IN VIVO TUMOUR INHIBITORY AND IMMUNOMODULATORY EFFECTS OF *CLINACANTHUS NUTANS* ETHANOLIC CRUDE EXTRACT

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ABSTRACT

Clinacanthus nutans (belalai gajah) has been used extensively by Asian community to treat various diseases, including cancers. Despite of that, studies on its potential toxicity effect and tumour inhibitory efficacy have not been fully validated and supported by scientific evidence. Therefore, the present study aims at determining the sub-acute toxicity effect and tumour inhibitory potential of *C. nutans* crude ethanolic extract. In sub-acute toxicity, mice were orally administered with *C. nutans* leaves extracts for 28 days and were monitored for any physical and histological changes. Meanwhile, tumour inhibitory potential was determined by administering TC-1 tumour-bearing mice with *C. nutans* extract. Tumour growth was monitored and the influence on immune response was also evaluated. From the results, it was shown that all treatment groups (50, 300, 2000 and 5000 mg/kg) displayed no significant changes in body weight, relative organ weight and feeding pattern when compared to control. However, histolological analysis revealed slight structural changes in liver and kidney of mice administered with *C. nutans* at concentrations 300 to 5000 mg/kg, thus suggesting the lowest observed adverse effect level (LOAEL) is 300 mg/kg. In addition, the present study demonstrated that mice administered with *C. nutans* at 50 mg/kg significantly delayed tumor growth compared to untreated group. This result was supported by a significant increase of apoptotic cells, as well as significant reduction of proliferating cell nuclear antigen (PCNA)-positive cells, compared to untreated mice. Despite no substantial changes in all hematological parameters, the level of pro-inflammatory cytokines; IFN-γ and IL-2, and anti-inflammatory cytokine, IL-10 in the treatment group was increased as compared to the control. In contrast, the level of chemokine MIP-2 was shown to decrease following treatment. The present findings also revealed the decreased level of $CD4^+$ T lymphocytes and increased level of $CD8^+$ T lymphocytes infiltrations following treatment as compared to control. In conclusion, results from this study highlight the therapeutic potential of *C. nutans* leaves extract in inhibiting tumour growth by mediating apoptosis, suppressing tumour cells proliferation and modulating immune response in tumour bearing mice.

ABSTRAK

Clinacanthus nutans (belalai gajah) telah digunakan secara meluas oleh masyarakat Asia untuk merawat pelbagai penyakit, termasuk kanser. Walau bagaimana pun, kajian berkenaan potensi ketoksikan dan keberkesanan perencatan tumor masih belum disokong sepenuhnya secara saintifik. Justeru, kajian ini bertujuan untuk menentukan kesan toksisiti sub-akut dan potensi perencatan tumor oleh ekstrak etanol kasar *C. nutans*. Untuk menguji ketoksikan sub-akut, mencit telah diberikan ekstrak daun *C. nutans* secara oral selama 28 hari kemudiannya dipantau untuk sebarang perubahan fizikal dan histologi. Sementara itu, potensi perencatan tumor telah diuji dengan memberikan ekstrak *C. nutans* kepada mencit yang mempunyai tumor TC-1. Pertumbuhan tumor kemudiannya dipantau dan kesan terhadap tindak balas imun juga dinilai. Hasil kajian menunjukkan bahawa kesemua kumpulan rawatan (50, 300, 2000 dan 5000 mg/kg) tidak mempamerkan perubahan ketara pada berat badan, berat organ relatif dan corak pemakanan apabila dibandingkan dengan kumpulan kawalan. Bagaimanapun, analisis histolologi menunjukkan sedikit perubahan pada struktur hati dan buah pinggang mencit yang diberikan *C. nutans* pada kepekatan 300 hingga 5000 mg/kg, mencadangkan tahap kesan buruk yang rendah (LOAEL) adalah 300 mg/kg. Di samping itu, kajian ini menunjukkan pertumbuhan tumor yang paling perlahan pada mencit yang diberikan ekstrak *C. nutans* pada kepekatan 50 mg/kg berbanding dengan kumpulan kawalan. Hasil ini disokong oleh peningkatan signifikan bilangan sel apoptotik serta pengurangan signifikan sel positif antigen nukleus sel terproliferasi (PCNA), berbanding dengan mencit yang tidak dirawat. Walaupun tiada perubahan besar dalam semua parameter hematologi, paras sitokin pro-keradangan; IFN-γ dan IL-2, dan sitokin anti-keradangan, IL-10 dalam kumpulan rawatan telah meningkat berbanding dengan kawalan. Sebaliknya, tahap kemokin MIP-2 telah berkurangan berikutan rawatan. Penemuan ini juga mendedahkan pengurangan limfosit $CD4^+$ T dan peningkatan limfosit $CD8^+$ T selepas rawatan berbanding dengan kawalan. Kesimpulannya, hasil kajian ini menunjukkan potensi terapeutik ekstrak daun *C. nutans* dalam menghalang pertumbuhan tumor dengan mengaruh apoptosis, menghalang proliferasi tumor dan memodulasi tindak balas imun pada mencit yang mempunyai tumor.

