ANTICANCER STUDY OF PORCUPINE BEZOAR EXTRACTS ON HUMAN BREAST AND LUNG CANCER CELLS

BY

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ABSTRACT

Breast and lung cancer have highest mortality rate among female and male respectively. Even though with the availability of advanced treatment, the mortality rate is still increasing. Thus, this study aims to elucidate the potential Malayan porcupine bezoar (PB) in exhibiting anticancer effects on the breast (MCF7) and lung (A549) cancer cells. Porcupine Bezoar extracts were tested with optimized $IC5_0$ for its potential ability in inhibiting cell growth, changing morphology, inducing apoptosis and cell arrest. The PB extract showed the growth inhibitory effect with IC_{50} value at 19.0 µg/ml (MCF7) and 13.5 µg/ml (A549) respectively. The morphology assessment upon PB extract treatment at 72 hours displayed possible apoptosis features of pyknosis and karyorrhexis. Further investigation with Annexin-V/7AAD revealed PB treated cell induced early and late apoptosis with a presence of dead cells in 72 hours. Further test has shown, PB may have altered cell cycle regulation and arrest in G1 for both cells. Possible signaling pathway at a molecular level for both cells was investigated. The result revealed induction of apoptosis following intrinsic pathway by initiating the release of *Cytochrome C* from mitochondria thereby activating caspase cascade. The above claims were supported by downregulation of *Bcl-2* which suggest PB extracts triggered apoptosis. Furthermore, the investigation was conducted on cell cycle regulator genes, revealed the up-regulation of *p21* and down-regulation of *cyclin D* suggesting cell arrest in G1 phase. Additionally, finding demonstrate PB extracts was selective in inducing significant effect on cancer cells MCF7 and A549 compared to normal fibroblast 3T3-L1 and HGF-1. This research report unveil the potential medicinal properties of porcupine bezoar in anticancer perspectives.

خلاصة البحث

أعلى معدل وفيات بين الإناث والذكور، سرطان الثدي وسرطان الرئة على التوالي. وعلى الرغم من توافر العلاج المتقدم، فإن معدل الوفيات ما زال في ازدياد. وهكذا، تهدف هذه الدراسة إلى توضيح فعالية porcupine bezoar (PB) الماليزي في تأثيره كمضاد لسرطان الثدي (MCF7) والرئة(A549) . تم اختبار مستخلصات porcupine bezoar مع IC_{50} الأمثل لقدرتها المحتملة في تثبيط نمو الخلايا السرطانية، وتغيير التشكل، وتحريض الخلايا على القتل المبرمج وتوقيف الخلايا. أظهر مستخلص PB التأثير التثبيطي على نمو الخلايا مع قيمة IC_{50} عند 19.0 ميكروغرام / مل (MCF7) و 13.5 ميكروغرام / مل (A549) على التوالي. تقييم مورفولوجيا الخلايا المعالجة ب PB خلال 72 ساعة عرض ملامح موت الخلايا المبرمج مع أشكال من pyknosis and karyorrhexis. كشف مزيدا من التحقيق مع أنيكسين V / 7AADالمعالجة ب PB وقتا مبكرا ومتأخرا في موت الخلايا المبرمج مع وجود خلايا ميتة في 72 ساعة. وقد أظهرت اختبارات أخرى، أن PB غيرت تنظيم دورة الخلية وحدث الاعتقال في G1 لكلا من الخلايا. وقد تم التحقيق في مسار الإشارات المحتملة على المستوى الجزيئي لكل من الخلايا. وكشفت النتيجة تحريض موت الخلايا المبرمج بعد المسار الجوهري من خلال الشروع في الافراج عن السيتوكروم C من الميتوكوندريا وبالتالي تفعيل caspase cascade. وقد دعمت النتائج المذكورة أعلاه حدوث تناقص في تنظيم Bcl-2 التي تشير إلى أن مستخلصات PB تسبب في موت الخلايا المبرمج. وعلاوة على ذلك، أجري التحقيق على الجينات المنظمة لدورة الخلية، وكشف عن تنظيم أعلى من P21 والتناقص في تنظيم cyclin D مما يشير إلى اعتقال الخلايا في مرحلةG1 . بالإضافة إلى ذلك، تعتبر مستخلصات PB انتقائية في إحداث تأثير كبير على الخلايا السرطانية MCF7 و A549 مقارنة مع الخلايا الليفية العادية 3 T3-L1و HGF-1. هذا التقرير البحثي كشف النقاب عن الخصائص الطبية المحتملة ل porcupine bezoar من وجهات النظر المضادة للسرطان.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Science.

