



ANTI-CANCER ACTIVITY OF
TRITERPENOIDS ISOLATED FROM *LUVUNGA*
SCANDENS AGAINST MCF-7 CELLS

BY

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ABSTRACT

Luvunga scandens belong to the family of Rutaceae which usually inhabits tropical and moist environment. This plant is known as ‘Mengkurat Jakun’ among locals and used traditionally to treat fever and fatigue via decoction. The study aimed to elucidate the compounds from *L. scandens* stem, which were isolated using bioactivity guided isolation together with their mechanism of action on human breast cancer cell (MCF-7). The bioactivity-guided isolation of cytotoxic agent of the stem of *L. scandens* resulted in the isolation and characterization of a new triterpenoid from this species, 3-oxotirucalla-7,24-dien-21-oic-acid (**2**), along with a known triterpenoid, flindissol (**1**). The isolation was conducted using chromatographic techniques on silica gel and sephadex LH-20. The structures of the isolated compounds were elucidated on the basis of spectroscopic analysis including UV, IR, NMR, MS and 2D NMR. The cytotoxic activity of the plant on the proliferation of MCF-7 cell line was evaluated by MTT, WST-1 assay, scanning electron microscope (SEM), flow cytometry and RT-qPCR. The cytotoxic evaluation of the extracts showed that IC₅₀ value of the dichloromethane (LSC-SD) and methanol (LSC-SM) extracts from the stem were 75.0 and 77.0 µg/mL respectively. Whereas IC₅₀ value of *n*-hexane (LSF-H), *n*-hexane:DCM (LSF-HD), DCM (LSF-D) and DCM:MeOH (LSF-DM) fraction extracts were 97.5, 35, 98.9 and 94 µg/mL respectively. The IC₅₀ value of MeOH (LSF-M) fraction could not be extrapolated since none of the concentration of this extract was able to reduce the proliferation activity to 50 %. Compound **1** and **2** showed potent cytotoxicity against MCF-7 cell line with IC₅₀ values of 13.8 µM and 27.5 respectively after 24 h of treatment. Doxorubicin was used as a positive control drug with IC₅₀ values of 6.21 µM. Morphological analysis of the cells surface exhibit the apoptosis results after 24 h treated with LSC-SD extract, **1** and **2**, where the cells were rounded up, shrank and lost contact with neighboring cells. Apoptosis of MCF-7 cells treated with both compounds were confirmed by flow cytometry. The results show that compound **1** and **2** exert their anti-proliferative effect on MCF-7 cells through inhibiting cell cycle where the distribution of cells at Sub G₁ phase was 7.7 and 9.3 % respectively. Further evident of apoptosis induction by compound **1** and **2** towards MCF-7 cell lines were assayed by RT-qPCR on the expression of *PUMA*, *caspase-8* and *caspase-9*. In this assay, both compounds showed the increased of the expression of *PUMA*, *caspase-8* and *caspase-9* gene. In conclusion, both triterpenoids, 3-oxotirucalla-7,24-dien-21-oic-acid (**2**) and flindissol (**1**) have a potential to be developed as anticancer agents against breast cancer.

