GREEN SYNTHESIS OF ZINC OXIDE SILVER NANOPARTICLES USING *PUNICA GRANATUM* FOR ANTIBACTERIAL AND ANTICANCER APPLICATIONS

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DEDICATION

This thesis is dedicated to my family, friends, lecturers and most importantly myself. It was definitely not an easy journey but I am proud that I made it to the finish line.

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ABSTRACT

The problem of antimicrobial resistance (AMR) is expected to become a much worse issue as more microbes have developed resistance to conventional drugs. Thus, making medical treatments more complicated than usual. Similarly, cancer disease has been projected to affect a large number of human populations in the next few decades, resulting in increased morbidity and mortality worldwide. The field of nanotechnology has been thriving as it shows potential to ameliorate these growing concerns. Metallic nanomaterials such as zinc oxide nanoparticles (ZnO-NPs) and silver nanoparticles (Ag-NPs) are interesting materials to study as they possess great physical and chemical properties that could contribute in antibacterial and anticancer applications. Individual ZnO-NPs and Ag-NPs exhibit excellent activities in various field but there is still a lack of information on the efficiency of biologically synthesized coupled zinc oxide silver nanoparticles (ZnO-Ag-NPs) for use as antibacterial or anticancer agent. In this research, the "green" synthesis of ZnO-NPs and ZnO-Ag-NPs in the presence of *Punica granatum* (pomegranate) fruit peels extract under different parameters is reported. *P. granatum* fruit peels extract contains an abundance of phytochemicals that can act as a reducing or stabilizing agent during NPs synthesis process. Characterization of synthesized samples were carried out using X-ray diffraction (XRD), UV-visible (UV-vis) spectroscopy, High Resolution Transmission Electron Microscopy (HR-TEM) and Fourier Transform Infrared spectroscopy (FTIR). Sol-gel as well as combustion method was adopted for the synthesis of ZnO-NPs and the effect of calcination temperatures (400, 500, 600 and 700 °C) on the physico-chemical properties of ZnO-NPs was investigated. Crystallinity and particle sizes of the biosynthesized ZnO-NPs increased proportionally with calcination temperature. Subsequently, ZnO-Ag-NPs was synthesized using precipitation method in acidic, neutral and alkaline medium reaction (pH 5, pH 7 and pH 9 respectively) with *P. granatum* F. peels extract as a reducing/stabilizing agent. The role of pH during synthesis process of ZnO-Ag-NPs was evaluated and discussed accordingly. XRD confirmed the production of highly pure ZnO-Ag-NPs with increased crystallinity for samples in higher pH level. UV-vis analysis displayed shifting of absorption peaks of ZnO-Ag-NPs towards higher wavelength and a decrease in band-gap energy compared to pure ZnO-NPs, further confirming the coupling of Ag-NPs onto ZnO-NPs. For antibacterial studies, ZnO-Ag-NPs showed significantly high inhibition activities of both Gram-positive and Gram-negative bacterial strains compared to pure ZnO-NPs. The proposed mechanism of this enhanced antibacterial activities was further elaborated. *In vitro* cytotoxicity studies of the biosynthesized ZnO-NPs and ZnO-Ag-NPs against human colorectal cancer cell and normal colon cell were investigated but results showed non-selective killing of cells for both NPs. To conclude, pomegranate-mediated ZnO-Ag-NPs proves to be efficient as antibacterial agent, however, further modifications are required to give the NPs better specificity for cancer killing activities.