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

ATCC	American type culture collection
A549	Lung cancer cells
BLAST	Basic local alignment search tool
Bax	Bcl-2-associated X protein
Bcl2	B-cell lymphoma 2
BID	BH3-interacting-domain death
CASPASE	Cysteine aspartic acid protease
CDKs	Cyclin-dependent kinases
CDKI	Cyclin dependent kinases inhibitors
CDNA	Complementary DNA
CGM	Complete growth media
CIP/KIP	CDK Interacting protein/kinase inhibitor protein
CO2	Carbon dioxide
ddH2O	double-distilled water
DNA	Deoxyribonucleic acid
DMEM	Dulbecco‟s Modified Eagle Medium
DMSO	Dimetyl sulphoxide
E	Efficiency
FADD	Fas-associated death domain protein
FasL	Fas ligand
FBS	Fetal bovine serum
IC_{50}	Inhibition concentration (reduces the effect by 50%)
IAP	Inhibitor of apoptosis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Guanine-cytosine (DNA base pairing)
KIP	Kinase inhibitory proteins
LC50	Lethal concentration
mRNA	messenger RNAs
MIQE	Minimum information for quantitative polymerase chain reaction publication experiments
MCF7	Breast cancer cells
NCBI	National center for biotechnology
PB	Porcupine bezoar
PBS	Phosphate buffer saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI	Propidium Iodide
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Real-Time
7AAD	7-Aminoactinomycin D

LIST OF SYMBOLS

α	Alpha
β	Beta
Δ	Delta
Cq	Quantification cycle
Ct	Threshold cycle
g	Gram
G	Gravity
G	Gap
hpf	Hours post fertilization
M	Mitosis
S	Synthesis
µl	Microliter
µg/ml	Microgram per millilitre
°C	Degree Celsius
%	Percent
-	To
>	More than
<	Less than
±	Plus-minus
x	Times
=	Equal to
*	Statistical significance denotation

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Cancer is one of major concern nowadays as it is continuously reported to be the second leading cause of death worldwide (Hashim et al. 2016; Park et al. 2008; Siegel, Miller, and Jemal 2016; Xu et al. 2016). Study reported cancer is expected to grow worldwide, especially in underdeveloped countries which contribute to 82% of the world's population (Alberg, Brock, and Samet 2005; American Cancer Society 2014; Park et al. 2008; Siegel et al. 2016; Torre et al. 2015; Yip, Taib, and Mohamed 2006). Additionally, a study by Torre et.al reported in 2012 there were 14.1 million new cancer cases and 8.2 million deaths due to cancer condition globally. In Asia alone, the incidence from 6.1 million in 2008 to 10.6 million in 2030 of cancer cases was estimated to expand as Asia contributed 60% of the world total population (Sankaranarayanan, Ramadas, and Qiao 2014).

In Malaysia, the cumulative cancer risks in peninsular Malaysia were up to 18% of 26,089 cancer patients, in which 54.7% were females and 45.3% were males (Onn and Stats 2002). The statistic also showed that the risk of getting cancer is very high with a probability of 1 from 4 Malaysian will have the potential of getting cancer in their lifetime. Furthermore, the report revealed that the highest occurrence cancer for males was lung cancer by 13.9%, while for the woman was breast cancer 30.4%. Different incidence was documented in 2016 where the leading cancer for males was

prostate (21%) followed by lung or bronchus cancer by 14.0% and for woman it is breast cancer by 29.0% subsequently lung or bronchus with 13.0% (Siegel et al. 2016).

In addition, the occurrence of cancer is becoming an enormous burden when available cancer therapies, such as surgery, chemotherapy and radiotherapy, are showing defective prognosis and various side effects (American Cancer Society 2014; Mariotto et al. 2007; De Moor et al. 2013; Siegel et al. 2013). Therefore, the exploration of anti-cancer agent with minimal toxicity and highly specific becomes urgent to avert increasing cancer cases throughout the world every year.

