UNIVERSITI PUTRA MALAYSIA

ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTI-PROLIFERATIVE EFFECTS OF METHANOLIC LEAF EXTRACT OF Muntingia calabura L. ON COLON CANCER

NUR LIANA BINTI MD NASIR

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements 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 requirement for the Degree of Doctor of Philosophy

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Chairmain : Associate Professor Zainul Amiruddin bin Zakaria, PhD
Faculty : Medicine and Health Sciences

Worldwide known as '*Jamaican cherry*' and to the Malay as '*kerukup siam*', *Muntingia calabura* is not fully utilized in the Malay traditional medicine, despite the medicinal uses of various part of this plant by other tribes. The present study evaluated the cytotoxicity of methanol extract of *M. calabura* leaves (MEMC) towards human colorectal cancer cell line (HT-29) and cancer chemopreventive property of this extract. MEMC was partitioned into 3 partitions: petroleum ether extract (PEMC), ethyl acetate extract (EAMC), and aqueous extract (AQMC). All these extracts underwent antioxidant study using 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), superoxide dismutase scavenging activity assay (SOD), oxygen radical absorbance capacity assay (ORAC) and total phenolic content (TPC). MEMC were screened for cytotoxicity towards HT-29 and 3T3 cell lines using MTT assay. The anti-inflammatory study using lipoxygenase (LOX) and xanthine oxidase (XO) assays was also performed. For *in vivo*, assessment of toxicity of MEMC at 50, 250 and 500 mg/kg was done for 90 days of oral administration in male Sprague Dawley rats. The parameters for toxicity include changes of body weight, relative organ weight, haematological and biochemical profile, and gross histology of vital organs. In chemopreventive study, rats were randomly divided into 5 groups of 7 rats (two control groups and 3 treatment groups). All group received azoxymethane (AOM) injection (15 mg/kg) except for the group 1 (Normal control). Rats in treatment groups (group 3, 4 and 5) received 50, 250 and 500 mg/kg of MEMC, while rats in AOM control (group 2) only received AOM injection. Rats were euthanized after 8 weeks of treatment. Further evaluation on MEMC partition extracts was done using *in vitro* model. All the partitions (PEMC, EAMC and AQMC) were tested against HT-29 cell line and anti-inflammatory study. The induction of apoptosis was further confirmed by examining the morphological changes of cells under phase contrast field of fluorescent microscope and staining using acridine orange/propidium iodide. The extracts were screened on the bioactive

compounds that are responsible for pharmacological properties using high performance liquid chromatography (HPLC). From the antioxidant assays, MEMC and EAMC demonstrated strongest activity in SOD, DPPH and TPC assay. *In vitro* study of MEMC showed inhibition against HT-29 cells with IC_{50} of 90.8±5.8 µg/mL. Nevertheless, MEMC also showed strong anti-inflammatory action against LOX activity with inhibition of 87.65±4.21% and moderate inhibition against XO, 51.89±4.58%. In toxicity study, The No Observed Adverse Effect Level (NOAEL) for MEMC was greater than 500 mg/kg. For chemoprevention study, MEMC significantly reduced the number of aberrant crypt foci (ACF) ($p < 0.05$) ranging from 20.77%, 29.43% and 55.13% in rats fed with 50, 250 and 500 mg/kg of MEMC, compared to the AOM control group. For antioxidant assay, treatments with 500 mg/kg of MEMC significantly increase all antioxidants level with 2.526±0.0878 U/g tissue for SOD, 108.1±4.942 U/g tissue for CAT and 32.06±1.782 mM/g tissue for GSH as compared to the AOM control group. Among MEMC partitions, EAMC showed highest cytotoxicity effect towards HT-29 with IC_{50} of 58±12.9 µg/mL and HT-29 cells exhibit characteristics of apoptosis such as cell membrane blebbing and cell membrane rupture after stained with AO/PI. Phytochemical screening and HPLC analysis demonstrated the present of flavonoid-based compounds in MEMC and EAMC namely rutin and quercetin. In conclusion, MEMC exerted potential anti-colon cancer activity that partly attributed to its antioxidant and anti-inflammatory activity. EAMC was considered to have the best activity among partition extracts, which warrant further investigation such as *in vitro* study to confirm mode of cell death, *in vivo* chemoprevention study and isolation of bioactive compounds.