ABSTRAK

Masalah rintangan antimikrobial dijangka bertukar menjadi isu yang lebih teruk apabila lebih banyak mikrob membina daya tahan terhadap ubat-ubatan konvensional, menjadikan rawatan lebih rumit daripada biasa. Tambahan pula, penyakit kanser telah dijangka akan menjejaskan sebilangan besar populasi manusia dalam beberapa dekad akan datang, mengakibatkan peningkatan morbiditi dan kematian di seluruh dunia. Bidang nanoteknologi telah berkembang maju kerana ia menunjukkan potensi untuk memperbaiki kebimbangan yang semakin meningkat ini. Bahan nano logam seperti nanozarah zink oksida (ZnO-NPs) dan nanozarah perak (Ag-NPs) adalah bahan yang menarik untuk dikaji kerana ia mempunyai sifat fizikal dan kimia yang hebat dan boleh menyumbang dalam aplikasi antibakteria dan antikanser. ZnO-NPs dan Ag-NPs secara individu mempamerkan aktiviti cemerlang dalam pelbagai bidang tetapi masih terdapat kekurangan maklumat tentang kecekapan nanozarah zink oksida perak (ZnO-Ag-NPs) yang disintesis secara biologi untuk digunakan sebagai agen antibakteria atau antikanser. Dalam penyelidikan ini, sintesis hijau ZnO-NPs dan ZnO-Ag-NPs dengan kehadiran ekstrak kulit buah *Punica granatum* (delima) dengan parameter yang berbeza telah dilaporkan. Ekstrak kulit buah *P. granatum* mengandungi banyak fitokimia yang boleh bertindak sebagai agen pengurangan atau penstabil semasa proses sintesis NPs. Pencirian sampel yang disintesis telah dijalankan menggunakan pembelauan sinar-X (XRD), spektroskopi UV-visible (UV-vis), Mikroskop elektron Transmisi Resolusi Tinggi (HR-TEM) dan spektroskopi Inframerah Transformasi Fourier (FTIR). Sol-gel serta kaedah pembakaran telah digunakan untuk proses sintesis ZnO-NPs dan kesan suhu pengkalsinan (400, 500, 600 dan 700 °C) pada sifat kimia-fizik ZnO-NPs telah dikaji. Kehabluran dan saiz zarah ZnO-NP meningkat secara berkadar dengan suhu pengkalsinan. Selepas itu, ZnO-Ag-NPs telah disintesis menggunakan kaedah pemendakan dalam tindakbalas berasid, neutral dan alkali (pH 5, pH 7 dan pH 9) dengan ekstrak kulit *P. granatum* F. sebagai agen pengurangan/penstabil. Peranan pH semasa proses sintesis ZnO-Ag-NPs dinilai dan dibincangkan dengan sewajarnya. XRD mengesahkan penghasilan ZnO-Ag-NPs yang sangat tulen dengan peningkatan kehabluran untuk sampel yang disintesis dalam tahap pH yang lebih tinggi. Analisis UV-vis menunjukkan peralihan puncak penyerapan ZnO-Ag-NPs ke arah panjang gelombang yang lebih tinggi dan pengurangan tenaga jurang jalur berbanding ZnO-NPs tulen, seterusnya mengesahkan gandingan Ag-NPs ke ZnO-NPs. Untuk kajian antibakteria, ZnO-Ag-NPs menunjukkan aktiviti perencatan tinggi yang ketara bagi kedua-dua strain bakteria Gram-positif dan Gram-negatif berbanding ZnO-NPs tulen. Mekanisme yang dicadangkan untuk aktiviti antibakteria yang dipertingkatkan ini dihuraikan dengan lebih lanjut. Kajian sitotoksisiti *in vitro* ZnO-NPs dan ZnO-Ag-NP terbiosintesis terhadap sel kanser kolorektal manusia dan sel kolon normal telah dikaji tetapi keputusan menunjukkan pembunuhan tidak selektif sel untuk kedua-dua NP. Untuk membuat kesimpulan, ZnO-Ag-NP yang dimediasi delima terbukti cekap sebagai agen antibakteria, walau bagaimanapun, pengubahsuaian lanjut diperlukan untuk memberikan kekhususan nanozarah yang lebih baik untuk aktiviti pembunuhan kanser.

