UNIVERSITI PUTRA MALAYSIA

CHEMOPREVENTIVE POTENTIAL OF WATER AND METHANOLIC EXTRACTS OF Clinacanthus nutans (Burm.f) . LINDAU ON COLON CANCER In Vitro AND In Vivo

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IB 2015 30

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By

MARIAMU NYAKO KURA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

September 2015

DEDICATION

I dedicate this MSc thesis and give special thanks to my parents Vice Admiral Murtala H. Nyako and Hajiya Zainab M. Nyako. Their constant love, support and encouragement have been a blessing throughout my life.

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

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Chairman : Prof Maznah binti Ismail PhD
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Chemoprevention is a promising approach in controlling the occurrence of colorectal cancer (CRC). In this study the effects of extracts of *Clinacanthus nutans* (Cn) on colorectal cancer *in vitro* and *in vivo* was investigated. Cn Leaf and stem were extracted using sonication. Cytotoxicity testing of the extracts on the human colon adenocarcinoma cell line HT- 29 was determined. For the *in vivo* study, male Sprague Dawley rats were randomly assigned to six groups. Rats in treatment groups were administered 100mg/kg 1, 2- dimethylhydrazine (DMH) by gavage twice in the first week. The extracts were administered for eight weeks. After sacrifice, full blood count and histological analysis of the colons and livers of experimental animals was then carried out. The extracts were also analyzed qualitatively for the presence of certain phytochemicals.

IC_{50} values of 771.28±0.35µg/ml, 911.8±0.06µg/ml and 622.63±0.2µg/ml were observed for the water, hot water and methanolic extracts of the leaves of Cn after 72h treatment respectively. The number of aberrant crypt foci and crypt multiplicity significantly decreased in the colons of rats in the 500mg/kg water extract treated group. The 500mg/kg water extract also significantly decreased hepatic steatosis and significantly increased mRNA expression of SOD1antioxidant gene in the liver of rats. The extracts induced a certain amount of toxicity in blood of treated rats (anemia, decreased total White blood cell counts, decreased thrombocyte counts and decreased monocyte counts). Phytochemical screening of the extracts of also revealed the presence of saponins, tannins, alkaloids, flavonoids, carbohydrates, phenols and diterpenes. Though it induced toxicity in the blood of treated rats, the water extract of the leaf showed the most chemopreventive potential among the 12 extracts of *Clinacanthus nutans* studied therefore this merits the further study of this extract for clinical trials.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains.

POTENSI KEMOPREVENTIF DARIPADA EKSTRAK AIR DAN METANOL DARIPADA *Clinacanthus nutans (*Burm.f*).*LINDAU PADA KANSER KOLON *In Vitro* DAN *In Vivo*

Oleh

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Pencegahan kimo adalah satu pendekatan yang menjanjikan harapan dalam mengawal kejadian kanser kolorektal (CRC). Dalam kajian ini kesan ekstrak Clinacanthus nutans (Cn) ke atas kanser kolorektal in vitro dan in vivo telah dikaji. Daun dan batang Cn telah diekstrak menggunakan kaedah sonikasi. Ujian sitotoksik ekstrak ke atas sel kolon adenokarsinoma manusia HT- 29 telah ditentukan. Untuk kajian in vivo, tikus jantan Sprague Dawley dibahagikan kepada enam kumpulan secara rawak. Tikus dalam kumpulan rawatan telah diberikan 100mg / kg 1, 2- dimethylhydrazine (DMH) secara gavage sebanyak dua kali dalam minggu pertama. Ekstrak telah diberikan selama lapan minggu. Selepas tikus dibunuh, kiraan darah lengkap dan analisis histologi daripada kolon dan hati haiwan eksperimen telah dijalankan. Ekstrak juga dianalisis secara kualitatif bagi menentukan kehadiran fitokimia tertentu. Nilai IC_{50} untuk ekstrak air, ekstrak air panas dan ekstrak metanol daripada daun Cn selepas rawatan selama 72 jam masing-masing adalah 771.28 ± 0.35µg/ml, 911.8 ± 0.06µg/ml dan 622.63 ± 0.2µg/ml. Bilangan "abberant crypt foci" dan "crypt multiplicity" menurun dengan ketara dalam kolon tikus yang dirawat menggunakan 500mg/kg ekstrak air. 500mg/kg ekstrak air juga menurunkan steatosis hati dan meningkatkan ekspresi mRNA gen antioksidan SOD1 dalam hati dengan signifikan. Ekstrak Cn menyebabkan kesan toksik dalam darah tikus yang dirawat (anemia, pengurangan jumlah bilangan sel darah putih, pengurangan bilangan thrombocyte dan pengurangan bilangan monosit). Penyaringan fitokimia ke atas ekstrak juga mendedahkan kehadiran saponin, tanin, alkaloid, flavonoid, karbohidrat, fenol dan diterpenes. Walaupun menyebabkan kesan toksik dalam darah tikus yang dirawat, ekstrak air daun Cn menunjukkan kesan pencegahan kimo yang paling berpotensi antara 12 ekstrak Clinacanthus nutans yang dikaji, justeru memberi merit ke atas kajian lanjut ekstrak ini bagi ujian klinikal.

