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BIOSYNTHESIS OF ZINC OXIDE NANOPARTICLES BY *Pichia kudriavzevii* GY1 AND THEIR BIOMEDICAL PROPERTIES

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In the field of nanotechnology, the use of various biological materials instead of toxic chemicals for the synthesis of nanoparticles, has merited substantial attention. The use of toxic compounds limits their applications especially in biomedical field. To overcome the problem of toxicity in synthesis, safe eco-friendly green procedures have a major role for producing nanoparticles. Among the many possible bio resources, biologically active products from fungi and yeast represent high potential for this purpose. The present research aims to study the potential ability of a new yeast strain, *Pichia kudriavzevii*, in the synthesis of zinc oxide nanoparticles (ZnO-NPs) in order to evaluate their physical and biomedical properties.

Zinc oxide nanoparticles (ZnO-NPs) were synthesized by a green method using simple precursor from the solution consisting of zinc acetate and the fungal cell-free filtrate of *Pichia kudriavzevii* GY1.The effect of reaction time (12, 24 and 36 h) on the structure of the resulting ZnO nanoparticles was investigated. The samples were characterized using Fourier transform infrared (FTIR), ultraviolet-visible (UV-vis), x-ray diffraction (XRD), transmission electron microscope (TEM), field emission scanning electron microscope (FESEM), Photoluminescence (PL) and biomedical tests as free radical scavenging activity, cytotoxicity, antibacterial and anticancer activities.

From the XRD and TEM results, the ZnO-NPs with a hexagonal wurtzite structure and a particle crystal size of ~10–61 nm were formed at different reaction times. The most favourable nanosized structure with the lowest defect concentration was obtained at intermediate duration (24 h). The ZnO-NPs showed strong DPPH free radical scavenging and a dose dependent toxicity with non-toxic effects on Vero cells for concentrations below 190 µg/mL. The antibacterial tests reveal a favourable antibacterial effect for those ZnO-NPs prepared at intermediate duration.

The anticancer activity of biosynthesized ZnO-NPs on breast cancer cells (MCF-7) was determined using MTT assay. Cell cycle analysis and the mode of cell death were evaluated using a flow cytometry instrument. Quantitative real-time-PCR (qRT-PCR) was employed to investigate the expression of apoptosis in MCF-7 cells. ZnO NPs were cytotoxic to the MCF-7 cells in a dose-dependent manner. The 50% growth inhibition concentration (IC_{50}) of ZnO NPs at 24 h was 121 µg/mL. Cell cycle analysis revealed that ZnO NPs induced sub-G_1 phase (apoptosis), with values of 1.87% at 0 µg/mL (control), 71.49% at IC_{25}, 98.91% at IC_{50}, and 99.44% at IC_{75}. Annexin V/propidium iodide (PI) flow cytometry analysis confirmed that ZnO NPs induce apoptosis in MCF-7 cells. The pro-apoptotic genes *p53*, *p21*, *Bax*, and *JNK* were upregulated, whereas anti-apoptotic genes *Bcl-2*, *AKT1*, and *ERK1/2* were downregulated in a dose-dependent manner. The arrest and apoptosis of MCF-7 cells were induced by ZnO NPs through several signalling pathways. Generally, the study has successfully contributed to the knowledge on the discovery of using fast-growing microorganism as a novel green approach in producing nanoparticles as new materials for the current innovation of bionanotechnology field particularly for medical applications.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

BIOSINTESIS NANOPARTIKEL ZINK OKSIDA OLEH *Pichia Kudrivzevii* GY1 DENGAN PENCIRIAN BIOPERUBUATAN

Oleh

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Dalam bidang nanoteknologi penggunaan pelbagai bahan biologi dan bukannya bahan kimia toksik bagi sintesis nanopartikel telah mendapat perhatian besar. Penggunaan sebatian toksik mengehadkan aplikasi mereka terutama dalam bidang bioperubatan. Bagi mengatasi masalah keracunan dalam sintesis, prosedur yang selamat dan mesra alam berperanan penting bagi penghasilan nanopartikel. Di antara kepelbagaian sumber biologi, produk bioaktif daripada kulat dan ragi mempunyai potensi tinggi untuk tujuan ini. Penyelidikan ini bertujuan untuk mengkaji potensi strain yis baru, *Pichia kudriavzevii*, dalam sintesis nanopartikel zink oksida (ZnO-NPs) bagi menilai sifat fizikal dan bioperubatan.

Nanopartikel zink oksida (ZnO-NPs) disintesis melalui kaedah mesra alam iaitu menggunakan prekursor mudah dari larutan yang terdiri daripada zink asetat dan filtrat bebas-kulat filtrat Pichia kudriavzevii GY1. Kesan masa tindakbalas (12, 24 dan 36 jam) pada struktur nanopartikel ZnO yang dihasilkan telah dikaji. Sampel dianalysis menggunakan Fourier transform infrared (FTIR), ultraviolet-visible (UV-vis), x-ray diffraction (XRD), transmission electron microscope (TEM), field emission scanning electron microscope (FESEM), Photoluminescence (PL) dan ujian biomedikal iaitu pengujian penangkapan radikal bebas, sitotoksik, aktiviti antibakteria dan antikanser.

