EFFECTS OF CHEMICALLY SYNTHESIZED KAVA-KAVA
(Piper methysticum G.Forst) FLAVOKAWAIN A AND B ON
THE APOPTOTIC AND METASTATIC PROCESS OF MCF-7
AND MDA-MB231 CELLS IN VITRO AND 4T1 CELLS IN VIVO

NADIAH ABU

FBSB 2014 27
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APOPTOTIC AND METASTATIC PROCESS OF 
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By

NADIAH ABU

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

December 2014
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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

EFFECTS OF CHEMICALLY SYNTHESIZED KAVA-KAVA (*Piper methysticum* G. Forst) FLAVOKAWAIN A AND B ON THE APOPTOTIC AND METASTATIC PROCESS OF MCF-7 AND MDA-MB231 CELLS *IN VITRO* AND 4T1 CELLS *IN VIVO*

By

NADIAH ABU

December 2014

Chairperson: Assoc. Prof. Noorjahan Banu Alitheen, PhD
Faculty: Biotechnology and Biomolecular Sciences

In Malaysia, breast cancer is becoming a more prominent health issue among women today. Unfortunately, most malignant breast cancer will tend to metastasize to distant locations and form secondary tumors. This is usually the main cause of cancer-related deaths. Therefore, it is imperative to not actually treat cancer but to halt the metastatic process altogether. Though a number of approaches can be used to treat this disease, the prognosis tends to be unfavorable due to unwanted side effects and development of resistance. Natural products still remain one of the most sought after sources to find the perfect cure for cancer. The kava-kava (*Piper methysticum*) plant has been well known to aid illnesses and harness remedies since ancient times, especially in the pacific region. There are two classes of molecules that can be extracted from this kava-kava plant, kavalactones and chalcones. Chalcones can be divided into three types, flavokawain A, flavokawain B and flavokawain C. This project aims to study the effects of flavokawain A and B in the apoptotic and metastatic process in, MCF7 and MDA-MB231. Notably, both flavokawain A and B were non-toxic in both *in vitro* and *in vivo* experiments using Balb/C mice after 28 days of treatment. Through the MTT assay, it was found that both flavokawain A and B were cytotoxic in both breast cancer cell lines. Both flavokawain A and flavokawain B managed to induce apoptosis significantly as evidenced by these assays; double staining acridine orange/propidium iodide, flow cytometry cell cycle analysis, Annexin V analysis, JC-1, Caspase 8/9 fluorometric assay and BrdU cell proliferation assay. The results suggest that both flavokawain A and B induce G2/M arrest and apoptosis in both cell lines. Additionally, metastasis-related assays were also conducted such as; wound healing assay, migration and invasion assay, HUVEC tube formation and rat aortic ring assay. Flavokawain A and flavokawain B were shown to possess promising anti-metastatic potential. To further elucidate the apoptotic and anti-metastatic mechanism of flavokawain A and
B at the molecular level, real time polymerase chain reaction and western blot were conducted. Even though both molecules pose similar mechanism of action, flavokawain B is more potent and active than flavokawain A in terms of the induction of cell death and inhibition of metastasis. This notion was put to test in an in vivo setting whereby the compounds were used to treat 4T1 cells (mouse breast cancer) in mice. Based on the results, both flavokawain A and flavokawain B reduced the size of the tumor in vivo. In conclusion, flavokawain B is seemingly a better candidate as an anti-cancer agent than flavokawain A as evidenced by the in vitro assays. Moreover, based on the metastatic potential, flavokawain B was also a much more potent agent than flavokawain A. This concept was also proven by the in vivo assays using 4T1-breast cancer cell challenged mice. This study was able to elucidate the mechanism of action of both flavokawain A and flavokawain B in terms of its anti-cancer properties.
KESAN FLAVOKAWAIN A DAN FLAVOKAWAIN B KAVA-KAVA (Piper mehysticum G.Forst) YANG DISINTESIS SECARA KIMIA TERHADAP PROSES ANTI-BARAH DAN ANTI-METASTATIK PADA MCF-7 DAN MDA-MB231 IN VITRO DAN SEL 4T1 IN VIVO

Oleh

NADIAH ABU

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I certify that a Thesis Examination Committee has met on (date of viva voce) to conduct the final examination of Nadiah Abu on her thesis entitled EFFECTS OF CHEMICALLY SYNTHESIZED KAVA-KAVA (*Piper methysticum*) FLAVOKAWAIN A AND B ON THE APOPTOTIC AND METASTATIC PROCESS ON TWO BREAST CANCER CELL LINES, MCF-7 AND MDA-MB231 CELLS *IN VITRO* AND 4T1 CELLS *IN VIVO* 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 Doctor of Philosophy

