INVESTIGATION OF EFFECTS OF GAHARU DISTILLATES ON LUNG CANCER CELLS

BY

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ABSTRACT

Agarwood or Gaharu is the fragrant, resinous heartwood that results when some trees, such as *Aquilaria malaccensis*, are attacked by fungi. This highly-prized heartwood contains a wide array of chemical compounds that fall under various classes of healthy phytochemicals such as terpenoids (monoterpenes, diterpenes and sesquiterpenoids), alkaloids, and flavonoids. Previous studies have shown promising results when Gaharu essential oils are studied for anticancer effects. However, very little information can be found on Gaharu distillates including their therapeutic effects. This study aims to determine the potential anticancer effects of Gaharu distillates on lung cancer. Calu-3 lung cancer cells were used as model cell line. They were cultured in Eagle's Minimum Essential Medium (EMEM), supplemented with 10% (v/v) Fetal Bovine Serum (FBS). Two factor- Face-centred Central Composite Design was used to study the effects of Gaharu distillate amount and time of exposure on cell attachment (via trypan blue dye exclusion assay) as well as cytotoxicity (via MTT assay). It was found that Gaharu distillates of *Aquilaria malaccensis* possess both anti-attachment and cytotoxic effects on Calu-3 lung cancer cells. The best IC_{50} value obtained for both assays was 20, 000 ppm at 12 hours exposure time. A linear model was developed for anti-attachment effects, and a quadratic model was developed for cytotoxic effects with exposure duration being significant in both cases. This study also presents and compares profiling data from Gas Chromatography Mass Spectrometry (GCMS) and headspace Solid Phase Microextraction-Gas Chromatography Mass Spectrometry (SPME-GCMS) of the Gaharu distillates. From these profiles, 1-tricosene and 16-hentriacontanone are some of the compounds that may possess anticancer potential. In conclusion, Gaharu distillates hold a great potential to be further studied as source of anti-cancer compounds.

ملخص البحث

العود أو Gaharu هو الخشب العطر الراتنجي الذي ينتج عندما تتعرض بعض الأشجار مثل Aquilaria للهجوم عن طريق الفطريات. يحتوي هذا الخشب الغالي على مجموعة كبيرة من المركبات الكيميائية التي تندرج تحت فئات مختلفة من المواد الكيميائية النباتية الصحية مثل تيربينويدس (تربينات أحادية وتربينات ثنائية وتربينات ثلاثية وأشباه القلويات وفلافونيدات. وقد أظهرت دراسات سابقة نتائج واعدة عند دراسة الزيوت العطرية للعود وآثارها المضادة للسرطان، ومع ذلك فالمعلومات المتوفرة عن نواتج العود المقطرة وآثارها العلاجية قليلة جداً. تهدف هذه الدراسة إلى تحديد الخاصية المضادة للسرطان المحتملة في نواتج العود المقطر على سرطان الرئة. واستخدمت خلايا سرطان الرئة Calu-3 كخط الخلية نموذج. حيث تم استنباتهم في Eagle's Minimum Essential Medium (EMEM) و استكملت بـ ١٠٪ Fetal Bovine Serum (FBS) (v/v). تم استخدام تصميم الوجه المركزي للمجمع المحوري–ثنائي العامل لدراسة آثار تركيز نواتج العود المقطر ووقت تعرضه على الخلية المرافقة (عن طريق المقايسة بصبغ التريبان الأزرق)، وكذلك السمية الخلوية (عبر فحص MTT). وقد تبين أن هذا نواتج العود المقطر من Aquilaria malaccensis تمتلك كلا من الخواص المضادة المرافقة والآثار السامة للخلايا على خلايا سرطان الرئة Calu-3 . وكانت أعلى قيمة لد IC50 تم الحصول عليها لكلا المقايسات ٢٠ مايكرولتر/ مل، في ١٢ ساعة من وقت التعرض. تم تطوير نموذج خطي ذو تأثير كبير للآثار المضادة المرافقة وفي الوقت نفسه تم اعداد نموذجا تربيعياً للتأثيرات السامة للخلايا مع مدة التعرض هامة في كلتا الحالتين. بالاضلافة لما سبق فإن هذه الدراسة تقدم وتقارن البيانات الناشئة من تحاليل Gas Chromatography Mass Spectrometry(GCMS) وSolid Phase Microextraction-GCMS(SPME-GCMS) لنواتج العود المقطر. من هذه التشكيلات، اكتشف أن ١–tricosene و١٦ hentriacontanone هي بعض المركبات التي قد تمتلك إمكانات مضادة للسرطان. ختاماً، هناك امكانيات كثيرة لمقطرات العود كمصدر من المركبات المضادة للسرطان والتي بحاجة الى المزيد من الدراسات.