Daripada hasil XRD dan TEM, ZnO-NP dengan struktur wurtzit heksagon dan ukuran kristal partikel di antara ~ 10-61 nm terbentuk pada masa tindakbalas yang berbeza. Struktur nanosaiz yang paling baik dengan kepekatan kecacatan terendah didapati pada tempoh pertengahan (24 jam). ZnO-NPs memperlihatkan penangkapan

radikal bebas DPPH dan ketoksikan yang bergantung kepada dos dengan kesan tidak toksik kepada sel Vero untuk kepekatan di bawah 190 µg / mL. Ujian antibakteria menunjukkan kesan antibakteria yang baik untuk ZnO-NPs adalah pada tempoh pertengahan.

Aktiviti antikanser oleh ZnO-NPs pada sel-sel kanser payudara (MCF-7) ditentukan menggunakan assay MTT. Analisis kitaran sel dan mod kematian sel telah dinilai menggunakan instrumen aliran cytometry. *Quantitative real-time-PCR* (qRT-PCR) digunakan untuk menyiasat ekspresi apoptosis dalam sel-sel MCF-7. ZnO-NPs adalah sitotoksik kepada sel MCF-7 di mana bergantung kepada nilai dos. Nilai Kepekatan perencatan 50% pertumbuhan (IC50) oleh ZnO-NPs pada 24 jam adalah 121 µg / mL.. Analisis kitaran sel menunjukkan bahawa ZnO-NPs menggalakkan fasa sub-G1 (apoptosis), dengan nilai 1.87% pada 0 µg / mL (kawalan), 71.49% pada IC25, 98.91% pada IC50, dan 99.44% di IC75. Analisa aliran sitometri Annexin V / propidium iodide (PI) mengesahkan bahawa ZnO-NPs telah menggalakkan apoptosis di dalam sel MCF-7. Gen-gen pro-apoptotik p53, p21, Bax, dan JNK dikawal selaras, manakala gen anti-apoptotik Bcl-2, AKT1, dan ERK1/2 dikurangkan dengan cara yang bergantung kepada dos. Penangkapan dan apoptosis sel MCF-7 yang digalakkan oleh ZnO-NPs dilaksanakan melalui beberapa laluan isyarat sel. Secara umumnya, kajian ini telah berjaya menyumbang kepada pengetahuan dalam penemuan menggunakan mikroorganisma yang tumbuh secara cepat sebagai kaedah hijau yang baharu bagi penghasilan partikel nano sebagai bahan baharu untuk inovasi terkini bidang nanobioteknologi secara khususnys untuk aplikasi perubatan.

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LIST OF ABBREVIATIONS

DMSO	Dimethyl sulfoxide
NPS	Nanoparticles
ZnO	Zinc Oxide
h	Hour
PDA	Potato dextrose agar
PDB	Potato dextrose broth
mg	Milligram
min	Minutes
mL	Milliliter
PI	Propidium IODID
ROS	Reactive oxygen species
rpm	Rotation per minutes
TEM	Transmission electron microscopy
FESEM	Field emission scanning electron microscopy
ICP-AES	Inductively Coupled Plasma-atomic Emission Spectroscopy
XRD	X-ray Diffraction Pattern
UV	Ultraviolet
MTT	3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
DPPH	2,2-diphenyl-1-picrylhydrazyl
SDS	sodium dodecyl sulphate
°C	Degree Celsius
μ	Micro
%	Percentage
ATCC	American Type Culture Collection
FITC	Fluorescein isothiocyanate
IC_{50}	Median lethal concentration
μg	Microgram
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
DMEM	Dulbecco's Modification Eagle's Medium
PCR	Polymerase Chain Reaction

CHAPTER 1

INTRODUCTION

Nanotechnology provides many possibilities in various scientific and technological fields (Adibkia et al., 2007). Researchers are becoming increasingly interested in pharmacological nanotechnology due to its widespread applications. Nanoparticles can be produced by either chemical, physical and biotic processes (Alani et al., 2012). Green synthesis is an eco-friendly method for the production of nontoxic and biocompatible nanoparticles. These methods of NP synthesis are simple and efficient, and the products are safe for medical applications (Gopalakrishnan et al., 2012). In addition, molecular components of biological materials have great affinity for the surface of nano-structures, stabilize and prevent aggregation while improving the biological effects of NPs (Shabestarian et al., 2017). Biological procedures for nanoparticle synthesis using microorganisms (Tarafdar & Raliya, 2013; Gericke & Pinches, 2006; Liu et al., 2014; Ahmad et al., 2003), plants (Sadeghi & Gholamhoseinpoor, 2015), and alga (Singaravelu et al., 2007) have been proposed as feasible eco-friendly alternatives to chemical and physical approaches, because these methods are hazardous and costly (Sinha et al., 2009).