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<tr>
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<td>Alkaline Phosphatase</td>
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<td>ALT</td>
<td>Alanine aminotransferase</td>
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<td>AO</td>
<td>Acridine Orange</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>C-X-C Chemokine receptor type 4</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
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</table>
ECM: Extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
ER: Estrogen Receptor
FACS: Fluorescence-activated cell sorting
FBS: Fetal Bovine Serum
FITC: Fluorescein isothiocyanate
FKA: Flavokawain A
FKB: Flavokawain B
FOXM: Forkhead box protein M1
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GLUT: Glucose Transporter
H&E: Hematoxylin and eosin
HEGF: Human endothelial growth factor
HPRT: Hypoxanthine-guanine phosphoribosyltransferase
HRP: Horseradish peroxidase
HSP: Heat shock protein
IC50: Inhibitory Concentration 50
ICAM-1: Intercellular Adhesion Molecule
IL: Interleukin
IFN: Interferon
INOS: Inducible nitric oxide synthase
JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MG/KG BW</td>
<td>Mg/kg body weight</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP9</td>
<td>Matrix metalloproteinase 9</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRP-1</td>
<td>Multidrug resistance protein 1</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NAOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NF-KB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>P-AKT</td>
<td>Phosphor AKT</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PLK</td>
<td>Polo-like kinase</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>QPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>S.E.M</td>
<td>Standard error of means</td>
</tr>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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# LIST OF APPENDICES

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<td>170</td>
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<td>D</td>
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A Synthesis of Flavokawain A and Flavokawain B
B Preparation of Cell Culture Reagents
C Validation of the primers used in qPCR
D Preparation of Western Blot Reagents
CHAPTER 1

INTRODUCTION

Cancer has become a global burden as the number of new cases increases year by year (Siegel et al., 2013). In women today, breast cancer has been one of the leading causes of cancer-related fatalities. This unfortunate incident has been linked to various factors including lack of a viable treatment, late screening for cancer patients and the lack of awareness among women to self-screen. In Malaysia, the number of new cases of breast cancer patients has also been increasing annually (Hisham et al., 2004; Yip et al., 2006). It is estimated that in 1 out of 16 women will be diagnosed with breast cancer at some point in their lives (Siegel et al., 2013). Although conventional treatments including chemotherapy and surgery are widely used, these methods have several drawbacks including physical pain, increased relapse and lower survival rate (Bauma et al., 2005; Ganz et al., 2011).

One of the main reasons of administering anti-cancer agents in cancer patients is to eliminate cancer cells; it is also favorable that it inhibits the metastatic process as well (Zijl et al., 2011). Metastasis is a process whereby primary tumor cells migrate and invade to form secondary tumors at a distant site, or secondary location (Zijl et al., 2011). Metastasis accounts for more than 90% of cancer-related fatalities (Finger et al., 2010; Lu et al., 2009). There are several steps in the metastasis cascade including extravasation, migration, tissue invasion, angiogenesis and circulation (Fidler, 2000; Finger et al., 2010). The most common sites of breast cancer metastasis is the lung, bone and liver (Weigelt et al., 2005).

Natural products have played an important part in search for new drugs, even some of the most famous widely used drugs are derived from natural sources (Newman et al., 2012; Rocha et al., 2001). Kava-kava (Piper methysticum) plant is an evergreen shrub that is widely consumed in the pacific region (Dharmaratne et al., 2002; Lebot et al., 1997). Moreover, this plant is largely known to be involved in a wide spectrum of biological activities including, anti-inflammation, anti-bacterial and most importantly, anti-cancer (Tang et al., 2008). Intriguingly, there has been a correlation between the consumption of kava-kava and the incidence of cancer (Steiner, 2000).

There are several interesting components that can be found in the kava root extracts, including chalcones (Dharmaratne et al., 2002; Tang et al., 2008). Chalcones are open ring flavonoids that are widely synthesized in the plant kingdom (Batovska et al., 2010). Flavokawain A is a chalcone and has been reported to possess promising anti-cancer and anti-inflammatory activities (Tang et al., 2008). Additionally, flavokawain A was found to inhibit the growth of bladder cancer cell lines in vitro (Tang et al., 2008). Based on
the preliminary study, flavokawain A was found to have similar potential cytotoxic activities in breast cancer as in bladder cancer cells. Flavokawain B on the other hand, is a much better studied chalcone as compared to flavokawain A. It has been put forward that flavokawain B possess promising anti-inflammatory and antinociceptive properties (Kuo et al., 2010; Kwon et al., 2013). The promising anti-cancer properties of flavokawain B have also been tested in oral carcinoma, synovial sarcoma and liver cancer (Kuo et al., 2010; Sakai et al., 2011; Tang et al., 2010). Nevertheless, though as hopeful as flavokawain A and flavokawain B may seem, further in depth mechanism as well as the anti-metastatic values is still yet to be discovered, especially in breast cancer. Moreover, the safety profile of the flavokawain A and B should also be tested even though the anti-cancer activities are promising. To achieve the objectives of this study several bioassays were attempted such MTT analysis, flow cytometry analysis, real-time PCR and western blot.

The objectives of this study were:
1. To assess and compare the in vitro toxicity and in vivo immunomodulatory potential of both flavokawain A and flavokawain B.
2. To investigate the cytotoxic effects and anti-metastatic potential of flavokawain A in two breast cancer cell lines, MCF-7 and MDA-MB231 in vitro.
3. To assess the anti-cancer mechanism of flavokawain B in terms of induction of cell death and anti-metastatic abilities in MCF-7 and MDA-MB231 in vitro.
4. To evaluate the anti-cancer activity of flavokawain A in an in vivo setting; 4T1-breast cancer challenged mice.
5. To determine the anti-cancer activity of flavokawain B in 4T1-breast cancer challenged mice in vivo.


Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F. L., Fevr, T., Clark, L., Bayan, N., Coppe, J.-P., Tong, F., Speed, T., Spellman, P. T., Devries, S.,