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 dissertation for the degree of Master of Science (Biotechnology Engineering).

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Md. Noor bin Salleh
Dean, Kulliyyah of Engineering

DECLARATION

I hereby declare that this dissertation is the result of my own investigation, 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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This dissertation is dedicated to my beloved parents

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LIST OF SYMBOLS

c	Live cell concentration (cells/mL)
n	Average number of live cells for the two chambers (cells)
v	Volume counted (mL)
μ	Specific growth rate at a given time point (day $^{-1}$)
t	Culture time (hours)
X	Viable cell number (cells)
t_d	Doubling time (days)
μ_{max}	Maximum specific growth rate (day -1)
NC	Negative control viable cell number or OD_{570} reading
N	N is viable cell number or OD570 reading after application of Agarwood distillate
A	Distillate amount (µL/ml complete medium)
B	Exposure duration (hours)

LIST OF ABBREVIATIONS

ASCO	American Society of Clinical Oncology
ANOVA	Analysis of Variance
BSLA	Brine Shrimp Lethality Assay
CARIF	Cancer Research Initiatives Foundation
CITES	Convention on International Trade in Endangered Species
CLA	Conjugated Linoleic Acid
CT	Computed Tomography
DMEM/F12	Dulbecco's Modified Eagle Medium/ Nutrient F12 Ham
ELISA	Enzyme-linked Immuno-Sorbent Assay
EMEM	Eagle's Minimum Essential Medium
FAME	Fatty Acid Methyl Esters
FBS	Fetal Bovine Serum
FIGO	International Federation of Gynecology and Obstetrics
FRAP	Ferric Reducing Antioxidant Power
GC-MS	Gas Chromatography- Mass Spectrometry
HIV	Human Immunodeficiency Virus
HPV	Human Papillomavirus
IARC	International Agency for Research on Cancer
LC-MS	Liquid Chromatography- Mass Spectrometry
MEM	Minimum Essential Medium
MRI	Magnetic Resonance Imaging
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide
NSCLC	Non-Small Cell Lung Carcinoma
NSCLC-NOS	Non-Small Cell Lung Carcinoma Not Otherwise Specified
PBS	Phosphate Buffered Saline
PET	Positron Emission Tomography
SCLC	Small Cell Lung Carcinoma
SPME	Solid Phase Microextraction
SRB	Sulforhodamine B
TNM	Tumor Node Metastasis
TRT	Thoracic Radiotherapy
TS	Total Solids
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Agarwood is a highly valued resin obtained from the heartwood of trees of *Aquilaria* species and other trees of the Thymelaeaceae family in response to attack by fungi. The resulting resin makes the heartwood dark in color and with a very strong scent that is highly prized in the perfume industry, hence Gaharu is also known as black gold.

This high demand for Gaharu has caused some *Aquilaria* species to be considered threatened and in need of protection. As a result, many efforts have been focused on preserving the trees by systematic cultivation and reforestation thus reducing illegal logging. Aside from its value as perfume oil, many medicinal claims have been associated with the Gaharu itself. Of particular interest, both Gaharu oil and distillates may possess anticancer activity against MCF-7 breast cancer cells, in terms of cell attachment and cell viability (Hashim, Phirdaous, & Azura, 2014; Phirdaous, et al., 2014). As Gaharu oil itself is in major demand for perfumery, our attention turns to Gaharu distillates, which are obtained from the waste of the distillation processes that produce the oil. In fact, distillates are often found to have more components than the primary oil of the distillation.