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LIST OF ABBREVIATIONS

AMR	-	Antimicrobial Resistance
ATCC	-	American Type Culture Collection
ATR	-	Attenuated total reflection
B. subtilis	-	*Bacillus subtilis*
CCD112	-	Normal colon cell line
CFU/mL	-	Colony-forming units per milliliter
CLSI	-	Clinical and Laboratory Standards Institute
DI	-	Deionized
DMEM	-	Dulbecco's Modified Eagle's medium
DNA	-	Deoxyribonucleic Acid
E. coli	-	*Escherichia coli*
E. coli K1	-	*Escherichia coli K1* strain
EPR	-	Enhanced permeability and retention
FBS	-	10 % fetal bovine serum
FDA	-	Food and Drug Administration
FTIR	-	Fourier Transform Infrared
GRAS	-	Generally Recognized As Safe
HCT116	-	Human colorectal cancer cell
HR-TEM	-	High Resolution Transmission Electron Microscopy
IC_{50}	-	The minimum inhibitory concentration needed to inhibit 50% of cell growth
MDR	-	Multidrug Resistance
MHA	-	Mueller Hinton agar
MHB	-	Mueller Hinton broth
MIC	-	Minimum Inhibitory Concentration
MIC_{50}	-	The minimum inhibitory concentration needed to inhibit 50% of microorganism growth
MOF	-	Metal-organic frameworks
MRSA	-	Methicillin-resistant *Staphylococcus aureus*
MSW	-	Municipal solid waste
MTS	-	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MyAP-AMR	-	The Malaysian Action Plan on AMR

NPs	-	Nanoparticles
OD	-	Optical density
P. granatum F.	-	*Punica granatum* Fruit
PPL	-	Priority Pathogens List
PSO	-	Pomegranate seed oil
R & D	-	Research and Development
ROS	-	Reactive oxygen species
S. aureus	-	*Staphylococcus aureus*
S. enterica	-	*Salmonella enterica*
SPR	-	Surface plasmon resonance
TB	-	Tuberculosis
UV	-	Ultraviolet
UV-vis	-	Ultraviolet visible
WHO	-	World Health Organization
XRD	-	X-Ray Diffraction
Z-4	-	ZnO-NPs calcined at 400 °C
Z-5	-	ZnO-NPs calcined at 500 °C
Z-6	-	ZnO-NPs calcined at 600 °C
Z-7	-	ZnO-NPs calcined at 700 °C
ZA-0	-	ZnO-Ag-NPs synthesized in original pH of reaction medium (without addition of NH_4OH)
ZA-7	-	ZnO-Ag-NPs synthesized in pH 7 of reaction medium
ZA-9	-	ZnO-Ag-NPs synthesized in pH 9 of reaction medium

LIST OF SYMBOLS

mA	-	Milliamperes
kV	-	Kilovolt
M	-	Molarity
λ	-	X-ray wavelength of radiation
θ	-	Diffraction angle
β	-	Full width at half maximum
μ	-	Micro

CHAPTER 1

INTRODUCTION

1.1 Research Background

Presently, the issue of antimicrobial resistance (AMR) has been growing as a dangerous global threat to multiple sectors especially the healthcare industry. AMR occurs when a microorganism develops natural resistance to an antimicrobial drug that was originally effective for the treatment of infections caused by it (Mariappan *et al.*, 2021). These drug-resistant microbes can then grow and spread, leading to the emergence of ‘superbugs’, which are extremely difficult to treat with limited existing medicines. Previous data report already suggested that more than 700,000 people die every year currently from drug-resistant strains of common bacterial infections such as tuberculosis (TB) and malaria (Neill, 2016). Without proper actions to combat this issue, it is estimated that AMR will cost up to 10 million lives by the year of 2050 with more than four million deaths in Asia continent alone as shown in Figure 1.1 (Neill, 2016). In an effort to address this extremely concerning matter, World Health Organization (WHO) has published a Global Priority Pathogens List (PPL) to urge more research and development (R&D) of novel antimicrobial agents, in particular antibacterial drugs.