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

ANTIOKSIDAN, ANTI-RADANG DAN ANTI-PROLIFERATIF KESAN EKSTRAK METANOL DARIPADA DAUN *Muntingia calabura* L. KE ATAS KANSER KOLON

Oleh

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Mac 2017

Pengerusi : Profesor Madya Zainul Amiruddin bin Zakaria, PhD
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Dikenali di serata dunia sebagai "Jamaican cherry' dan di kalangan Melayu sebagai kerukup siam, *Muntingia calabura* tidak dimanfaatkan sepenuhnya dalam perubatan tradisional melayu walaupun nilai perubatannya digunakan oleh suku kaum yang lain. Kajian ini menilai kadar toksik ekstrak methanol daripada M. calabura (MEMC) terhadap sel kanser kolon manusia (HT-29) dan keupayaannya dalam mencegah kanser. MEMC dibahagikan kepada 3 bahagian: ekstrak petroleum eter (PEMC), ekstrak etil asetat (EAMC), ekstrak akueus (AQMC). Semua ekstrak melalui kajian antioksidan menggunakan cerakin 2,2- difenil-1-picrylhydrazyl radikal (DPPH), pengujian perangkap aktiviti superoxide dismutase (SOD), cerakin penyerapan oksigen radikal kapasiti (ORAC) dan kandungan jumlah fenol (TPC). Ketoksikan MEMC terhadap sel HT-29 dan 3T3 diperiksa menggunakan ujian MTT. Kajian anti-radang menggunakan analisa aktiviti lipoxygenase (LOX) dan xanthine oxidase (XO) turut dijalankan. Dalam kajian *in vivo,* penilaian kadar toksik MEMC pada 50, 250, 500 mg/kg dilakukan selama 90 hari secara pemakanan oral kepada tikus jantan Sprague Dawley. Parameter ketoksikan adalah termasuk perubahan kepada berat badan, berat relatif organ, profail hematologi dan biokimia serta histologi organ penting. Dalam kajian kemopencegahan, tikus dibahagikan secara rawak kepada 5 kumpulan yang terdiri daripada 7 ekor tikus setiap kumpulan. (2 kumpulan kawalan dan 3 kumpulan rawatan). Semua kumpulan subjek menerima suntikan (15mg/kg) azoxymethane (AOM) kecuali kumpulan I (kawalan normal). Tikus di dalam kumpulan rawatan (kumpulan 3, 4 dan 5) menerima 50, 250 dan 500 mg/kg MEMC, sementara itu, tikus dalam kumpulan kawalan AOM (kumpulan 2) hanya menerima suntikan AOM. Tikus dibunuh selepas 8 minggu rawatan. Penilaian lanjutan ke atas bahagian ekstrak MEMC dijalanakan menggunakan model *in vitro*. Kesemua bahagian (PEMC, EAMC dan AQMC) diuji ke atas cell HT-29 dan kajian anti-radang dilakukan. Kesan aruhan apoptotik telah dikaji dengan lebih lanjut melalui pemeriksaan perubahan morfologi sel dibawah fasa kontras mikroskop 'fluoresce'

dan pewarnaan oren acridine/propium iodida (AO/PI). Bioaktif kompenen dalam ekstrak tersebut yang bertanggung jawab dalam nilai farmakologi disaring menggunakan kromatografi cecair berprestasi tinggi (HPLC). Melalui ujian antioksidan, MEMC dan EAMC menunjukkan aktiviti yang kuat dalam memerangkap radikal melalui ujian SOD, DPPH dan TPC. Walau bagaimanapun, MEMC juga menunjukkan aktiviti anti-radang yang kuat terhadap aktiviti LOX dengan perencatan sebnyak 87.65±4.21% dan kesan perencatan serdehana terhadap XO, 51.89±4.58%. Dalam kajian toksisiti, 'No Observed Adverse Effect Level' (NOAEL) bagi MEMC lebih daripada 500 mg/kg. Melalui kajian kemopencegahan, MEMC menunjukkan penurunan bilangan ACF yang ketara ($p < 0.05$) dalam julat dari 20.77%, 29.43% dan 55.13% pada tikus diberi dengan 50, 250 dan 500 mg/kg MEMC berbanding dengan kumpulan AOM. Untuk ujian antioksidan, rawatan dengan ekstrak MEMC sebanyak 500 mg/kg meningkatkan kesemua Aktiviti antioksidan sebanyak 2.526±0.0878 U/g tisu untuk SOD, 108.1±4.942 U/g tisu untuk CAT dan 32.06±1.782 mM/g tisu untuk GSH berbanding dengan kumpulan AOM. Di antara bahagian MEMC, EAMC menunjukkan ciri-ciri apoptosis seperti 'membrane blebbing' dan 'cell membrane rupture' selepas diwarna dengan AO/PI. Saringan fitokimia dan analisis HPLC menunjukkan kewujudan flavonoid di dalam MEMC dan EAMC iaitu 'rutin' dan 'quercetin'. Kesimpulannya, MEMC berpotensi sebagai anti-kanser kolon yang sebahagiannya mungkin bergantung kepada antioksidan dan anti-radang aktiviti. EAMC dianggap mempunyai aktiviti yang terbaik di antara pecahan ekstrak yang memerlukan siasatan lanjut seperti ujian *in vitro* untuk memastikan jenis mod kematian sel, kemopencegahan dan pengasingan/penulenan sebatian bio-aktif.

