UNIVERSITI PUTRA MALAYSIA

***In Vitro* INVESTIGATION OF CYTOTOXIC AND ANTIOXIDATIVE ACTIVITIES OF SELECTED MALAYSIAN PLANTS**

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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 Veterinary Science

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Chairman : Arifah Binti Abdul Kadir, PhD
Faculty : Veterinary Medicine

Mammary cancer cases continue to increase yearly due increasing of the population. The incident rate of mammary cancer amongst humans and animals are high. The drug resistance and long-term side effects issues eventually necessitate scientist to discover and explore new source of treatment such as from herbs that perhaps can encounter the issues. This study was conducted to screen the cytotoxic effect of hydromethanolic crude extract from leaves of *Ardisia crispa* (HEAC), *Tetracera indica* (HETI), *Aquilaria Malaccensis Lam* (HEAM) *and Syzygium Polyanthum (Wight) Walp* (HESP), towards animal and human mammary cancer cell lines. The cytotoxic effect of hydromethanolic leaves extracts was determined with MTT assay against various mammary cancer cell lines (4T1, MCF-7, CMT-Stylo, MDA-MB-231). Among mammary cancer cell lines tested, 4T1 and MCF-7 showed significant reduction of cell viability with IC_{50} value of 42.26 ± 1.82 µg/mL and 52.41 ± 3.49 µg/mL, respectively after 72 hours of treatment. Thus, HEAC was further evaluated for its anti-mammary cancer and antioxidant properties. HEAC was partitioned using ethyl acetate and aqueous to obtain ethyl acetate (EAEAC) and aqueous (AQEAC) fractions of *A. crispa*, respectively. The EAEAC and AQEAC were screened for cytotoxic effects using MTT assay against 4T1, MCF-7, CMT-Stylo and MDA-MB-231 and the IC_{50} values results were compared with HEAC. Selectivity index (SI) of HEAC, EAEAC and AQEAC were also estimated by comparing IC_{50} value of extracts of 4T1 against CC_{50} value of extracts against NIH3T3 (mouse fibroblast cell line). HEAC had the highest SI value indicating that HEAC can be classified as a potential agent for anti-mammary cancer, being toxic only to the cancer cells but not to the normal cells. Antioxidant capacity of the HEAC, EAEAC and AQEAC were assessed using DPPH (1,1-diphenyl-1-picrylhyrazyl) and ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) free radical scavenging assays. Phytochemical screening, total phenolic content (TPC) and total flavonoid content (TFC) were also determined and analysed. Generally, *A. crispa* has good antioxidant capacity contributed with the presence of high phenolic and flavonoid compounds. The mechanism of cell death was assessed using Acridine Orange/Propidium iodide (AO/PI) and Annexin V-FITC/PI on 4T1 cells. The morphology results of AO/PI showed that

majority of untreated cells emitted green fluorescence colour whereas in treated cells, there were mixtures of colour including orange which indicated apoptosis and red fluorescence colour indicated cell death. The confirmation of cell death event which was mainly apoptosis was further proven using Annexin V-FITC/PI staining through flow cytometer. The cells were labelled and differentiated into four quadrate based on the stage of the cells; viable, early apoptosis, late apoptosis and necrosis. Apoptosis occurred as early as 6 hours. The percentages (%) of early and late apoptosis were 8.30 ± 1.11 and 0.70 ± 0.42, respectively. There was no apoptosis event (0%) in untreated cells (control). In conclusion, HEAC showed potential anti-mammary cancer effect and antioxidative activity. The extract able to inhibit cancer cell proliferation through apoptosis, suggesting cancer cell cycle arrest at sub G0/G1 phase.

