APOPTOSIS EFFECT OF *LUVUNGA SCANDENS* AGAINST HUMAN SKIN CANCER A431 CELL LINE

BY

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INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

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A thesis submitted in fulfilment of the requirement for the degree of Master in Pharmaceutical Science (Pharmaceutical Technology)

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ABSTRACT

Epidermal carcinoma cell line is one of the neoplastic cell that are characterized by abnormalities in cell differentiation and growth. Epidermal carcinoma is reported as one of the most common types of cancer with increasing numbers of occurrence. *Luvunga scandens* is one of the plants found in Malaysia, this plant is known to possess many bioactivities and general health effects, yet its anti-proliferative effect is generally under reported and need to be scientifically evaluated. The aim of this study is to evaluate the anti-proliferative and apoptotic effects of *Luvunga scandens* plant leaves against human epidermal carcinoma cell line. MTT assay was used to assess the cytotoxicity of the plant against human epidermal carcinoma cells in addition to the safety assessment for normal cell lines (HaCaT and HDF). Scratch assay was carried out to monitor the cell growth. The morphological changes of *L. scandens* treated epidermal carcinoma cells has been confirmed by SEM, and the apoptosis of the plant against epidermal carcinoma cells has been tested using caspase 3/7 assay, followed by cell cycle analysis done using a flow-cytometer on epidermal carcinoma cells treated with the IC_{50} does of *L. scandens* plant. Western blot was preformed to confirm the anti-carcinogenic effect of *L. scandens* against human epidermal carcinoma cells. The results of the tests done illustrated that the extract and compound possesses cytotoxic effect against epidermal carcinoma cells with IC_{50} readings; (methanol= 37.5 μg/mL, DCM= 38 μg/mL, hexane= 37.5 μg/mL and compound= 27.5 μM), and no cytotoxic activity in both HaCaT and HDF cells. The IC_{50} dose of *L. scandens* can restrict the growth of epidermal carcinoma cells more than Cisplatin (anti-cancer drug), the SEM results demonstrate that *L. scandens* treated cells showed an overall change in the cell shape, alteration of surface morphology, absence of microvilli and appearance of blebs. Caspase 3/7 assay results shows that *L. scandens* DCM extract produce the highest level of apoptosis against epidermal carcinoma cell. For cell cycle analysis, all the *L. scandens* treated epidermal carcinoma cells show high readings in the sub-G_1 phase. For western blot the *L. scandens* extract and compound show high apoptosis effects against human epidermal carcinoma cells. This *in vitro* study has proved that *L. scandens* plant exhibit anti-proliferative effects against human epidermal carcinoma cells, hence, it can be considered as a new promising potential anti-cancer therapy.