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LIST OF ABBREVIATIONS

ADI	-	Acceptable Daily Intake
APAF-1	-	Apoptotic Protease-Activating Factor-1
APC	-	Antigen Presenting Cells
Bcl-2	-	B Cell Lymphoma-2
Cyt c	-	Cytochrome C
CIN	-	Cervical Intraepithelial Neoplasia
CTL	-	Cytotoxic T Cells
CAM	-	Complementary and Alternative Medicine
DC	-	Dendritic Cells
DAB	-	Diaminobenzedine
DAPI	-	4′,6-Diamidino-2-Phenylindole
DISC	-	Death-Inducing Signaling Complex
ELISA	-	Enzyme-Linked Immunosorbent Assay
EDTA	-	Ethylenediaminetetraacetic Acid
FADD	-	Fas Associated Death Domain
H&E	-	Hematoxylin-Eosin
HGB	-	Hemoglobins
HRP	-	Horseradish Peroxidase
H_2SO_4	-	Sulphuric Acid
HCl	-	Hydrochloric Acid
HPV	-	Human Papilloma Virus
IFN	-	Interferon
IL	-	Interleukin
LYM	-	Lymphocytes
LD_{50}	-	Lethal dose, 50%
MHC	-	Major Histocompatibility Complexes
MIP	-	Macrophage Inflammatory Protein
MOM	-	Mitochondrial Outer Membrane
NOAEL	-	No Observed Adverse Level
NEUT	-	Neutrophils

NK	-	Natural Killer
NO	-	Nitrogen Oxide
OECD	-	Organisation for Economic Co-operation and Development
PBS	-	Phosphate Buffered Saline
PLT	-	Platelets
PAP	-	Papanicolaou
PCNA	-	Proliferating Cells Nuclear Antigen
RIPA	-	Radioimmunoprecipitation Assay
ROW	-	Relative Organ Weight
ROS	-	Reactive Oxygen Species
RBC	-	Red Blood Cell
SDS	-	Sodium Dodecyl Sulfate
STDs	-	Sexually Transmitted Diseases
TNF	-	Tumour Necrosis Factor
TBS	-	Tris Buffered Saline
TCR	-	T Cell Receptor
Th	-	Helper T Cells
Treg	-	Regulatory T Cells
TILs	-	Tumour-Infiltrating Lymphocytes
TUNEL	-	Terminal Deoxynucleotidyl Transferase-Mediated Dutp Nick-End Labeling
USM	-	Universiti Sains Malaysia
UKM	-	Universiti Kebangsaan Malaysia
UPM	-	Universiti Putra Malaysia
WBC	-	White Blood Cell