Gaharu distillates are the clear, by-products of the Gaharu distillation process that possess a characteristic smell that can be associated with Gaharu. To date, there is very scarce information on the Gaharu distillate. In one such study by Abdullah and Moosa (2010), Gaharu distillates from two different extraction facilities were characterized to provide physical, chemical and microbiological information. The pH

value of Gaharu distillates ranged from 3.62 to 4.53. From the four antioxidant assays conducted, namely total phenolic content assay, ABTS (2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity, DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging activity, and ferric reducing antioxidant power (FRAP), it was found that Gaharu distillates possessed antioxidants, though the positive controls in the experiment had performed better than Gaharu distillates. The fungal population of the distillates was also studied, by plating on Sabouraud Dextrose Agar. No fungi were found to grow. Its antibacterial activities against *Staphylococcus aureus* and *Pseudomonas aeruginosa* were assessed via well diffusion and disk diffusion assays and the inhibition of bacterial growth was found to be insignificant (Abdullah & Moosa, 2010).

Meanwhile, another study on the potential of Gaharu distillates for human consumption showed them to have a pH value of 3.60. The Total Solids (TS), Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were found to be 0.46 mg/ L, 0.01 mg/L, and 0.053 mg/L, respectively. These values are much lower than the standards set by National Water Quality Standards for Malaysia (Hashim et al., 2013). Furthermore, the study also included a cytotoxicity assay using brine shrimp larvae to determine if Gaharu distillates are safe for human consumption. The Brine Shrimp Lethality Assay (BSLA) had determined the drink to be safe for consumption with the highest lethal amount (LC_{50}) recorded of 39.8 % (v/v) (equivalent to 398,000 ppm). The value was notably similar to that of caffeine for example, which is (306 mg/mL) 306,000 ppm (Meyer et al., 1982).

Research has so far only shown that Gaharu distillates possess anticancer activity against one cancer cell line *i.e.* breast cancer MCF-7 (Phirdaous, et al., 2014). Furthermore, little is known about the composition of the Gaharu distillates produced

in Malaysia. In this research, the effectiveness of Gaharu distillates against lung cancer (Calu-3) cells and the effective dosage were investigated. The composition of Gaharu distillates was determined via GCMS and SPME-GCMS, and the findings of these two techniques were compared.

1.2 PROBLEM STATEMENT

Gaharu distillates are already in use in traditional and alternative medicine for their anticancer effects as well as against lung diseases such as pleurisy and asthma. However, there have been very limited scientific findings to support these uses. It is imperative, therefore, to study Gaharu distillates for the benefit of humanity whether that means discovery of new anticancer compounds or dismissing false claims in order to protect consumers.

1.3 RESEARCH OBJECTIVES

The overall objective of the study is to determine if Gaharu distillates possess any cytotoxicity towards Calu-3 lung cancer cells.

The specific objectives of this study are:

1. To profile the compounds found in Gaharu distillates via Gas Chromatography Mass Spectrometry (GCMS) and headspace Solid Phase Micro Extraction (SPME)-GCMS and compare the findings of these two methods.
2. To determine the potential of Gaharu distillates against Calu-3 cells by means of a cytotoxicity assay and cell attachment assay.
3. To determine the effects of exposure time and amount of Gaharu distillates on Calu-3 cell inhibition of attachment and viability.

1.4 SCOPE OF THE STUDY

This research is limited to *in vitro* studies of anti-cancer activity of Gaharu distillates. It does not aim to identify and extract the anticancer compound(s) that could be present in the Gaharu distillates.

1.5 SIGNIFICANCE OF THE STUDY

If Gaharu distillates can be shown to possess cytotoxic effects against lung cancer cells, their value increases as they have already been shown to be safe for consumption and do not pose cytotoxicity towards normal cells (Phirdaous, et al., 2014). As the distillates makeup an inevitable waste product, their commercialization would be an implementation of a zero-waste policy for the industry and Malaysia as well. Furthermore, as people have already consumed these distillates prior to any commercialization or scientific evidence to support claims of benefits, it is vital to prove or disprove these claims for the benefit of society.

