SYNERGISTIC EFFECT OF THE COMBINATION OF FICUS DELTOIDEA AND GYNOCHTHODES SUBLANCEOLATA EXTRACT ON NORMAL, BREAST AND PROSTATE CANCER CELL LINES

by

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A549	pulmonary carcinoma
AR	Androgen Receptor
ATP	Adenosine Triphosphate
BRCA1 and BRCA2	Breast Cancer genes 1 and 2
CaEs-17	esophageal squamous carcinoma
CO_2	Carbon dioxide
DMEM	Dulbeco Minimum Essential Medium
DMSO	Dimethyle Sulfoxide
DNA	Deoxyribonucleic Acid
DU145	Prostate cancer cell line
ECM	Endothelial Cell Medium
EB	Ethediumbromid
ECACC	European Collection of Cell Cultures
EDTA	Ethylene Diamine Tetra Acidicacid
EGCG	Epigallocatechin-3-gallate
FBS	Foetal Bovine Serum
GST	Glutathione S-Transferase
H_2O_2	Hydrogen Peroxide 30%
HIFBS	Heat Inactivated Fetal Bovine Serum
HONE-1	Nasopharyngeal carcinoma cells
HPV	Human Papilloma Virus
HT29	Colorectal carcinoma cells
HUVEC	Human Umbilical Vein Endothelial Cell
ICPCG	International Consortium for Prostate Cancer Genetics

KB	Oral epidermoid carcinoma cells
$KBrO_3$	Potassium bromate 1M
LDL	Low-Density Lipoprotein
LPS	Lipopolysaccharides
MARDI	Malaysian Agricultural Research and Development Institute
MCF-7	Breast Cancer Cell Line
MOLT 4	human T lymphoblast; leukemia
Mouse 4T1	Mouse breast tumor model
MPC-1	Monoyte Chemoattractant Protein-1
MTT	3-(4,5 Dimethylthiozol-2-yl)-2,5-Diphenyletetrazolium
NF-KB	nuclear factor kappa-light-chain enhancer of activated B cells
P53	Tumor protein gene
PBS	Phosphate Buffer Saline
PC3	Human Prostate Cancer Cell Line
RPIM	Roswell Park Memorial Institute medium
PTEN/MMAC1	Phosphatase and tensin homolog /mutated in multiple advanced cancers
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SGC- 701	gastric carcinoma
TFC	Total Flavonoid Content
TNF	Tumor Necrosis Factor
tNOX	a tumor-selective target for cancer therapies
TPC	Total Phenolic Content

UDP-GT	Uridine Diphosphate Glucuronyl trans ferase
UV	Ultra Violet Rays
UVB	Ultra Violet B

Kesan sinergistik penggabungan ekstrak *Ficus deltoidea* dan *Gynochthodes sublanceolata* pada sel kultur normal, kanser payudara dan kanser prostat

