BIOLOGICAL ACTIVITIES AND TOXICITY EFFECTS OF Clinacanthus nutans (Burm.f.) Lindau LEAF EXTRACTS

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BIOLOGICAL ACTIVITIES AND TOXICITY EFFECTS OF *Clinacanthus nutans* (Burm.f.) Lindau LEAF EXTRACTS

By

YU GUK TING

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Clinacanthus nutans (*C. nutans*) which also known as Sabah snake grass or Pokok Belalai gajah has long been used as traditional medicines in Thailand, China and Indonesia to treat various types of diseases. Recently, *C. nutans* has become a popular in Malaysia as a folk medicine to treat cancer and kidney failure. Side effects arise from the used of synthetic drugs for the treatments of chronic diseases have brought about the urge to search for alternative medicine derived from natural products. Thus, the objective of this study was to determine the biological activities of the *C. nutans* leaf extracts. Leaves of *C. nutans* were extracted sequentially using hexane followed by dichloromethane, ethyl acetate, acetone and lastly distilled water. Antioxidant activity test showed that dichloromethane extract demonstrated highest free radical scavenging ability (IC_{50} value 106.41 ± 1.32 µg/ml). For the FRAP assay, acetone extracts exhibited highest activity with 193.00 ± 2.63 mg TE/g extract. The total phenolics contents, the acetone extract exhibited the highest value, 66.67 ± 13.85 mg GAEs/mg extract. While the dicholoromethane extract gave the highest total flavonoid contents with the value of 851.82 ± 78.06 mg QEs/mg extract. The leaf extracts have shown no inhibition on the tyrosinase activity. Anti-inflammatory activity was determined using Griess assay on nitric oxide inhibitory activity upon IFN-γ/LPS stimulated RAW 264.7 cell line showed various extracts of *C. nutans* have high anti-inflammatory effects with the IC_{50} value < 20 µg/ml except distilled water extract. Further study of *in vitro* toxicity test using five cultured cancer cell lines and MTT assay showed *C. nutans* leaf extracts exhibited selective inhibition towards the cultured cancer cell lines. Dichloromethane extract exhibited highest anti-proliferative effects on murine melanoma (B16/F10) cell line with LC_{50} value 48.08 µg/ml, human breast cancer (MCF-7) cell line, LC_{50} value 97.46 µg/ml, human colorectal carcinoma (HT-29) cell line, LC_{50} value of 98.06 µg/ml, human hepatocellular carcinoma cell line (HepG2), LC_{50} value of 99.59 µg/ml but weakly inhibited human neuroblastoma (SY-SY5Y) cell line, LC_{50} value156.34 µg/ml but was not toxic towards human normal Chang liver cell line with LC_{50} value of > 250 µg/ml. For *in vivo* toxicity study, the various leaf extracts tested on zebrafish embryos showed the acetone extract exhibited highest toxicity towards the embryos with LC_{50} value of 88.33 ± 0.58 µg/ml followed by dichloromethane extract LC_{50} value of 141.73 ± 3.46 µg/ml. At the higher concentration (>125 µg/ml), the embryos and larvae exhibited teratogenic effect such as coiled body, bended tail and/or tail tip, pericardial and/or yolk sac oedemas. Collectively, the study showed that the *C. nutans* leaf extracts possessed high antioxidant and anti-inflammatory activities as well as anti-proliferative properties

against cultured cancer cell lines. This suggests the preventive and therapeutic potential of *C. nutans* leaf to be used as alternative medicines for various types of diseases including cancer in the future.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

AKTIVITI BIOLOGI DAN KESAN TOKSIKSITI EXTRAK DAUN *Clinacanthus nutans (Burm.f.)* Lindau

