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Genotoxicity and apoptotic activity of biologically synthesized magnesium oxide nanoparticles against human lung cancer A-549 cell line

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Abstract
The study focussed on the synthesis of magnesium oxide (MgO) nanoparticles from an aqueous extract of *Penicillium* species isolated from soil. A suitable amount of magnesium nitrate (Mg(NO₃)₂) was mixed with the aqueous extract of *Penicillium*. Then the colour of the solution changed due to the formation of MgO nanoparticles. These nascent formed MgO nanoparticles were further confirmed by using UV spectrophotometry which showed the maximum absorption (λ<sub>max</sub>) at 215 nm indicating the formation of MgO nanoparticles. Fourier transform infrared spectroscopy (FTIR) was used to find the possible functional groups and proteins involving the stabilization of MgO nanoparticles. Transmission electron microscopy (TEM) study revealed the size, the shape as well as the dispersity of the prepared MgO nanoparticles and showed that they were well dispersed around 12–24 nm (scale 200 nm). The anticancer activity against A-549 cell line of these green synthesized MgO nanoparticles was evaluated. The result showed good anticancer effect after 24 h of incubation. Nevertheless these MgO nanoparticles showed less effect on normal Vero cells. Further apoptotic study clearly displayed the effect of MgO nanoparticles on cancer cells. The effect was observed through chromatin condensation by forming apoptotic bodies using propidium iodide, acridine orange and ethidium bromide (AO/EB) staining technique. The DNA was isolated to confirm the DNA damage; the observation clearly showed DNA damage when compared with DNA ladder.

Keywords: magnesiuim oxide nanoparticles, *Penicillium* sp., TEM, FTIR, apoptotic activity, anticancer activity
Classification numbers: 2.03, 4.02, 5.08

1. Introduction
Cancer, a very dreadful disease characterized by the uncontrolled and abnormal growth of cells, was mostly treated by using radiation therapy, surgery and chemotherapy [1]. These therapies are very good in destroying the uncontrolled growth of cancer cells, but at the same time they destroy also the normal cells [2]. Nowadays above-mentioned therapies are getting outdated due to the advancement in the field of nanobiotechnology and nanomedicine with specific targeted drug delivery which can inhibit the cells at different target sites [3]. Nanobiotechnology is the area in which nanoparticles are engineered for its biological and biomedical application. Nanotechnology has immense potential for therapeutic and imaging for early tumour detection...
and effective treatment of various cancers [4]. Nowadays nanotechnology especially nanomedicine showed the application in personalized medicine and for the detection of various cardiovascular disease [5, 6].

Nowadays various nanomaterials are synthesized like ZnO, MnO and MgO, which have immense potential in an increase in image contrast, can be used for diagnosis of diseases [7, 8]. Among various synthesized nanoparticles the CaO, ZnO and MgO nanoparticles are highly interested, because they are extremely stable in very harsh conditions [9, 10]. Magnesium oxide has large bandwidth with a wide range of applications, especially in toxic waste treatment, catalysis, anti-reflecting and reflecting coating, adsorbents, refractory materials [11, 12]. MgO nanoparticles showed good antibacterial and anticancer activities [13] and can be used for the treatment of heartburn, stomach sore and bone regeneration [14, 15].

Various approaches were used for the synthesis of nanoparticles like physical, chemical and biological methods, but the method using biological living organisms as biofactories like plants, bacteria, and fungi has the advantage because it is eco-friendly, cost-effective, easily available and straightforward [16–18].

This study aims to perform the biological synthesis of MgO nanoparticles from an aqueous extract of Penicillium species (Penicillium sp.), to characterize synthesized MgO nanoparticles by various microscopic techniques and to investigate the effect of these nanoparticles on A-549 cell line. The apoptotic activity of the cells and the DNA damage are also studied by using different stains PI (propidium iodide), AO, EB and gel electrophoresis.

2. Material and methods

2.1. Sample collection

The soil sample was collected from Universiti Kuala Lumpur Royal College of Medicine Perak (UniKL RCMP). The sample was transferred to sterile petri plate. Then, the plate was covered and was taken to Research Laboratory of UniKL RCMP and incubated at room temperature for drying purpose for further process.

2.2. Isolation of fungal culture

Serial dilution technique and spread plate method were used for the isolation of fungal culture. One gram of dried soil sample was diluted in 10 ml of sterilized distilled water. Then, the dilution was serially diluted to prepare a dilution with concentration range from $10^{-1}$ ml until $10^{-5}$ ml. 0.5 ml volume of dilution from the concentration range of $10^{-3}$ ml until $10^{-5}$ ml was transferred aseptically and spread on the potato dextrose agar (PDA) plates respectively. The plates were placed in dark condition and incubated at room temperature for three days. To prepare a pure culture of Penicillium sp. the isolated fungi were sub cultured on the Sabouraud Dextrose Agar (SDA) plates which can identify by the morphology. The purely isolated Penicillium sp. was maintained at 4°C for further studies.