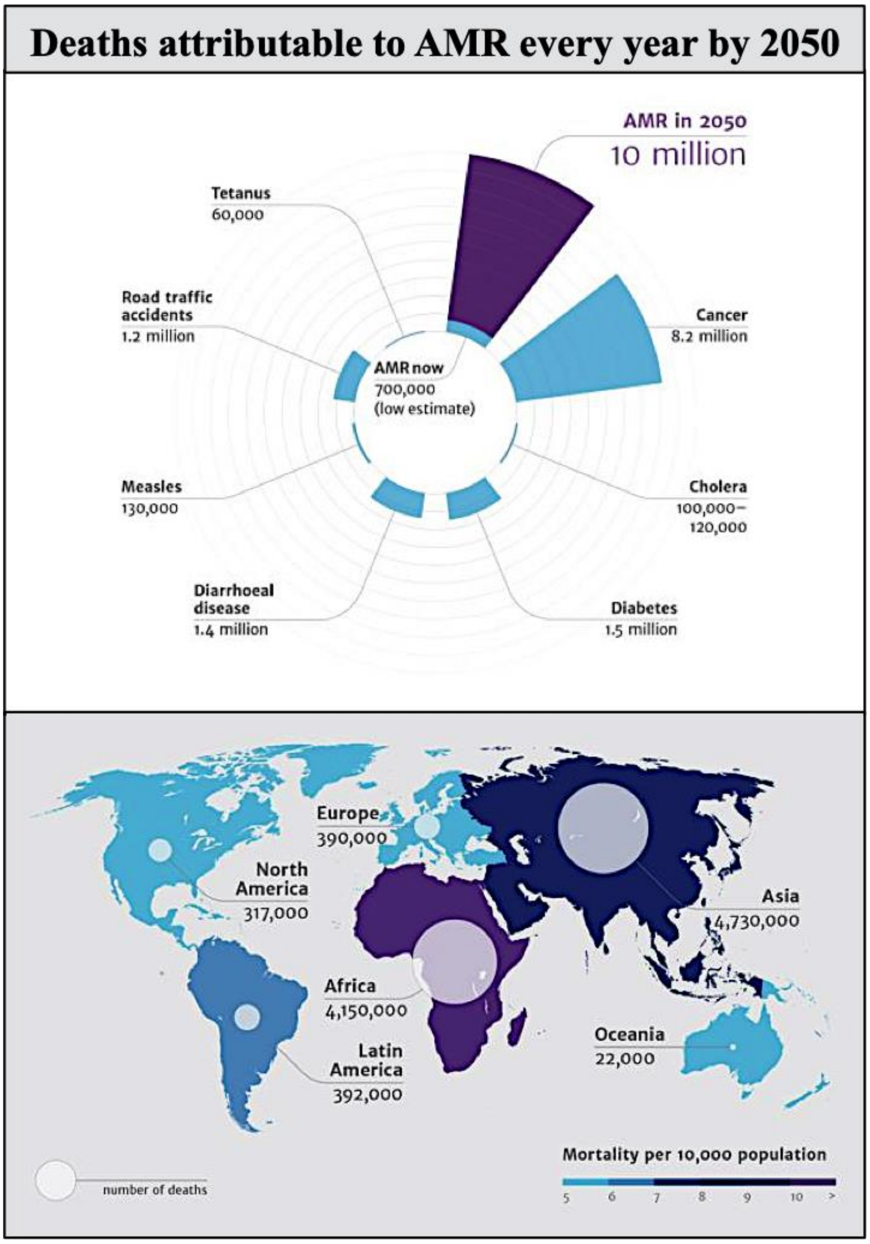

Figure 1.1 Estimated deaths worldwide due to AMR by the year 2050 (Neill, 2016)

Another major disease and leading cause of deaths that has long been burdening the human population is cancer. Cancer is a well-known illness triggered by genetic mutations in cellular DNA, damaging the mechanism regulating cell death and cell division, leading to uncontrolled proliferation of cells in human body (Doughty *et al.*, 2019). Various types of cancer forms have been identified up until now but the most common ones are formation of tumor masses in specific parts of the body. Figure 1.2 shows the top 10 most common cancer types and their estimated global incidence count in the year of 2020 . For male of all ages, lung cancer (14.3 % of all cases) is the most frequently diagnosed cancer, closely followed by prostate cancer (14.1 % of total cases) and colorectum cancer (10.6 % of total cases). Meanwhile, breast cancer (24.5 % of total cases) takes a major lead as the most commonly diagnosed cancer for female of all ages followed by colorectum cancer (9.4% of total cases) and lung cancer (8.4 % of total cases). It can be observed that lung and colorectum cancers make up a large part of the cases affecting both genders.

