INDUCTION OF APOPTOSIS VIA JNK AND AKT/mTOR SIGNALING PATHWAYS BY Alternanthera sessilis (L.) R.Br. ex DC. IN COLORECTAL CANCER IN VITRO AND IN VIVO

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By

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

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements of the degree of Doctor of Philosophy

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Chairman : Associate Profesor Norhaizan Mohd. Esa, PhD
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Incidence rates vary 10-fold globally for colorectal cancer (CRC). Asia has lower rates than Western countries, but as the Western life-style becomes more prevalent in economically developing Asian countries like Malaysia, rates are increasing. Dietary phytochemicals have been drawing increasing attention for CRC prevention and treatment due to their chemical diversity, biological activity, easy availability, lack of toxic effects, and ability to modulate various signal transduction pathways and cell processes. *A. sessilis* is a well-known medicinal perennial herb in traditional medicine it is used for different therapeutic purposes, such as venereal diseases, eye diseases, cancer and gastrointestinal related ailments. Despite its beneficial uses, no studies on its chemo-preventive potential have been reported. Thus, this study was designed to focus on the elucidation of the putative anticancer potential of *A. sessilis* extracts *in-vitro* human colorectal cells (HT-29) and *in-vivo* Sprague-Dawley (SD) rats by elucidating the effect of the extract on intermediate biomarkers which can be used as effective predictors of CRC. The preliminary *in-vitro* work examined the effects of extracts from three different plant parts (whole plant, leaf and stem) via cytotoxicity assay (MTT, colonogenic, cell motility and AOPI assay). All three plant extracts exhibited dose- and time-dependent killing capabilities in HT-29 cell line.Whole plant and leaf extract showed IC_{50} above 150 µg/mL, followed by 500 µg/mL for stem extract.The killing activity of *A. sessilis* leaf extract was more specific toward HT-29 cells, as the leaf extracts had reduced effect (IC_{50} >200 µg/mL) on healthy murine cells, 3T3 with higher cytotoxic potency on HT-29 cells. The bioactive composition profiling of *A. sessilis* leaf extract by GC-MS revealed presence of hexadecanoic acid, carbonic acid, octadecadienoic acid, linoleic acids, neophytadiene, phytol, heptadecanone, octadecyne, dodecanetetrol, decanoic acid and oxirane which is an essential medicinal compound owing properties such as anticancer, anti-inflammatory and antioxidant. The mechanism involved in the cytotoxic effect of the extract was then evaluated in terms of apoptosis by caspase -3,

-8 and -9 colorimetric kit assay, mitochondrial membrane potential, cell cycle, Annexin V-FITC/PI staining, ROS and western blot analysis. The caspase assay data showed an increased level of all the tested caspase, MMP distruption was up to 70% in the highest concentration (200 µg/mL) when compared to control with only 2 %, cell cycle results indicate arrest in G_2/M phase arrest, generation ROS was increased dose dependently, Anexin V FITC indicate that *A. sessilis* leaf extract successfully induce apoptosis in HT-29 cells with increased total apoptotic cells (16 %) compared to control group with only 2.8% and western blot analysis exhibited elevation of administration pro-apoptotic protiens with reduced anti-apoptotic protein upon treatment with *A. sessilis* leaf extract. *In-vivo* evaluation of azoxymethane (AOM)-induced CRC in SD rats assessing *A. sessilis* leaf extract efficacy, clinical assessment, histopathological and molecular studies were performed for pre-clinical colon cancer diagnosis. The results indicated that *A. sessilis* leaf extract oral and offered no side effects such as weight loss, behavior changes or changes in kidney, and liver functions were observed. These results may indicate that active doses of *A. sessilis* leaf extract are not toxic. Additionally, *A. sessilis* leaf extracts significantly inhibited colorectal carcinogenesis induced by AOM in SD rats by the reduction in the number of Abberant crypt Foci (ACF). The mechanistic studies demonstrate *A. sessilis* leaf extract strongly inhibits AOM induced CRC in SD rats by activating JNK signaling pathway via Akt inhibition. On the basis of these findings, *A. sessilis* leaf extract could be used to the development of new and efficient strategies for treatment of human CRC.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