ملخص البحث

Luvunga scandens تنتمي إلى عائلة Rutaceae والتي تنمو في العادة بمحيط استوائي ورطب. هذه النبتة تسمى كذلك 'Mengkurat Jakun' ضمن السكان المحليين وهي تستخدم تقليدياً لعلاج الحمى والتعب عن طريق استخلاصها بالغلي في الماء. الهدف من هذه الدراسة كان معرفة تأثير المركبات والتي تمّ استخلاصها من *L. scandens* ثمّ عزلها باستخدام تقنية جذع العزل عن طريق النشاط الحيوي الموجّه بالإضافة إلى معرفة آلية العمل على خلايا الثدي السرطانية البشرية (MCF-7) تقنية العزل عن طريق النشاط الحيوي الموجّه للعامل السام بالنسبة للخلايا *L. scandens* أدى إلى عزل وتمييز ثلاثي لجذع تيرينويد جديد من (2) 3-oxotirucalla-7,24-dien-21-oic-acid، بالإضافة إلى آخر معروف (1) flindissol سلالة عملية العزل تمّت بواسطة تقنيات كروماتوغرافية على جيل السيليكا LH-20. بنيّة المركبات المعزولة تمّ تحليلها والسيفاديكس باستخدام الأشعة الطيفية بالإضافة إلى UV، IR، NMR، MS، 2D NMR النشاط السام للخلايا الخاص بالنبتة ضدّ MCF-7 تمّ تقييمه بتقنيات MTT، WST-1، ميكروسكوب التحليل الإلكتروني خلايا تقنية التدفق الخلوي وتقنية RT-qPCR، التقييم السام للخلايا الخاص بالمستخلصات أظهر IC₅₀ الخاصة بمستخلصات الديكلوروميثان (LSC-SD) والميثانول بأنّ قيم (LSC-SM) من الجذع كانت تعادل 77.0 و 75.0 مكغ \مل على التوالي. حيث كانت قيمة IC₅₀ الخاصة بـ ن-هيكسان، (LSF-H)، ن-هيكسان (LSF-HD)، وكذا (LSF-DM) DCM:MeOH من أجزاء المستخلصات تعادل 97.5، 98.9 و 94 مكغ \مل على التوالي. قيمة IC₅₀ الخاصة بجزء الميثانول (LSF-M) لم يتمّ تقدير قراءتها بما أنّ تركيزات هذا المستخلص لم تتمكّن من تخفيض نشاط تكاثر الخلايا إلى % المركبات 1 و 2 أظهرت فعالية عند النشاط السام للخلايا ضدّ خلايا MCF-7 بقيم IC₅₀ تعادل 13.8 و 27.5 مكغ \مل على التوالي بعد 24 ساعة من العلاج. تمّ استخدام دوكتوروبيسين كدواء دليل إيجابي بقيم IC₅₀ تعادل 6.21 مكغ. التحليل المورفولوجي لسطح الخلايا أظهر نتائج موت الخلايا بعد 24 ساعة من العلاج LSC-SD 1 و 2 بمستخلص. حيث كانت الخلايا كروية، منكمشة وفقدت التواصل مع الخلايا المجاورة لها. موت MCF-7 المبرمج المعالجة بكلا المركبين تمّ تأكيده بواسطة تقنية التدفق الخلوي. النتائج تظهر بأنّ المركبين 1 و 2 خلايا. يطرحان خصائص مضادة للتكاثر على خلايا MCF-7 عن طريق منع الدورة الخلوية حيث كان تقسيم الخلايا عند مرحلة Sub G₁ 7.7 و 9.3 بالمئة على التوالي. الدليل الموالي على وجود موت مبرمج للخلايا من طرف المركبين 1 و 2 ضدّ خلايا تمّت معايرته عن طريق تقنية RT-qPCR حول التعبير عن PUMA، الكاسباس 8 والكاسباس 9. كخلاصة فإنّ الترابتيرونيويدات 3-oxotirucalla-7,24-dien-21-oic-acid (2) والفلينديسول (1) يملكان خصائص يمكن تطويرها كعلاج ضدّ السرطان وبالأخصّ سرطان الثدي.

