



APOPTOSIS EFFECTS OF *LUVUNGA SCANDENS*
LEAVE EXTRACTS AND ITS COMPOUND ON
HUMAN SKIN CANCER A431 CELL LINE

BY

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ABSTRACT

Skin cancer is reported as one of the most common types of cancer with increasing numbers of occurrence. *Luvunga scandens* is one of the medicinal 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 skin cancer cell line. MTT assay was used to test the cytotoxicity effect of *L. scandens* on human skin carcinoma cells together with its effect against normal cell lines (HaCaT and HDF). Scratch assay was carried out to evaluate the cell growth. The morphological changes of *L. scandens* treated skin cancer cells was confirmed by scanning electron microscopy, and the apoptotic effect of the plant against skin cancer cells was tested using caspase 3/7 assay, followed by cell cycle analysis done using a flow cytometer on skin cancer cells. Western blot was preformed to investigate the anti-carcinogenic effect of *L. scandens* against human skin cancer cells. The results showed that the extract and a compound 3-oxotirucalla-7,24-dien-21-oic acid possesses cytotoxic effect against skin cancer cells, and it was no cytotoxic activity in both of HaCaT and HDF cells. The scanning electron microscopy 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 showed that *L. scandens* dichloromethane (DCM) extract exhibited the highest level of apoptosis against skin cancer cells. For cell cycle analysis, all the *L. scandens* treated skin cancer cells show high readings in the sub-G₁ phase. For western blot the *L. scandens* extract and compound show high apoptosis effects against human skin cancer cells. This *in vitro* study has proven that *L. scandens* plant exhibit anti-proliferative effects against human skin cancer cell hence, it can be considered as a promising natural source for anticancer therapy.

ملخص البحث

خلايا سرطان الجلد الحبيثة هي واحدة من الخلايا الورمية التي تتميز بتشوهات في تمايز الخلايا والنمو. ذكرت معظم الدراسات السابقة ان سرطان الجلد يعد واحدا من أكثر أنواع السرطان شيوعا و نسبه الافراد المصابين في تزايد ملحوظ. لفونجا سكاندس هي احد النباتات المنتشرة في ماليزيا، هذه النبتة 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 الفونجا سكاندس. لقد قمنا بعمل اختبار ال م.ت.ت لتقييم مقدار السمية الناتجة من النبتة لخلايا سرطان الجلد، بالإضافة إلى نوعين آخرين من خلايا جسم الانسان الطبيعيه (خلايا الجلد الليفية والخلايا الكيراتينية) لتحديد ما إذا كانت هذه النبتة امنه للاستخدام مع باقي خلايا الجسم الطبيعيه. نفذ اختبار الخدش لمراقبة نمو الخلايا ، بواسطة المجهر الالكتروني كان بوسعنا مشاهدة التغييرات الشكلية لخلايا سرطان الجلد الناتجة عن معالجة الخلايا بالنبات المستخلص. باستخدام فحص كاسباس ٧/٣ لقد تمت عملية مراقبة موت الخلايا المبرمج تلك المعالجة بنبات الفونجا سكاندس. بعد ذلك تم تحليل دورة الخلايا باستخدام جهاز تدفق الكريات لخلايا سرطان الجلد المعالجة بنسبة محددة من نبات ال ل. سكاندس. استخدمنا أيضا لطخة وسترن كأحد التقنيات لتأكيد تاثير النبات في مكافحة خلايا الجلد السرطانية. نتائج الاختبارات التي أجريت أوضحت أن مستخلص و مركب نبات ال ل. سكاندس يمتلك تأثير سمي ضد خلايا سرطان الجلد الحبيثة، بالنسبة للخلايا الطبيعية (خلايا الجلد الليفية و الخلايا الكيراتينية) فمركب النبات و مستخلصه اظهروا قراءات امنه. 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. سكاندس دي. سي. ام هو الأكثر فعالية بتحفيز موت الخلايا المبرمج لدى خلايا سرطان الجلد الحبيثة. بالنسبة لتحليل دورة الخلية النتائج أظهرت بان نبتة ال ل. سكاندس بمستخلصاتها الثلاثة و المركب المعزول رفعت قرائة المرحله ما قبل ال ج ١ في دورة الخلايا السرطانية. نتائج اختبار لطخة وسترن أتت بان ال ل. سكاندس قد حقق الموت المبرمج لخلايا الجلد السرطانية البشرية. وقد أثبتت هذه الدراسة التي تم تنفيذها في المختبر أن نبات ال ل. سكاندس اظهر تأثيرات مضادة لخلايا سرطان الجلد الحبيث، وبالتالي، فإنه من الممكن ان تكون هذه النبتة 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 Sciences (Pharmaceutical Technology).

