ANTICANCER STUDY OF PORCUPINE (*Hystrix brachyura)* BEZOAR ON MELANOMA AND THE IDENTIFICATION OF ITS ACTIVE COMPOUNDS

BY

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ABSTRACT

Hystrix brachyura bezoar (PB) has been traditionally used as an alternative medicine to treat cancer. However the anticancer effect of PB is yet to be explored. Therefore this study aims to evaluate PB anticancer potential using metabolomics approach and molecular docking. Four different PBs namely PB-A, PB-B, PB-C and PB-D were procured and used in this study to evaluate the anticancer effect. All PBs were treated with water through ultrasonication-assisted extraction technique to obtain aqueous extracts. The melanoma cells (A375) were chosen for the evaluation of further anticancer effects. A375 cells were subjected to cell proliferation, colony formation, apoptosis, cell cycle arrest, cell migration, cell invasion assays followed by molecular pathways using real time polymerase chain reaction (qPCR) and *in vivo* antiangiogenesis using zebrafish (*Danio rerio*) larvae. Additionally, all four PBs were also evaluated for their toxicity effect using zebrafish embryos. The anticancer compounds of PB were putatively identified using liquid chromatography–mass spectrometry (LC-MS) and liquid chromatography–mass spectrometry (LC-MS) based metabolomics approaches. All the compounds identified were then docked to BCL-2, cyclin B/CDK1 complex, VEGF and NM23 crystal structures to predict the ligand-protein interaction. Lastly, the study also developed a validated regression model using Fourier transform infra-red (FT-IR) spectroscopy to predict the anticancer activity of new PB extract to ensure its quality as an effective anticancer agent. All PB extracts exhibited significant cytotoxicity on A375 cells. Further analysis revealed that PB-A, PB-C and PB-D had a good inhibitory effect on cell proliferation, colony formation, cell cycle, cell migration, cell invasion, angiogenesis and apoptosis inducer. PB-A, PB-C and PB-D revealed apoptosis through intrinsic pathway, arresting the cell cycle at G2/M phase by downregulating cyclin B and CDK1. PB-A, PB-C and PB-D at molecular level were showed to suppress NM23, E-cadherin, MMP2 and MMP9. The toxicity assessment showed the morphological developmental defects caused by all four PB extracts such as deformed brain section, contorted backbone with deformities in somites and notochord, deformities in soft tissues (yolk sac, pericardial edema and swim bladder). Additionally, the PB extracts were showed to affect the cardiovascular systems via presence of heart edema, downregulating the heart rate and blood flow. The LC_{50} values at 96 hpf were <100 µg/mL. Chemical profiling analyses of PB extracts through GC-MS and LC-MS based metabolomics approached identified 4-androsten-4-ol-3,17-dione, acetate cholest-7-en-3-ol, gallic acid, isolongifolol, mangiferin and propafenone as the active principles. The docking results predicted the interaction of the active principles with BCL-2, cyclin B/CDK1 complex, VEGF and NM23 majorly via hydrophobic interaction with protein residues. The docking results showed good binding affinity to the crystal structures of BCL-2, cyclin B/CDK1 complex, VEGF and NM23 for 4-androsten-4-ol-3,17-dione, cholest-7-en-3beta-ol,4,4-dimethyl-, acetate and mangiferin, suggesting the roles of these compounds as the potential anticancer agents. Moreover, the study has also generated a validated statistical model to predict the anticancer activity of new PB extracts. Conclusively, the study has revealed the anticancer activity of *Hystrix brachyura* bezoar, active principles, *in vitro* mechanism of action and *in vivo* toxicity effect using zebrafish model for the first time. Results of this study further support the traditional claims for the use of *Hystrix brachyura* bezoar as an anticancer agent in Malaysia.