ABSTRAK

Luvunga scandens tergolong dalam keluarga Rutaceae yang kebiasaannya mendiami persekitaran tropika dan lembap. Pokok ini dikenali sebagai 'Mengkurat Jakun' dalam kalangan penduduk tempatan dan digunakan secara tradisional untuk mengubati demam dan keletihan dengan meminum air rebusan. Kajian ini bertujuan untuk menguraikan sebatian dari *L. scandens* batang yang diasingkan berdasarkan pengasingan berpadukan bioaktiviti bersama-sama tindak balas sebatian terhadap sel kanser payudara manusia (MCF-7). Pengasingan berpandukan bioaktiviti daripada batang pokok *L. scandens* menghasilkan pemencilan dan pencirian triterpenoid baru, 3-oxotirucalla-7,24-dien-21-oic-acid (**2**) serta triterpenoid yang diketahui iaitu flindissol (**1**). Pengasingan telah dijalankan dengan menggunakan teknik kromatografik di atas gel silica dan sephadex LH-20. Struktur sebatian yang telah diasingkan itu telah diuraikan berdasarkan analisis spektroskopi termasuk UV, IR, NMR, MS dan 2D NMR. Kesan sitotoksik daripada pokok ini terhadap pertumbuhan sel MCF-7 telah dijalankan menggunakan ujian MTT, WST-1, scanning electron microscope (SEM), flow cytometry dan RT-qPCR. Ujian sitotoksik telah menunjukkan nilai IC_{50} diklorometana (LSC-SD) dan metanol (LSC-SM) ekstrak dari batang masing-masing adalah 75.0 dan 77.0 $\mu\text{g/mL}$. Manakala nilai IC_{50} *n*-heksana (LSF-H), *n*-heksana: DCM (LSF-HD), DCM (LSF-D) dan DCM: MeOH (LSF-DM) ekstrak masing-masing adalah 97.5, 35, 98.9 dan 94 $\mu\text{g/mL}$. Nilai IC_{50} daripada MeOH (LSF-M) tidak boleh dikenalpasti kerana ekstrak ini tidak dapat merencat 50 % daripada aktiviti pertumbuhan sel. Sebatian **1** dan **2** menunjukkan kesan sitotoksik yang kuat terhadap sel MCF-7 dengan nilai IC_{50} 13.8 dan 27.5 μM selepas 24 jam rawatan. Doxorubicin telah digunakan sebagai ubat kawalan positif dengan nilai IC_{50} 6.21 μM . Analisis morfologi permukaan sel selama 24 jam menunjukkan kesan apoptosis selepas rawatan dengan ekstrak LSC-SD, sebatian **1** dan **2**, di mana sel-sel itu telah membulat, mengecut dan kehilangan hubungan dengan sel bersebelahan. Apoptosis daripada sel MCF-7 yang dirawat dengan kedua-dua sebatian telah disahkan dengan flow cytometry. Keputusan menunjukkan bahawa sebatian **1** dan **2** memberi kesan anti-proliferasi pada sel MCF-7 melalui pengencatan kitaran sel di mana pembahagian sel di fasa Sub G_1 adalah masing-masing 7.7 dan 9.3 %. Bukti yang lagi jelas tentang apoptosis oleh sebatian **1** dan **2** terhadap sel MCF-7 telah diuji oleh RT-qPCR pada ekspresi gen *PUMA*, *caspase-8* dan *caspase-9*. Dalam pengujian ini kedua-dua sebatian menunjukkan peningkatan ekspresi pada gen *PUMA*, *caspase-8* dan *caspase-9*. Kesimpulannya, keputusan kajian menunjukkan triterpenoids, 3-oxotirucalla-7,24-dien-21-oic-acid (**2**) dan flindissol (**1**) berpotensi untuk dijadikan agen menentang kanser payudara.

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 Pharmaceutical Science (Pharmaceutical Technology)

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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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CHAPTER ONE

INTRODUCTION

1.1 GENERAL OVERVIEW

Most plants consist of phytochemicals or known as secondary metabolites which have disease preventive properties. Plants produce these chemicals to protect themselves from any possible harm in the ecological environment. Recent scientific researches show that phytochemicals have the potential to protect human against various diseases including cancer, heart disease, diabetes and high blood pressure (Liu, 2004; Lodish, Berk, Zipursky, Matsudaira, Baltimore, & Darnell, 2000).