INDUKSI APOPTOSIS MELALUI MEKANISME JNK DAN AKT / MTOR OLEH *Alternanthera sessilis* DALAM KANSER KOLOREKTAL *IN VITRO* DAN *IN VIVO*

Oleh

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Kadar insiden kanser kolorektal di seluruh dunia berbeza 10 kali ganda. Walaupun Asia mempunyai kadar yang lebih rendah berbanding negara-negara Barat, namun disebabkan gaya hidup Barat yang semakin berleluasa di negara ekonomi membangun seperti Malaysia, kadarnya semakin meningkat. Diet fitokimia telah menarik banyak perhatian dalam pencegahan dan rawatan bagi kanser kolorektal kerana kepelbagaian bahan kimia, aktiviti biologi, mudah diperolehi, kurang kesan toksik, dan keupayaan untuk modulasi pelbagai mekanisme dan proses sel. *A. sessilis* adalah herba yang terkenal dalam perubatan tradisional, digunakan untuk tujuan terapeutik yang pelbagai, seperti penyakit kelamin, penyakit mata, kanser dan penyakit berkaitan gastrousus. Walaupun penggunaannya amat bermanfaat, tetapi tiada kajian saintifik tentang potensi pencegahan kemoterapi telah dilaporkan. Oleh itu, kajian ini memberi tumpuan kepada potensi *A. Sessilis* sebagai anti-kanser dalam model sel-sel kolorektal manusia (HT-29) dan tikus Sprague-Dawley (SD) untuk mengilustrasi kesan ekstrak pada biomarker yang boleh digunakan sebagai peramal berkesan kanser kolorektal. Analisis awal *in-vitro* mengkaji kesan ekstrak dari tiga bahagian *A. Sessilis* yang berbeza (keseluruhan pokok, daun dan batang) melalui ujian ketoksikan kepada sel (MTT, kolonogenik, motilitas sel dan ujian AOPI). Ketiga-tiga ekstrak mempamerkan keupayaan membunuh sel HT-29 secara bergantung dos dan masa dengan IC_{50} melebihi 150 µg / mL untuk ekstrak pokok keseluruhan dan pucuk setiapnya diikuti dengan 500 µg/mL untuk ekstrak batang. Kesan aktiviti kematian akibat daripada pendedahan kepada ekstrak daun *A. sessilis* adalah lebih spesifik terhadap sel-sel HT-29, kerana ekstrak daun tidak toksik kepada sel tikus yang sihat (IC_{50} >200 µg/mL), 3T3. Komposisi bioaktif daun *A. sessilis* melalui kaedah GC-MS mendedahkan kehadiran asid heksadekanoik, asid karbonik, asid *octadecadienoik*, asid linoleik, *neofytadiena*, *phytol*, *heptadecanone, oktadecyne, dodecanetetrol*, *asid decanoik* dan *oxirane* yang merupakan kompaun penting yang mempunyai sifat-sifat perubatan seperti anti-kanser, anti-radang dan anti-oksidan. Mekanisme yang terlibat dalam kesan sitotoksik ekstrak daun *A.*