خلاصة البحث

تم استخدام *Hystrix brachyura* bezoar (PB) بشكل تقليدي كدواء بديل لعلاج السرطان. علماً أنه مع ذلك, لم يتم بعد استكشاف التأثير المضاد للسرطان من PB. ولذلك كانت هذه الدراسة تهدف إلى تقييم إمكانيات مكافحة السرطان عن طريق مضاد السرطان PB باستخدام نهج الأيض والالتحام الجزيئي. تم اقتناء أربعة PBs مختلفة وهي PB-A و PB-B و PB-C و PB-D واستخدامها في هذه الدراسة لتقييم التأثير المضاد للسرطان. تمت معالجة جميع مركبات ثنائي الفينيل متعدد البروم بالماء من خلال تقنية الاستخراج بمساعدة الموجات فوق الصوتية للحصول على مستخلصات مائية. تم اختيار خلايا سرطان الجلد (A375) لتقييم المزيد من التأثيرات المضادة للسرطان. تم تعريض خلايا A375 لعمليات تكاثر ألخلايا, وتشكيل المستعمرات، واستماتة الخلايا، وإيقاف دورة الخلية، وهجرة الخلايا ، واختبارات غزو الخلية متبوعة بمسارات جزيئية باستخدام تفاعل سلسلة البلمرة في الوقت الحقيقي (qPCR) وتولد الأوعية في الجسم الحي باستخدام يرقات الزرد (*Danio rerio*). بالإضافة إلى ذلك، تم تقييم جميع ال PBs الأربعة أيضًا لتأثيرها على السمية باستخدام أجنة الزرد. تم تحديد المركبات المضادة للسرطان من PB بشكل افتراضي باستخدام اللوني السائل - مطياف الكتلة (LC-MS) و كروماتوغرافيا السائل - مطياف الكتلة (LC-MS) على أساس نهج التمثيل الغذائي. تم بعد ذلك إرساء جميع المركبات التي تم تحديدها على مركب BCL-2 و cyclin B / CDK1 و VEGF و NM23 البلوري للتنبؤ بتفاعل بروتين ليجاند. وأخيرًا، طورت الدراسة أيضًا نموذج انحدار تم التحقق منه باستخدام مطياف تحويل فورييه بالأشعة تحت الحمراء (FT-IR) للتنبؤ بالنشاط المضاد للسرطان لمستخلص PB الجديد لضمان جودته كعامل مضاد للسرطان فعال. أظهرت جميع مستخلصات PB سمية خلوية كبيرة على خلايا A375. كشف التحليل الإضافي أن PB-A و PB-C و PB-D كان له تأثير مثبط جيد على تكاثر الخلايا وتشكيل المستعمرة ودورة الخلية وهجرة الخلايا وغزو الخلايا وتولد الأوعية ومحفز موت الخلايا المبرمج. كشفت PB-A و PB-C و PB-D موت الخلايا المبرمج من خلال المسار الجوهري، مما أدى إلى إيقاف دورة الخلية في المرحلة G2 / M عن طريق خفض تنظيم cyclin B و CDK1. تم إظهار PB-A و PB-C و PB-D على المستوى الجزيئي لقمع NM23 و E-cadherin و MMP2 و MMP9. أظهر تقييم السمية العيوب النمائية الشكلية التي تسببها جميع مقتطفات PB الأربعة مثل قسم الدماغ المشوهة, العمود الفقري الملتوي مع التشوهات في الجسيدات و notochord، التشوهات في الأنسجة الرخوة (الكيس المحي، وذمة التامور والمثانة). بالإضافة إلى ذلك، تبين أن مستخلصات PB تؤثر على أنظمة القلب والأوعية الدموية من خلال وجود وذمة القلب، مما يقلل من معدل ضربات القلب وتدفق الدم. كانت قيم LC50 عند 96 ساعة بعد التخصيب (hpf) أقل من 100 ميكروجرام / مل. اقترب التحليل الكيميائي من مقتطفات PB من خلال استقلاب GC-MS و LC-MS الذي تم تحديده androsten-4-ol-3-4،17-dione، خلات cholest-7-en-3-ol، حمض الغاليك، isongifolol، mangiferin وبروبافينون كمبادئ فعالة. تنبأت نتائج الالتحام بتفاعل المبادئ النشطة مع مركب BCL-2 و cyclin B / CDK1 و VEGF و NM23 بشكل رئيسي عن طريق التفاعل مع رهاب الماء مع بقايا البروتين. أظهرت نتائج الإرساء ألفة ربط جيدة للهياكل البلورية لمركب BCL-2 و cyclin B / CDK1 و VEGF و NM23 لـ androsten-4-ol-3-4،17-dione و cholest-7-en-3beta-ol، 4، 4-dimethyl- acetate و mangiferin، مما يشير إلى أدوار هذه المركبات كعوامل مضادة للسرطان محتملة. علاوة على ذلك، أنتجت الدراسة أيضًا نموذجًا إحصائيًا تم التحقق منه للتنبؤ بالنشاط المضاد للسرطان لمستخلصات PB الجديدة. أخيراً، كشفت الدراسة عن نشاط مضاد للسرطان من Hystrix brachyura bezoar، والمبادئ النشطة، وآلية العمل في المختبروتأثير السمية في الجسم الحي باستخدام نموذج الزرد (زيبرا فيش) لأول مرة. تدعم نتائج هذه الدراسة أيضًا الادعاءات التقليدية لاستخدام Hystrix brachyura bezoar كعامل مضاد للسرطان في ماليزيا.