1.2 STATEMENT OF THE PROBLEM

Researchers had been struggling after years in finding new potential anticancer agents to manage cancer patients as current treatment leaves side effects for a lifetime and are not targeted specifically on the cancer cells (Amin et al. 2009). Thus, exploration on alternative strategy using a natural resources is crucial to overcome cancer treatment challenges.

One of long forgotten natural resources that once known as the prince of antidote are porcupine bezoar (PB). Bezoar is a stone which consists of lump undigested organic and inorganic material, which hardened into calcareous concretions in the gastrointestinal tract (Barroso 2014; Duffin 2013; Mori and Sforzi 2013). It is reported that bezoar can be existed in any kind of mammals, however the most famous bezoar is PB which is due to its medicinal value. Porcupine bezoar was documented of its medicinal value as early 8^{th} century from Persian (Barroso 2014). Between the years of 968 and 977AD, Abu Mansur Muwaffak in his famous work Materia Medica mentioning about bezoars, its medicinal values and it was categorized under precious

stone (Duffin 2013). It was described to have the ability to treat pestilent disease such as cholera, plague, malignant diseases, quartan fevers, acute febrile illness, small pox, measles, jaundice, bilious, kidney stones, pleurisy, palpitations of the heart, heart diseases, epilepsy, intestinal worms, the bloody flux, internal abscesses, leprosy, intestinal obstructions, and chickenpox (Duffin, 2013& Barroso, 2014).

Despite all these medicinal values, no scientific investigation has been done with regard to its medicinal value. Additionally, there are PB consumers in Malaysia which believe PB can cure cancer. Therefore, this study intended to discover the PB extracts effects on cancer using in vitro model to understand its effect and mechanism.

1.3 RESEARCH OBJECTIVES

Considering the inadequate information on the PB and its efficacy as anticancer agents, the general aim of this study is to explore the anticancer property of PB using in vitro models. The specific objectives are:

i. To determine 50% inhibitory concentration (IC_{50}) of PB on human breast cancer cells (MCF7) and human lung cancer cells (A549).

ii. To determine the effect of PB extracts on normal cell lines, mouse embryo fibroblast (3T3-L1) and normal human gingival fibroblast (HGF-1).

iii. To observe apoptosis related morphological changes upon treatment with PB extracts on MCF7 and A549 cells.

iv. To investigate the effect of PB extracts on apoptosis and its markers (*Bax, Bcl-2, Bid, caspases, Cytohrome C*) on MCF7 and A549 cells.

v. To investigate the effect of PB extracts on cell cycle regulation and its markers (cyclins, cyclin dependent kinases) on MCF7 and A549 cells.

1.4 RESEARCH QUESTIONS

i. Does IC_{50} of PB extracts induce significant effects on MCF7 and A549 cells?

ii. Do PB extracts induce significant toxicity to 3T3-L1 and HGF-1?

iii. Does PB extracts able to induce apoptosis morphology on MCF7 and A549 cells?

iv. Does IC_{50} of PB extracts induce significant effects of apoptosis on MCF7 and A549 cells?

v. Does IC_{50} of PB extracts induce significant effects of arresting cell cycle on MCF7 and A549 cells?

1.5 RESEARCH HYPOTHESES

i. PB extracts induce significant 50% inhibitory at low concentration.

ii. PB extracts can be toxic to 3T3-L1 and HGF-1 only at extremely high concentration.

iii. PB extracts able to induce morphological changes of apoptosis on MCF7 and A549 cells.