sessilis kemudiannya dinilai dari segi apoptosis melalui kit ujian caspase -3, -8 dan -9, potensi membran mitokondria, kitaran sel, Annexin V-FITC / PI, ROS dan analisis *Western Blot.* Keputusan dari ujikaji caspase menunjukkan tahap peningkatan untuk semua caspase (3, 8 dan 9) yang diuji, MMP adalah sehingga 70% pada dos kepekatan tertinggi (200 µg / mL) berbanding dengan kumpulan kawalan dengan hanya 2%, keputusan kitaran sel menunjukkan penangkapan dalam penangkapan fasa G2/M, kadar ROS meningkat, keputusan anexin V-FITC menunjukkan bahawa ekstrak daun *A. sessilis* berjaya mencetus apoptosis dalam sel HT-29 dengan peningkatan jumlah sel apoptosis (16%) berbanding kumpulan kawalan dengan hanya 2.8%. Analisis dari *Western Blot* menunjukkan kadar pro-apoptosis yang tinggi berbanding dengan protein anti-apoptosis yang dikurangkan setelah rawatan dengan ekstrak daun *A. sessilis*. Keputusan ini menunjukkan bahawa ekstrak daun *A. sessilis* berjaya mengaktifkan apoptosis dalam sel HT-29. Dalam ujikaji *in-vivo* tikus SD yang diaruh CRC dengan *azoxymethane* (AOM), keberkesanan ekstrak daun *A. Sessilis* diukur melalui penilaian klinikal, kajian histopatologi dan molekul untuk diagnosis kanser kolon di peringkat pra-klinikal. Keputusan menunjukkan bahawa ekstrak daun *A. sessilis* melalui kaedah oral tidak mengakibatkan kesan toksik seperti tiada penurunan berat badan, perubahan tingkah laku atau perubahan pada fungsi buah pinggang dan hati yang dilihat. Keputusan ini menunjukkan bahawa dos aktif ekstrak daun *A. sessilis* mungkin tidak beracun. Selain itu, ekstrak daun *A. sessilis* dengan ketara menghalang penyebaran kanser kolorektal yang disebabkan oleh AOM dalam tikus SD dengan pengurangan bilangan *Abberant crypt Foci* (ACF). Kajian mekanistik menunjukkan ekstrak daun *A. sessilis* menghalang dengan berkesan perkembangan CRC dalam tikus SD yang diaruh AO dengan mengaktifkan JNK melalui perencatan Akt. Atas dasar penemuan ini, menunjukkan ekstrak daun *A. sessilis* boleh digunakan sebagai strategi baru untuk rawatan kanser kolorektal manusia.

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

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%	Percentage
˚C	Degree celcius
<	Less than
>	More than
×	Dilution/ Times
≥	More than and equal to
µm	Micrometer
µM	Micromolar
ACF	Abberant Crypt Focci
AJ	Adherence junctions
AOPI	Acridine Orange Propodium Iodide
AOM	Azoxymethane
APS	Ammonium Persulphate
CRC	Colorectal Cancer
CT	Computerized Tomography
DCF	Dihydrodichlorofluorescein Diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
Eg	Example
EGFR	Epidermal Growth Factor Receptor
FBS	Fetal Bovine Serum
FITC	Fluorescein

g	Gram
G1	Group 1
G2	Group 2
G3	Group 3
G4	Group 4
GC-MS	Gas Chromatography-Mass Spectrometry
GIT	Gastrointestinal Tract
h	Hour
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IBD	Inflammatory Bowel Disease
JNK	c-Jun N-Terminal Kinase
L	Liter
LCC	Left-Sided Colon Carcinoma
MAPK	Mitogen-Activated Protein Kinase
mg/L	Miligram per Liter
min	Minute
mL	Milliliter
mm	Millimeter
mm^3	Millimeter cube
MMP	Mitochondrial Membrane Potential
MMR	Mismatch Repair
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear Factor Kappa Beta
NIST	National Institute Standard and Technology
OS	Overall Survival

PBS	Phosphate Buffer Saline
PFS	Progression Free-Survival
PH	Pleckstrin Homology
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PS	Phosphatidylserine
RCC	Right-Sided Colon Carcinoma
ROS	Reactive Oxygen Species
RR	Resection Rate
RTKs	Receptor Tyrosine Kinases
SDS	Sodium Dodecyl Sulfate
A. sessilis	*Alternanthera sessilis*
SSA	Sessile Serrated Adenoma
TEMED	Tetramethylethylenediamine
TJ	Tight Junctions
TNF	Tumor Necrosis Factor
TSA	Traditional Serrated Adenoma
TTR	Time-To-Recurrence
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background

Among all form of cancer, colorectal cancer (CRC) is considered as one of the most avertible disease. Almost 2/3 of CRC may be averted by exercise and nutrition alone. Thus, CRC is an excellent option for chemoprevention combined chemotherapeutic, since CRC involves a long pre-cancerous stage, individuals owe an opportunity to impede, afore adenomas advance to cancer. Countless dietary agents have been assessed for their anticancer properties towards CRC. Unlike other cancer cells, CRC cells are very responsive to any agents as colonic environment are exposed directly to dietary alteration (Fung et al., 2013). A favorable diet strategy can avert CRC based on a high consumption of vegetables, fruits and fibers. Statistically, people that include a high level of natural products in their diets, have a lowers risk of cancer. For an example, Asian countries with high turmeric consumption are reported to have low rate of CRC incidence. This medicinal plant founded on culinary dietary system, remains to play an vital part of the health care, with almost 80% of the world's populations trusting largely on traditional medicines for their primary health care and safety (Desai et al., 2008).