The potential use of yeasts in NP biosynthesis is promising because of the ease of handling yeasts under laboratory conditions, their abundant enzyme synthesis and rapid growth without the need of complex nutrients (Kumar et al., 2011; Yan et al., 2009). Economic longevity and capacity for employing biomass is yet another advantage of using the green approach employing yeasts to produce metallic NPs. Furthermore, numerous yeasts species grow at accelerated rates and therefore producing cultures as well as their storage in the laboratory is very practical (Dameron et al., 1989). Yeast biomasses are capable of producing metal nanoparticles and nanostructures through the reduction of proteins in enzymes either intracellularly or extracellularly (Castro, Blázquez et al., 2014). The reductive properties of proteins are related to many functional groups such as $-NH_2$, –OH, C=O, C–O–C, C–N–C, and –S–S–, which enable the donation of electrons to form NP from metal ions.

The biosynthesized ZnO-NPs are suitable to be used as drug carriers, cosmetics, and fillings in medicinal materials (Nagarajan & Kuppusamy, 2013). Zinc oxide (ZnO) shows biocompatibility compared to different materials (Arakelova et al., 2014). ZnO is an inorganic compound listed as "Generally Recognized as Safe" (GRAS) by the USA Food and Drug Administration (FDA) (21CFR182.8991) (Espitia et al., 2012).

Breast cancer is one of the most frequent types of cancers in women, and overall it is the second most common form of cancer worldwide. As many as 25% of cases of cancer (1.67 million cases) and 15% of deaths in women (522,000 individuals) were caused by forms of breast cancer, as reported by GLOBOCAN (Wolff et al., 2013).

The balance lost between the proliferated cells and apoptosis is a hallmark that intensifies the failure of damaged cells to be wiped out via apoptosis. Activating apoptotic paths in cells affected by tumour is a crucial approach to cancer therapy. Several natural products that are considered potentially powerful sources of anticancer drugs apply anti-tumour effects by inducing apoptosis (Elkady et al., 2012). Additionally, the deregulations that cause the initiation and development of cancers represent hundreds of genes or signalling cascades (Teiten et al., 2010). Furthermore, numerous genes like *p53*, *p21*, *JNK*, *Bax*, *Bcl-2*, *AKT*, and *ERK1/2* engage in apoptotic pathways.

ZnO nanoparticles with sizes less than 100 nm has been reported to possess good antimicrobial (Sirelkhatim et al., 2015), antioxidant (Nagajyothi et al., 2015) and antitumor (Selvakumari et al., 2015) activities. The technique for synthesizing ZnO nano-sized materials is a challenge for attaining standard medical therapies. Green and eco-friendly processes for the synthesis of ZnO-NPs are believed to be safe, nontoxic, and bio-compatible, for pharmaceutical and bio-medical applications as compared to chemical and physical methods which are hazardous and expensive. Moreover, the biologically active molecules involved in the green synthesis of NPs act as functionalizing ligands leads to the occurrence of succeeding different surface effects, controlling the size and structure of nanoparticles making these NPs more suitable for biomedical applications (Lu et al., 2007).

The main purpose of this study was used a new yeast strain, *Pichia kudriavzevii*, in the synthesis of ZnO-NPs through a green method for biomedical applications. In this study, it demonstrated a simple one-step green process to synthesize ZnO-NPs at different reaction durations using a newly isolated yeast strain, *Pichia kudriavzevii* GY1. *Pichia kudriavzevii* is synonymously known as *Issatchenkia orientalis* and is an anamorph of *Candida krusei* (Carlotti et al., 1996; Kurtzman et al., 1980). This yeast strain has been isolated from food and fruit sources, such as sourdoughs (Meroth et al., 2003), fermented butter-like products (Ongol & Asano, 2009) and the starter culture of Tanzanian fermented togwa (Mugula et al., 2003).

To our knowledge, there is no report on the green synthesis of ZnO-NPs using *P. kudriavzevii* GY1 in order to evaluate their antibacterial, antioxidant, and antitumor activities.As a result, a better insight into the anticancer activities of ZnO NPs and the cytotoxic effects of their constituents can contribute to facilitating the improvement of auspicious cancer therapeutics for use in nanomedicine. Nanomedicine has recently emerged as a better choice for treating some common cancers, resulting in many nanoparticles being used as treatment in cancer cell lines.

Hence, the specific objectives were as follows:

1. To isolate and characterize the yeast strain of *Pichia kudriavzevii* GY1.
2. To determine the effect of biosynthesis reaction on the characterization of ZnO-NPs.
3. To evaluate the antibacterial, antioxidant and anticancer activity of biosynthesized ZnO-NPs.
4. To evaluate the gene regulation of biosynthesized ZnO-NPs on the MCF-7 cell line.

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LIST OF PUBLICATIONS

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