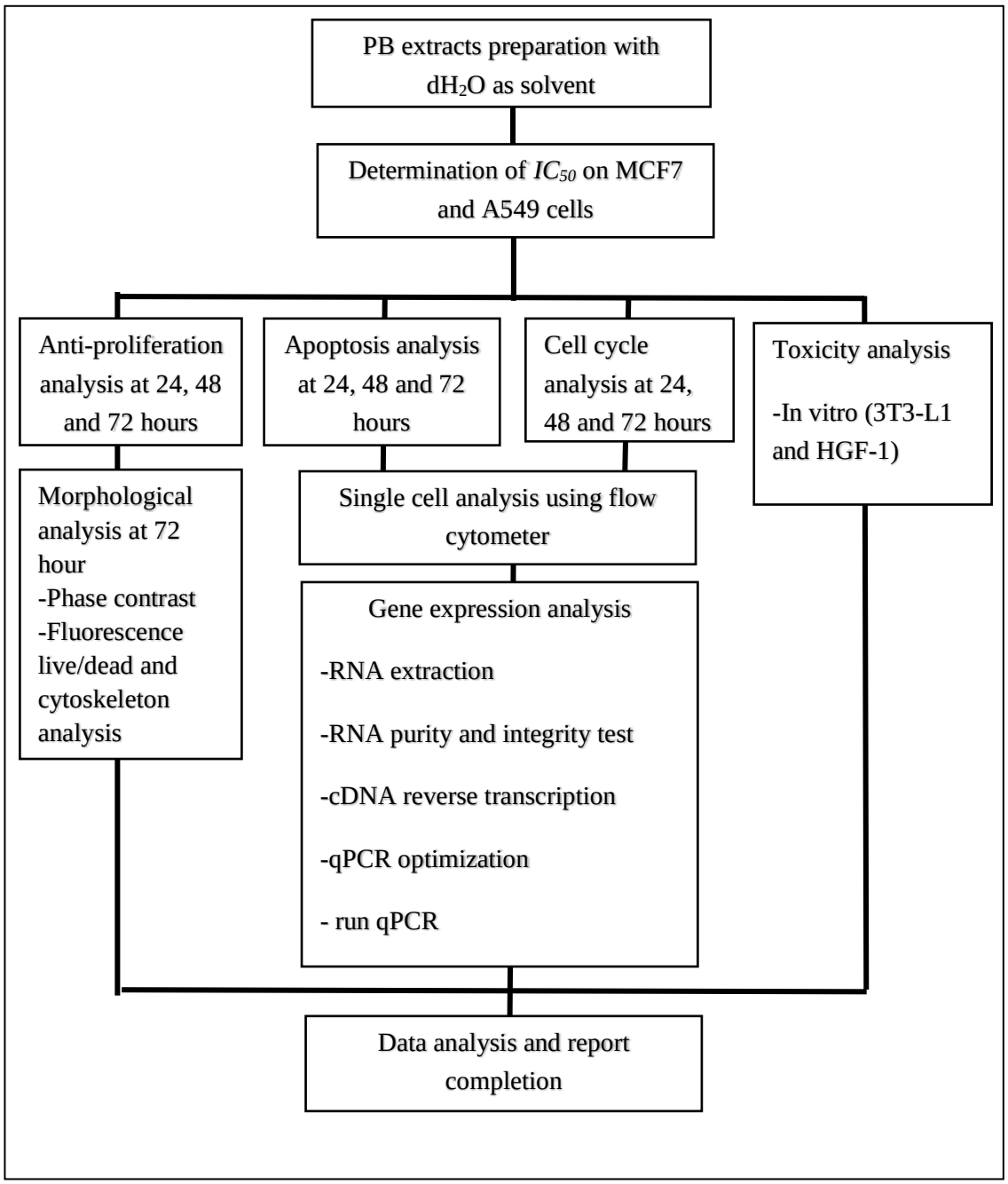
iv. PB extracts trigger apoptosis on MCF7 and A549 cells.

v. PB extracts promote cell cycle arrest on MCF7 and A549 cells.

1.6 SIGNIFICANCE OF THE STUDY

For the first time, the present study will provide scientific data on the effect of PB extract on cancer cells, MCF7 and A549. The cell cycle profile changes and type of cell death induced by PB extract also crucial as a part of anticancer candidate development. In addition, the molecular part of the result will provide more information in understanding of how PB extracts react towards MCF7 and A549 cells at the molecular level. Furthermore, a study done in normal cells and preliminary findings in zebrafish will provide better understanding on the therapeutic and toxic dosage of PB extracts. Overall, this study will provide a better insight of PB and the strategy that can be established to direct the study further as potential anticancer agents in the future.

1.7 RESEARCH FRAMEWORK



1.8 CHAPTER SUMMARY

This chapter has presented and discussed the background of the study consisting of the global, Asia and Malaysia statistic on cancer cases that occurred previously. Explained in the research background and problem statement section the importance of finding anticancer agents which give justification to conduct the study. Additionally, the statement of the problem was discussed, as this study was undertaken to investigate PB effects on breast and lung cancer cells. The research objectives, questions and hypotheses were also outlined in this chapter, followed by the significance of study and finally the research framework used in this study was also presented.