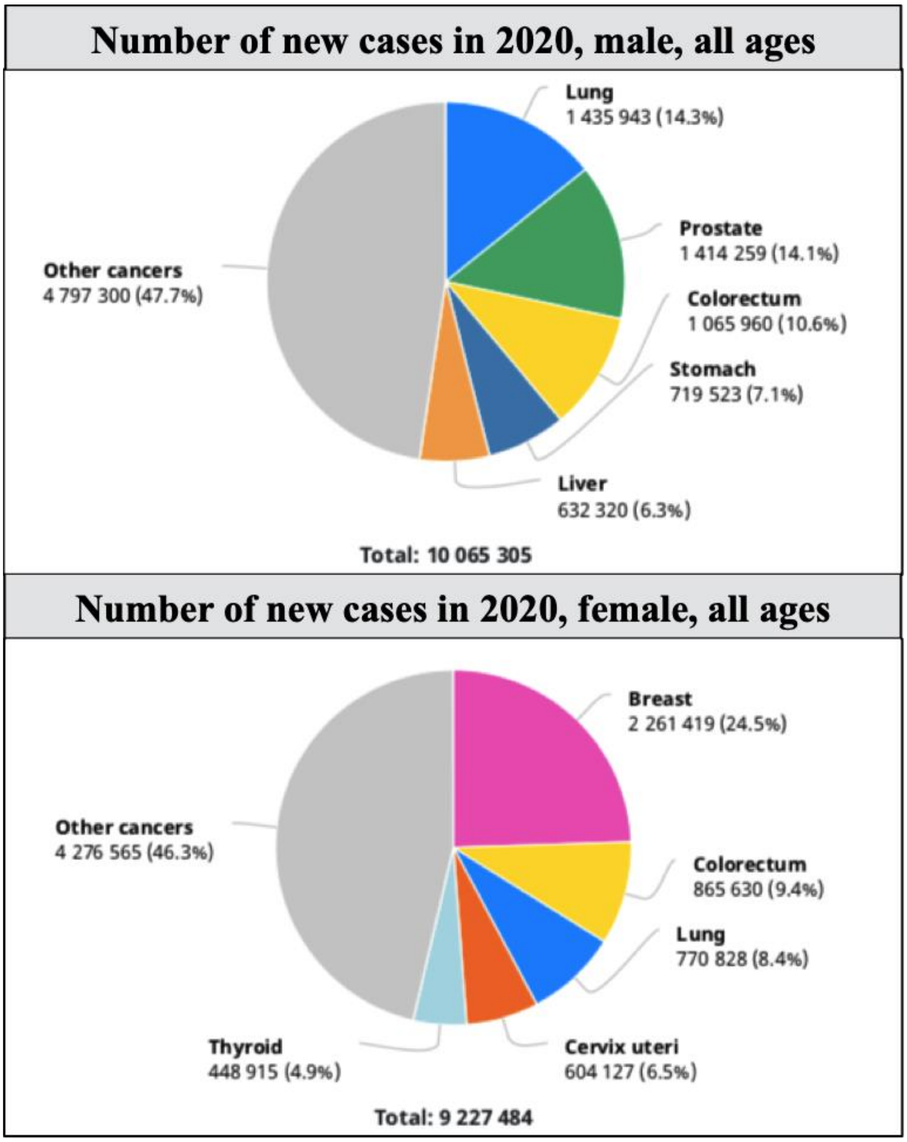

Figure 1.2 Global cancer incidence in the year 2020 for both sexes (Sung *et al.*, 2021)

Cancer patients can be treated accordingly based on the type and stage of their disease. Conventional cancer treatments such as surgery, radiotherapy and chemotherapy are effective, however, they come with severe side effects that reduce patient’s quality of life. In addition, the development of resistance to various chemotherapeutic drugs, a phenomenon known as multidrug resistance (MDR), is one of the most significant obstacles in treating cancer.

