MODULATORY EFFECTS OF *Clidemia hirta* AGAINST CARBON TETRACHLORIDE (CCl_4) INDUCED FULMINANT HEPATIC FAILURE AND NECROSIS IN MICE

NURUL BINTI AMZAR

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2017

MODULATORY EFFECTS OF *Clidemia hirta* AGAINST CARBON TETRACHLORIDE (CCl_4) INDUCED FULMINANT HEPATIC FAILURE AND NECROSIS IN MICE

NURUL BINTI AMZAR

THESIS SUBMITTED IN THE FULLFILMENT FOR THE DEGREE OF MASTER OF SCIENCE

BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
2017

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN TESIS

JUDUL : _____
_____
_____

IJAZAH : _____
_____

SAYA : _____ **SESI PENGAJIAN** : _____
(HURUF BESAR)

Mengaku membenarkan tesis *(LPSM/Sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis adalah hak milik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

☐ SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)

☐ TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

☐ TIDAK TERHAD

Disahkan oleh:

_____ _____
(TANDATANGAN PENULIS) (TANDATANGAN PUSTAKAWAN)

Alamat Tetap: _____
_____
_____
_____

_____
(NAMA PENYELIA)

TARIKH: _____ TARIKH: _____

Catatan:
*Potong yang tidak berkenaan.
*Jika tesis ini SULIT dan TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.
*Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana Secara Penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM).

DECLARATION

I hereby declare that the material in this thesis is of my own effort except for the quotations, excerpts, equations, references and summaries which have been duly acknowledged and cited clearly it sources.

16th May, 2017

NURUL BINTI AMZAR

MZ1321006T

CERTIFICATION

NAME	:	**NURUL BINTI AMZAR**
MATRIC NO	:	**MZ1321006T**
TITLE	:	**MODULATORY EFFECTS OF *Clidemia hirta* AGAINST CARBON TETRACHLORIDE (CCl_4) INDUCED FULMINANT HEPATIC FAILURE AND NECROSIS IN MICE**
DEGREE	:	**MASTER OF SCIENCE (BIOTECHNOLOGY)**
VIVA DATE	:	**14th MARCH, 2017**

CERTIFIED BY:

SUPERVISORS

1. Associate Professor Dr. Mohammad Iqbal _____

2. Brig. Jen. Prof. Datuk Seri Panglima Dr. Kamaruzaman Hj. Ampon _____

ACKNOWLEDGEMENT

I would like to convey my deepest gratitude to Allah (Azza' Wa Jalla) for the strength along my journey in making my Master studies in Universiti Malaysia Sabah a possible.

Through it all, Assoc. Prof. Dr Mohammad Iqbal, my supervisor that always has been the source of strength in constant supervising, encouraging and guiding me. I am more grateful to him than he will ever know. I am also thankful to Puan Zarina Amin, Director of Biotechnology Research Institute (BRI) for giving me the opportunity to be part of this developing scientific community. I also would like to thank to Ministry of Higher Education, Malaysia for providing grant-in-aid No.: FRGS0411-SG-1/2015 throughout the research project and offering me the funding for my studies through MyBrain15 scholarship programme.

I believe that I am greatly indebted and fortunate to have worked with these outstanding individuals, Charles Gnanaraj and Dg Syahidah Nadiah. They have been there for tirelessly, providing me with ideas, endless guidance, encouragement when the times got rough and never fail to cheer me up during my ups and downs. I can't thank enough for all the staff that have been there throughout my requests and complain since day first especially Miss Salmi Afidah, Vidarita Maikin and Mr. Moni Mian.

Not forgetting Miss Marilyn Jane Yong from FPSK for the assistance and building comments in histopathology. Dr. Lee Ping Chin and Miss Pang Shing Yi from FSSA for the mice management and handling technique. Mr. Johnny Gisil from IBTP for the taxonomy identification of the plant sample.

My completion of this project could not have been accomplished without the support of my wonderful friends in BRI especially in Postgraduate Room 1 and FSSA that have been assisting me through the long struggles of thesis writing with their help and moral support direct and indirectly.