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

KAJIAN SECARA *In Vitro* KESAN SITOTOSIK DAN ANTIOKSIDAN AKTIVITI DARIPADA TUMBUHAN MALAYSIA YANG TERPILIH

Oleh

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Kes kanser payu dara tetap meningkat saban tahun disebabkan pertambahan populasi dunia. Kadar berlaku kejadian kanser payu dara dalam kalangan manusia dan haiwan adalah tinggi. Masalah kerintangan dan kesan sampingan ubat kanser terhadap pesakit membuatkan keperluan untuk para saintis meneroka rawatan baru antaranya daripada herba. Kajian ini dijalankan untuk menyaring kesan sitotosik hidrometanol ekstrak daripada *Ardisia crispa* (HEAC), *Tetracera indica* (HETI), *Aquilaria Malaccensis Lam* (HEAM) and *Syzgium Polyanthum (Wight) Walp* (HESP), terhadap sel kanser payu dara haiwan dan manusia. Kesan sitotosik ekstrak hidrometanol daripada daun telah disaring dengan menggunakan MTT asai terhadap pelbagai jenis sel kanser payu dara (4T1, MCF-7, CMT-Stylo, MDA-MB-231). Di antara sel kanser payu dara yang diuji, 4T1 and MCF-7 menunjukkan kadar penurunan kebolehhidupan sel kanser terendah dengan nilai IC_{50} adalah 42.26 ± 1.82 µg/mL dan 52.41 ± 3.49 µg/mL masing masingnya selepas 72 jam rawatan. HEAC menunjukkan potensi sitotosik yang terbaik berbanding ekstrak yang lain yang diuji dan telah dipilih untuk kajian selanjutnya terhadap antikanser dan antioksidan aktiviti. HEAC telah diasingkan polariti dengan menggunakan etil asetat and akueus untuk mendapatkan pecahan etil asetat (EAEAC) and akueus (AQEAC) daripada hidrometanol ekstrak *A. crispa* dan kemudian disaring juga untuk kesan sitotosiknya terhadap 4T1, MCF-7. CMT-Stylo and MDA-MB-231 dan keputusan ujian (IC_{50}) telah di bandingkan dengan HEAC. Selektif index (SI) untuk EAEAC dan AQEAC diperoleh dengan mengira nisbah nilai IC_{50} ekstrak 4T1 terhadap nilai CC_{50} ektrak NIH3T3 (sel fibroblast tikus). HEAC mempunyai nilai SI tertinggi, menunjukkan HEAC boleh diklasifikasikan sebagai anti kanser payu dara agen yang bagus, hanya memilih toksik kepada sel kanser tetapi kurang toksik kepada sel normal. Kapasiti antioksidan yang terkandung daripada HEAC, EAEAC dan AQEAC diukur dengan menggunakan asai radikal bebas iaitu DPPH (1,1-diphenyl-1-picrylhyrazyl) dan ABTS2 (2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid). Pemeriksaan fitokimia, kandungan total sebatian fenolik, dan kandungan total sebatian flavonoid telah dilaksanakan. Secara keseluruhannya, *A. crispa* mempunyai kandungan antioksidan yang bagus, disumbangkan oleh sebatian fenolik dan flavonoid. Mekanisma kematian sel telah dinilai menggunakan 'akridina jingga/propidium idorenida' (AO/PI), ujian 'Annexin V-

FITC/PI' terhadap 4T1 sel kanser. Melalui ujian AO/PI, morfologi majoriti sel yang tidak dirawat membalikkan floresen warna hijau manakala untuk sel yang dirawat, terdapat campuran warna termasuk warna oren iaitu merujuk sel mengalami apoptosis dan warna merah pula menunjukkan sel telah mati. Untuk kepastian proses kematian sel cancer telah dilanjutkan dengan menggunakan stain Annexin V-FITC/PI dan diukur melalui mesin sitometri aliran. Sel telah dilabel dengan stain Annexin V-FITC/PI dan keputusan ujian diasingkan kepada 4 bahagian berdasarkan keadaan sel ketika itu sama ada; hidup; apoptosis awal; apoptosis akhir and nekrosis. Apoptosis berlaku seawal 6 jam. Peratusan apoptosis awal dan akhir masing masing adalah 8.30 ± 1.11 dan 0.70 ± 0.42. Untuk sel yang tidak dirawat (kawalan), tiada kejadian apoptosis (0%). Tuntasnya, HEAC menunjukkan anti kanser payu dara dan antioksidan aktiviti. Ekstrak mampu merencatkan pertumbuhan kanser sel melalui apoptosis, mencadangkan hentian kitaran hidup kanser sel pada fasa sub G0/G1.

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I certify that a Thesis Examination Committee has met on 16 February 2017 to conduct the final examination of Muhammad Luqman bin Nordin on his thesis entitled "*In Vitro* Investigation of Cytotoxic and Antioxidative Activities of Selected Malaysian Plants" 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 Veterinary Science.