Nanoparticle-based therapy has emerged as a promising alternative option to resolve both antimicrobial resistance problem and improving treatment of cancer diseases. The unique and distinctive physico-chemical features of nanomaterials give them various advantages when interacting with biological systems. Amongst different types of nanomaterials, metallic nanoparticles (NPs) are known to have excellent antimicrobial and anticancer activities. Metallic NPs offer special qualities, such as enhanced properties due to high surface-to volume ratio, increased mobility because of their small size and bioavailability as they can be used for specific drug targeting.

In the process of fabricating NPs, researchers can adopt either ‘top-down’ or ‘bottom-up’ approach. ‘Top-down’ approach involves breaking down bulk materials into nanostructures using mainly physical techniques such as sonication, mechanical milling and laser ablation (Sadhasivam *et al.*, 2020). However, this method is quite elaborate, time consuming and generates larger particles as opposed to nano-sized particles. In ‘bottom-up’ approach, atoms or molecules self-assemble into nuclei and then into particles of nano-sized range (Yadi *et al.*, 2018). This type of method is usually favored as it gives better control of particle sizes and shapes. Generally, ‘bottom-up’ approach is carried out through chemical or biological synthesis. Although both methods are effective, chemical synthesis mainly use toxic and dangerous chemicals which then generate hazardous by-products to be released to the environment (Yadi *et al.*, 2018). With increasing public awareness on environmental pollution and sustainability, the focus on biological synthesis of nanomaterials has been consistently growing as it makes use of green resources such as plants, fruits, and microbes. Therefore, biological method is a much more simple, environmentally-friendly and cost-effective way to fabricate NPs.

Zinc oxide (ZnO) is a renowned inorganic material that has been used widely in everyday applications. Compared to other metal oxides, ZnO is comparably inexpensive and deemed non-toxic since the Food and Drug Administration (FDA) has listed it as a generally recognized as safe (GRAS) material (Espitia *et al.*, 2012). Currently, ZnO is regularly used as a UV blocker in sunscreen and as food additives (Sadhasivam *et al.*, 2020). Based on previous studies, ZnO in nanoscale perform considerably well in various biomedical applications and drug delivery systems due to its biocompatibility nature (Sadhasivam *et al.*, 2020). Despite promising reports of ZnO-NPs efficiency in nanomedicine, there are still intrinsic limitations such as UV-dependent optical absorption, instability in biological fluids and unpredictable cytotoxic effects that need to be improved (Carofiglio *et al.*, 2020). To overcome the said limitations of ZnO-NPs and further boost its use as an antibacterial or anticancer agent, coupling ZnO-NPs with another metallic NPs seems like a promising solution.

Silver (Ag) has been utilized since 5000 years back when the Egyptians, Persians, Greeks and Romans used Ag in numerous forms to store food items (Tarannum, Divvya and Gautam, 2019). Similarly, various dinasties have been using silverwares for drinking and eating during the ancient period. This extensive usage of Ag in daily household items and activities is probably driven by the knowledge of antimicrobial action of this superior material. According to published literatures on Ag-NPs, it is widely accepted that Ag-NPs can demonstrate remarkable protection against a wide range of Gram negative and Gram positive bacteria (Abinaya *et al.*, 2016; Nguyen *et al.*, 2019). In addition, several papers also reported good cytotoxic activities of Ag-NPs against different types of cancer cells (Długosz *et al.*, 2020; Elena *et al.*, 2020).