ملخص البحث

خلايا سرطان الجلد الخبيثة هي واحدة من الخلايا الورمية التي تتميز بتشوهات في تمايز الخلايا والنمو. ذكرت معظم الدراسات السابقه ان سرطان الجلد يعد واحدا من اكثر أنواع السرطان شيوعا و نسبه الافراد المصابين في تزايد ملحوض. لفونجا سكاندس هي احد النباتات المنتشره في ماليزيا، هذه النبته تحتوي الكثير من المنشطات الحيويه و تساعد عامه في تحسين الصحة، لكن لا توجد دراسات حول تأثير هذه النبتة ضد السرطان، لذلك من المهم ان نقوم بتقييم تأثير هذا النبات ضد السرطان بشكل علمي. الهدف من هذه الدراسة هو لتقييم التأثير المضاد لخلايا سرطان الجلد الناتج من أوراق اللفونجا سكاندس. لقد قمنا بعمل اختبار ال م.ت.ت لتقييم مقدار السمية النابجة من النبتة لخلايا سرطان الجلد، بالإضافة إلى نوعين اخرين من خلايا جسم الانسان الطبيعيه (خلايا الجلد الليفية والخلايا الكيراتينيه) لتحديد ما إذا كانت هذه النبته امنه للاستخدام مع باقي خلايا الجسم الطبيعيه. نفذ اختبار الخدش لمراقبة نمو الخلايا ، بواسطه المجهر الالكتروني كان بوسعنا مشاهدة التغييرات الشكليه لخلايا سرطان الجلد الناتجه عن معالجة الخلايا بالنبات المستخلص. باستخدام فحص كاسباس ٧/٣ لقد تمت عملية مراقبة موت الخلايا المبرمج تلك المعالجه بنبات اللفونجا سكاندنس. بعد ذلك تم تحليل دورة الخلايا باستخدام جهاز تدفق الكريات لخلايا سرطان الجلد المعالجة بنسبة محددة من نبات ال ل. سكاندنس. استخدمنا أيضا لطخة وسترن كاحد التقنيات لتاكيد تاثير النبات في مكافحة خلايا الجلد السرطانية. نتائج الاختبارات التي أجريت أوضحت أن مستخلص و مركب نبات ال ل. سكاندنس يمتلك تأثير سمي ضد خلايا سرطان الجلد الخبيثة مع قراءات أي سي ٥٠؛ (الميثانول = ٣٧.٥مايكروجرام / مليلتر و دي.سي.ام = ٣٨ مايكروجرام / مليلتر و الهكسان = ٣٧.٥ مايكروجرام / مليلتر والمركب = ٢٧.٥مايكروموليي)، بالنسبة للخلايا الطبيعية (خلايا الجلد الليفية و الخلايا الكيراتينيه) فمركب النبات و مستخلصه اظهروا قراءات امنه. اظهرت نتائج اختبار الخدش ان جرعة ال أي سي ٥٠ من النبات يمكن أن تحد من نمو خلايا سرطان الجلد الخبيث أكثر من سيسبلاتين (علاج مستخدم ضد السرطان) ، نتائج المجهر الالكتروني أظهرت تغير شامل في شكل الخلية السرطانية و تغيير في مورفولوجيا السطح وغياب الزغيبات الصغيرة وظهور الفقاعات. فحص الكاسباس ٧/٣ اظهر ان مستخلص ال ل. سكاندنس دي. سي. ام هو الأكثر فعالية بتحفيز موت الخلايا المبرمج لدى خلايا سرطان الجلد الخبيثه. بالنسبه لتحليل دورة الخليه النتائج أظهرت بان نبتة ال ل. سكاندس بمستخلصاتها الثلاثه و المركب المعزول رفعت قرائة المرحله ما قبل ال ج١ في دورة الخلايا السرطانيه. نتائج اختبار لطخة وسترن أتت بان ال ل. سكاندنس قد حقق الموت المبرمج لخلايا الجلد السرطانيه البشريه. وقد أثبتت هذه الدراسة التي تم تنفيذها في المختبر أن نبات ال ل. سكاندنس اظهر تأثيرات مضادة لخلايا سرطان الجلد الخبيث، وبالتالي، فإنه من الممكن ان تكون هذه النبته كعلاج جديد لمكافحة السرطان في المستقبل.

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 in 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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Signature ..... Date .....

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First of all, praise is due to almighty Allah S.W.T with His compassion and mercifulness to allow me to complete this research, and may His peace and blessings be upon our beloved prophet Muhammad (S.A.W).

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
Cis	Cisplatin
DCM	Dichloromethane
dH_2O	Distilled water
DTT	Dithiothreitol
DLS	Dichloromethane *Luvunga Scandens*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECL	Enhanced Chemiluminescence
FBS	Fetal Bovine Serum
HLS	Hexane *Luvunga Scandens*
HMDS	Hexamethyldisilazane
MLS	Methanol Luvunga Scandens
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide
ODO	3-oxotirucalla-7,24-dien-21-oic acid
PBS	Phosphate buffer solution
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene Fluoride
Rpm	Revolutions per minute
SD	Standard deviation
SEM	Scanning Electron Microscope
TBS	Tris-Buffer Saline
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Cancer is a disease that is characterized by abnormal cell differentiation and maturation, uncontrolled cell growth, and is the most significant cause of death in recent years (Ginestier et al., 2007).