ACKNOWLEDGEMENTS

All praise is due to Allah the most gracious and merciful. I would like to use this opportunity to express my sincere gratitude to everyone who supported me throughout the course of my MSc. I would first like to thank my supervisor Professor Maznah binti Ismail for her guidance, constructive criticism and invaluable advice during the project. I would also like to thank my co- supervisor Prof Madya Norhaizan binti Mohd Esa for all the encouragement and guidance. I would like to thank Dr Mustapha Umar Imam who was always willing to help and give suggestions. Many thanks to my fellow students and the staff of the Laboratory of Molecular Biomedicine for all their help, encouragement and advice. Finally, I would like to thank my husband for all the encouragement and advice.

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

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

AC	Aberrant crypts
ACF	Aberrant crypt foci
AO	Acridine orange
AOM	Azoxymethane
Apc	Adenomatous polyposis coli
ATCC	American type culture collection
ANOVA	Analysis of variance
BA	Bile acids
BMI	Body mass index
CD	Crohn' s disease
Cn	*Clinacanthus nutans*
CRC	Colorectal cancer
CVD	Cardiovascular disease
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified eagle's medium
DMH	1, 2- dimethylhydrazine
DOC	Deoxycholic acid
DPPH	1, 1- diphenyl- 2- picrylhydrazyl
DSS	Dextrane sodium sulphate
EGCG	Epigallocatechin- 3- gallate
EPIC	European prospective investigation into cancer and nutrition
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FDA	Food and drug administration
5- Fu	5- Fluorouracil
FTIR	Fourier transform infrared spectroscopy
Gpx1	Glutathione peroxidase 1
GSH	Glutathione
HCA	Heterocyclic amines
HCL	Hydrochloric acid
Hmox1	Heme oxygenase (decycling) 1

HNPCC	Hereditary non- polyposis colon cancer
HSV	Herpes simplex virus
HFD	High fat diet
IACUC	Institutional Animal Care and Use Committee
IC_{50}	50% inhibitory concentration
IFD	Inflammatory bowel disease
IR	Infrared spectroscopy
Keap1	Kelch- like ECH- associated protein 1
LCA	Lithocholic acid
M1	250mg/ kg 80% methanol extract
M2	500mg/ kg 80% methanol extract
MAM	Formmethylazoxymethanol
MNNG	N- methyl- N'- nitro- N- nitrosoguanidine
MNU	Methylnitrosourea
MTT	3- (4, 5- dimethylthiazol- 2- yl)- 2, 5, diphenyl tetrazolium bromide assay
NC	Normal/ negative control
NCD	Non- communicable chronic disease
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2- related factor 2
NSAIDS	Non- steroidal anti- inflammatory drugs
Nqo1	NAD(P)H dehydrogenase (quinone) 1
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PS	Phosphatidyl serine
RONs	Reactive oxygen/ nitrogen species
ROS	Reactive oxygen species
RONS	Reactive nitrogen species
RT	Reverse transcription
SD	Standard deviation
Sod1	Superoxide dismutase 1
Sod2	Superoxide dismutase 2
TE	Tris- EDTA

UC	Ulcerative colitis
VDR	Vitamin D receptor
VZU	Varicella- zoster virus
W1	250mg/ kg water extract
W2	500mg/ kg water extract
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 General introduction