Phytochemicals are present in smaller quantities in plants which include alkaloids, steroids, flavonoids, terpenoids, tannins and many others. Nearly about 50% of drugs used in medicine are plant origin and only a small fraction of plants with medicinal activity has been assayed. Based on the previous research studies and findings, phytochemicals have been reported to play an important role in the prevention of cancer. It can be proven via revelation of new anticancer compound, Taxol. Taxol is originally isolated from the stembark of *Taxus brevifolia*. It is a new antitumor drug approved by FDA for the treatment of breast, ovarian and non-small-cell lung carcinomas (Peteros & Uy, 2010).

Breast cancer is the second most frequently diagnosed cancer in the developed and developing countries. Breast cancer occurs due to the out-of-control growth of normal cells in breast. Instead of dying like normal cells, breast cancer cells can grow and invade other tissues. Different types of cancer can behave very differently. They

grow at different rates and respond to different treatments. That is why people with cancer need treatment that is targeted their own kind of cancer.

This research study was focused to evaluate the effect of plant-derived terpenoids specifically known as triterpenoids from *Luvunga scandens* against human breast adenocarcinoma (MCF-7) cell lines. These plant-derived triterpenoids were targeted to inhibit the breast cancer cells via apoptosis pathway.

1.2 OBJECTIVES OF THE STUDY

1.2.1 General Objective

To evaluate the cytotoxic effects of *L. scandens* on human breast adenocarcinoma (MCF-7) cell line through bioactivity-guided isolations of active compounds.

1.2.2 Specific Objectives

1. To evaluate the cytotoxic activity of *L. scandens*'s stem extracts against MCF-7 cells.
2. To isolate anticancer compounds from the active fractions.
3. To observe the morphological changes of MCF-7 cells treated with different concentrations of isolated compounds.
4. To analyze cell cycle profile of MCF-7 treated with *L. scandens* compounds.
5. To determine the gene expression of *PUMA*, *caspase-8* and *caspase-9* genes at mRNA level in MCF-7 cell lines treated with *L. scandens* compounds.