Skin cancer, which includes both melanoma and non-melanoma are considered among some of the most common types of cancer among the white population. Skin cancer has now reached epidemic proportions. In Australia, studies have showed that there are over 50 new cases of melanoma skin cancer per 100,000 people, and the incident rate is 2% basal cell carcinoma and 1% squamous cell carcinoma in the male population (Diepgen & Mahler, 2002).

The main cause for this malignancy is not specifically known or identified, however like other types of cancer two main factors contribute in its development. These two factors are the external factor (environment) and the internal factor (genetics) (Houghton & Polsky, 2002). Though it has been proven that sunlight does play a big role in skin cancer whereby the ultraviolet (UV) rays of sunlight is very carcinogenic and is considered the main factor in squamous cell carcinoma (SCC), it has been studied that sunlight-related tumor progression could be caused by mutations that can only be precipitated by UV. It has been determined that 58% of invasive SCC have mutations in the p53 tumor suppressor gene, affecting the amino acid sequence. To indicate that the tumor mutations are caused by UV light, we observed CC----TT double-base change occurring at dipyrimidine sites, and high a frequency of C----T substitution. The p53 mutations in

other malignancies that are not UV related, such as those in breast cancer, does not show these UV related factors (Brash et al., 1991).

There are three main types of skin cancers; basal cell carcinoma (BCC), squamous cell carcinoma (SCC)/epidermoid carcinoma and melanoma. It is believed that these three types of cancers are caused by the exposure to sunlight (UV), and it is recorded that the occurrence is higher in individuals with fairer and more sensitive. Epidermoid carcinoma/SCC and BCC both occur 18 to 20 times more than malignant melanoma (Leiter & Garbe, 2008). As a prevention of skin cancer; habits like sun protection and decreased sun exposure should be practiced (Armstrong & Kricker, 2001).

After studying these facts, a serious thought leading to safe treatment should be considered. For many years various types of human cancer cell lines have been broadly used as an *in vitro* model to understand the mechanism of carcinogenesis and to discover new treatment methods. Even once involving the use of natural compounds which are assumed to have anticancer effects and at the same time safe towards the healthy cells of the human body (Shukla & Mehta, 2015; Prakash et al., 2013).

Plants are being used with a wide range of biologically active compounds in the treatment of mild to serious diseases since ancient times. This has provided researchers today with a new sight for the use of natural products on its own, or by adding it to synthetic drugs or products, to modify them for better results and less serious side effects. “Drug discovery from natural products for confronting cancer has brought in the rational opportunity to attain the newest clinical applications of plant secondary metabolites and their derivatives” (Hamedeyazdan et al., 2012).

The very first study on anticancer agents from plants was carried out in the 1950s on vinca alkaloids, vinblastine, and vincristine. Since then three thousand plant species were tested and used

for its anticancer properties and for its cancer treatment ability. Nowadays, natural sources are the main focus and play a major role in discovering anticancer agents (Ashraf et al., 2013).

Discovering and testing for new and better ways for treatment is an obligation of every true scientist, and as God created a cure for every disease it is our responsibility to find that cure.

Abu Baker Al Sedeeq, may Allah be pleased with him, reported: Prophet Mohamad (SAW) said;

> "Ask Allah for forgiveness and health, for after being granted certainty, one is given nothing better than health" (Al- Albani, 1988).

And health we shall seek and it is where we are heading to in this research which is based on finding a cure for cancer and that is not impossible.

Abi Hurairah may Allah be pleased with him, reported: Prophet Mohamad (SAW) said;

> “God did not come down with any disease but revealed its healing, his knowledge of his knowledge and his ignorance of his ignorance, and He did not put any disease but put its cure, or medication” (Al- Askalani, 1986).

Medicine of plant origin is known to be safe and effective because it is and has been used in conventional medicine. Which is important and widely used worldwide.