 5). The cell line was subjected to various concentrations (1, 10 and 100 ng; and 1, 10 and 100 µg) of the Asp-Mg-ZnO nanobiocomposite, and cytotoxicity was observed and compared with cell control. The percentage of cytotoxicity was calculated as the optical values. Fig. 5 shows the decline in viability of the HepG2 cells with a continuous increase in the nano-biocomposite concentration. This shows that the Asp-Mg-ZnO nanobiocomposite exhibits the property of an anticancer drug. Where the lowest concentration of 1ng showed cell cytotoxicity of 4% while the highest concentration of 100 µg, showed percentage cytotoxicity to be 67%. The IC_{50} value, the value at which 50% of cell death occurs was found to be 100 µg for the synthesized Asp-Mg-ZnO nano-biocomposite which is efficient when compared to copper-based nanoparticle which has an IC value of 185.1 µg and starch-based nickel nanoparticle with an IC_{50} value of 118 µg[24,25]. Figure 6 depicts microscopic images of Asp-Mg-ZnOnanobiocomposite action on the morphology of the HepG2 cell line under various concentrations of Asp-Mg-ZnO nanobiocomposite. In comparison with the control, cell cycle perturbation can be observed in the cell line when nano-biocomposite was added. The cell proliferation is hindered and the morphology of cells becomes elongated due to stress at 1 µg of nanobiocomposite. However, at 100 µg of nanobiocomposite, complete clearance of cells with an appearance of the feature suggesting apoptotic cell death was observed. This ensures that the synthesized nanobiocomposite has anti-cancerous therapeutic value when an optimum concentration of 100 µg is supplied.

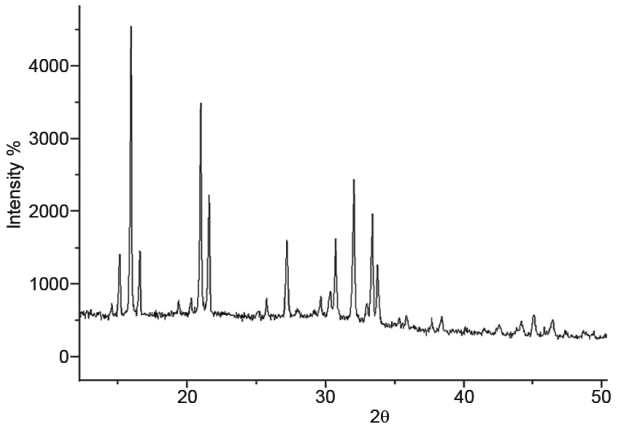

Fig. 5 — Anticancer activity of Asp-Mg-ZnOnanobiocomposite on HepG2 cell line

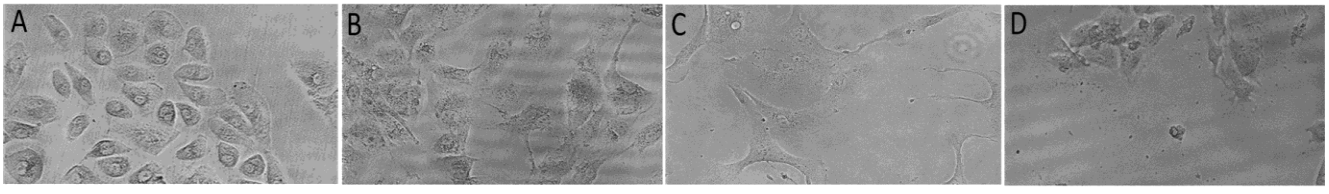

Fig. 6 — Microscopic images of anticancer activity of Asp-Mg-ZnOnanobiocomposite on HepG2 cell line under various concentrations (A) control; (B) 1 ng; (C) 1µg; and (D) 100 µg

Conclusion

The Asp-Mg-ZnO nanobiocomposite was synthesized using green synthesized Mg-ZnO nanocomposite as the carrier. The FT-IR analysis confirmed the presence of amide bond, which confirmed the presence of asparaginase immobilized onto the nanocomposite of Mg-ZnO. The SEM-EDAX characterization confirmed the heterogeneous nature of the Asp-Mg-ZnO nanobiocomposite due to the immobilization of the asparaginase. The elemental composition of Zn – 21.29%, Mg – 16.98%, and O – 61.88% were found in the Asp-Mg-ZnO nanobiocomposite. The synthesized nanobiocomposites average size was calculated to be between 40 nm and 60 nm. The XRD data finally confirmed the crystalline nature of the Asp-Mg-ZnO nano-biocomposite with cubic blended wurtzite structure along with an average size determined to be 44.6 nm. The IC_{50} value for the HepG2 cell line was found to be 100 µg which is efficient when compared to other nanocomposites. The Asp-Mg-ZnO nano-biocomposite was found to be effective and reliable, as well as a potential anti-cancer drug.

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