UNIVERSITI PUTRA MALAYSIA

IN-VITRO COMPARISON OF ANTICANCER ACTIVITY BETWEEN PURE GALLIC ACID AND GALLIC ACID - IRON OXIDE COATED WITH POLYETHYLENE GLYCOL NANOPARTICLES

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IB 2018 38

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

October 2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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October 2018

Chairman: Associate Professor Datin Sharida Fakurazi, PhD
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Cancer poses one of the biggest health threats in the world. Lung cancer, breast cancer and colorectal cancer were the top three leading cause of cancer mortality worldwide, both in 2012 and 2015. Chemotherapy has been the most commonly used treatment to treat cancer but the side effects of conventional chemotherapy has proven to be detrimental to the body, as surrounding healthy normal cells are unnecessarily affected due to lack of selectivity, poor aqueous solubility and rapid degradation of the anticancer drug. This calls for a need of a safer anticancer drug and a more specific and sustained drug delivery system. Gallic acid is a bioactive polyphenol with anticancer and cytoprotective properties that can be found in plants and foods such as blueberries and walnuts. Polymeric magnetite nanoparticles, iron oxide-polyethylene glycol (FPEG), was used as a nanocarrier for gallic acid, synthesizing a gallic acid-iron oxide coated with PEG nanocomposite. This study compared the anticancer activity between pure gallic acid (GA) and the nanocomposite (FGPEG) to prove the significance of nanoparticles in ensuring a safer, bio-responsive and more efficient drug delivery. The 3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay result showed that both GA and FGPEG exhibited time and dose-dependent cytotoxicity in human lung cancer cells (A549), human breast cancer cels (MCF-7) and human colon cancer cells (HT-29), while no cytotoxicity was seen in normal fibroblast cells (3T3). 3T3 has been one of the most widely used normal cell lines in anticancer studies. IC_{50} values caused by FGPEG were much lower in all cancer cell lines compared to those of GA alone. With the lowest IC_{50} value, HT-29 was the most responsive to FGPEG, followed by MCF-7 and A549. Acridine orange propidium iodide (AOPI) double staining showed that both GA and FGPEG were found to trigger morphological features related to apoptosis such as membrane blebbing, chromatin condensation and

nuclei fragmentation. FGPEG treated cells showed distinctive morphological changes and higher rate of apoptosis than cells treated with GA, evident by the increased cell blebbing and the yellowish orange stained cells indicating late apoptosis. High resolution transmission electron microscopy (HRTEM) revealed intact cell membrane and nucleus, chromatin condensation, increased vacuolation, dilated mitochondria, elongated rough endoplasmic reticulum and nuclear shrinkage in both GA and FGPEG treated cells. However, the presence of autophagosomes, residual bodies, increased number of dilated organelles and lipid droplets seen only in FGPEG treated cells indicated cells were in late apoptosis progressing to secondary necrosis. Since FGPEG treated cells have displayed more distinguishable morphological characteristics stipulating higher rate of apoptosis than GA alone despite being subjected to the exact same prolonged *in vitro* conditions, the significant advantage of nanoparticles FPEG as a nanocarrier was evident. Our results demonstrated that polymeric magnetite nanoparticles are indeed advantageous over pure drug alone in anticancer drug delivery system, forming a compelling justification for the utilization of this design as a platform for a safer, more specific and sustained chemotherapeutic drug delivery.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PERBANDINGAN *IN-VITRO* AKTIVITI ANTIKANSER ANTARA ASID GALLIC TULEN DAN ASID GALLIC-ZARAH NANO OKSIDA BESI YANG DISALUTI DENGAN ZARAH NANO POLYETHYLENE GLYCOL