Medicinal plants and their therapeutic values have been explored, since the beginning of human civilization. The phytochemicals in the plants are biologically active compounds used by the plants to ward off attacks from animals and environmental insults, give off fragrances, colors and indeed toxicity. These same molecules give plants their medicinal properties. A plant with medicinal properties is a plant which, in one or more of its organs, holds constituents that can be utilized for therapeutic purposes or serve as starting material in the development of drugs in modern medicines (Kooti et al., 2017). Phytochemicals in the field of anti-cancer research has made significant achievements, over 60% of the clinical use of anti-cancer drugs originate from medicinal plants. A remarkable amount of commercial drugs have been developed from natural sources in anticancer therapies. First accomplishment in curing human cancer was attained from Vinca alkaloid from Catharanthus roseus. Podophyllotoxin and few other compounds (known as lignans) isolated from the common mayapple (Podophyllum peltatum) and Paclitaxel (Pacific Yew tree), ultimately led to the development of effective drugs for lung, ovarian, breast and colon cancer (Song et al., 2015).

Historically, traditional medicine practiced taking the whole plant or extracts. The phytochemicals in the plant may work together concurrently, thus their uses can either augment or nullify their probable medicinal effects to give the plant's active compound its therapeutic effect (Shaukat et al., 2016). Furthermore, active compounds extracted from the medicinal plants present with better tolerance and safety when linked to other chemical entities. This will aid in reducing the usage of

the synthetic remedies (conventional treatment) when the disease is already present i.e., reduce the side effect of synthetic treatment. The synergetic effects of plant phytochemicals present higher effectiveness, differing from prevention to protection from gene-damaging effects, elevated anti-inflammatory and antioxidant properties (Soobrattee et al., 2006).

Clarification of plants effectiveness might lead to the establishment of an alternative and complementary medicine for cancer treatment. Withal, researches on ethnopharmacology have lately been accountable for the explosive improvement in drug discovery, separation and extract preparation techniques, physicochemical measurements and new concepts. Numerous natural compounds extracted from medicinal plants are being used as anti-cancer agents and are presently undergoing medical development. With the above background and considering the importance of phytochemicals for therapeutic point of view for effective management of CRC, the identification of unexplored medicinal plants with chemopreventive and anticancer potentials from the plethora of traditional and indigenous knowledge is necessary to validate the efficacy in experimental cancer models (Kim et al, 2015).

1.2 Problem Statement

CRC is a very complex disease to treat due to its ability to escape the immune system recognition and to immortalize and endure division infinitely. A large number of therapies are available to minimize the harmful side effects of drugs on the neighbouring cells and tissues, increasing drug delivery and targeting system. Though the five-year survival statistic for CRC has endured a fairly stagnant rate, the standard therapy still remains non-specific; conventional drugs that is effective only in certain patients, yet is accompanied with side effects (David and Gordon, 2016). In addition, the traditional mono-target therapy protocol that is used in the treatment of many cancers is becoming ineffective and may lead to the development of cancer acquired drug resistance. This resistance is mainly related to the complex signaling pathways involved in cancer and due to genetic redundancy. Nowadays, there is a growing trend in cancer studies to use combination therapy for treating solid tumors. Thus, treating cancer cells by employing mono-target chemical agent is not an effective method.

Given, the high frequencies of occurrence and recurrence of the CRC following treatment, new candidate compounds are necessary to improvise the success of CRC therapy. Phytochemicals from medicinal plant are expected to revolutionize CRC treatment. They have shown to exhibit chemoprevention and chemotherapeutic effects not only in cell lines but also in some clinical trials (Mishra et al., 2012). However, the main concern about the role of phytochemicals in health is insufficient data on the transport mechanisms for phytochemicals metabolism in human body to its target sites for exerting the health benefits. Thus, a complete and systematic exploration of phytochemicals should be accomplished considering its bioavailability, because single or clustered dietary phytochemicals are contributing

to both chemoprevention and chemotherapeutic action will be a significant assessment in the future.