Although there have been extensive research done on pure individual metallic NPs such as ZnO-NPs and Ag-NPs, there are still lack of studies reported for coupled ZnO-Ag-NPs fabricated using biological synthesis. In this research, we report the preparation of ZnO-NPs and ZnO-Ag-NPs through biological synthesis by utilizing the extract of *Punica granatum* fruit (*P. granatum* F.), more familiarly known as pomegranate. The extract was obtained from pomegranate fruit peels, which make up about 49 % to 55 % of the fruit weight and are typically disposed as agro-waste (Waste *et al.*, 2020). Studies shown that *P. granatum* F. peels extract contains a substantial amount of beneficial phytochemical compounds that play a key role as a reducing or capping agent during the synthesis process of NPs (Boroumand *et al.*, 2015; Salem, Albanna and Awwad, 2016). Therefore, it can replace the usage of conventional toxic reducing agents as well as enhance biomedical activities of the NPs. The biosynthesized ZnO-NPs and ZnO-Ag-NPs were then analysed for their antibacterial and cytotoxic efficiency against a wide range of Gram positive and Gram negative bacterial strains as well as normal and cancerous colorectal cells.

1.2 Problem Statements

Antimicrobial resistance is considered as a global health concern that should be taken seriously before it turns into a devastating problem in the near future. If not tackled accordingly, AMR situation could exacerbate, crippling our healthcare system and negatively affecting world's economy. WHO in their published Global PPL, has highlighted the concerning number of bacterial resistance presently threatening public health. Besides AMR, another disease that has been consistently contributing to the high number of mortality worldwide is cancer. It is predicted that by the end of 2040, the number of deaths caused by cancer will increase up to three times than it is now (Andleeb *et al.*, 2021). One of the most commonly diagnosed cancer types affecting both male and female worldwide is colorectal cancer (Sung *et al.*, 2021). Currently, conventional cancer therapies used in treatment frequently induce various negative side effects, significantly reducing patient's quality of life. Additionally, another issue that keeps limiting patients from efficient treatment is the development of drug resistance towards multiple chemotherapeutic drugs (Kulshrestha and Khan, 2018). In an effort to resolve these extremely alarming problems, it is evident that there is an urgent need to develop a highly effective antibacterial and anticancer agent by utilizing nanomaterials.

Typically, chemical methods are used to synthesize ZnO-NPs but this technique requires the use of hazardous chemicals such as sodium borohydride, sodium dodecyl sulfate and hydrazine as the reducing agent (Sadhasivam *et al.*, 2020). When released as by-products, these toxic chemicals can cause serious environmental pollution and cause adverse reaction to human health. Due to this, biological or green synthesis has emerged as an alternative method in the fabrication process of nanomaterials. Biological synthesis is preferable as it makes use of natural resources such as plants, fruits and microbes instead of toxic substances. However, microbial-mediated synthesis is usually associated with tedious protocols and expensive instruments. On the other hand, plant-mediated synthesis of nanomaterials, particularly those that uses agro-wastes as the green material is more desirable as it makes the overall process more environmental-friendly, simple and cost-effective.

While pure ZnO-NPs possess many interesting physical and chemical properties, there are still several limitations that reduce its effectiveness as an antibacterial and anticancer agent. As ZnO-NPs has a large band gap, it can only be photoactivated under UV light radiation (Tri *et al.*, 2019). Additionally, ZnO-NPs has a low photoenergy conversion efficiency, is said to be unstable in biological fluids and has uncontrolled cytotoxic activities (Carofiglio *et al.*, 2020). In order to improve the performance of ZnO-NPs, coupling it with a superior material such as Ag can further enhance its role in biomedical applications.

1.3 Objectives

The main aim of this project is to biofabricate ZnO-NPs and hybrid ZnO-Ag-NPs with antibacterial as well as anticancer properties. Several research objectives can be derived as listed below:

(a) To green synthesize ZnO-NPs and ZnO-Ag-NPs mediated by *Punica granatum* fruit peels extract in different parameters.

(b) To characterize and evaluate physico-chemical properties of biosynthesized ZnO-NPs and ZnO-Ag-NPs.

(c) To determine the antibacterial and cytotoxic efficacy of biosynthesized ZnO-NPs and ZnO-Ag-NPs.