Oleh

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Kanser adalah salah satu ancaman kesihatan terbesar di dunia. Kanser paru-paru, kanser payudara dan kanser kolon merupakan 3 penyebab utama kematioan akibat kanser di seluruh dunia pada tahun 2012 dan 2015. Kimoterapi merupakan rawatan kanser yang paling banyak digunakan sekarang. Namun begitu, kimoterapi konvensional banyak membawa kesan buruk kepada tubuh badan kerana ubat kimoterapi turut serta membunuh sel-sel normal disekeliling sel kanser. Ini adalah disebakan kelemahan dalam aksi pemilihan, kekurangan daya larutan akueus dan degradasi ubat yang pantas. Oleh yang demikian, sistem penyampaian ubat yang lebih selamat, spesifik dan mampan adalah diutamakan. Asid galik (GA) adalah sebatian polifenol bioaktif yang mempunyai ciri-ciri antikanser dan bersifat sitoperlindungan yang boleh didapati di dalam makanan seperti beri biru dan kekacang. Polimer magnetik nanozarah-besi oksida-poliethilin glaikol (PEG) telah digunakan sebagai nanopembawa untuk asid galik, lantas menghasilkan asid galik-besi oksida yang diselaputi PEG nanokomposit (FGPEG). Kajian ini telah membandingkan aktiviti antikanser antara GA dan FGPEG nanokomposit bagi membuktikan manfaat penggunaan nanozarah dalam penyampaian ubat yang lebih selamat, bio-responsif dan lebih efisyen. Keputusan ujian 3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) telah menunjukkan kedua-dua GA dan FGPEG mempamerkan sitotoksik mengikut masa dan dos ke atas sel kanser paru-paru (A549), sel kanser payudara (MCF-7) dan sel kanser kolon (HT-29) tetapi sitotoksik tidak berlaku keatas sel fibroblas normal (3T3). Nilai IC_{50} yang disebabkan FGPEG adalah jauh lebih rendah bagi kesemua sel-sel kanser berbanding rawatan GA semata-mata. Dengan nilai IC_{50} yang terendah, HT-29 adalah sel kanser yang mempunyai reaksi paling tinggi terhadap FGPEG, diikuti oleh MCF-7 dan A549. Melalui ujian pewarnaan akridin oren propidium iodida untuk melihat mod kematian sel, kedua-dua GA

dan FGPEG didapati mencetuskan ciri-ciri morfologi yang berkaitan dengan apoptosis seperti pelecetan membran, kondensasi kromatin dan kepecahan nuklei. Sel yang dirawat dengan FGPEG menunjukkan perubahan morfologi yang lebih ketara dan menakjubkan serta mempamerkan kadar apoptosis yang lebih tinggi daripada sel yang dirawat dengan GA. Ini kerana kenaikan di dalam pelecetan membran dan juga warna sel yang kuning keorenan telah membuktikan tahap apoptosis yang lebih tinggi. Keputusan mikroskopi elektron transmisi dengan resolusi tinggi menunjukkan membran dan nukleus sel yang masih utuh, kondensasi kromatin, kenaikan vakuol, kelebaran mitokondria, kepanjangan endoplasmik retikulum kasar dan pengecutan nuklear di dalam sel yang dirawat dengan FGPEG dan GA. Walaubagaimanapun, autofagosom, badan-badan baki, kenaikan dalam jumlah organel yang lebar dan kenaikan jumlah titisan lipid yang didapati hanya di dalam sel yang dirawat dengan FGPEG telah menandakan bahawa sel tersebut di tahap apoptosis yang lewat dan menuju kepada nekrosis sekunder. Memandangkan sel yang dirawat dengan FGPEG telah menunjukkan perubahan morfologi yang lebih banyak dari sel yang dirawat dengan GA semata-mata, walaupun pada dari permulaan kedua-dua kumpulan sel berada di dalam keadaan *in-vitro* yang sama, ini telah membuktikan manfaat penggunaan naokomposit FGPEG sebagai nanopembawa. Keputusan kajian ini telah menunjukkan bahawa polimer magnetik nanozarah adalah benar-benar bermanfaat dari GA semata-mata dalam sistem penyampaian ubat antikanser. Ini telah membentuk satu justifikasi yang meyakinkan bagi penggunaan reka bentuk nanozarah ini untuk memastikan penyampaian ubat kimoterapi yang lebih selamat, spesifik dan mampan.

ACKNOWLEDGEMENTS

First and foremost, all praises to Allah SWT for I have been blessed with a sound mind, good health and the willpower to power through my Master of Science journey. It was by no means the easiest and smoothest journey of my life but there are a lot of people who have helped me to make the experience so much better, fulfilling and ultimately successful.