ABSTRAK

Tumbuhan perubatan tradisional digunakan secara meluas untuk mencegah atau menyembuhkan penyakit-penyakit di kebanyakkan negara di seluruh dunia. Malaysia, yang merupakan salah satu daripada negara berkenaan, telah menyediakan pengetahuan termaju berkaitan dengan tumbuhan dan herba perubatan tradisional. Oleh itu, kajian ini telah dijalankan di Malaysia untuk menilai perbandingan diantara aktiviti antioksidan, jumlah kandungan fenolik dan flavonoid (TPC dan TFC) terhadap daun betina dan daun jantan *Ficus deltoidea* (Mas Cotek). Ia adalah salah satu daripada tumbuh-tumbuhan awam dan terkenal di Malaysia. Aktiviti antioksidan telah ditentukan dengan menggunakan aktiviti pemerangkap radikal bebas yang diukur oleh DPPH 1, 1-diphenil-2-picrilidrazil dan asai pemerangkap hidrogen peroksida (H_2O_2) yang dibandingkan dengan Vitamin C sebagai standard. Jumlah kandungan fenolik dan flavonoid kemudiannya dibandingkan untuk menentukan yang mana satu mempunyai kandungan yang lebih tinggi. Keputusan ini menunjukkan bahawa daun betina *Ficus deltoidea* mempunyai aktiviti antioksidan yang lebih tinggi dan kandungan TFC dan TPC yang lebih tinggi. Aktiviti antioksidan-antioksidan yang telah ditentukan dengan ujian yang sama seperti dinyatakan di atas telah kemudiannya dinilai dengan menggunakan *Gynocthodes sublanceolata* secara individu dan secara kombinasi dengan daun betina *Ficus deltoidea* dan *Gynocthodes sublanceolata* (50%:50%). 3-(4,5 Dimethilthiozol-2-yl) -2,5-Diphenilетetrazolium (asai MTT) sitotoksisiti dan kajian morfologi juga dijalankan bagi dua tumbuhan perubatan secara individu dan penggabungan. Ekstrak daun *Ficus deltoidea* telah menunjukkan aktiviti antioksidan yang lebih tinggi dalam asai pemerangkapan DPPH dan H_2O_2, TPC dan TFC berbanding dengan ekstrak daun Gynocthodes sublanceolata. Gabungan ekstrak-ekstrak ini adalah lebih tinggi berbanding dengan setiap individu di dalam kesemua ujian. Hasil keputusan tertinggi ditunjukkan untuk gabungan TPC dan TFC yang masing-masing adalah 170.12 µg /mg dan 51.19 µg /mg. Untuk asai (MTT), ujian ini telah menggunakan dua kultur sel kanser seperti payudara manusia MCF-7, adenokarsinoma PC3 prostatik dan sel kultur urat pusat endothelial manusia (HUVEC) sebagai sel kultur normal. Penggabungan itu menunjukkan kesan perencatan terbaik pada sel kultur kanser dan kesan pengabaian pada sel kultur normal berbanding dengan keputusan individu. Keputusan-keputusan menunjukkan bahawa penggabungan tumbuh-tumbuhan itu telah diuji dan menunjukkan aktiviti sitotoksisiti yang luarbiasa melebihi daripada 63% pada 200 µg / ml. Perencatan individu pada sel kultur kanser telah dipertingkatkan, manakala perencatan pada sel kultur normal masih kurang berbanding dengan sel kultur kanser. IC_{50} *Ficus deltoidea* untuk PC3 adalah 100 µg/mL dan IC_{50} *Gynocthodes sublanceolata* adalah 138 µg/mL, dan IC_{50} penggabungan adalah 83 µg/mL. Bagi MCF-7, IC_{50} *Ficus deltoidea* adalah 125 µg/mL, IC_{50} *Gynocthodes sublanceolata* adalah 159 µg/mL dan IC_{50} penggabungan adalah 95 µg/mL. Sel kultur HUVEC menunjukkan IC_{50} 180 µg/mL untuk *Ficus deltoidea* dan IC_{50} 168 µg / mL untuk Gynocthodes sublanceolata, tetapi untuk penggabungan tersebut adalah IC_{50} 196µg/mL. Di samping itu, ekstrak tumbuh-tumbuhan itu menunjukkan kesan anti-proliferatif yang ketara ke atas sel kultur kanser payudara manusia (MCF-7) dan prostat (PC3) secara individu, tetapi menunjukkan sitotoksisiti yang sederhana kepada sel

kultur normal yang diuji. Walaubagaimanapun, untuk penggabungan herb terhadap sel kultur normal adalah melindungi, manakala pertumbuhan sel kultur kanser adalah terbantut. Dua ekstrak secara individu menunjukkan kesan perubahan morfologi terhadap sel-sel. Sebaliknya, penggabungan itu menunjukkan lebih kesan perubahan morfologi terhadap sel-sel kanser.

Synergistic effect of the combination of *Ficus deltoidea* and *Gynochthodes sublanceolata* extracts on normal, Breast and Prostate Cancer cell lines