Finally, grace and love to my parents that never fail to keep me in the warmest hand and being my backbone in every step I have taken since the very first day with all those support emotionally and mentally.

Nurul Binti Amzar
16th May, 2017

ABSTRACT

Liver diseases still represent a major health burden worldwide. Moreover, medicinal plants have gained popularity in the treatment of several diseases including liver. *Clidemia hirta* possesses many medicinal properties in healing several diseases and for health care maintenance. However, hepatoprotective effect and antioxidative potential of *C. hirta* has not fully investigated. Thus, the present study was to evaluate the hepatoprotective and antioxidative potential of aqueous extract of *C. hirta* leaves against carbon tetrachloride (CCl_4)-induced liver injuries and oxidative damage in mice. Various biochemical changes associated with liver damage and oxidative stress were measured. Phytochemical screening showed the presence of saponin, flavonoid, steroid, tannins and cardiac glycosides of *C. hirta.* Total phenolic content was 610.24 mg/g GAE and flavonoid was 91.67 mg/g CAE. The DPPH free radical scavenging activity showed inhibition of 94.62% at 620 µg/ml and inhibition concentration (IC_{50}) was 45.48 µg/ml for *C. hirta.* For *in vivo* studies, the mice were pre-treated for 14 consecutive days with aqueous extract of *C. hirta* (150 mg/kg body weight, 300 mg/kg body weight and 600 mg/kg body weight) followed by two dosages of CCl_4 (1.0 ml/kg body weight) orally on day 14 and 15. All of these animals were sacrificed 24 hours after the last dose of CCl_4 or saline. Blood and liver tissues were taken quickly for biochemical and histopathological studies to assess the derangement in the functioning of liver. The development of the oxidative stress was observed through the escalation of hepatic lipid peroxidation, depletion of reduced glutathione and antioxidant enzymes (glutathione peroxidase, glutathione reductase, catalase, glutathione S-transferase and quinone reductase). Hepatic damage was evaluated by measuring serum transaminase (ALT and AST). In addition, CCl_4-mediated hepatic damage was further evaluated by histopathological examination. However, most of these changes were ameliorated by pretreatment of mice with *C. hirta* in a dose dependent manner. Biochemical improvements after *C. hirta* treatment were paralleled by histopathological findings. The results of the present study indicated that hepatoprotective effect of aqueous extract of *C. hirta* might be ascribable to its antioxidant and free radical scavenging properties.