1.6 ORGANIZATION OF THE DISSERTATION

This dissertation is comprised of five chapters. Chapter One provides a background of the study and its main goals. Chapter Two is a deeper review of recent, related literature concerning this research. Chapter Three provides the materials and the detailed methodology employed in this research. Chapter Four is on the findings from this study. Chapter Five is the conclusion and provides recommendations pertaining to the research.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter provides a critical review of recent researches on the subject. It is divided into three main sections, namely cancer, plants as a source of anticancer compounds, and Gaharu, in that order for easy comprehension.

2.2 CANCER

In 2012, 8.2 million deaths were associated with cancer while 14.1 million adults were diagnosed with cancer (World Health Organization, 2015). Cancer.org estimates that for 2015, there will be an estimated 1,658,370 new cancer cases diagnosed and 589,430 cancer deaths in the US alone. These statistics are horrifying proof that, despite progress in our understanding of the disease and improvements in aspects of diagnosis and treatment, cancer is still a major concern that needs our attention. (Cancer Staging Fact Sheet, 2015)

It is important to understand what normal cells are like before one can understand cancer. Cells may first be classified as prokaryotic or eukaryotic. The most notable difference between the two types of cells is that eukaryotic cells (those of complex organisms) possess membrane-bound organelles.

Eukaryotic cells duplicate and divide via the cell cycle which consists of the interphase, consisting of G1 (Gap 1), S (synthesis), and G2 (Gap 2); and the mitotic phase, M (mitosis). During interphase, the cell grows, replicates cellular DNA (S), and then prepares to divide (G2). It is here that the cell enters M phase, which consists of the mitosis and cytokinesis stages. Each phase of the cell cycle is well regulated, and

checkpoints exist to detect potential DNA damage and allow for repair, or apoptosis (a form of programmed cell death) in the case of no possible repair. The first checkpoint is at the end of G1, making the decision if a cell should enter S phase and divide, delay division, or enter G0, a dormant or senescent state of no division. The second checkpoint, at the end of G2, triggers mitosis if conditions are suitable. The cell cycle is illustrated in Figure 2.1.

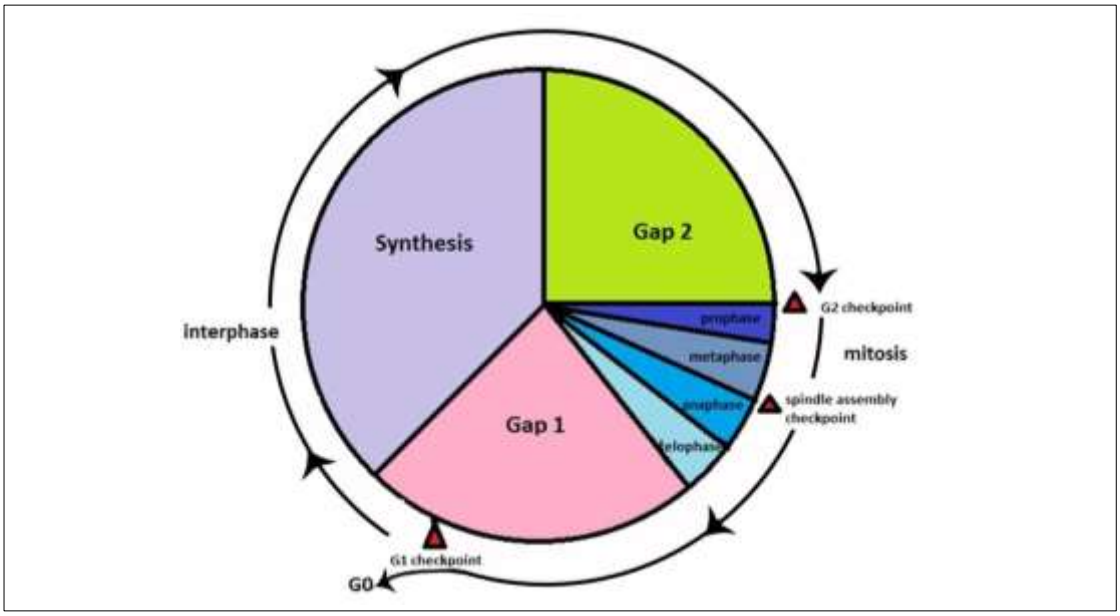

Figure 2.1 The cell cycle (Cooper, 2000)