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
Cis	Cisplatin
DCM	Dichloromethane
dH ₂ O	Distilled water
DTT	Dithiothreitol
DLS	Dichloromethane <i>Luvunga Scandens</i>
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECL	Enhanced Chemiluminescence
FBS	Fetal Bovine Serum
HLS	Hexane <i>Luvunga Scandens</i>
HMDS	Hexamethyldisilazane
MLS	Methanol <i>Luvunga Scandens</i>
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, it is known as the most significant cause of death worldwide 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, namely 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 skin. 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. The new treatment methods have been involving the use of natural compounds which are assumed to have anticancer effects and at the same time they are considered 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. “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 around 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).

In this research health is what we are seeking by finding a cure for cancer and that is not impossible as 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).

80% of the world’s population depend on conventional medicine obtained from plants or of plant origin. 95% of traditional medicine prepared with plants, and 90% of people in the rural areas depend on traditional medicine (Mir et al., 2014).

1.2 PROBLEM STATEMENT AND SIGNIFICANCE OF THE STUDY

The main reason of conducting this study is to find an effective cure for cancer, specifically for human epidermoid carcinoma one, of the most common types of skin cancer. It is not easy to cure skin cancer and it's hard to go to the surgical option especially in the case of spreading. Chemotherapy and radiation therapy treatments have many debilitating side effects that leave the body drained and fatigue. These side effects include nausea, loss of appetite, mouth sores, urinary dysfunction, hormonal changes, and hair loss. It is also possible for these side effects to affect the production of healthy blood platelets, red and white blood cells, in addition to pain and aches around the whole body. All those side effects would affect the emotional status of the patient and can cause depression and anxiety which slows down the healing process (Chintamani et al., 2011).

In view of that, researchers all around the world are searching for effective drugs which are potent to the cancer cells but with fewer side effects. Many medicinal plants used in folk and traditional medicine and their biologically active derivatives isolated from natural sources are being increasingly used in clinical trials for anticancer activity, as they have fewer side effects than conventional therapeutic procedures at relatively low cost. Herbal medication has been successfully demonstrated to have anti-neoplastic potential and is the source of many chemotherapeutic agents. The bioactivities of *Luvunga scandens* extract have been reported as previous studies have suggested that *Luvunga scandens* extract has many health and beneficial effects such as anti-fungal effects and general health effects. However, to this day no studies have confirmed whether the extract of *L. scandens* possess anticancer activities on the human epidermal carcinoma A 431 cell line. This *in vitro* study will focus on the cytotoxic and anti-proliferative potential of *Luvunga*

scandens on human epidermoid carcinoma A 431 cell line.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objective

To evaluate the anti-proliferative and the apoptotic effects of *Luvunga scandens* plant leaves against human skin cancer A431 cell line.

1.3.2 Specific Objectives

1. To investigate the cytotoxicity effect of *Luvunga scandens* extract and isolated compound on A431 skin cancer cell line.
2. To evaluate the effect of *Luvunga scandens* extract and isolated compound on HaCaT and HDF normal cell lines.
3. To determine the apoptosis effects of the crude extract and isolated compound on the A431 skin cancer cell line.

1.4 RESEARCH HYPOTHESIS

Luvunga scandens extract (methanol, DCM and hexane) and isolated compound (3-oxotirucalla-7,24-dien-21-oic acid) may inhibit the growth of human skin cancer cell.

1.5 RESEARCH METHODOLOGY

The major steps involved are presented in Figure 1.1 and is briefly outlined as below.

Detailed material and methodology are described in chapter three.