My utmost gratitude goes to my supervisor Associate Prof. Dr. Sharida Fakurazi, whose guidance, knowledge and wisdom have enriched my journey as a postgraduate student and pushed me to greater limits. Thank you very much for not just being an incredible mentor, but also a motherly figure to me. Special thanks to my co-supervisors; Prof. Mohd Zobir Hussein, and Dr. Sandra Maniam, who has been so amazing, understanding and very helpful in making my time as a postgraduate student teasier.

Of course, my research and thesis would not be completed without the tremendous support from various departments and laboratories. My sincerest thank you to MAKNA Cancer Research namely Mrs Tommini Salleh for providing cancer cell lines, Mrs Emi Nadia Mohd Thani for helping me with my AOPI and Mrs Norlela Ahmad for aiding to smoothen the microscope booking process. Not forgetting those from the Microscopy Unit like Mr Rafiuz Zaman Haroun, Mrs Irmazian Abd Shukor, Mrs ZahidahMuhamed and especially to Prof. Tengku Azmi Tengku Ibrahim who has taught me so much on distinguishing cell morphologies and what to look out for under TEM, and also for going through my TEM results thoroughly. I would like to extend my gratitude to the staff of LIVES such as Mrs Norhafiza Azwa Ghozali, Mrs Norhaszalina Md Isa, Mrs Nancy Liew Woan Charn, Dr. Tan Sheau Wei and Mrs Noor Nadia Mohd Sohaili. My appreciation goes to Dr. Saifullah Bullo for his assistance with my very first paper and also for providing the nanocomposite for my research. A big thank you to my fellow laboratory mates like Dr. Aminu Kura, Khaleel Badran, Taufik, Tan Woan Sean,Hasfar Amynurliyana, Nur Syafinaz, Kalaivani Buskaran, Hani, Karthi and Suleiman for not only teaching me the correct ways and the right ethics working in a laboratory but also for always sharing laughter and worries with me.

Last and certainly not the least, words cannot express my appreciation for all the things my family has done for me. To my mother, Nor Aini Ab Shukor and my father, Rosman Abdullah, thank you ever so much for setting such a remarkable example for me to emulate, both in the academic field and in becoming a well-rounded human being. Finally ,my thoughts and prayers go to my grandparents especially my grandmother Hajah Che Mah Abu Bakar, who is lost but never forgotten.

I certify that a Thesis Examination Committee has met on 12th October 2018 to conduct the final examination of Raihana Rosman on her thesis entitled "In-vitro Comparison of Anticancer Activity Between Pure Gallic Acid and Gallic Acid - Iron Oxide Coated With Polyethylene Glycol Nanoparticles" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

3T3	Normal fibroblast cell line
A549	Human lung cancer cell line
AO	Acridine orange
ATCC	American Type Culture Collection
Bcl-2	B-cell lymphoma 2
DAMP	Damage-associated molecular pattern
DDS	Drug delivery system
DISC	Death inducing signalling complex
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
EPR	Enhanced permeability and retention
ER	Endoplasmic reticulum
FADD	Fas-associated death domain protein
Fas	Stimulating apoptotic fragment
FasL	Stimulating apoptotic fragment ligand
FBS	Fetal bovine serum
FGPEG	Gallic acid-iron coated with PEG nanoparticles
FPEG	Iron oxide-PEG nanocarrier
GA	Gallic acid
HRTEM	High resolution transmission electron microscopy
HT-29	Human colon cancer cell line
IC_{50}	Half maximal effective concentration
LNCaP	Human prostate cancer cell line
MCF-7	Human breast cancer cell line
MGDD	Magnetically guided drug delivery
MMP	Mitochondrial membrane potential
MRI	Magnetic resonance imaging
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	Nanocarrier
NP	Nanoparticle
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PI	Propidium iodide
RER	Rough endoplasmic reticulum
RIP	Receptor-interacting serine
RPMI	Roswell Park Memorial Institute 1640 medium
ROS	Reactive oxygen species
SPION	Superparamagnetic iron oxide nanoparticle
TNF-α	Tumor necrosis factor-α
TNF	Tumor necrosis factor
TNFI	Tumor necrosis factor inhibitor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated death domain protein
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Research background

Cancer is a group of disease where it affects all living cells, at all ages and in both genders. Cancer cells are formed when healthy cells lose their normal regulatory mechanisms that control cell growth. They become rogue cells and often lose the special characteristics that differentiate one type of cell from another, for example a liver cell from a blood cell (Lin et al., 2012; Ohno et al., 1999). This is called loss of differentiation, which results in a cancerous growth and later may become malignant through metastasis. It is these malignant cancers which are life threatening. Cancer is not one disease and despite having common genetic themes, every cancer is different (Zaghloul Salem, 2015).