In the previous paragraph, apoptosis was mentioned as a form of cell death. Necrosis is another form of cell death and it is associated with acute cellular stress, and can be caused by toxins, infection, or injury. In addition to necrosis and apoptosis, there are other specialized forms of programmed cell death. Anoikis, for instance, is a form of programmed cell death that is associated with detachment of cells from their extracellular matrix (Paoli, Giannoni, & Chiarugi, 2013). Pyroptosis (from ‘pyro’,

Greek for heat, denoting fever) on the other hand, is a proinflammatory form of cell death associated with microbial infection (Fink & Cookson, 2005).

In summary, normal cells have the characteristics of being specialized in their function, they grow and divide finitely, and finally they undergo programmed cell death. Cancer cells, on the other hand, often grow and divide unchecked, and are immortal. Cancer cells also do not perform what their equivalent of normal cells would do. Furthermore, they often make growth factors that would stimulate other cells to grow as well, and the cancer may metastasize *i.e.* spread to other organs. Fortunately, however, not all abnormal growths are harmful. A tumor, which is the mass of tissue formed by new growth of cells, may be either benign or malignant. A benign tumor is one that stops growing by itself, and does not metastasize. An example of benign tumor is lipoma. Cancer can then be defined as follows:

> Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host (Ruddon, 2007).

The process of transformation of normal cells to cancer cells is known as carcinogenesis, literally the creation of cancer. Carcinogenesis is made up of the initiation, promotion, and progression phases (McKinnell, 1998). In the initial phase, by preventing interaction of chemical carcinogens with DNA through induction of phase I and II enzymes, detoxification of the carcinogen is possible and this prevents formation of cancer. Hence, this approach is known as chemoprevention. On the other hand, once it is in the promotion phase, treatment aims to inhibit tumor cell proliferation, accelerate tumor cell death rate, and induce tumor cell differentiation. Having said so, chemopreventive agents, such as antioxidants, may be used as adjuvants to chemotherapy or surgery (Mehta, 2014).

2.2.1 Cancer causes

Cancer causes are both environmental and genetic (Ruddon, 2007). A normal gene may undergo mutation and become stuck on 'ON', making it an oncogene. It may also be a tumor suppressor gene, *i.e.* a gene that suppresses tumor by preventing growth of injured or mutated cells, but it then underwent mutation and therefore stopped functioning. Cancer genes may be inherited, such as breast cancer 1, early onset (*BRCA* 1), and breast cancer 2, early onset (*BRCA* 2), which are normal tumor suppressor genes that are normally expressed in the cells of the breast. Many mutations of these genes have been identified and found to cause increased risks of cancer and produce very high rates of breast and ovarian cancer, amongst others (Pal, et al., 2005).

According to an online publication by the Lowell Center for Sustainable Production (Clapp, Howe, & Lefevre, 2005), environmental factors that are related to the development of cancer include:

- Diet (e.g. acrylamide, meat that is cooked at high temperatures, artificial sweeteners, etc.),
- Age,
- Lifestyle (e.g. body weight and obesity, physical activity, food, stress, etc.)
- Toxic chemicals and air pollutants (e.g. carcinogens such as asbestos, formaldehyde, smoking cigarettes, etc.),
- Radiation exposure (e.g. UV, X-ray, Nuclear, Magnetic fields, Radon, etc.),
- Hormones (e.g. estrogen),
- Viral and bacterial infections (e.g. HIV, HPV, *Helicobacter pylori*), and
- Lowered immunity