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%	Percentage
° C	Degree Celsius
ē	Electron
·OH	Hydroxyl radical
µL	microliter
4T1	Mouse mammary cancer
ABTS	2, 2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)
$AlCl_3$	Aluminium Chloride
ANOVA	Analysis of variance
AO/PI	Acridine Orange/Propidium iodide
AQEAC	Aqueous Extract of *Ardisia Crispa*
ATCC	American Type Culture Collection
BRCA1	Human genes tumor suppressing proteins type 1
BRCA2	Human genes tumor suppressing proteins type 2
CAFs	Carcinoma associate fibroblasts
CGM	Complete growth media
CO_2	Carbon dioxide
CC_{50}	Minimum cytotoxic concentration that allow 50% of cells growth
C_6H_6O	Basic structure of phenolic compound
CDK	Cyclin-dependent kinases
CMT-Stylo	Canine mammary cancer
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid

DPPH	1, 1-diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
ER	Oestrogen receptor
EC_{50}	Effective concentration
ETC	Mitochondrial Electron Transport Chain
EAEAC	Ethyl Acetate Extract of *Ardisia Crispa*
FMN	Flavin mononucleotide
$FeCl_3$	Ferric Chloride
FBS	Foetal Bovine Serum
GAE	Gallic Acid Equivalent
G_0	Resting phase of cell cycle
G_1	Growth one phase of the cell cycle
G_2	Growth two phase of cell cycle
HEAC	Hydromethanolic extract of *Ardisia crispa*
HEAM	Hydromethanolic extract of *Aquilaria malaccensis*
HepG2	Hepatocellular Carcinoma Cell
HESP	Hydromethanolic extract of *Syzygium polyanthum Lam*
HETI	Hydromethanolic extract of *Tetracera indica*
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric Acid
IBS	Institute of Bioscience
IC_{50}	minimum inhibitory concentration
$K_2S_2O_8$	Potassium Persulphate
mm	milimeter
M	Mitosis phase of the cell cycle

MCF-7	Human mammary cancer
mL	milliliter
MTT assay	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
$NAHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NO	Nitrite oxide
NIH3T3	Normal mouse fibroblast cell line
O_2^-	Superoxide anion
$ONOO^-$	Peroxynitrite
PBS	Phosphate Buffer Saline
PP 2A	protein phosphatase 2A
p-Akt	phopho-Akt
PCD	Programmed cell death
PS	Phosphatidylserine
g	Gram
mg	Milligram
QE	Quercetin Equivalent
RE	Rutin Equivalent
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPMI 1640	Rosselle's Park Memorial Institute Medium
S	Synthesis phase of the cell cycle
TAMOX	Tamoxifen
TEAC	Trolox equivalent antioxidant capacity

TFC	Total Flavonoid Content
TPC	Total Phenolic Content
Trolox	6-hydroxyl-2,5,7,8-tetramethyichroman-2-carboxylic acid
UPM	Universiti Putra Malaysia
USNCI	United State National Cancer Institute
WHO	World Health Organization
µg/mL	microgram per mililitre

CHAPTER 1

GENERAL INTRODUCTION

The increasing prevalence of cancer nowadays, relapse and the long term side effect from the cancer treatment eventually lead scientist to discover a new therapeutic approach that could solve the problem. Cancer is a complicated disease, occurrs in human and animal, that arises from gene disturbance, generally known as mutation. Many anticancer drugs develop drug resistance toward cancer (Zahreddine and Borden, 2013; Townsend and Tew, 2003). Moreover, the adjuvant treatment such as from chemotherapy is very painful and provided adverse effects (American Cancer Society, 2016). Unhealthy life styles such as smoking, unhealthy diets and physical inactivity as well as long exposure to UV radiation and sunlight would predispose host toward gene mutation (Jemal *et al.*, 2011). Generally, the body would able to repair any mismatch of the gene code, but once the damaging rate is more than the repairing rate, proliferation of cells would occur along with mutation gene. Women continue to bear a high risk of mammary cancer. A report by Jemal *et al.* (2011) stated that the prevalence of human mammary cancer is 25% from total cancer cases and is the most frequent cancer among women in the world and as the population continues to rise, the prevalence increases.

In animals, female intact dogs and cats also have high prevalence of mammary cancer which account to 52% and 17%, respectively (Magalhães *et al.*, 2012; Misdorp *et al.*, 1999; Moulton, 1990). Mammary cancer that occur in animals share similarities in clinical, histopathological and molecular features with human mammary cancer (Salas *et al.*, 2015; Gupta *et al.*, 2012). For that reason, study on animal model of mammary cancer is very relevant especially to correlate prognostic and therapeutic values before entering to clinical trials.

