UNIVERSITI PUTRA MALAYSIA

ANTINOCICEPTIVE EFFECTS AND MECHANISMS OF ACTION OF Clinacanthus nutans LINDAU LEAF METHANOLIC AND PETROLEUM ETHER EXTRACTS IN MICE

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By

MOHAMMAD HAFIZ BIN ABDUL RAHIM

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DEDICATION

This thesis is dedicated to my parents, who believe in the richness of learning, their love and support has enabled me to achieve my goals and finish what I have started.

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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

Clinacanthus nutans (*C. nutans*) Lindau is a shrub widely cultivated in the South East Asian region including Malaysia. It has traditionally been used for the treatment of various ailments including pain–mediated diseases. Although various pharmacological activities of this plant have been reported, its pain–relieving activity has been neglected. Therefore, the objective of the present study is to determine the chemical constituents, acute and subchronic toxicity, and antinociceptive effects of the *C. nutans* leaf extracts. Phytochemical screening and chromatography methods analysis, i.e., High Performance Liquid Chromatography (HPLC), Ultra High Performance Liquid Chromatography–Electrospray Ionisation (UHPLC–ESI) and Gas Chromatography–Mass Spectrometry (GC–MS), standard procedures were carried out to determine the presence of bioactive compounds. Acute (14 days) and subchronic (28 days) oral toxicity tests were performed according to the Organisation for Economic Co–operation and Development (OECD) guidelines. The antinociceptive effect of *C. nutans* leaf methanol extract (MECN) was investigated using acetic acid–induced abdominal constriction, hot plate, and formalin–induced paw licking tests. The antinociceptive effects of its partitions, i.e., petroleum ether (PECN), ethyl acetate (EACN), and aqueous (AQCN) partitions, were evaluated using acid–induced abdominal constriction test. The PECN, which was the most effective in acetic acid–induced abdominal constriction test, was further subjected to hot plate and formalin–induced paw licking tests. Furthermore, the MECN and PECN also were subjected to the rota–rod test in order to determine non–specific sedative effects. The roles of capsaicin, glutamate, phorbol 12–myristate 13–acetate (PMA), bradykinin, various non–opioid and opioid receptors, L–arginine–nitric oxide (NO)–cyclic Guanosine Monophosphate (cGMP) and potassium (K^+) channels pathway in MECN and PECN–induced antinociception were also evaluated. Phytochemical Screening of MECN and PECN revealed the presence of flavonoids, saponins, steroids and triterpenes. Further analysis, i.e., HPLC, UHPLC–ESI, and GC–MS, of the extracts

have revealed the presence of polyphenolic compounds such as phenolic acid and flavonoid–based compounds as major components. In the acute toxicity test, the median lethal dose (LD_{50}) estimated for *C. nutans* leaf was more than 5000 mg/kg body weight, whereas in subchronic toxicity test, the no–observed–adverse–effect levels (NOAELs) estimated was more than 2500 mg/kg body weight/day. Oral administration of 100, 250, and 500 mg/kg body weight MECN and PECN produced significant ($p < 0.05$) inhibition in acetic acid–, formalin–, capsaicin–, glutamate–, PMA–, bradykinin–induced nociception, while in hot plate test, only the highest dose showed significant ($p < 0.05$) pain inhibition. In the rota–rod test, 500 mg/kg body weight extracts did not show any significant ($p > 0.05$) effect on motor coordination. The antinociceptive activity of 500 mg/kg body weight extracts were significantly ($p < 0.05$) reversed by pre–treatment with L–arginine, while PECN but not MECN significantly ($p < 0.05$) reversed by pre–treatment with 1H–[1,2,4]oxadiazole[4,3–a]quinoxaline–1–one (ODQ). The present study also showed that 500 mg/kg body weight MECN and PECN produced significant ($p < 0.05$) antagonised following pre–treatment with non–opioid and opioid receptor antagonists, i.e., yohimbine, pindolol, caffeine, haloperidol, atropine, β–funaltrexamine, naltrindole, and nor–binaltorphimine, and various K^+ channels blockers, i.e., glibenclamide, apamin, charybdotoxin and tetraethylammonium chloride. In conclusion, MECN and PECN exert antinociceptive activity at both central and peripheral pain pathways through the modulation of the vanilloidergic, glutamatergic, bradykininergic, noradrenergic, serotonergic, adenosinergic, dopaminergic, cholinergic, and opioidergic receptors, protein kinase C, NO–cGMP–independent or dependent, and K^+ channels pathways systems. The synergistic actions of the bioactive compounds contribute to the antinociceptive activity of MECN and PECN.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN ANTINOSISEPTIF DAN MEKANISME TINDAKAN EKSTRAK METANOL DAN ETER PETROLEUM DAUN *Clinacanthus nutans* LINDAU PADA MENCIT