1.3 Significance of the Study

With the scientific and technological development on chemical and pharmacological research, dietary phytochemicals are widely used as pharmaceuticals beneficial for human health. There are several theoretical advantages to the screening of phytochemicals for the discovery of anti-cancer medicines. The use of phytochemicals as templates for combinatorial chemistry enables the generation of libraries of phytochemicals' analogs, which might have enhanced drug-like properties. They provide chemical diversity with structural complexity and biological potency which will certainly lead to the discovery of novel chemical entities for CRC. Consequently, exploring new chemical entitles from phytochemicals will remain the predominant way to provide the leads and scaffolds for elaboration into efficacious anti-cancer medicines (Rafieian-Kopaie and Nasri, 2015).

The synergetic effects of the plant phytochemicals concoction are present with abundant level of interference throughout chemoprevention and chemotherapeutic regimens. These regimens aimed prior to the onset of cancer diagnosis and also apply to treat and preventing cancer recurrence, thus little to no toxicity can be tolerated. Unlike conventional cancer drug therapies which is difficult to engineer a chemically-derived drug which is non-toxic to normal cells and is specific to cytotoxicity of cancer cells, the clarification of effectiveness of plants phytochemicals could direct the establishment of an alternative and complementary technique against CRC (Yudraj et al., 2016).In line with that, it was an attempt to venture into natural based therapy yet an assessable source to study its regulatory potential towards the factors affecting the therapeutic process. Therefore, *Alternanthera sessilis* was chosen as the sample study.

A. sesillis may represent as an option for treating CRC offer an effective and viable alternative based on reported literature. It is commonly eaten as vegetable in many countries around the world. Besides, it has been used as a traditional remedy by folk healers for the past few centuries for the cure of many illnesses, such as headache, snake bites, febrifuge, and made into decoction to be taken orally to treat cough and gastrointestinal problem such as diarrhea. It has an excellent coolant effect that was commonly used for hair, eyes and skin health in general (Walter et al., 2014). The whole plant of *A. Sesillis* has numerous numbers of biologically active compounds (β-carotene, ricinoleic acid, myristic, palmitic, stearic, oleic and linoleic acids, uronic acid, flavonoids, triterpenoids, steroids and β-sitosterol, stigmasterol, campesterol and lupeol) which are of significant attention because of their bioactivities, with prevention and therapy of CRC. This naturally occurring compound are currently undergoing medical development and is used as anti-cancerous against tumorigenic effects *in-vitro* and *in-vivo* conditions by repressing the proliferation in cancer cells (Roy and Saraf, 2008; Lian-Wen et al., 2010).

Despite various study conducted on the pharmacological properties of *A. Sesillis*, the inhibitory effects and mechanism of anti-cancer activity of this plant is not been reported. For the previous conducted study on the toxicity studies, it involved the usage of leaf part of the plant and it was tested on animal model, thus parting a gap of research on the action mechanism of anti-cancer on different types of cancer cell lines and carcinogenesis factors associated with therapeutic parameters by targeting the signaling pathway. Moreover, edible plants based therapy is also believed to be minimally toxic (Chiang et al., 2017). Therefore, this examination possibly will bridge the therapeutic gap that is important for chemotherapeutic agent. With the information from previous studies, it is believed that *A. sessilis* is a potential candidate to be further studied for the assessment of anti-cancer activity in colon cancer.

1.4 Objectives

In accordance with the preceding approach, the present investigation is taken up with the following objectives:

1.4.1 General Objective

To evaluate the anti-cancer activity of *A. sessilis* extracts in colorectal cancer using *in-vitro* and *in-vivo* experimental models.

1.4.2 Specific Objectives

i. To identify and select the active extracts from *A. sessilis* plant parts through investigation of cytotoxicity assays against colon cancer cells, HT 29.
ii. To determine the cell death induces by *A. sessilis* through modulation of apoptotic pathway in colon cancer cells, HT 29.
iii. To examine the *in-vivo* apoptosis cell death induced by *A. sessilis* active extract through modulation of Akt/mTOR and JNK pathways in AOM induced colorectal cancer experimental model.

1.5 Hypothesis

1.5.1 Null Hypothesis

A. sessilis has no significant role to induce apoptotis in human colorectal cancer

1.5.2 Alternative Hypothesis

A. sessilis may play an imperative role and effective targets in developing colon cancer killing agents.

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