The Non- Communicable diseases (NCDs) comprising mainly of diabetes, obesitycardiovascular disease and the cancers are the leading cause of death globally. The World Health Organization (WHO) estimates that 36 million of the 57 million deaths that occurred in 2008 were due to the NCDs (Alwan et al., 2011). Four main behavioral risk factors have been recognized as the causes of NCDs, a combination of changes in lifestyle and dietary patterns, including the harmful use of alcohol, unhealthy diet, tobacco use and a sedentary lifestyle. The public health sectors in nations worldwide have been under continuous strain due to the increase in the morbidity and mortality brought on by these diseases. Available data demonstrates that almost 80% of deaths caused by NCDs occur in low- and middle-income countries. Malaysia is no exception to this trend. Poor nutritional choices and a sedentary lifestyle have also increased the morbidity and mortality of these diseases in Malaysia (Ministry of Health Malaysia., 2010; Alwan et al., 2011).

Colorectal cancer (CRC) is caused by modifiable and non- modifiable risk factors. Non- modifiable risk factors include genetic predisposition, age, male sex and previous colonic polyps. Modifiable risk factors are mostly associated with a western lifestyle consisting of a diet rich in processed and red meats, sugars, refined starches, saturated and trans- fatty acids; and poor in whole grains, fiber, fruits, vegetables, calcium, vitamin D and fish oils. A high body mass Index, visceral adiposity, alcohol consumption and physical inactivity among other risk factors are strongly implicated in the etiology of CRC. Malaysian government strategies to fight the increasing burden of this disease on the public health sector have not produced the desired impact so other alternative strategies need to be explored (Bhalla et al., 2009; MOH Malaysia., 2010).

Chemoprevention involves the use of phytochemicals, dietary bioactives, pharmacological or even whole plant extracts to block or arrest the cellular and molecular events that lead to neoplastic transformation (Neergheen et al, 2010). Preclinical testing is an important part of the anti- cancer drug development process. This includes both *in vitro* (the use of human cancer cell lines) and *in vivo* techniques (using animal models) (Cree, 2011).

Human cancer cells lines are important tools in cancer research as they are commercially available and exhibit unrestrained growth which makes them well suited for *in vitro* studies. They are also relevant models for the *in vivo* situation because they show epigenetic changes that are compatible with those found in primary carcinomas. The HT29 (human colon adenocarcinoma cell line) is one of those human cancer cell lines (Ahmad et al., 2013; Gillet et al., 2013). Chemopreventive studies involving animal models to test the efficacy of many compounds in inhibiting the initiation and progression of the disease with some

compounds showing good efficacy is a promising strategy in the fight against colorectal cancer (CRC) (Takayama et al, 2009).

Experimental models like the 1,2-dimethylhydrazine (DMH) model are histologically, morphologically and anatomically similar to human colonic epithelial neoplasms (Perse et al, 2011). DMH and its metabolite azoxymethane (AOM) induce colon cancer through a multistep process similar to that observed in human colon cancer. There are three stages to the carcinogenesis process: initiation, promotion and progression. The initiation stage, which this study was focused on, involves the formation of a mutated, preneoplastic cell from a genotoxic event (Femia and Caderni., 2008; Klaunig and Kamendulis., 2004).

Aberrant crypt foci (ACF) are preneoplastic lesions of the colonic mucosa. Large numbers of ACF are commonly observed in familial adenomatous polyposis and sporadic CRC patients. ACF are also one of the early morphological changes observed in DMH induced CRC in rodents. The DMH model is a good model to use when trying to understand the molecular alterations that arise in human CRC (Perse et al, 2011).

Equilibrium between cell proliferation and death needs to be maintained within tissues or the result will be carcinogenesis. The carcinogenesis process involves endogenous and exogenous factors influencing DNA damage, cell growth and cell death. An important role for reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the cancer process is supported by experimental evidence. An increase in ROS/ RNS production either through physiological modification or exposure to a chemical carcinogen contributes to the multistep carcinogenesis process. This could be through modification of gene expression or genotoxic effects resulting in oxidative DNA adducts being formed (Klaunig and Kamendulis., 2004). Under normal physiological conditions, cells have the ability to counterbalance the production of ROS/ RNS with antioxidants. Endogenous cellular antioxidant defenses are mainly enzymatic and include catalase, super oxide dismutase and glutathione peroxidase (Klaunig and Kamendulis., 2004).