ABSTRACT

Traditional medicinal plants are widely used to prevent or cure diseases in many countries all over the world. Malaysia, which is one of the countries, has provided advanced knowledge of traditional medicinal plants and herbs. Therefore, the present study was conducted in Malaysia to evaluate a comparison of antioxidant activity, the total phenolic and flavonoid content of the female and male leaves of *Ficus deltoidea* (Mas Cotek.). It is one of the public and famous plants in Malaysia. The antioxidant activity was determined using free radical scavenging activity measured by DPPH 1, 1-diphenyl-2-picrylydrazyl and H_2O_2 hydrogen peroxide scavenging assay compared to Vitamin C as a standard. The total phenolic and flavonoid content (TPC and TFC) were then compared to determine which one has higher amount. The results of this showed that female leaves of *F.deltoidea* have higher antioxidant activity and higher TFC and TPC content. The antioxidants activity determined by the same test mentioned above was then evaluated using *Gynocthodes sublanceolata* individually and in combination with female leaves of *F.deltoidea* with *G. sublanceolata* (50%:50%). Cytotoxicity 3-(4,5 Dimethylthiozol-2-yl)-2,5-Diphenyletetrazolium (MTT assay) and morphological study of two medicinal plants individually and in combination. *F. deltoidea* leaves extract were indicated to have a higher antioxidant activity in DPPH and H_2O_2 scavenging assay TPC and TFC than *G. sublanceolata* leaves extract. The combination for these extracts is higher than from each one individually with all tests. The highest result indicated the combination for TPC and TFC at 170.12 µg/mg and 51.19µg/mg, respectively. For (MTT) assay, this test used two cancer cell lines such as Human Breast MCF-7, Prostatic PC3 adenocarcinoma and Human Umbilical vein endothelial cell line (HUVEC) as normal cell lines. The combination revealed the best inhibition on cancer cell lines and ignorable effect on normal cell lines compared to those work individually. The results indicated that the combination of plants was tested and exhibited a remarkable cytotoxic activity more than 63% at 200 µg /ml. Individual inhibition on cancer cell lines was enhanced, whereas inhibition on normal cell lines was still less than that on cancer cell lines. PC3 for *F.deltoidea* IC_{50} 100 µg/mL, and *G. sublanceolata* was IC_{50} 138 µg/mL, and combination was IC_{50} 83 µg/mL. On MCF-7, *F.deltoidea* IC_{50} 125 µg/mL *G. sublanceolata* was IC_{50} 159 µg/mL, and combination was IC_{50} 95 µg/mL. HUVEC cell line showed IC_{50} 180 µg/mL for *F. deltoidea* and that for *G. sublanceolata* IC_{50}168 µg/mL, but that for combination was IC_{50}196 µg/mL. In addition, the plants extract showed a significant anti-proliferative effect MCF-7 and PC3 cancer cells individually, but indicated mild-cytotoxic to the tested HUVEC normal cells. However, for the combination of the herbs on normal cell lines have preserved while the cancer cell lines growth was inhibited. Two extract individually showed morphological changes on the cells. On the other hand, the combination showed more morphological changes on cancer cells.

CHAPTER 1

INTRODUCTION

1.1 Overview

The use of plants in medical remedies has been a part of the history of medicine for a long time. Indeed, the ancient writings in Egypt and China highlight the importance and the usage of plants in medicine. The term herbal medicine is defined as medicine including herbal material and herbs, and the production of herbal medicine and commercial herbal products refer to the use of materials of plants for methodologies on research and evaluation of traditional medicine (Zhang & World Health Organization (WHO), 2000).

In recent years, a number of people suffer from serious common health problems that are associated with a wide range of effects at both molecular and cellular levels. It is the fact that research statistics showed 20% of all the death in the world, resulting from cancer, which affects more than one third of the world population (Center, Siegel, & Jemal, 2011). As known, cancer belongs to a group of malignant diseases characterized by uncontrolled proliferation of abnormal cells due to the unbalance between cell division and cell differentiation, leading to a progressive increase in the number of dividing cells that mass together to form a tumor, or proliferate throughout the body, initiating abnormal growth at other sites by invading other tissues through blood and/or the lymph system. Undoubtedly, prostate and breast cancer is more common types of cancer which is one of the leading causes of death and increases human's illness in the world (Cancer Treatment Centers of America, 2015).

Therefore, it is necessary to emphasize the important role of natural treatment due to the chemotherapy caused many side effects. For this reason, the novel anticancer agents are needed because many cancer patients developed resistance to standard anticancer agents during the treatment. It was found that toxic is the main cause of undesired side effect and hypersensitive reactions. It is estimated that less than one percent of 250000 species of higher plants, which exist on the earth, have bioactivity property. Therefore, (Ali Omer, 2013) asserted that it is possible to determine new compounds that have potential bioactivity.