ABSTRAK

KESAN PEMULIHAN *C. HIRTA* TERHADAP KEGAGALAN HEPATIK AKIBAT INDUKSI KARBON TETRAKLORIDA (CCl_4) *TERHADAP* TIKUS

Penyakit berkaitan hati masih menjadi antara masalah kesihatan utama di dunia. Di samping pelbagai masalah kesihatan yang kian diperkatakan, tumbuhan berasaskan perubatan kian meraih perhatian populasi dunia dalam rawatan pelbagai penyakit termasuk hati. *Clidemia hirta* mengandungi pelbagai ciri perubatan dalam penyembuhan beberapa penyakit dan bagi penyelenggaraan penjagaan kesihatan. Walau bagaimanapun, kesan perlindungan hepatik dan potensi antioksida *C. hirta* masih belum disiasat sepenuhnya. Oleh itu, kajian ini bertujuan untuk menilai kesan perlindungan hepatik dan potensi antioksidan *C. hirta* terhadap kerosakan hati dan tekanan oksidatif oleh karbon tetraklorida (CCl_4) pada tikus. Pelbagai perubahan biokimia berkenaan kerosakan hati dan tekanan oksidatif di ukur dan di nilai. Kehadiran saponin, flavanoid, steroid, tannins dan Kardiak gligosida diperhatikan dalam *C. hirta*. Kandungan jumlah fenolik di dapati sebanyak 610.24 mg/g GAE. Manakala bagi kandungan jumlah flavanoid pula ialah 91.67 mg/g CAE. Bagi aktiviti radikal bebas DPPH pula, rencatan setinggi 94.62% dilihat pada kepekatan 620 µg/ml. Kepekatan perencatan pada 50% (IC_{50}) bagi aktiviti radikal bebas DPPH *C. Hirta* sebanyak ialah 45.48 µg/ml. Bagi kajian *in vivo*, tikus telah di pra-rawat selama 14 hari berturut-turut dengan ekstrak akueus pada dos yang terpilih (150mg/kg berat badan, 300mg/kg berat badan dan 600mg/kg berat badan diikuti dengan induksi dua dos CCl_4 (1.0ml/kg bw) secara oral pada hari 14 dan 15. Model haiwan di eutanasi secara berperikemanusian selepas dos terakhir CCl_4. Pembentukan tekanan oksidatif diperhatikan melalui peningkatan pengoksidaan lipid hepatik, pengurangan glutation, enzim antioksidan (glutation peroksida, glutation reduktase, katalase, glutation s-transferase dan quinone reduktase). Kerosakan hepatik dinilai dengan mengukur transaminase serum (ALT dan AST). Di samping itu, kerosakan hepatik oleh induksi CCl_4 dinilai dengan pemeriksaan histopatologi. Walau bagaimanapun, sebahagian besar daripada perubahan ini telah diatasi dengan rawatan awal ekstrak akueus *C. hirta*. Pembaikpulihan pada tahap biokimia dapat dilihat seiring dengan penemuan histopatologi. Hasil kajian ini menunjukkan bahawa kesan perlindungan hepatik oleh ekstrak akueus *C. hirta* mungkin berkaitan rapat dengan aktiviti antioksidan dan penghapusan radikal bebas**.**

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOS

LIST OF ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
µg/ml	:	Microgram per milliliter
	:	Microliter
µm	:	Micrometer
µmol	:	Micromol
•CCl_3	:	Trichloromethyl free radical
•OH	:	Hydroxyl radical
2,4-DNPH	:	2,4-dinitrophenylhydrazine
Abs	:	Absorbance
	:	
ALT	:	Alanine aminotransferase
ANOVA	:	One-way analysis of variance
AST	:	Aspartate aminotransferase
ATP	:	Adenosine triphosphate
BSA	:	Bovine serum albumin
C. hirta	:	*Clidemia hirta*
Ca^{2+}	:	Calcium ion
CAE	:	Catechin equivalent
CAT	:	Catalase
CCl_4	:	Carbon tetrachloride
Cl^-	:	Chloride ion
cm	:	Centimeter
CNS	:	Central nervous system
CO_2	:	Carbon dioxide
$COCl_2$	:	Carbon dichloride oxide
COX	:	Cyclooxygenases
Cu^{2+}	:	Copper (II) ion
DCPIP	:	2,6-Dichloroindophenol
df	:	*Dilution factor*

DPPH	:	2,2-Diphenyl-2-picrylhydrazyl
DPX	:	Di-N-butyl phthalate in xylene
DTNB	:	bna
e^-	:	Electron
E.C.	:	Enzyme classification
EDTA	:	Ethylenediamine tetraacetic acid
FAD	:	Flavin adenine dinucleotide
FCR	:	Folin- Ciocalteu reagent
Fe^{2+}	:	Ferum (II) ion
Fe^{3+}	:	Ferum (III) ion
$FeCl_3$	:	Ferric chloride
FeO_2	:	Iron oxide
g	:	Gram
g/mol	:	Gram per mole
GAE	:	Gallic acid equivalent
GR	:	Glutathione reductase
GSH	:	Reduced gluthatione
GSH-PX	:	Glutathione peroxidase
GSSG	:	Glutathione oxidized
GSSH	:	Glutathione disulphide
GST	:	Glutathione S-transferase
H	:	Hydrogen
h	:	Hour
H & E	:	Haematoxylin and eosin
H_2O	:	Water
H_2O_2	:	Hydrogen peroxide
HIV	:	*Human immunodeficiency virus*
HOCl	:	Hypochlorite
IC_{50}	:	Inhibition concentration at 50%
IU/L	:	International units per liter
KCI	:	Potassium chloride
kg	:	Kilogram
LOX	:	Lipoxygenases