1.3 EXPERIMENTAL DESIGN

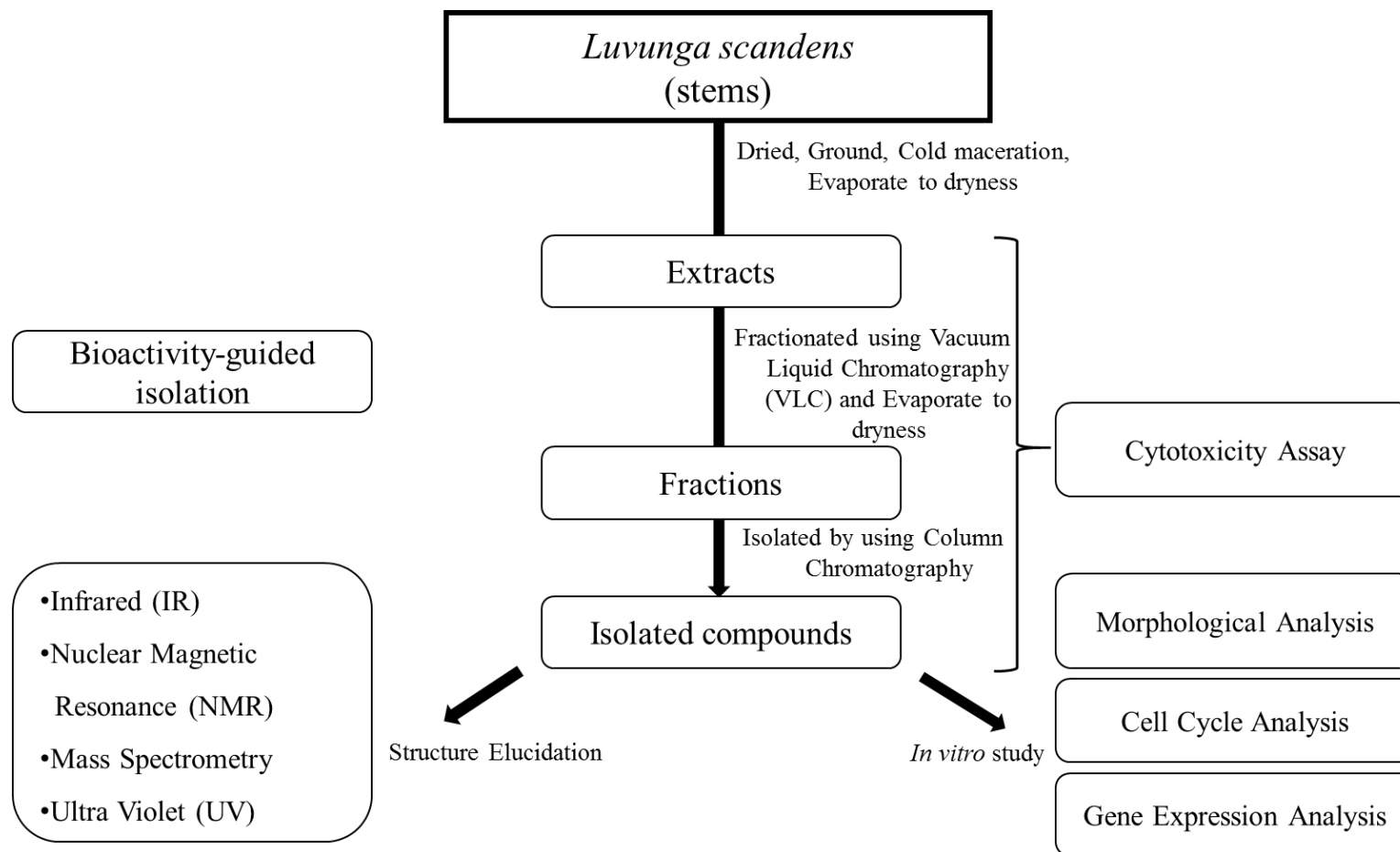


Figure 1.1 Flow chart of the study.

CHAPTER TWO

LITERATURE REVIEW

2.1 NATURAL PRODUCT AS ANTI-CANCER AGENT

Natural products, especially from plants, have been used for the treatment of various diseases for thousands of years. They have also played an important role in the development of several clinical useful anticancer agents (Shoeb, 2006). Over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and microorganisms (Cragg & Newman, 2005). Example of natural product based drug is doxorubicin or known as Adriamycin derived from *Streptomyces peucetius*, the daunomycin producing microorganism (Arcamone, Cassinelli, Fantini, Grein, Orezzi, Pol, & Spalla, 1969), have been used in oncologic practice since the late 1960. It is a powerful drug in the fight against cancer that includes breast and esophageal carcinomas, osteosarcoma, Kopsi's sarcoma and others (Singal & Iliskovic, 1998). Other plant derivatives used in cancer therapy are listed in Table 2.1.

2.1.1 Secondary Metabolites in Natural Products

Bioactive natural products play a vital role in order to find the novel therapeutic agents. Over 40 % of the medicines have been originated from natural products. Plant and marine sources are among the natural products from the nature and have existed since the beginning of life. This is the evidence that works related to natural products continue to develop and involve the researchers from various scientific backgrounds throughout the world (Hadi, Duru, & Martin-Diana, 2013).

Table 2.1
List of plant derivatives used in cancer therapy.