Ficus deltoidea is called Mas Cotek, which can be found in Moraceae family multiple cancer preventive, anti- inflammatory and cancer therapeutic effects can be found from several species of Ficus. Thus, in recent years, these species known as herb have been studied by a number of researchers of traditional medicine and phytotherapy. They have therapeutic property used for regulating the circulation of blood. More especially, in Malaysia, their leaves are traditionally used as medicine for females after their giving birth period rather than any other traditional herb (Ali Omer, 2013).

Pitang leaves are known as *Gynocthodes sublanceolata* hail from the Rubiaceae family; these leaves are a rare plant. This plant has many active compounds such as xanthophyll, chlorophyll, flavonol, flavones, carotene and anthocyanin (Mamat & Arif, 2011). Although very few investigations on the anticancer properties of *G. sublanceolata* extract were conducted, other potential properties of the plant can be found based on the presence of phytochemicals.

It is believed that two-thirds of the world's plant species have medicinal properties, and almost all of them have excellent antioxidant potentials. The interest in the exogenous plant antioxidants was first excited by the discovery and subsequent

isolation of ascorbic acid from plants. Since then, the antioxidant potential of plants has received a great deal of attention. In fact, increased oxidative stress is a main cause of diseases including cancer, neurodegenerative and cardiovascular diseases. In addition, the supplement with exogenous antioxidants or boosting of endogenous antioxidant defense of the body is a promising method of countering the undesirable effects of oxidative stress (Kasote, Katyare, Hegde & Bae, 2015).

According to a report by Pandey and Rizvi (2009) the consumption of fruit, vegetables, spices, beverages, cereal all contains a high amount of flavonoids (flavonols and flavones),which is integral to the improvement of health due to their effectiveness in different diseases. These potential properties could prevent many degenerative diseases including cancer. study has been conducted on the subject of polyphenol intake with different populations, and their results indicated an inverse association between the risk of cancer and high intake of polyphenol (Xie, Huang, & Su, 2016).

Today, bioactivity of plant extracts is found based on cell based assay which is known as an alternative method. This method is used to test animals in toxicology laboratories due to the simplicity of homogenous methods and correlation with in vitro cytotoxicity data. The effect of plant extract on cell viability or cytotoxicity in vitro is investigated, and cell morphology is observed due to the technology of cell culture (Freshney, 2005). As a standard colorimetric method MTT assay is used to determine the biochemical activity of the extracts when the live and dead cells are distinguished, and when critical biochemical function is disrupted (Van Meerloo, Kaspers & Cloos, 2011).

Anti-cancer synergy can help scientists due to the presence of phytochemicals and polyphenols in plants. Both of them can interact with one another to create a higher anti-cancer activity than that works individually (Działo et al., 2016).

The combination of two or more phenolic compounds has shown the signs of synergistic anticancer activities. This is true for plant extracts which are rich in polyphenol. In traditional medication, most of these herbal products are usually consumed a combination of herbs instead of individually.

1.2 Problem Statement and Motivation

To provide a proper anticancer treatment for the patient at a minimal cost and lower complexity procedure, the treatment procedures with safe, efficient, and less side effects but without pain for the patient are needed. Today, the initial anticancer treatment is chemotherapy whose procedure fails to kill all infected cells but damages normal cells. In fact, this procedure is very painful and sometimes dangerous for the patient due to the immunity degradation; for example, fall of hair follicles, bone pain and negative psychology can occur. When chemotherapy procedure fails, doctors resort to the surgery procedure. Although this method is accurate, it is very painful and dangerous for the patient. Therefore, the usage of natural source of antioxidant is significant to the reduction of chemotherapy side effect and maintenance of a normal cell from damage. In this research project, synergy between phytochemicals which is present in plants can interact with one another to create a higher anti-cancer activity than that works individually. The cytotoxicity effect of combination from *F.deltoidea* and *G. sublanceolata* towards cancer and normal cells is, therefore, explored and investigated. Polyphenols, compounds accumulated in plants have antimicrobial, anti-inflammatory, antiviral, immune modulatory properties and anticancer, all of which are advantageous to human health (Fantini et al., 2015).Therefore, the present study aims to compare the antioxidants, total phenols and flavonoids in *Ficus deltoidea* (male and female). The highest one was then selected to be combined with *G. sublanceolata* extract. Next, all tests mentioned above were carried out on each extract individually and in combination. After that, cytotoxicity test on cancer and normal cell lines for leaves extract of these plants collaboratively and individually was carried out to determine their anti-proliferative properties and morphological changes in these conditions.