2.3. Identification of Penicillium species

Penicillium sp. was observed by colony morphology concerning its size, colour, shape and nature of colony. The observation was conducted through microscopically and method was compared with available literature in the laboratory.

2.4. Biosynthesis of magnesium oxide nanoparticles

The purely isolated Penicillium sp. was employed for the synthesis of magnesium nanoparticle through extracellular method. Fungal biomass of Penicillium sp. was grown aerobically by adding the fungal spore in PDA broth and incubated at 25°C on rotary shaker fixed at 160rpm for 72 h. After 72 h, the fungal biomass was filtered by using Whatman filter paper No.1 and washed two to three times by using distilled water to remove the residual part. The fungal biomass was put into
a conical flask containing 100 mL of distilled water and incubated at 25 °C on rotary shaker fixed at 160 rpm for next 72 h. After 72 h of incubation on the rotary shaker, the biomass was filtered by using Whatman filter paper No. 1. The fresh, clean, and cell-free extract was taken into the clean conical flask for further study purpose. A suitable amount of magnesium nitrate (Mg(NO₃)₂) was added into the conical flask and kept at 25 °C on rotary shaker fixed at 160 rpm for 72 h in the dark condition. The colour and appearance change during storage in dark room was observed.

2.5. Characterization of MgO nanoparticles

The colour change of solution was observed by optical appearance after the addition of Mg(NO₃)₂ and was further confirmed by UV–vis spectrophotometric analyser with the wavelength of 300 nm to 600 nm to find the wavelength of maximum absorbance (λ_max). FTIR spectroscopy analysis has confirmed the essential functional groups and proteins which plays a significant role in stabilization of MgO nanoparticle. The powder form of nanoparticles was mixed with potassium bromide (KBr) to form a solid mixture of transparent film by using hydraulic presser and put in holder and subject for FTIR spectroscopy analysis. The MgO nanoparticles were centrifuged at 20000 rpm for 20 min, then supernatant of liquid was drained out and dried solid was converted into pellet was analyzed for structure through TEM analysis.

2.6. Antitumor activity of MgO nanoparticles

2.6.1. Cell line and cell culture. The alveolar lung cancer cell A-549 and Vero cell were obtained from the National center of cell science (NCCS) Poona India. Both cell lines were classically grown in MEM as monolayer supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) glutamine, 100 units ml⁻¹ streptomycin, 100 units ml⁻¹ penicillin at 37 °C under 5% carbon dioxide environment.

2.6.2. In vitro assay of cell viability. Biologically synthesized MgO nanoparticles were evaluated for its toxicity on normal Vero and A-549 lung cancer cell lines by MTT assay. The MgO nanoparticles were dissolved in double distilled water (ddH₂O) and different concentration of nanoparticles were used such as 60 μg ml⁻¹, 80 μg ml⁻¹, 100 μg ml⁻¹, 120 μg ml⁻¹, 140 μg ml⁻¹ and 160 μg ml⁻¹ on cell culture grown on 96-well microplates and incubate for 24 h at 37 °C. After the incubation cells were washed in phosphate buffer solution followed by 100 ml of 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide, tetrazolium MTT solution was added properly to all 96 wells and kept for incubation for 2–3 h at 37 °C. 100 μl dimethyl sulphoxide (DMSO) was added to each 96-wells microplate to solubilize the crystals formed by using MTT dye and after put in the shaker for 10–15 min so that all crystals of Vero and cancer cells will solubilize properly observed by producing the color. The viability of all cells was measured by using spectrophotometric analysis at 570 nm. Viability of cells was measured by using the following relation

\[
\text{Percentage of Cell viability} = \frac{\text{Optical density (OD) of MgO nanoparticles}}{\text{Optical density (OD) of Control}} \times 100. \tag{1}
\]

The toxicity of MgO nanoparticles on cancer and normal vero cells was expressed the amount of drug that kills 50% of cells which can compared with the 50% of untreated cells as IC50 value. This biological experiment was done in triplicate and DMSO was kept as negative control.