Surgery remains the main approach for interventation of mammary cancer followed with treatment of adjuvant therapies such as chemotherapy, hormonal therapy and radiation therapy. The selection of adjuvant therapy depends on the individual status such as age, current health condition and stage of the cancer. Despite many modern therapeutic approaches, unfortunately, the side-effects and prognosis of the treatment still consistently remain the major topic of discussion among practitioners. Currently, the ability of cancer cells to undergo mutagenesis during treatment leads to a relapse, thus making the treatment more complicated.

In ancient immemorial times, medicines from herbal and natural products were used widely in every culture throughout the world. Medicinal plants generally known as herbs have played a significant role in the drug development and have shown a promising outcome. Examples of plant derived chemotherapy drugs include paclitaxel from *Taxus brevifolia* plant species, vincristine and vinblastine from *Catharanthus roseus* plant species. To the date, up to 70,000 plant species have been screened for biological activities (Veeresham, 2012) and about 70% end up as a commercial drug (Steenhuysen, 2007). Up to now, scientists and medical professionals show interest in these natural based-product therapies. They recognize the true health benefits of these remedies with

the fewer side effects and a very good potential resource in the drug discovery field (Helmstädter and Staiger, 2014). Currently, 80% of the population from developing and non-developing countries still used natural product as an alternative therapeutic source (Bandaranayake, 2006). During 2002, the trades of natural product worldwide is approaching 60 million US Dollar (WHO, 2008). With the increasing population and health conscious minded amongst people, the demands and pharmaceutical investment on herbs as a healthcare option is believed to have increased tremendously (WHO, 2008).

In Malaysia, medicinal plants or herbs have been used widely because of their prized aromas and taste, which add variety and flavour to foods. Traditionally, many of these plants are also used to treat different human ailments such as cancer and diabetes, due to its broad range of bioactive constituents (Shoeb *et al.,* 2010). The demand of using herbs as a medication and supplimentation among Malaysians also have rised due to the high incidence of side effects derived from commercial drug (Fazirah *et al.,* 2015). In fact, in 1999, the natural product domestic trades market was reported to be RM 4.4 billion (Nordin *et al.,* 2008; Aziz, 2003) while in 2009, the natural products trades was approximately RM 777 billion and the growth rate is estimated to be about 15% annually until 2020 (Farizah *et al.,* 2015).

In the view of exploration for an alternative medicine, particularly antimammary cancer studies, four selected native plants *Ardisia crispa* (Thunb.) A. DC, *Tetracera Indica Merr*, *Aquilaria Malaccensis Lam* and *Syzygium Polyanthum (Wight) Walp)* were investigated *in vitro*. These plants were believed by a traditional practitioner to have medicinal properties in preventing, curing and maintaining body health care for example in treating cancer patients but there is still lack of proper scientific evaluation to prove this claim (Ismail, 2007). As such, further meticulous study is needed to provide scientific evidences of their efficacy and safety, thus they can be nominated as future candidates for antimammary cancer therapy.

1.1 Problem statement

Mammary cancer is very well known non-communicable disease and become a serious illness worldwide. Like the other types of cancer, mammary cancer also having the same issues of long term side effects and relapse. The treatment principally able control cancer from metastasising but difficult to cure. Relapse and side effects are major challenge during a course of treatment. Relapse attributable mainly by the alteration in genes substitution and loss of oestrogen receptor expression (Alluri *et al.,* 2014; Ring and Dowsett, 2004; Fuqua *et al.,* 2000).

Considering the idea that oxidative stress form free radicals is one of the factors that initiate many cancer developments (Scott *et al.,* 2014), and herb often provide an awesome natural source of antioxidant (Chusri *et al.,* 2014), discovering of anticancer and antioxidative activities of the potential plant, would provide relevant information to link the relationship between mammary cancer and antioxidant. Hence, the unearthing of new effective and safer for mammary cancer treatments especially derived from plants need to be clearly elucidated. Moreover, despite modern and advanced science and

technology nowadays, there are still presences of several issues regarding regimes are plagued with numerous side effects and relapse of cancer (Anthony and Jones, 2016). Therefore, the research of new therapeutic approach for curing mammary cancer is highly recommended to overcome the problem.