1.4 Scopes of Study

In order to achieve and complete all of the listed objectives in this project, the research scopes complementing each objective are further explained in detail as follows:

(a) Both ZnO-NPs and ZnO-Ag-NPs were biosynthesized with *P. granatum* fruit peels extract as the reducing agent in the reaction. ZnO-NPs was produced via sol-gel and combustion method and the effect of different calcination temperature (400 °C, 500 °C, 600 °C and 700 °C) on the properties of samples was investigated. ZnO-Ag-NPs was then generated through precipitation method under varying pH level of experimental reaction (ori pH, pH 7 and pH 9).

(b) All biosynthesized samples underwent characterization analysis using ultraviolet visible (UV-vis) spectroscopy, X-Ray Diffraction (XRD), Fourier Transform Infrared spectroscopy (FTIR) and High Resolution Transmission electron Microscopy (HR-TEM) in order to observe the changes in their physico-chemical properties under different experimental conditions.

(c) Evaluation of antibacterial activity for all biosynthesized ZnO-NPs and ZnO-Ag-NPs was done using Minimum Inhibitory Concentration (MIC) Assay against Gram Positive and Gram Negative bacterial strains. On the other hand, cytotoxic efficacy against human colorectal cancer cell (HCT116) and normal colon cell line (CCD112) was assessed using MTS Assay.

1.5 Significance of research

Antimicrobial resistance and cancer are two major problems that have been and will be affecting a major group of the human population worldwide. Nano-based therapeutic agents have emerged as an alternative solution to combat these growing issues as they show great potential for bacterial growth inhibition and cancer killing activities. Recent focus on clean and green process for environmental sustainability has shed light towards the practice of biological synthesis for fabricating nanomaterials. In this work, *P. granatum* F. peels extract is utilized to biosynthesize ZnO-NPs and ZnO-Ag-NPs, eliminating the usage of toxic chemicals reagents that would be released into the environment as waste by-products. Besides that, fruit peels are considered as agro-wastes and it contributes to a staggering 45% of the total municipal solid waste (MSW) in Malaysia (Jun, Bashir and Sayuti, 2021). These organic wastes are disposed into landfills, further contributing to the generation of polluting greenhouse gases. Therefore, by utilizing these agro-wastes as green materials for nanomaterials production, it indirectly helps in environmental sustainability. To the best of our knowledge, the biological synthesis of ZnO-Ag-NPs using *P. granatum* F. peels extract for biomedical applications has not been reported before. Our research also studies on the effect of pH level in the biosynthesis of ZnO-Ag-NPs and comparing its antibacterial and cytotoxic efficiency against pure ZnO-NPs.

1.6 Outline of the Thesis

This thesis contains five chapters starting from introduction, literature review, methodology, results and discussion as well as conclusion. In Chapter 1, the background of research will be introduced to give a general overview of the main topics in this work. Subsequently, the problem statements of this research are addressed and all important objectives to be achieved are highlighted. The next section explains further in detail about the scopes of study executed in order to accomplish each objective and lastly the research significance is elaborated to emphasize the importance of this work.

Chapter 2 specifically focuses on literature review of previous scientific findings by other scholars who work in this area. The subtopics include in-depth discussions on biological synthesis of nanomaterials, particularly ZnO-NPs and ZnO-Ag-NPs, the uses of nanomaterials in antibacterial and anticancer applications as well as the interesting properties of *P. granatum* (pomegranate) for nanoparticles fabrication. The purpose of this chapter is to find the research gap in this study by analyzing past results published by fellow researchers and come up with an improved procedure to solve it.

Chapter 3 comprehensively describes the experimental methodology including a research flowchart, list of chemicals and detailed explanations on the instruments and methods used for characterization analysis.

In Chapter 4, all experimental results obtained throughout the whole research are presented through pictures, tables, illustrations and plotted graphs. These include findings from preparation and characterization of *P. granatum* F. peels extract, biosynthesis and characterization of ZnO-NPs and ZnO-Ag-NPs, antibacterial activities of the NPs samples and finally their cytotoxic efficacy. These results were carefully analyzed and discussed accordingly with supporting data.

Final chapter of this thesis summarizes the findings achieved from this project and concludes whether the objectives have been successfully accomplished or not. In addition, recommendations for future studies are also proposed to improve the quality of forthcoming studies.

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