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Thank you so much.

I certify that a Thesis Examination Committee has met on 29 March 2017 to conduct the final examination of Nur Liana binti Md Nasir on her thesis entitled "Antioxidant, Anti-Inflammatory and Anti-Proliferative Effects of Methanolic Leaf Extract of *Muntingia calabura* L. on Colon Cancer" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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The thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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ACF	Aberrant crypt foci
AO	Acridine orange
AOM	Azoxymethane
APC	Adenomatous polyposis coli
AQMC	Aqueous extract of *Muntingia calabura*
CAT	Catalase
COX	Cyclooxygenase
DCC	Deleted in colorectal carcinoma
DMEM	Dulbecco's modified Eagle's medium
DMH	1,2-dimethylhydrazine
DPPH	2,2-diphenyl-1-picrylhydrazyl
EAMC	Ethyl acetate extract of *Muntingia calabura*
FAP	Familial adenomatous polyposis
GSH	Glutathione
HNPCC	Hereditary nonpolyposis colon cancer
HPLC	High performance liquid chromatography
HT-29	Human Colonic Cancer
IC_{50}	Median inhibitory concentration
LOX	Lipoxygenase
MDA	Malondialdehyde
MEMC	Methanol extract of *Muntingia calabura*
MNCR	Malaysian National Cancer Registry
N-CAM	Neural cell adhesion molecule

NOAEL	No observed adverse effect level
ORAC	Oxygen radical absorbance capacity
PEMC	Petroleum ether extract of *Muntingia calabura*
PI	Propidium iodide
ROS	Reactive oxygen species
ROW	Relative organ weight
SOD	Superoxide dismutase
TNF	Tumor necrosis factor
TPC	Total phenolic content
WHO	World health organization
XO	Xanthine oxidase

CHAPTER 1

INTRODUCTION

1.1 Research background

Malaysia is one of the Asian countries, which hosts a huge diversity of plant species. There are approximately 12,500 species of flowering plants that can be found in this country (Napis *et al*., 2001). Since the beginning of human civilization, herbs derived from plants have been an integral part of society, valued for both culinary and medicinal properties. There has been tremendous drug discovery from natural sources since World War I, but only 10% of it has been studied for medical purpose (McChesny *et al*., 2007). Despite Malaysia being blessed as a 'jungle of pharmacy', only a few thousands of plants have been tested and identified for its medicinal values, leaving behind many species awaiting to be therapeutically explored (Ahmad *et al*., 2015). One of the plant species that has potential medicinal activities is *Muntingia calabura* (*M. calabura*).

Locally known as 'Kerukup Siam' and 'Buah ceri', *M. calabura* belongs to the Elaeocarpaceae family. This plant possesses sweet red fruits and its leaves are covered with sticky hair. This flowering plant is native to southern Mexico, Caribbean, Central America, and western South America (Sani *et al*., 2012). Peruvian traditional medicine practice belief *M. calabura* leaves can treat several types of illness including headache, gastric ulcer and swelling of prostate gland by boiling or steeping the leaves in water (Zakaria *et al*., 2007). Previously, *M. calabura* has been reported for its promising pharmacological properties such as antioxidant activity (Preethi *et al*., 2010; Karthyaini and Suresh 2012), hepatoprotective activity (Mahmood *et al*., 2014), antiulcer activity (Ibrahim *et al*., 2012; Balan *et al*., 2013), antiproliferative activity (Zakaria *et al*., 2011), cytotoxic activity (Kaneda *et al*., 1991; Chen *et al*., 2004; Sufian *et al*., 2013) and others. Despite of many scientific findings, its anti-cancer properties towards colon cancer have not yet been thoroughly explored.