For centuries plants and humans have sustained each other. In every culture there is definable plant knowledge about medicinal, ceremonial and edible plants. Modern conventional medicine is derived mostly from work done by the first botanists who were physicians that kept personnel herb gardens for treating the sick (Warber et al, 2006). Malaysia is blessed with untapped floral and faunal biodiversity. It also has a rich tapestry of traditional healing techniques and plants that are yet underutilized. Plant phytochemicals are receiving much attention due to their huge potential in reducing cancer risk (Neergheen et al., 2010; Yadav and Agarwala., 2011; Sengottuvelan et al., 2006; Russo et al., 2010). The public is disillusioned with conventional medication due to problems regarding side effects and have fueled an intense interest in herbal preparations.

Plants contain phytochemicals such as terpenoids, phenolic acids, tannins, flavonoids, alkaloids and other metabolites that are rich in antioxidant activity. Studies have shown many of these antioxidant phytochemicals possess anti-inflammatory, antitumor and anticarcinogenic effects. Reduced risk of cancers and

other chronic diseases has been associated with the ingestion of natural antioxidants (Aiyegoro and Okoh., 2010).

Clinacanthus nutans (Cn) is a small shrub found throughout south-east Asia. Its English name is Sabah snake grass and its Malay name is Belalai gajah. It is reported to have anti- inflammatory, immune modulatory, antimicrobial, hepatoprotective and antioxidant effects (Chelyn et al., 2014; Yong et al., 2013).

1.2 Statement of research problem

Colorectal cancer is the fourth most common cancer among both sexes in south-east Asia and the second most common cancer among both sexes in Malaysia. Mortality rates for the disease are unacceptably high. In Malaysia, the disease has a 5- year prevalence rate of 12.2% and 10.6% mortality for both sexes (WHO/IARC, 2012).
It is doubtful that incidence and mortality rates will change because current therapies are unsatisfactory, associated with severe side effects and unsuccessful remission rates. There is an urgent need in finding alternatives to current therapies and an even greater need for prevention. Chemoprevention is an alternative that is strongly being advocated. Chemoprevention as a strategy has been used successfully in reducing the incidence and mortality associated with cardiovascular disease. Defined as the use of natural or pharmacological agents to reverse or delay carcinogenesis at an early stage, chemoprevention is gaining popularity both among clinicians and the general public (Steward and Brown., 2013).

Phytochemicals from both dietary and non- dietary (medicinal plants or herbs) sources are currently been studied for their potential benefits against an array of cancers. The Federal Drug law enforcement Agency (FDA) in the US has several drugs under consideration as potential chemopreventive agents; some are presently undergoing clinical trials. Some positive results have been attained for non-steroidal anti- inflammatory drugs (NSAIDs) in colorectal adenoma and carcinoma prevention, vitamin C for bladder cancer and finasteride for prostate cancer (Russo, 2007).

Clinacanthus nutans is easily available in Malaysia and research on its anti-colorectal cancer effects have yet to be determined. Therefore due to the public health burden of colorectal cancer in Malaysia there is a strong need to find readily accessible and inexpensive medicinal plants that could possibly help in the fight against this disease.

1.3 Significance of study

The focus of this study was to investigate the effects of extracts of *Clinacanthus nutans* (Cn) on cytotoxicity of CRC cells in vitro and also to investigate the ability of the extracts to stop the initiation stage of DMH induced cancer *in vivo*. This study will endeavor to investigate the anti- colon cancer effects of the leaf and stem of Cn. Due to Cn been readily available and inexpensive; it could be beneficial in the fight against colorectal cancer in Malaysia.

1.4 General objective

To study the chemopreventive potential of different extracts of the stem and leaves of Cn.

1.4.1 Specific objectives

1. To determine the cytotoxic effect of extracts of *Clinacanthus nutans* leaves and stems on HT29 and NIH/3t3 cells.

2. To determine the ability of candidate extracts to inhibit the formation of aberrant crypt foci in a

   DMH induced CRC model in rats.

3. To determine the mechanistic basis for the anti-oxidative effect of extracts.

4. To determine phytochemicals present in the extracts of Cn.

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