1.2 Justification for selection of herbs and extraction methods

Previous studies on *Ardisia crispa*, *Tetracera Indica Merr*, *Aquilaria Malaccensis Lam* and *Syzgium Polyanthum (Wight) Walp* showed that the biological activity of the plant such as anti-inflammatory that can be relevant to antimammary cancer activity (Hamsin *et al.,* 2013; Endringer *et al.,* 2010; Zhou *et al.,* 2008). Since anti-inflammation often associated with inhibition of angiogenesis (Hamsin *et al.,* 2013), its together regulate the activation of cell chemotaxis, migration, and proliferation, therefore has the potential to suppress tumour growth and metastases. Hence, the inhibition of angionesis is one of the most promising strategies in the development of novel anti-cancer therapies, and in the treatment of other human diseases associated with angiogenesis. Furthermore, few bioactive compound from plants have been tested clinically to treat mammary cancer in human and providing a promising results, for instance, beta-lapachone from Tabebuia (Li *et al.,* 2000), vindesine from Catharanthus roseus (Cragg *et al.,* 2005), taxol from Taxus brevifolia (Kingston *et al.,* 2007) and genistein from Glycin max (Kinghorn *et al.,* 2009).

Taking all these reports into consideration, thus far, no one has reported on the antimammary cancer effect together with antioxidative properties of the selected plants. Therefore, this study was conducted with the intention to discover the true potential local herbs for antimammary cancer activity, perhaps could reduce the adverse effects of current treatment or could be used synergistically with the available ones; and subsequently improve their pharmacological and toxicological effect and prognosis of the treatment. Furthermore, complementary methods such as using herbs or vitamins to treat cancer or relieve side effects of cancer is not something new (American Cancer Society, 2016).

Phytochemicals, especially phenolic and flavonoid compounds, present in many herbs have received much attention in recent years due to their many health benefits, including anticancer, antioxidant and anti-inflammatory activities. Differences in attachment of aromatic ring to the hydroxyl group (-OH) in phenolic compounds structure determine their solubility in solvents of different polarity and also antioxidative properties. Moreover, the phenolic compounds have many subclassess and usually the compound attached with another compound such as saponin, glycosides, chlorophyl, terpenoids and lipid (Druzynska *et al.,* 2007). So, the solubility of phytochemical compounds depends on the type of solvent and its polarities. Therefore, type of solvent system may have a significant impact on biological activities and the yield of extraction of polyphenols from herbs (Złotek *et al.,* 2016; Sultana *et al.,* 2007). A mixture of methanol or ethanol extract was commonly being used and has greater efficiency to pull out phytochemical compound especially phenolic compound from medicinal plant as compared to pure methanol/ethanol (Seo *et al.,* 2014; Aktumsek *et al.,* 2013; Anwar *et al.,* 2012; Shabir *et al.,* 2011; Sultana *et al.,* 2007). Hence in this study, a mixture of (80 methanol:20 water,

v/v) based on Casagrande *et al.* (2014) was chosen as primary solvent based on that mixture of alcoholic solvent with water is preferable solvent especially in extracting phytochemical components from herbs. Considering from previous report that the phenolic content was well known related to antioxidative properties (Kumar and Pandey, 2013), and moreover oxidative stress is also one of the pathway of carcinogenesis (Babu *et al.*, 2013), therefore, hydromethanolic solvent is chosen as the primary solvent for cytotoxic screening evaluation. In the next step, in order to discover the effect of solvent with different polarities against extracting yield of compunds, ethyl acetate solvent (semipolar chemical characteristic) and aqueous solvent (polar chemical characteristic) were used in the partitioning section. The separation of compound based on polarities would give idea which compound that responsible to the anticancer, antioxidative and apoptosis activities.

1.3 Hypothesis

The hypothesis of the study is *Ardisia crispa, Tetracera Indica Merr, Aquilaria Malaccensis Lam* and *Syzygium Polyanthum (Wight) Walp* will have cytotoxic effect against mammary cancer cell lines and antioxidative effect *in vitro*.

1.4 General objective

The general objective of the study is to investigate anti-mammary cancer and antioxidative activities of hydromethanol extraction of selected Malaysian plants.

1.5 Specific objectives

Specific objectives of the study are :

1. To assess the cytotoxic effect of hydromethanolic crude extract of *Ardisia crispa, Tetracera Indica Merr, Aquilaria Malaccensis Lam* and *Syzygium Polyanthum (Wight) Walp* against four types of mammary cancer cell lines (4T1, MCF-7, CMT-Stylo, and MDA-MB-231) and normal cell line (mouse fibroblast cell line, NIH3T3).
2. To determine the phytochemical constituents and antioxidative properties of the most potential antimammary cancer plant extract *in vitro*.
3. To determine the mode of cell death of the extract and the cell cycle arrest of 4T1 cancer cells.

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