2.6.3. Analysis of apoptotic activity by PI/AO/EB dual staining. The propidium iodide (PI), acridine orange (AO) and ethidium bromide (EB) staining was used analyze the morphological changes induced by MgO nanoparticles in human alveoli lung cancer A-549 cell line. For that A-549 cells were separately plated at 5 · 10⁴ cells/well into a 6-well chamber plate. At >90% confluence and cells were treated with particular concentration of nanoparticles and incubate for 24 h. After incubation cells were washed with phosphate buffer saline (PBS) fixed in methanol: acetic acid (3:1, v/v) for 10 min and stained with 50 μg ml⁻¹ of PI for 20 min. For determining the mode of cell death, the cells were seeded in 6-well plates and treated with inhibitory concentration (IC50) of nanoparticles. To determine the nuclear properties, the monolayer of cells was washed with the PBS and stained with 5 μl of AO (100 μg ml⁻¹) and 5 μl of EB.
4 (100 µg ml\(^{-1}\)). The morphological and topological changes in the stained cells and their apoptotic nuclei were observed by fluorescent microscopy.

2.6.4. Analysis of DNA fragmentation. To determine the actual effect of nanoparticles on the DNA of A-549 (1 × 10⁶ cells ml\(^{-1}\)) cancer cells were plated per well in 6-well plates with the Dulbecco’s modified eagle medium (DMEM) contained 10% FBS. The cells were then incubated for 24 h under 5% CO\(_2\) at 37 °C. Then the medium was removed, washed with PBS; fresh serum free medium was added and kept in a CO\(_2\) incubator at 37 °C for 1 h.

2.6.5. Isolation of DNA. The starved cells were washed with PBS and then add 1 ml of lysis buffer mix properly and put in centrifuge tube. The whole mixture was incubated for 1 h at 37 °C and after incubation again add 5 µl of proteinase K incubated at 50 °C for 3 h. After incubation add pheno/chlo-roform isopropyl alcohol (25:24:1). The aqueous layer was gently transferred to the new Eppendorf tube and the absolute chilled alcohol (ethanol) was added and mix along with stand followed by centrifugation at 13000 rpm for 10 min. After centrifugation the supernatant was discarded and the pellet was detached and treated 70% ethanol to remove the impurities.

10 µg ml\(^{-1}\) of DNA samples were electrophoresed in 1% agarose gel containing ethidium bromide in a gel tank containing Tris-Borate EDTA (TBE) buffer for 1 h under 90 V. The gels were examined under a UV transilluminator.

Figure 4. Transmission electron microscopy (TEM) of MgO nanoparticles synthesized from Penicillium sp. with size range of (12 nm–24 nm). where (A)–(C) are the magnification of samples at different angles while (D) is the magnification at 200 nm scale.

Figure 5. Cytotoxic effect MgO nanoparticles on A549 cancer cells; where (A)—control A549 cancer cells, (B)—after incubated with MgONPs—Morphological changes in the cells showing membrane damage.

Figure 6. Graphical representation of cytotoxic effect MgO nanoparticles on Cancer cells.
towards normal cells (figure 7). Figures 6 and 8 show thepared with dosage on cancer cells that means it has less effectby the higher concentration of MgO nanoparticles when com-
tition of the DNA inside the cell [24]. The vero cell line affectedby producing the oxidative distress that results in the destruc-
species (ROS) which affected the essential enzymes in the cell
adherence to the surface due to the release of reactive oxygen
ology of the cells can be observed by the shrinking, and non-
toxic towards normal cells (figure 6). The change in morph-
alysis showed that MgO nanoparticles look spherical, prop-
shape and dispersity of MgO nanoparticles. The TEM anal-
alysis showed that MgO nanoparticles contain more apoptotic cells when compare with untreated
cells and damage of cells was observed by using 100 µg ml−1
concentration of nanoparticles increase the DNA damage and
apoptotic and necrotic cells would be fragmented and stained
DNA is intact. Early apoptotic cells will have fragmented DNA
visible observed by cell membrane damage on a dose dependent manner [22, 23] (as shown in figure 5).
Moreover Vero cell line incubated with MgO nanoparticles showed the IC50 value at 140 µg ml−1 which it showed less
toxicity towards normal cells (figure 6). The change in morph-
ology of the cells can be observed by the shrinking, and non-
-adherence to the surface due to the release of reactive oxygen
species (ROS) which affected the essential enzymes in the cell
by producing the oxidative distress that results in the destruc-
tion of the DNA inside the cell [24]. The vero cell line affected
by the higher concentration of MgO nanoparticles when com-
pared with dosage on cancer cells that means it has less effect
towards normal cells (figure 7). Figures 6 and 8 show the

3. Results and discussion
The aqueous extract of Penicillium sp. was utilized for the syn-
thesis of MgO nanoparticles. When the cell filtrate was chal-
enged with magnesium nitrate (MgNO3) the color of solution
becomes white cloudy indicates the synthesis of nanoparticles
(figure 1). These MgO nanoparticles were characterized by
UV–vis spectroscopy which showed the absorption peak at 215 nm confirms the presence of MgO nanoparticles due to
surface plasmon vibration [19, 20] (figure 2).