LIST OF SYMBOLS

g	-	gram
g	-	Gravitational Force
hr	-	Hour
M	-	Molar
mg	-	Miligram
min	-	Minutes
ml	-	Mililitre
mM	-	Milimolar
nm	-	Nanometre
s.c.	-	Subcutaneous
v	-	Volume
µg	-	Microgram
µl	-	Microlitre
µM	-	Micrometer
%	-	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Background Study

Cancer is a complex disease characterized from the abnormality of the cell growth (Nezafat *et al.*, 2015). In general, this incidence can be seen through the manifestation of mass formation following its elevated and deregulated proliferation activity that is beyond the ability of the normal cell (Evan and Vousden, 2001). These masses can be classified into two types, benign and malignant where the latter is known to be associated with cancer as it is made up of cancerous cells. It has the potential to metastasize to the other sites of the body, thus is deadly to the patient due to the interruption of normal body function.

Cancer prevalence has become a public health concern worldwide where there were approximately 18.1 million new cases and 9.5 million deaths reported globally in 2018 (Bray *et al.*, 2018). In Malaysia, cervical cancer was ranked as the second leading cancer among woman with an estimation of 2,145 new cases being diagnosed annually (Bruni *et al.*, 2017). This incidence is particularly associated with the infection of high-risk human papilloma virus (HPV) through the integration of its viral DNA in the host genome, thus causing mutation to the host cells (Hu *et al.*, 2015). However, not all HPV infection is lethal since most of them are able to resolve naturally (Chen *et al.*, 2014a). In minority cases, the persistent infection will only take place as the immune response fails to clear the virus effectively. Since there is no visible symptom that can be observed at the early stage, thus it is advisable for all women to undergo Pap smears screening as a precaution step (Othman *et al.*, 2016).

The past decades have seen the development of new treatment strategies in oncology field. These treatments includes the application of chemotherapy, radiotherapy and surgery (Sudhakar, 2010). In relation to this, cancer mortality rate

has been reported to decline steadily over the years (Hashim *et al.*, 2016). Despite of its positive achievement, the outcome of these approaches is still relatively poor. This is due to devastating effect caused by the treatments such as toxicity risk, infertility and bowel dysfunction (Alicikus *et al.*, 2011; Resnick *et al.*, 2013).

In Asian countries, complementary and alternative medicine (CAM) has been widely used among cancer patients (Thisoda *et al.*, 2013). One such practice is through the consumption of herbal based product. In the recent years, studies on the pharmacological activity of *Clinacanthus nutans* extract have shown intriguing results by suppressing the growth of various cancer cells *in vitro* (Yong *et al.*, 2013; Khiru Nasir and Mohd Bohari, 2015; Sulaiman *et al.*, 2015). As the therapeutic potentials of *C. nutans* have come into limelight, nowadays, people have begun to consume this plant in order to prevent cancer or as supplement for their health maintenance.

Therefore, an attempt to study the efficacy of *C. nutans* as a therapeutic agent and its adjuvant properties in enhancing immune system is conducted by referring on its potency through the existing literature. Besides, its possible toxicity risk with prolonged consumption was also elucidated through the sub-acute toxicity study. Results from this study eventually reflects on its therapeutic potential as well as validating its safety profile.

1.2 Problem Statement

The utilization of *C. nutans* has recently witnessed a growing interest among consumer. It is claimed to possess healing potential, thus has been used extensively as a traditional remedy for treating variety of diseases, including cancer (Alam *et al.*, 2016). Regarding to this, the commercialization of products derived from this plant has been intensified and marketed with health promoting properties (Zulkipli *et al.*, 2017). However, no standard measurement of *C. nutans* has been reported so far, making it a major issue of concern. Since *C. nutans* is commonly consumed over a long period of time, the consumers might be exposed to potential toxicity risk

following long term consumption. Therefore, the study of sub-acute toxicity of *C. nutans* extract is crucial as it may provide a safety profile of this plant through the evaluation of any adverse reactions associated with its repeated consumption.