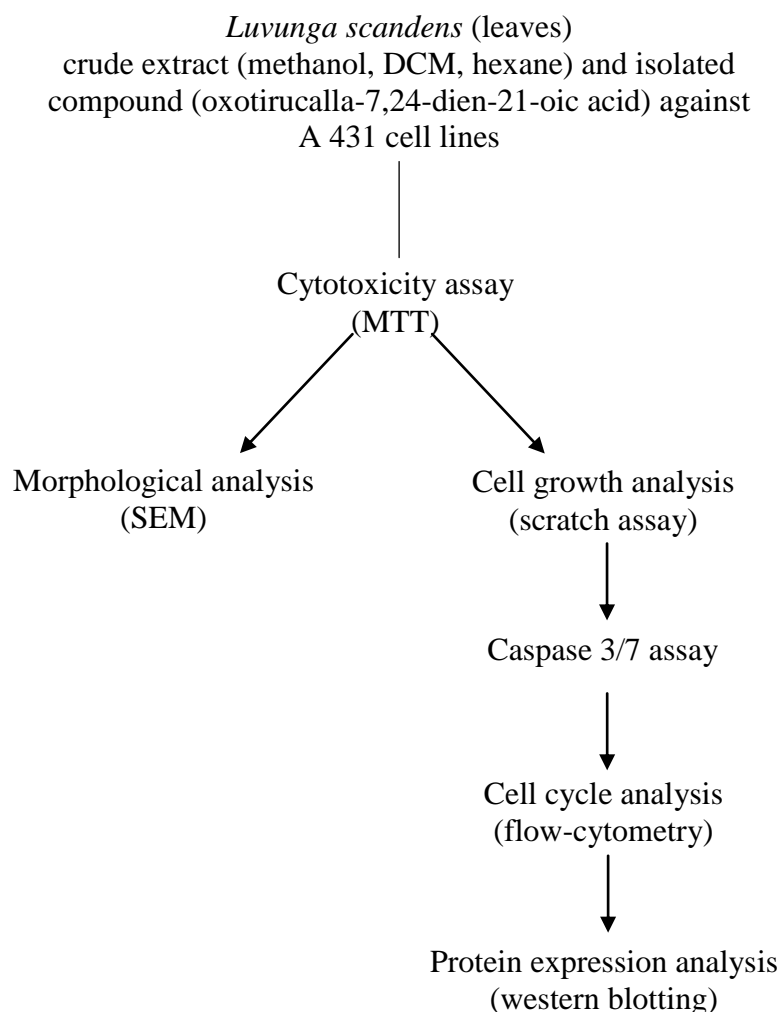


Figure 1.1 Flow chart of the study

1.6 SCOPE OF RESEARCH

The extracted leaves of *L. scandens* and isolated compound were used to screen for the presence of cytotoxic activity against A431 skin cancer cell line. Investigation on cytotoxic property of the extract and isolated compound was conducted through mammalian cell culture testing against skin cancer A 431 cells using *in vitro* screening assays which is (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT assay).

The growth of treated cells was tested with a scratch assay. The morphology of apoptotic cells was viewed via scanning electron microscopy (SEM). Caspase 3/7 analysis was done to determine the apoptosis of the treated cells using a caspase 3/7 kit. Cell cycle analysis using a flow-cytometer was done to identify the cell death phases on skin cancer cells treated with both the crude extract and isolated compound. Protein expression analysis was also done using the western blot method with the extraction of protein from the cells.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter of literature review begins with some introduction for the human skin cancer and how it occurs. Then we will review the methods of cancer treatment currently used including chemotherapy, radiotherapy, and surgical treatment methods. After that the topic will move on to natural products discussing the methods used for cancer prevention and treatment.

The chapter then will focus on the medicinal benefits of *Luvunga scandens* plant. Then, extraction method, and other methods commonly used to aid in determination of factors affecting the study.

2.2 SKIN CANCER

Skin cancer is considered to be a big public health concern as it shows a high incident rate especially in young adults and it collects a big number of lives worldwide (Guy & Ekwueme, 2011).

According to Bittner et al. (2000) skin neoplasms are the most common human cancers found, with a rising incident rate and low progress in non-surgical treatment of advanced stages. For the last 40 years' skin cancer incidents, has been increasing in three times the number it had begun with (Middleton et al., 2000).

The survival rate of skin cancer drops to a low number in cases of metastasis, as it makes it hard for the body to respond to chemotherapies and other drugs, and reduces the survival rate of patients to 6-9 months. If patients undergo the surgical option for metastasis the survival rate will prolong to 11-19 months. Other treatment