In addition, cancer develops very slowly over many years and as such, it reflects years of ongoing evolutionary selection. It is a very complex disease that humans only ever begin to treat it very late in its course (Wild, 2014).

.Non- conventional natural treatments have gained wide acceptance due to their promise of a cure with minimal or no side effects (Kura et al., 2014). Gallic acid (GA) is one of the phenolic acids and bioactive compounds that can be found in plants and foods such as white tea and witch hazel and it has been reported to possess antioxidant, anti-inflammatory and anticancer properties (Devi et al., 2014; Subramanian et al., 2015). It is also known for its protective activity on normal cells which makes it as a pivotal for cancer therapy (Devi et al., 2014; Liang et al., 2014). This bioactive compound alone is anticancer by itself but it may not be delivered effectively in conventional therapy due to limitations like non-specific drug distribution, poor drug efficacy due to shorter half-life and poor aqueous solubility among others, causing detrimental side effects. Therefore, the significant utilization of polymeric magnetite nanocarrier for targeted drug delivery system to improve the overall pharmacological properties of gallic acid in potential novel chemotherapy is emphasized.

Magnetically-assisted delivery of chemotherapeutic agents to the site of tumor, which is referred to as magnetic drug targeting, has proven to be a promising strategy for cancer therapy in a number of studies (Kralj et al., 2017; Tietze et al., 2015; Wang et al., 2013). Superparamagnetic iron oxide nanoparticles with proper surface architecture and biocompatibility in particular, have attracted extensive attention for drug delivery applications as nanocarrier (Nedyalkova et al., 2017; Valdiglesias et al., 2016).

On top of that, a polymeric coating of polyethylene glycol (PEG) gives way to a controlled release of the drug besides contributing to the drug sustainability by prolonging its circulatory half-life. PEG is also hydrophilic by nature which benefits towards greater aqueous solubility (Mishra et al., 2016; Yallapu et al., 2010). A previous study also reported that metallic oxide core encapsulated in a polymeric coating resulted in stable, biocompatible and biodegradable nanoparticles (Dorniani et al., 2014b).

For the stated advantages, iron oxide-polyethylene glycol (FPEG) nanoparticles are employed as the nanocarrier for novel anticancer agent gallic acid in this study, creating the gallic acid-iron oxide coated with PEG nanocomposite. This nanocomposite has the abilities to deliver the drug specifically to the targeted cells while extending the half -life of the drug. The impact of this study will pave a way to develop a safer, more specific and sustained drug delivery system to treat cancer more effectively without serious side effects.

1.2 Problem statement and justification of study

For both 2012 and 2015, lung cancer, breast cancer and colon cancers were the most common cancer globally. These three cancers were also among the top five deadliest cancers in the world for both years. Therefore, the development of effective cancer medications is one of the greatest health challenges mankind faces (Hare et al., 2017). A major problem in treating cancer is the fact that it is not a single disease. There are more than 200 different cancers resulting from different cellular defects (Patrick, 2017). Therefore, treatment that is effective in controlling one type of cancer may be ineffective on another.

The main goal of cancer treatment is to mitigate and kill cancer cells without overexerting the patients with undesirable side effects which could eventually lead to fatality (Estanqueiro et al., 2015). The current main cancer treatments include surgical excision, radiotherapy and chemotherapy (Hu et al., 2016). The choice of treatment normally depends on the type and severity of the disease but chemotherapy has been the most common line of treatment used to treat cancer to date (Huang et al., 2015; van der Meel et al., 2017). However, chemotherapy comes with a lot of detrimental side effects as chemotherapeutic drugs have a number of drawbacks such as lack of selectivity in the mechanisms of action, lack of specificity towards cancer tissues and the tendency of drug degradation in the body due to the low molecular weight, low circulation half-life and poor aqueous solubility of the drug (Lin et al., 2012; Partridge et al., 2001; Rathore et al., 2017).