Chemotherapy is one of the standard cancer care modalities focused primarily on curative purposes by getting rid of the cancerous cells (Arruebo *et al.*, 2011). The killing actions of chemotherapeutic drugs targeting on cancerous cells have been reported to cause DNA damage to healthy cells as well (Pearce *et al.*, 2017). This action somehow compromised with the patients' overall health and oftentimes leads to other complications such as chemobrain (Selamat *et al.*, 2014), nausea (Salihah *et al.*, 2016) and neutropenia (Lyman and Rolston, 2010). In addition, chemotherapy may also impair the host's immune system as it affects bone marrow function, making the patients susceptible to infection (Meir *et al.*, 2017). Therefore, the use of *C. nutans* as an alternative treatment is expected to be safer as it comes from natural sources. Its therapeutic properties may lessen the burden of illness suffered by the patients, thereby improving the quality of life and overall wellbeing of the patients.

Despite the long history of *C. nutans* as a traditional medicine for treating cancer, there is still limited reports documented on its safety and efficacy for anti-cancer treatment *in vivo*. Previous tumour inhibitory and oral toxicity studies conducted using this extract have only been tested on lower dosages of 3 mg/kg and 10 mg/kg and up to 250 mg/kg, respectively (Huang *et al.*, 2015; Nadia Asyura *et al.*, 2016). Therefore, this study is designed to explore its effectiveness in inhibiting tumour, particularly in TC-1 murine cervical cancer model as well as to test its safe dose level by testing on much higher dosages of up to 5000 mg/kg.

1.3 Objectives of Research

The objectives of this study are:

(a) To determine the sub-acute toxicity effect following oral administration of *C. nutans* crude extract in mice within 28 days consecutively.

(b) To evaluate the tumour inhibitory effect of *C. nutans* crude extract *in vivo* through the evaluation of tumour growth profile, histopathological and immunohistochemistry analysis.

(c) To examine the immunomodulatory activity of *C. nutans* crude extract *in vivo* by examining complete blood count, cytokines profiles and the infiltration of T lymphocytes in TC-1 tumour microenvironment.

1.4 Scope of Research

This research was conducted in three stages. The first stage involved a sub-acute toxicity study, in which *C. nutans* ethanolic extract was administered orally to the mice for 28 days consecutively and then followed by analyzing on the absolute body weight, relative organ weight and also histopathological analyses for organ tissue of the test mice. Next, the efficacy of the tumour inhibitory activity of *C. nutans* crude extract *in vivo* was evaluated by monitoring tumour growth profile, histopathological changes and the rates of apoptosis and cell proliferation marker of TC-1 tumour tissue sections. Whereas its effect to the immune system was elucidated by analyzing series of cytokines profiles, complete blood count and also the infiltration of T lymphocytes in murine cervical cancer model.

1.5 Significance of Research

Results from this study may provide evidence on the potential use of *C. nutans* as a supplement among cancer patients, particularly on the possible toxicity risk

associated with *C. nutans* administration, the tumour inhibitory activity as well as the effect of *C. nutans* to the immune system. Information gathered from this study might be useful for the advancement and efficient oncotherapy in the future.

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LIST OF PUBLICATIONS

Conference proceedings

1. **Roslan, N. A**., Kassim, N. I. I., Lim, V. and Jemon, K. (2017) 'Subacute Toxicity Study of *Clinacanthus Nutans* Ethanolic Extract *In Vivo'*, in *International Postgraduate Symposium in Biotechnology 2017*. Johor Bahru, Malaysia, pp. 3–6.

2. **Roslan, N. A**., Latif, N. A., Lim, V. and Jemon, K. (2018) 'Preliminary Study on *In Vivo* Anti-Cancer Activity of *Clinacanthus Nutans* on Murine Cervical Cancer Model', in *International Graduate Conference on Engineering, Science and Humanities (IGCESH)*. Johor Bahru, Malaysia