Cancer is a condition of uncontrolled growth and spread of cells. It can affect almost any parts of the body. The growth often invades surrounding tissues and can metastasize to distant sites (World Health Organization, WHO). According to Ferlay *et al*. (2014), in the year 2012, there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people were living with cancer worldwide. Colon cancer is the third most common cancer in both men and women with 102,480 cases are being predicted to occur in the year 2013 (American Cancer Society, 2013). In Malaysia, colon cancer is the second most common cancer affecting both males and females. Colon carcinogenesis is a multistage process involving initiation, promotion and progression. There are a number of factors that have the link with colon carcinogenesis such as lifestyle, chronic inflammatory bowel disease, aging and genetic hereditary (National cancer society Malaysia, 2016). Colon cancer is a

challenging disease to treat, as the cancer is curable if diagnosed during its early stages (Winawer, 2003). The most common treatments for this cancer are surgery (colectomy), radiotherapy and chemotherapy (Wu *et al*., 2008). The available treatments aiming to destroy cancer cells commonly give side effects to the patients such as nausea, diarrhea, immunosuppression and fatigue (Wu *et al*., 2008; Malaysia Oncology Society, 2016).

The major target of this study is to seek the potential of *M. calabura* leaves extract(s) in preventing and inhibiting the development of colon cancer using *in vitro* and *in vivo* methods, and might add as another candidate to the list of plants with potential anti-cancer property.

1.2 Justification of study

Cancer is a complex disease that comprised of a different number of subtypes, and due to this heterogeneity, every person's cancer is unique in its composition. Colon cancer was ranked as second most common cancer affecting Malaysian and advanced stage of colon cancer is commonly incurable hence, early detection of cancer is really important as it usually results in less extensive treatments and better outcome. Despite the advance growth of modern medicine, there are some difficulties faced by the public such as the side effects of the treatment, viability of drugs and high cost for the modern treatments. The search for alternative medicine using natural sources is highly recommended nowadays with the fact that natural based remedy is less toxic and widely available. Therefore, this study was conducted using natural plant, which aims to discover the potential of its chemopreventive property using *M. calabura* leaves.

Numerous folks' medications based on local herbs have been widely practiced in Malaysian households. *M. calabura* is a common roadside tree in Southeast Asia region including Malaysia. Other tribes all over the world (i.e. Peru, Colombia, Mexico, Vietnam and Philippines) uses of various part of M. calabura to treat several illness, however this plant's medicinal value is not properly documented in the Malays folklore medicine. Earlier cytotoxic study by Zakaria *et al*. 2011 and Sufian *et al*. 2013, revealed the potential of methanol extract of *M. calabura* leaves to inhibit the proliferation of human colon cancer, *in vitro*. In addition, several flavonoids (i.e. (2 S)-5'-hydroxy-7,3',4'-trimethoxyflavanone and 4'-hydroxy-7-methoxyflavanone) and chalcones (i.e. 2',4'-dihydroxychalcone, and 2',4'-dihydroxy-3'-methoxychalcone) isolated from the leaves of *M. calabura* also showed inhibition against HT-29 cell (Mahmood *et al*., 2014b). Scientifically, this plant is known to have high antioxidant activities which are important to scavenge free radicals that appear to be fundamental for inflammatory response and may lead to cancer development. In addition, in term of anti-inflammatory activity of *M. calabura* numerous data have shown the ability of anti-inflammatory agents to demonstrate anticancer activity (Henley, 2002; Rayburn *et al*., 2009).

At the present time, research about *M. calabura* is still limited regarding its effects towards colon cancer. Since *M. calabura* can be found easily in this country and in order to optimize the usage of this plant, this research has been carried out to explore its pharmacological benefits and to establish some of the basic pharmacological properties of *M. calabura* leaves against colon cancer *in vitro* and *in vivo*.

1.3 Objectives:

1.3.1 General objective:

To investigate the effect of methanol extract of *Muntingia calabura* leaves and its partitions on antioxidant, anti-inflammatory and anti-proliferative activity against colon cancer using *in vitro* and *in vivo* model.

1.3.2 Specific objectives:

1) To determine the antioxidant properties of methanolic extract of *M. calabura* leaves (MEMC) and its partitions; petroleum ether, ethyl acetate and aqueous.
2) To determine the anti-inflammatory and cytotoxicity of MEMC toward human colorectal cancer (HT-29) and mouse embryonic fibroblast cell line (3T3).
3) To evaluate the subchronic toxicity of MEMC using male Sprague Dawley rats.
4) To assess the chemopreventive effects of MEMC against azoxymethane (AOM)-induced colon cancer and to examine the involvement of antioxidants against colon cancer *in vivo*.
5) To screen the anti-inflammatory, cytotoxicity and mode of cell death induced by the most effective partition of MEMC.
6) To study the bioactive compound(s) present in MEMC and the most effective partition using high performance liquid chromatography (HPLC) technique as part of anti-colon cancer pathway.

1.4 Research hypothesis

It is hypothesized that methanol extract of *M. calabura* leaves (MEMC) possess an anti-colon cancer activity *in vitro* and *in vivo*, and one or more of its partition(s) is/are expected to have promising anti-colon cancer effect.

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