APPROVAL PAGE

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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
A375	Melanoma 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
CO^2	Carbon dioxide
dIH2O	Dionize distilled water
DNA	Deoxyribonucleic acid
DMEM	Dulbecco‟s Modified Eagle Medium
DMSO	Dimetyl sulphoxide
E	Efficiency
EC_{50}	Median efficient concentration
FADD	Fas-associated death domain protein
FasL	Fas ligand
FBS	Fetal bovine serum
IC_{50}	Median inhibition concentration
LC_{50}	Median lethal concentration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ACTB	Beta actin
GC	Guanine-cytosine (DNA base pairing)
LC50	Lethal concentration
mRNA	messenger RNAs
MIQE	Minimum information for quantitative polymerase chain reaction publication experiments
NCBI	National centre for biotechnology
NC	Negative control
NHDF	Normal human dermal fibroblast
PB	Porcupine bezoar
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PC	Positive control
PI	Propidium Iodide
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT	Real-Time
UT	Untreated
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 ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Natural products have played an important aspect in life since ancient time as it was integrated as natural resources into daily life for food and remedies for various reasons. Natural product-based medicines have been widely used and practised in Ayurveda, Traditional Chinese Medicine, Traditional Malay Medicine, Greek and Roman Medicine systems, and many more (Cragg et al., 2009; Ji et al., 2009; Rocha et al., 2001). Currently, researchers are exploring scientific findings of utilisations of natural products resources as alternative medicines to treat various illnesses, including cancer (Lahlou, 2013). Natural product had been the single most productive source of leads for the development of drugs where more than 100 new anticancer drugs were in clinical development (Harvey, 2017).

Besides, apart from using herbal or plant for remedies, traditional medicine had been using animal-based remedies for various medical condition. The phenomenon of using animal-based therapy exist until today due to deep rooted-historical origins and species geographical distribution (Alves & Rosa, 2005). The usage of animal-based includes body parts for instance fat, bile, brain, bones, fin, claws, horn, feather, scales, teeth, skin, eggs, milk, blood, bezoar or whole animal (Costa-neto, 2005; Alves et al., 2011; Ma et al., 2015). Despite the practice of animal-based worldwide, researchers had been neglecting the studies of the therapeutic use of animals and animal parts compared to plants. It is crucial to investigate the practice to verify the traditional claims, study the medicinal efficacy and its toxicity. Since there is very limited information on

animal-based medicine, the public tends to believe the history and traditional belief instead of using modern medicine.

The present study will investigate the usage of the animal derived-natural product, namely porcupine bezoar (PB) as alternative medicine/ supplement to treat cancer. Porcupine bezoars are concretions of undigested organic or inorganic compounds found in the gastrointestinal tract of porcupine (Mori & Sforzi, 2013). Bezoars are categorised into four classes which include phytobezoars, trichobezoar, lactobezoar and pharmacobezoar (Sanders, 2004). The bezoar is phytobezoar which is commonly composed of undigested fibres, skins, seeds, leaves, roots or stems of the plant depending on what the porcupine consumed (El Fortia et al., 2006; Sanders, 2004). Bezoar once was believed to be universal antidotes or panacea that can cure all kinds of deadly diseases or illness (Duffin, 2013; Barroso, 2014).

In Malaysia, the most common porcupine is *Hystrix brachyura*, a species of rodent from the Hystricidae family and is well known as a Malayan porcupine or Himalayan porcupine (Magintan et al., 2017). The PB is known to local aborigines as "*Geliga Landak*" and considered quite famous compared to other animal bezoar. The PB is considered as exotic medicine as porcupine numbers are decreasing and under the protection of Malaysian law. Moreover, PB is a rare material as less number of porcupine forms the bezoar. Therefore, poachers and traders tend to sell it implausibly at higher prices. Since the 8th century, numerous claims and myth were lingering around bezoar claiming it as the prince of antidotes capable of curing any illness. According to Shan et al., (2019) in Malaysia, the most famous story regarding PB comes from Chinese folklore, where they believed PB capable of treating cancer and as supplements post-cancer therapy. Despite the traditional claims, the anticancer properties of PB have never been investigated thoroughly. Therefore present study investigates PB

metabolomics profile, anticancer properties and its mechanism of action on melanoma, a malignant skin-derived cancer.