1.3 Research Objectives

This research has the following specific objectives:

i. To investigate the antioxidant activity of *F. deltoidea, G. sublanceolata* individually and in combination extracts between of them.

ii. To determine total phenolic and flavonoid content for two plant extracts individually and in combination.

iii. To evaluate cytotoxicity and morphological effects of plant extracts individually and in combination *(F. deltoidea* and *G. sublanceolata*) on the human Breast and prostate cancer cell lines.

iv. To evaluate protective effects of plant extracts individually and in combination *(F. deltoidea* and *G. sublanceolata*) on the Human Umbilical vein endothelial normal cell line.

1.4 Research Scope

This study is limited to identifying the antioxidant activity of extracts individually and in combination between *F.deltoidea* female leaves and *G. sublanceolata* leaves. Also, to determine the total flavonoid and phenolic content. Then the cytotoxic effects and morphological changes on cancer cells (Breast and Prostate carcinoma) and protective effects on normal cells are studied. Furthermore, the synergistic effect of extract combination is investigated. It provides sufficient evidence on whether the extract is cytotoxic against cancer cells and also cytoprotective against normal cells.

1.5 Contribution of the research

The main and significant contributions of this research are:

1. Anticipate that the combination of *Ficus deltoidea* and *Gynochthodes sublanceolata* leaves extracts produces higher antioxidant activities.
2. Combinations between two plant extracts illustrate higher TPC and TFC.
3. Combination between two plant extracts show higher inhibition on PC3 and MCF-7cancer cell lines.
4. Combination between two plant extracts show lees inhibition on HUVEC normal cell line.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Medicinal plants are used for the prevention and treatment of different types of human diseases. Today, over 50% of all drugs are used in clinical medicine made from natural products (plants or plant derivatives) (Kinghorn & Balandrin, 1993). A number of studies have been done in the field of natural products to examine whether it is biological or chemical and to find more effective agents used in therapies of diseases or novel drugs combinations with the stimulus of the plant products with the modern synthetic organic matter and medicinal chemistry (Baker et al., 1995).

2.2 Herbs in Medicine

In medical science, phytotherapy or herbal medicine refers to using plants in medicine for the treatment of diseases. According to World Health Organization, (2002) traditional medicine includes health practices, knowledge, approaches and beliefs incorporating plants, animals and mineral based medicines, manual techniques, spiritual therapies and exercises applied singularly or in combination to rest, diagnose and prevent illnesses or maintain well-being (World Health Organization, 2003). Therefore, it is significant to human's health if the researchers can find new medical plants for increasing antimicrobial activities. They need to acquire the resistance of infections agents with respect to chemotherapy and antibiotics. Today, over 120 drugs show a considerable significance to clinical uses that are introduced from traditional medicine all over the world (Abbott, 2014). According to Ekor (2014), in cases

pharmaceutical treatments are ineffective, a large number of patients tend to choose the treatment of their diseases by plants for many reasons as follows:

Firstly, the effects of this medicine are considerably acceptable compared to those are associated with pharmaceutical medicines because the chemistry of plants and human naturally occur. In this regard, the extracts of plants make it more compatible with human chemistry. Secondly, another slight difference between both types of medicine is how they work. For example, the pharmaceutical medicine works through isolating a single active ingredient. Meanwhile, for the plants medicine, the ingredients of the whole plant are used, which makes this type of medicine more effective due to the most synergistic action of each component of the plant.

One of the earlier examples of systematic pharmacy in the world is the isolation of Salicin from the bark of a Willow called Egyptian Salix Alba, which is used as a pain killer in combinations with quantified medical compound doses. As known, aspirin, one of the most popular drugs in the world today, is inspired by the functioning of Salicin and is made after the simplification of the salicylic acids (Jack, 1997). Moreover, other drugs are produced by the use of plants and their extracts known as the pain killer morphine from *Papaver sonniferum* and the anti-malaria quinine from *Cinchona officinalis* (Urban & Separovic, 2005). One of the most important sources in the formation of novel drug compounds is plants. The effect of the plants has two distinct parts (Abukakar, Ukwuani, & Shehu, 2008) based on which chemical structure of the new microbial is formed. They are also used for the treatment of diseases as phtyomedicine.