LPO	:	Lipid peroxidation
M	:	Molarity
MDA	:	Malondialdehyde
mg	:	Miligram
Mg/kg b.w	:	Miligram per kilogram body weight
Mg/ml	:	Miligram per mililiter
min	:	Minute
ml	:	Mililiter
mM	:	Milimolarity
N		Normality
Na_2CO_3	:	Sodium carbonate
NAC	:	N-Acetylcysteine
NaCI	:	Sodium chloride
$NADP^+$	:	B-Nicotinamide adenine dinucleotide phosphate
NADPH	:	B-Nicotinamide adenine dinucleotide phosphate reduced
NaH_2PO_4	:	Sodium dihydrogen phosphate
NaN_3	:	Sodium azide
3	:	
	:	
NFkB	:	Nuclear factor kappa light chain enhancer of activated B cell
nm	:	Nanometer
nmol	:	Nanomole
NO•	:	Nitroxyl radical
$O^{\bullet}{}_2^-$	:	Superoxide radical
O_2	:	Oxygen
O_3	:	Ozone
pH	:	Negative decimal logarithm of hydrogen ion activity in a solution
PMS	:	Postmitochondrial supernatant
PUFA	:	Polyunsaturated fatty acids
QR	:	Quinone reductase
RNA	:	Ribonucleic acid
RO•	:	Alkoxyl radical

ROO•	:	Peroxyl radical
ROS	:	Reactive oxygen species
RSA	:	Radical scavenging activity
s		Second
SGOT	:	Serum glutamic oxaloacetic transaminase
SGPT	:	Serum glutamate-pyruvate transaminase
SPSS	:	Statistical Package for the Social Sciences
SSA	:	Sulfosalicylic acid
TBA	:	Thiobarbituric acid
TBARS	:	TBA reactive substances
TCA	:	Trichloroacetic acid
TCM	:	Traditional chinese medicine
TFC	:	Total flavonoid content
TNF-α	:	Tumor necrosis factor-alpha
TPC	:	Total phenolic content
Tris-HCl		Tris (hydroxymethyl) aminomethane hydrochloric acid buffer
UMS		Universiti Malaysia Sabah
USA		United States of America
v/v	:	Volume over volume
w/v	:	Weight over volume
WHO	:	World health organization
α	:	Alpha
β		Beta
Δ Abs	:	Changes of absorbance
mM/L		Milimolarity per liter
BSL-3	:	Biosafety level 3
AP-1	:	Activator protein-1
cm^{-1}	:	Per centimetre
IL-1	:	Interleukin 1
M^{-1}	:	Per molarity
$•CCl_3O•_2$	:	Trichloromethyl peroxyl radical
$Na_2HPO_4.2H_2O$	:	Di-sodium hydrogen phosphate dehydrate

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of The Study

The usage of medicinal plants has been acknowledged to mankind since ages through numbers of historical discoveries. The Chinese and the Indians are amongst the earliest culture that develop their own medicinal system which are now widely known as Traditional Chinese Medicine (TCM) and Ayurvedic. Modern clinical studies have been used to investigate the claims of traditional practice; chemistry and chemical analysis were used for quality control of the TCM. Through chemical studies, connection has been distinguished between nature of herbal medicine and pharmacological activities, herbal tastes and chemical components. For example, odoriferous herbs contain essential oil; sour herbs contain acid and tannins; sweet herbs contain sugar, protein and amino acids; bitter herbs contain alkaloids and glycosides and salty herb contain inorganic salts (Ramzan, 2015).

The Malay culture also has their own healing tradition though it was not as well documented as the TCM and Ayurveda. Ahmad & Holdsworth (1994) in their compilation of the economic products of the Malay peninsula, documented that not less than 1,300 plants have been used in traditional medicine but larger number still remained undocumented especially those were used amongst native people that passed on verbally from one generation to another.

The knowledge of medicinal plants is normally passed on from generation to generation but this practice seems to be vanishing. The practice usually known to few elderly (Kulip, 2003).