Semisynthetic analogs of plant derivatives	Species and Genus name	Experiments on various cancer cells	References
Vindesine and vinorelbine	<i>Catharanthus roseus</i>	Leukemia, lymphomas advanced testicular cancer, breast cancer, lung cancer and Kaposi's sarcoma.	Cragg & Newman (2005)
Taxol®	<i>Taxus brevifolia</i> Nutt, <i>T. baccata</i>	Metastatic breast, ovarian, lung, prostate cancer and lymphoid malignancies	Kingston (2007)
Taxotere®	<i>T. brevifolia</i> Nutt, <i>T. baccata</i>	Used in patients resistant to Paclitaxel	Hait, Rubin, Alli, & Goodin (2007)
Topotecan	<i>Camptotheca acuminata</i>	Epithelial ovarian cancer and small cell lung cancer	Creemers, Bolis, Gore, Scarfone, Lacave, Guastalla, Despax, Favalli, Kreinberg, & Van Belle (1996)
Irinotecan	<i>C. acuminata</i>	Metastatic and colorectal cancer	Fuchs, Mitchell, & Hoff (2006)
Exatecan	<i>C. acuminata</i>	Potential anti-tumor activity both <i>in vitro</i> and <i>in vivo</i>	Ishii, Iwahana, Mitsui, Minami, Imagawa, Tohgo, & Ejima (2000)
Beberine	<i>Hydrastis canadensis</i> L., <i>Berberineeris</i> sp & <i>Arcungelisia</i> flav	Osteosarcoma, lung, liver, prostate and breast cancer	Patil, Kim, & Jayaprakasha (2010)
Beta-lapachone	<i>Tabebuia avellaneda</i>	Breast, prostate, pancreatic cancer and promyelocytic leukemia	Li, Li, Yu, & Pardee (2000)
Curcumin	<i>Curcuma longa</i>	Colorectal cancer, multiple myeloma and pancreatic cancer	Sa, Das, Banerjee, & Chakraborty (2010); Goel, Kunnumakkara, & Aggarwal (2008)

Semisynthetic analogs of plant derivatives	Species and Genus name	Experiments on various cancer cells	References
Diadzein and Genistein	<i>Lupinus</i> species, <i>Vicia faba</i> , <i>Glycine max</i> , <i>Psoralea corylifolia</i>	Genistein inhibits ovarian and breast cancers and also chemically induced cancers of stomach, bladder, lung, prostate, colon and blood.	Kaufman, Duke, Brielmann, Boik, & Hoyt (1997); Moon, Wang, & Morris (2006); Dixon & Ferreira (2002)
Flavopiridol	<i>Amoora rohituka</i> and <i>Dysoxylum</i> <i>binectariferum</i>	Colorectal, non-small cell lung cancer, renal cell carcinoma, non-Hodgkin's lymphoma, chronic lymphocytic leukemia and also solid tumor	Mans, da Rocha, & Schwartzmann (2000)
Harringtonine and Homoharringtonine	<i>Cephalotaxus harrintonia</i> , <i>Cephalotaxus hainanensis</i> and <i>Cephalotaxus qinensis</i>	Acute myeloid leukemia and chronic myeloid leukemia	Cragg & Newman (2005); Efferth, Li, Konkimalla, & Kaina (2007)
Pandimex TM	Saponins of gengseng	Advance cancer of breast, colon-rectum, lung, pancreas and solid tumor	Pan, Chai, & Kinghorn (2010)
Perillyl alcohol	Many plant species like mints, cherries, lavenders and many others	Non small lung cancer, prostate cancer, colon and breast cancer	Pan et al. (2010)
Schischkinnin	<i>Centaurea schischkinii</i>	Colon cancer lines <i>in vitro</i>	Shoeb, Celik, Jaspars, Kumarasamy, MacManus, Nahar, Thoo-Lin, & Sarker (2005)
Silvestrol	<i>Aglaia foveolata</i> Panell	Prostate, breast and lung cancers	Kinghorn, Carcache de Blanco, Chai, Orjala, Farnsworth, Soejarto, Oberlies, Wani, Kroll, & Pearce (2009); Kim, Hwang, Su, Chai, Mi, Kinghorn, Wild, & Swanson (2007)