Consequently, these drawbacks lead to implications like calling the need for higher drug concentration to achieve enough therapeutic effects, causing

failure in a uniform drug delivery to the whole tumor mass, reducing the efficacy and incurring significant damage to nearby healthy cells, especially those with a fast growth fraction such as hair cells. In fact, the trouble with chemotherapy is that it kills many more healthy cells than it does cancer cells (Pearce et al., 2017). These are causal factors for substantial short term and long term side effects notoriously associated with chemotherapy, namely systemic toxicity accretion towards healthy cells, immunosuppression, hair loss, infertility and the repercussive secondary tumour (Lin et al., 2012; Partridge et al., 2001; Rathore et al., 2017).

Although there are plenty of chemotherapeutic drugs that can combat the disease, they cannot cure cancer completely when detected at later stages (Subramanian et al., 2016). Additionally, these drugs are at risk of also killing rapidly dividing normal healthy cells, thus causing unwanted and often uncontrolled toxicity to normal cells(Partridge et al., 2001; Subramanian et al., 2016).

Owing to the unwanted side effects of chemotherapy, an important challenge in treating cancer using this method is to find a technology for a controlled and specific targeted drug delivery and release to maximize drug efficacy, thus eradicate tumour cells while sparing the healthy normal cells.

Despite the enormity of the challenge, nanotherapeutic and theranostic are gaining much attention to overcome the limitations of conventional drug delivery system. Various drug loaded nanocarriers which offer a predominantly unique set of chemical, physical and photonic properties for better drug delivery pr/and imaging diagnostic have been developed and reported.

In this study where the main focus is on targeted drug delivery system, attention is devoted to a novel anticancer drug delivery design in a form of a nanocompodite consisting of gallic acid-iron oxide coated with PEG nanoparticles for a number of reasons. Gallic acid is a bioactive polyphenol that has been hugely studied for its anticancer properties and protective activity towards normal cells (Devi et al., 2014; Subramanian et al., 2015, 2016).

Iron oxide has been one of the most extensively reported magnetic nanoparticles for targeted drug delivery system (Estelrich et al., 2015; Li et al., 2017). Key features of iron oxide such as the ability to exhibit magnetization only in an applied magnetic field and the ability to be guided to desired sites of tumour in the body by external magnetic force make it desirable as a nanocarrier for magnetically guided drug delivery (Laurent et al., 2014; Wang et al., 2013).

To enhance drug stability and to sustain its therapeutic effect by increasing their circulation time and also reducing their immunogenicity, hydrophilic polymeric coating is used and of the known hydrophilic synthetic polymers available, PEG has been most widely used as a hydrophilic drug carrier (Ulbrich et al., 2016; Yallapu et al., 2010).

Lung cancer (A549), breast cancer (MCF-7) and colon cancer (HT-29) cell lines have been chosen in this study in line with the fact that these three cancers have been the most common cancers in the world. In addition, the cancer cell lines were chosen as they have been predominantly used for the different types of cancer respectively in well-known studies (Dorniani et al., 2012; Hussein-Al-Ali et al., 2014; Maurya et al., 2011; Stevens et al., 2017; Tor et al., 2015). Normal fibroblast cell (3T3) cell line, being one of the most widely used normal cell lines against various cancer cell lines in anticancer studies (Barahuie et al., 2014; Hussein-Al-Ali et al., 2014), was chosen as the control cell line for this research.

1.3 Hypothesis

Gallic acid-iron oxide coated with PEG nanocomposite has higher anticancer activity than gallic acid alone due to the presence of superparamagnetic iron oxide and PEG nanocarrier that ensures a specific drug delivery and a sustained drug release.

1.4 Objectives

This study targets to compare the anticancer efficacies between pure gallic acid and gallic acid-iron oxide coated with PEG nanocomposite against cancer cells by exploring and analyzing the changes incurred between the treatments *in-vitro*.

The specific objectives are as follows:

1. To determine the cytotoxicity of gallic acid alone and the nanocomposite against human lung cancer (A549), breast cancer (MCF-7) and colon cancer (HT-29)
2. To determine the mode of cell death induced by 50% cytotoxic dose of gallic acid and the nanocomposite
3. To observe the morphological changes and localize the intracellular deposition of nanocomposite in cancer cells treated with IC_{50} dose of gallic acid and the nanocomposite

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