Oleh

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Clinacanthus nutans (*C. nutans*) yang juga dikenali sebagai 'Sabah snake grass' atau Pokok Belalai gajah telah lama digunakan sebagai ubat tradisional di Negara Thai China and Indonesia untuk mengubati pelbagai penyakit. Kebelakangan ini, *C. nutans* telah menjadi semakin popular di kalangan penduduk Malaysia sebagai ubat tradisional yang digunakan untuk mengubati penyakit kanser dan kerosakan ginjal. Kesan sampingan negatif yang timbul akibat dari penggunaan ubat-ubatan sintetik untuk mengubati penyakit kronik telah mendorong kepada usaha untuk mencari ubat-ubatan tradisional sebagai rawatan alternatif yang didapati dari tumbuhan semulajadi dan dikatakan tiada kesan sampingan. Tujuan penyelidikan ini dijalankan adalah untuk menentukan aktiviti biologi esktrak daun *C. nutans.* Daun tumbuhan ini diesktrak secara berturutan menggunakan pelarut seperti heksana, diikuti dengan diklorometha na, etil asetat, aseton dan air suling. Ujian antioksidan menunjukkan ekstrak diklorometha na mempunyai keupayaan memerangkap radikal bebas yang paling tinggi (IC_{50} 106.41 ± 1.32 µg/ml). Manakala, bagi ujian keupayaan menurun, ekstrak aseton pula menunjukkan keupayaan menurun yang tinggi dengan nilai 193.00 ± 2.63 mg TE/g ekstrak. Jumlah kandungan fenolik dan flavonoid dalam ekstrak menunjukkan ekstrak aseton mempunyai kandungan yang paling tinggi iaitu 66.67 ± 13.85 mg GAE/mg ekstrak. Manakala, ekstrak dikhloromethana menunjukkan kandungan flavonoid yang palang tinggi iaitu 851.82 ± 78.06 mg QEs/mg ekstrak. Tiada ekstrak daun *C. nutans* yang menunjukkan keupayaan merencat aktiviti enzim tyrosinase. Kesan anti radang menggunakan ujian Griess terhadap aktiviti perencatan nitrik oksida (NO) pada turunan sel RAW 264.7 yang diaktifkan oleh IFN-γ/LPS menunjukkan ekstrak daun *C. nutans* mempunyai kesan perencatan nitrik oksida yang tinggi dengan nilai IC_{50} < 20 µg/ml kecuali ekstrak air suling (IC_{50} > 500 µg/ml). Penyelidikan dilanjutkan dengan ujian toksisiti *in vitro* ke atas lima jenis turunan sel kanser berkultur menggunakan ujian MTT. Keputusan ujian ini menunjukkan ekstrak daun *C. nutans* menunjukkan kesan anti-proliferatif yang memilih. Ekstrak diklorometha na menunjukkan kesan anti-proliferatif yang tinggi terhadap turunan sel melanoma murin (B16/F10), nilai LC_{50} 48.08 µg/ml, turunan sel kanser payu dara manusia (MCF-7), nilai LC_{50} 97.46 µg/ml, turunan sel kanser kolorektal manusia (HT-29), nilai LC_{50} 98.06 µg/ml, turunan sel kanser hati (HepG2), nilai LC_{50} 99.59 µg/ml tetapi menunjukkan kesan perencatan yang lemah terhadap turunan sel neuroblastoma manusia (SY-SY5Y), nilai LC_{50} 156.34 µg/ml. Ujian toksisiti dilanjutkan dengan ujian

toksisiti *in vivo* yang menggunakan embrio zebrafish mendapati bahawa ekstrak aseton menunjukkan toksisiti yang paling tinggi dengan nilai LC_{50} 88.33 ± 0.58 µg/ml diikuti ekstrak diklorometha na, nilai LC_{50} 141.73 ± 3.46 µg/ml. Selain itu, pada kepekatan yang tinggi (>125 µg/ml), embrio dan larva zebrafish menunjukkan kesan teratogenik seperti bentuk badan melengkung, bengkok pada bahagian ekor dan/atau hujung ekor serta edema pericardial dan/atau pundi yolka. Secara keseluruhannya, penyelidikan ini menunjukkan pelbagai ekstrak daun *C. nutans* mempunyai aktiviti antioksidan dan anti radang yang tinggi serta sifat-sifat anti-proliferatif yang selektif terhadap turunan sel kanser berkultur. Ini mencadangkan bahawa daun *C. nutans* mempunyai potensi pencegahan dan terapi untuk digunakan sebagai ubat alternatif untuk pelbagai jenis penyakit termasuk kanser pada masa akan datang.

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Declaration by Members of Supervisory Committee

This is to confirm that;

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LIST OF ABBREVIATIONS

AAPH	2,2'-azobis-2-methyl-propanimidamide, dihydrochloride
AChE	Anti cholinesterase
ALP	Alkaline phosphatase
$AlCl_3$	Aluminium chloride
ALT	Alanine-aminotransferase
AST	Aspartate aminoteranferase
B16/F10	Murine melanoma cell
BW	Body weight
CAT	Catalase
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium
E2	Embryo medium
FBS	Fetal bovine serum
Fe^{3+}	Ferric (III) ion
Fe^{2+}	Ferric (II) ion
FRS	Free radical scavengers
GSH	Glutathione reductase
GPx	Glutathione peroxidise
HClO	Hypochlorous acid
HepG2	Human hepatocellular carcinoma cells
H_2O_2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible factor 1-alpha
HPLC	High performance liquid chromatography
HSV-1	Herpes simplex virus Type 1
HSV-2	Herpes simplex virus Type 2
HT-29	Human colorectal carcinoma cells
IC_{50}	Inhibitory concentration at 50%
IFN-γ	Interferon-gamma
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
LC_{50}	Lethal concentration at 50%
LDL	Low density lipoproteins
L-DOPA	L-3,4-dihydroxyphenylalanine
LD_{50}	Lethal dose at 50%
L-NAME	Nω-nitro-L-arginine methyl ester
MCF-7	Human breast cancer cells
$MgSO_4$	Magnesium sulphate
MPLC	Medium performance liquid chromatography
MPO	Myeloperoxidase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate

$NaHCO_3$	Sodium bicarbonate
NF-κB	Nuclear factor kappa-B
NO	Nitric oxide
O_2^-	Superoxide anion
OH^-	Hydroxyl ion
PBS	Phosphate Buffer Saline
RAW 264.7	Murine monocytic macrophage cells
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SH-SY5Y	Human neurobalstoma cells
SOD	Superoxide dismutase
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VZV	Varicella-zoster virus

LIST OF ANNOTATION

%	Percent
γ	Gamma
(v/v)	Volume per volume
(w/v)	Weight per volume
<	Lesser than
>	More than
±	Plus and/or minus
µ	Micro
Ml	Microlitre
µM	Micromolar
g	Gram
h	Hour
kg	Kilogram
L	Litre
mg/ml	Milligram per millilitre
µg/ml	Microgram per millilitre
U/ml	Unit per millilitre
min	Minute
mmol	Milimole
ºC	Degree Celsius
s	Second
α	Alpha
β	Beta

CHAPTER 1

INTRODUCTION

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) which were produced by various activities in the body including metabolism and inflammation are responsible for the development of variety of chronic diseases including cancer. Cancer has been the third most common cause of mortality in Malaysia and has always been the major health problem faced worldwide. Oxidative stress induced by the over production of free radicals have caused the deleterious damage effects of cellular lipid, proteins and DNA. These caused cell dysfunction, death or malignant transformations that involve genomics alteration and mutations which eventually promote carcinogenesis (Yong et al., 2013; Wang et al., 2011). Furthermore, the side effects that occurred during and after treatments of chronic diseases including cancer due to the use of the synthetics drugs has brought about the urge for the discovery of alternative medicine from the plant natural product in order to overcome the problems (Abdelwahab et al., 2011; Cooper, 2005). Antioxidants, the substances that play an important role in inhibiting the oxidation and thus preventing the damage of cells in the body (Chen et al., 2012; Tachakittirungrod et al., 2007) are found abundantly in plants especially fruits and vegetables. Natural antioxidants found in the plants are able to protect body from oxidative stress and associated diseases (Salganik, 2001; Halliwell, 1990).

Epidemiological studies have proven that antioxidant addition intake could retrieve cells from oxidative stress and prevent cancer growth and development. Several studies had revealed that medicinal plants contains more natural antioxidant sources such as phenolic acids, flavonoids and tannins possessed greater potential in antioxidant activities than common dietary plants (Li et al., 2008). Phytochemicals produced by the medicinal plant have been associated to many medicinal benefits to human health. They are safer, cheaper and readily available with fewer side effects compared to the synthetic drugs.

Clinacanthus nutans (Burm.f.) Lindau (*C. nutans*) which also known as Sabah snake grass (English) or Pokok Belalai Gajah (Bahasa Melayu) belongs to Acanthaceae family is a popular cultivated small shrub, native to tropical Asia. *C. nutans* is a well-known traditional medicine in Thailand for the treatment of various health problems such as skin rashes, snake bites (Thongharb et al., and Cherdchu et al., 1977), herpes infections (Jayavasu et al., 1992a), varicella-zoster virus (VZV) lesions (Wanikiat et al., 2008), anti-inflammatory activity (Satyavivad et al., 1996) anti-tumour angiogenesis (Duansak, 2007) and antioxidant activity (Pannangpetch, 2007). On the other hand, the whole plant of *C. nutans* has been used as anti-inflammatory agent for treating bruises, sprain and rheumatism in China. However, in Malaysia C. *nutans* which is a newly known herbs but becoming popular especially in the Chinese community due to its effectiveness in treating cancer (Yong et al., 2013) and kidney failure as a folk medicine.

Although in Thailand thorough studies have been done by the previous researchers on the beneficial effects of *C. nutans* plant, there are limited studies done in Malaysia due to its current popularity. However, previous studies done by Yong et al., (2013) and Sakdarat et al., (2009) reported that *C. nutans* has many chemical and biological activities has initiated the urge to establish more scientific information on its phytochemical benefits. Therefore, this research was carried out to evaluate the biological activities of the various extracts of *C. nutans* leaf.

Thus, the objectives of this study were:

1. to extract the leaf of *C. nutans* sequentially using five different solvents, hexane, dichloromethane, ethyl acetate, acetone and distilled water

2. to evaluate the biological activities of the crude extracts of the *C. nutans* leaf (antioxidant, anti-tyrosinase and anti-inflammatory activities)

3. to determine the toxicity effects of the crude extracts of *C. nutans* leaf on the cultured cancer cell lines using MTT assay (*in vitro*) and zebrafish embryos (*in vivo*)

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