FTIR spectroscopy was used to check and identify the biomolecules, functional groups and proteins which act as
capping and play the main role in the stabilization of MgO nan-
oparticles using the aqueous extract of fungal species. The
FTIR spectroscopy analysis showed the sharp absorption bands at 3508.22 cm−1 (OH-stretch phenols), 2770.03 cm−1
(C–H stretch aldehydes), 2428.57 cm−1 (C=N stretch),
1763.45 cm−1 (C=O stretch), 1624.78 cm−1 (N–H amines),
1560.29 cm−1 (N–O asymmetric stretch nitro), 1387.84 cm−1
(N–O asymmetric stretch nitro), 1355.19 cm−1 (C–H alkanes),
1012.07 cm−1 (C–N amines), 827.11 cm−1 (C–H alkenes),
708.24 cm−1 (C–H alkanes) [21] (figure 3).

A high-resolution TEM was used to determine the size,
shape and dispersity of MgO nanoparticles. The TEM anal-
ysis showed that MgO nanoparticles look spherical, proper-
dispersed and the size of nanoparticles is in the range of 12–24 nm (figure 4).

The cytotoxic effect of biologically synthesized MgO nanoparticles on A-549 human lung cancer cell line showed the
excellent cytotoxic effect on the cancer cells that can be observed by morphological changes in the cell membrane by
using various concentration of MgO nanoparticles. The results showed the IC50 value at 100 µg ml−1 upon 24 h of incubation
which can be visually observed by cell membrane damage
on a dose dependent manner [22, 23] (as shown in figure 5).
Moreover Vero cell line incubated with MgO nanoparticles showed the IC50 value at 140 µg ml−1 which showed a
graphical representation of the cytotoxic effect of MgO nanoparticles on normal Vero cell line.

Apoptotic activity of MgO nanoparticles was revealed by
PI on A-549 cells to determine the nuclear morphology con-
firmed by membrane permeable PI stain. The treated cells
contain more apoptotic cells when compared with untreated
cells and damage of cells was observed by using 100 µg ml−1
which can be change in size, decreased fluorescence confirming con-
densed nuclear chromatin in apoptotic cells and formation of
membrane blebs.

Acridine orange and ethidium bromide (AO/EB) dual
staining can show the overall morphological change in shape
and size to the treated cell when compare with non-treated cells
[25, 26]. The viable cell can give green colour which means the
DNA is intact. Early apoptotic cells will have fragmented DNA
which gives several green coloured nuclei. The DNA of late
apoptotic and necrotic cells would be fragmented and stained
orange and red. From the above data it is clear increase the
concentration of nanoparticles increase the DNA damage and
number of the viable cell will decrease which can be seen with
less viable cells with more apoptotic cells (figure 9).

Analysis of DNA fragmentation was carried out on the
A-549 lung cancer cells treated with MgO nanoparticles to
induce the apoptosis of the cells. The DNA fragmentation
and the damage of DNA can be analyzed through the death of
cancer cells [27]. In the current study the DNA was damaged
consequently no band was observed when compare with DNA
ladder. Different kinds of DNA fragments from cancer cells
S Majeed et al treated with MgO nanoparticles showed the DNA damage and hence no visualization of bands on the agarose gel electrophoresis was seen (figure 10).

4. Conclusions

In this study *Penicillium* species was first time used for biosynthesis of MgO nanoparticles. The TEM analysis showed the stability and nanosize of MgO nanoparticles and these MgO nanoparticles showed excellent anticancer activity and less effect towards normal cells. From this study it was showed how nanoparticles induced apoptotic activity and also DNA damage when treated with MgO nanoparticles. These MgO nanoparticles could be a good anticancer agent but needs further cytotoxicity study on animals before using as strong anticancer agent.

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Conflict of interest

There is no conflict of interest among authors.

References


Figure 9. AO/EB and PI staining of human alveoli A-549 cancer cells. where (A)—normal green colour live cell, (B)—orange colour cells are apoptotic bodies induced by MgO nanoparticles (C)—chromatin condensation induced by MgO nanoparticles.

Figure 10. DNA Fragmentation analysis against A549 cancer cells where L 4—Normal DNA ladder (control), L3—DNA of treated cells showing complete damage of DNA.