Melanoma arises from melanocytes, specialised pigmented cells that are found predominantly in the skin (Gray-schopfer, Wellbrock, & Marais, 2015). Melanocytes are specialised pigmented cells that are found predominantly in the skin and eyes, where they produce melanin, the pigments responsible for skin and hair colour (Tas, 2012). Melanoma has the greatest metastatic potential among other skin cancer. Though melanoma is diagnosed around 4% compared to all skin cancer, it contributed to 75% of skin cancer mortality (Arrangoiz et al., 2016). Moreover, metastatic melanoma has a poor five-year survival prognosis with inceasing incidence and mortality rate keep increasing (Rigel & Carucci, 2000; Chakraborty et al., 2013). Melanoma is an extremely aggressive type of cancer with high metastatic potential and notoriously high resistance to cytotoxic agents probably due to melanocytes originating from highly motile cells that have enhanced survival properties (Gray-schopfer et al., 2015). Furthermore, the study reported for the past 40 years, limited progress has been made in metastatic melanoma treatment (Chakraborty et al., 2013).

1.2 PROBLEM STATEMENT AND SIGNIFICANCE OF THE STUDY

Traditional medicine is the oldest health care used since ancient time to manage prevention, treating and curing disease or any medical condition. Each part of the world has its deep-rooted origins of traditional medicine to combat various health and life-threatening medical condition (Still, 2003). The traditional medicine advances have developed over the past centuries concerning methods of preparation, extraction, herbs selection, medicinal materials identification, the best time for obtaining different natural resources and prescription (Yuan et al., 2016). Though modern medicine had been well

developed, there are many societies still used traditional medicine as complementary or as alternative medicine.

In recent decades, the interest in evaluating the therapeutic effects of natural products has been increasing. However, most studies focused mainly on herbal and plants based medicine. Since traditional medicine includes the usage of materials from plants and animal, there is a huge gap in understanding traditional medicine in the past. Opposite to herbal medicine, animal-based medicine had less information. The preparation and prescription depend on traditional practice and belief rather than scientifically studied (Still, 2003). This situation caused excessive hunting by poachers as most of the animal-based medicine used exotic and wild animal hence leading to rapid extermination of numerous animal species.

According to Molur et al., (2005), *Hystrix brachyura* species in 2005 was enlisted as endangered animal in Bangladesh, and near threatened in India and Nepal. The same report mentions that the porcupine population had been declining by more than 10% for the last 20 years. The main reason for population declining are habitat loss and hunting for medicinal purpose. The porcupine population was declining in Malaysia as well it is now under the Malaysian government protection of Wildlife Act (1972). Although porcupines are under Malaysian law protection, however, the aborigines were permitted to continue hunting porcupine as it is a tradition passed down from their ancestor to consume and used the porcupine for medicinal purposes (Shan et al., 2019). Most of the PB was obtained from the aborigines and supplied to local traditional Chinese medicine store. The selling price of the PB in the Malaysian market is around RM 300-RM 1000 for 500mg of PB, due to difficulty in obtaining it. This circumstance

creates an overflow of fake PB and increasing numbers of porcupine killed for easy money.

There is an urgency to verify traditional claims of PB anticancer properties, as there is no scientific information on PB medicinal benefits to support. The present study will investigate PB anticancer properties from different porcupines and elucidate its mechanism of action on melanoma cells (A375) using Reverse Transcription Quantitative Polymerase Chain reaction (RT-qPCR). Toxicity profile of different PBs will be conducted using *Danio rerio* embryo to evaluate its toxicity profile. The PB metabolomics profile using gas chromatography-mass spectrometry (GC-MS) and quadrupole time of flight liquid chromatography-mass spectrometry (QTOF LC-MS) will be evaluated as well to determine possible active compounds and characterize PB extracts. The potential active compounds interaction with targeted proteins will be analysed using molecular docking approach. A validated regression model using FT-IR based fingerprinting will be developed to predict PB anticancer activity on melanoma cells (A375).

1.3 RESEARCH QUESTIONS

The present study aims to answer the following questions:

- Do PBs aqueous extract safe for consumption?
- Do different PBs aqueous extracts have anticancer properties on melanoma?
- What are the active compounds contributing to anticancer activities?
- Do active compounds identified in this study interact with similar targeted proteins?
- Do PB extracts from different forest have a similar anti-cancer effect?