More than 100 species of plants that are being used specifically by the native people throughout Sabah were medicinal plants. This includes wild fruit, handicraft materials, plants for social and religious purposes, and poisons (Kulip *et al.*, 2010).

To ensure these highly valuable knowledges will not be lost in time, National Biotechnology Policy (NBP) (accessed April,1, 2017) spells out nine key thrusts which includes Healthcare Biotechnology Development fall under second initiative which to capitalize on the strengths of biodiversity to commercialize discoveries in natural products as well as position Malaysia in the bio-generics market. Malaysia launched NBP in 2005, under the Ministry of Science, Technology and Innovation (MOSTI) to further develop healthcare economic sector as well as to support the growth of an enabling eco-system throughout the scientific, academic and business communities in the country. NBP spells out nine key thrusts that underpin these aspirations, and Healthcare Biotechnology Development fall under second initiative which to capitalize on the strengths of biodiversity to commercialize discoveries in natural products as well as position Malaysia in the bio-generics market. NBP envisions that biotechnology will be a new economic engine for Malaysia, enhancing the nation's prosperity and well-being by building a conducive environment for R&D and industry development whilst leveraging on the country's existing areas of strength.

Besides that, the World Health Organization (WHO), has develop a long-term strategy in promoting traditional medicines. It has produced a series of publications on global atlas such as Bodeker *et al.* (2005) which consist of reliable and evidence based information on the practice of traditional medicine in the world today. It also provides references and research tool for all those who are working to increase availability and accessibility to cost effective remedies as well as method of treatment in order to promote proper use, improve training and education on traditional medicine. In 2013, WHO has developed the latest version of WHO traditional medicinal strategy (2014-2023) to support and manage member states in harnessing the potential contribution of traditional medicine to health and wellness and promoting the safe and effective use of traditional medicines by regulating, researching and integrating traditional medicine products, practitioners and practice into the health systems.

The 33rd session of WHO South East Asia Advisory Committee on Health Research (SEA/ACHR) (2013) emphasized that health researches play a critical role in health development to improve health management especially in view of the current and emerging health challenges. Research is indeed needed to help face the challenges in the optimal cost-efficient and cost-effective manner. One of the particular area of research mentioned in the session is chronic liver disease.

Liver is one of the vital organs in human. It consists of multifunction lobes that are responsible in regulating most of biosynthetic, secretion, detoxification and metabolic processes. All these various processes depend upon energy and thus making the liver a highly aerobic, oxygen dependent tissue (Malhi & Gores, 2008). Metabolism of chemicals is one of the crucial metabolisms that take place mostly in the liver. This will highly account the susceptibility of the organ itself to metabolism-dependent, chemical/drug induced injury. The chemical/drug metabolites conceivably electrophilic chemicals or free radicals that promotes a variety of chemical reactions such as depletion of reduced glutathione by covalently binding to protein, lipid or nucleic acid and inducing lipid peroxidation (Kaplowitz, 2004; Malhi *et al.*, 2006).

Chemical-driven liver damage usually known as hepatotoxicity. Exposure to particular medical agents whether in a therapeutic or overdose range may induce the injury of the liver. Hepatotoxins are a chemical that responsible in causing liver injury (Pandit *et al.*, 2012). Drug induced liver toxicity is a frequently reported cause of liver injury. Every drug that associated with hepatotoxicity have its own characteristic signature regarding latency and pattern of injury (Kaplowitz, 2001). The pathogenesis of drug liver injury associates with the involvement of a toxic drug or metabolite that either induce an immune response or directly affects the biochemistry of the cells (Abboud & Kaplowitz, 2007). Various numbers of clinical researches have conducted and showed that mitochondrial dysfunction is a major or leading mechanism of drug induced liver injury, which involving the parent drug or reactive metabolite generated through cytochrome P450. The alteration emerged in the mitochondria sufficient enough to trigger mild to fulminant hepatic failure such as cytolysis and steatosis (lipid accumulation) (Begriche *et al.*, 2011).