Oleh

MOHAMMAD HAFIZ BIN ABDUL RAHIM

Julai 2017

Pengerusi : Profesor Madya Zainul Amiruddin Zakaria, PhD
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Clinacanthus nutans (*C. nutans*) Lindau adalah pokok renek ditanam secara meluas di rantau Asia Tenggara termasuk Malaysia. Ia secara traditional telah digunakan untuk rawatan pelbagai penyakit termasuk sakit–perantara penyakit. Walaupun pelbagai aktiviti farmakologi dari tumbuhan ini telah dilaporkan, aktiviti sakit–melegakan telah diabaikan. Oleh itu, objektif kajian ini adalah untuk menentukan juzuk kimia, ketoksikan akut dan subkronik, dan kesan antinosiseptif dari ekstrak daun *C. nutans*. Penyaringan fitokimia and analisis kaedah kromatografi, iaitu Kromatografi Cecair Prestasi Tinggi (HPLC), Kromatografi Cecair Prestasi Tinggi Ultra–Elektrosembur Ionisasi (UHPLC–ESI) dan Kromatografi Gas–Spektrometri Jisim (GC–MS), paiwaian prosedur telah dijalankan bagi menentukan kehadiran sebatian bioaktif. Ujian ketoksikan oral akut (14 hari) dan subkronik (28 hari) telah dijalankan mengikut garis panduan Pertubuhan Kerjasama Ekonomi dan Pembangunan (OECD). Kesan antinosiseptif ekstrak metanol daun *C. nutans* (MECN) telah disiasat menggunakan ujian asid asetik–teraruh pencerutan abdomen, piring panas, formalin–teraruh menjilat tapak kaki. Kesan antinosiseptif dari pecahannya, iaitu, pecahan eter petroleum (PECN), etil asetat (EACN), dan akueus (AQCN), telah dinilai menggunakan ujian asid asetik–teraruh pencerutan abdomen. PECN, yang paling berkesan dalam ujian asid asetik–teraruh pencerutan abdomen, telah dilanjutkan tertakluk kepada ujian piring panas dan formalin–teraruh menjilat tapak kaki. Tambahan pula, MECN dan PECN juga telah tertakluk kepada ujian 'rota–rod' bagi menentukan kesan sedatif yang bukan–spesifik. Peranan kapsaisin, glutamat, phorbol 12–myristate 13–asetat (PMA), bradikinin, pelbagai reseptor bukan–opioid dan opioid, laluan L–arginina–nitrik oksida (NO)–kitaran Guanosina Monofosfat (cGMP) dan saluran kalium (K^+) dalam MECN dan PECN–teraruh antinosiseptif juga telah dinilai. Penyaringan fitokimia MECN dan PECN mendedahkan kehadiran flavonoid, saponin, steroid dan triterpenes. Analisis selanjutnya, iaitu, HPLC, UHPLC–ESI, and GC–MS, ekstrak telah menunjukkan kehadiran sebatian polifenol seperti asid fenolik dan flavonoid–

berasaskan sebatian sebagai kompenen utama. Dalam ujian ketoksikan akut, dos maut median (LD_{50}) dianggarkan untuk daun *C. nutans* adalah lebih daripada 5000 mg/kg berat badan, sedangkan dalam ujian ketoksikan subkronik, tiada–tahap–kesan–buruk–yang diperhatikan (NOAELs) dianggarkan lebih daripada 2500 mg/kg berat badan/hari. Pemberian oral 100, 250, dan 500 mg/kg berat badan MECN dan PECN menghasilkan signifikan ($p < 0.05$) perencatan dalam asid asetik–, formalin–, kapsaisin–, glutamat–, PMA–, bradikinin–teraruh nosiseptif, manakala dalam ujian piring panas, hanya dos tertinggi menunjukkan signifikan ($p < 0.05$) perencatan kesakitan. Dalam ujian 'rota–rod', 500 mg/kg berat badan ekstrak tidak menunjukkan sebarang signifikan ($p > 0.05$) ke atas koordinasi motor. Aktiviti antinosiseptif 500 mg/kg berat badan ekstrak secara signifikan ($p < 0.05$) dibalikkan oleh pra–rawatan dengan L–arginina, manakala PECN tetapi tidak MECN secara signifikan ($p < 0.05$) dibalikkan oleh pra–rawatan dengan dengan 1H–[1,2,4]oxadiazole[4,3–a]quinoxaline–1–one (ODQ). Kajian ini juga menunjukkan bahawa 500 mg/kg berat badan MECN dan PECN menghasilkan signifikan ($p < 0.05$) antagonis berikutan pra–rawatan dengan antagonis reseptor bukan–opioid dan opioid, iaitu, yohimbina, pindolol, kafeina, haloperidol, atropine, β–funaltrexamine, naltrindole, and nor–binaltorphimine, dan pelbagai penyekat saluran K^+, iaitu, glibenclamide, apamin, charybdotoxin and tetraethylammonium klorida. Kesimpulannya, MECN dan PECN menjalankan aktiviti antinosiseptif di kedua–dua laluan kesakitan pusat dan periferal menerusi modulasi sistem reseptor vanilloidergik, glutamatergik, bradikininergik, noradrenergik, serotonergik, adenosinergik, dopaminergik, cholinergik, dan opioidergik, kinase protein C, NO–cGMP–tidak bergantung atau bergantung, dan laluan saluran K^+. Tindakan synergistik dari sebatian bioaktif menyumbang kepada aktiviti antinosiseptif MECN dan PECN.

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I certify that a Thesis Examination Committee has met on 20 July 2017 to conduct the final examination of Mohammad Hafiz bin Abdul Rahim on his thesis entitled "Antinociceptive Effects and Mechanisms of Action of *Clinacanthus nutans* Lindau Leaf Methanolic and Petroleum Ether Extracts in Mice" 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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LIST OF ABBREVIATIONS

12–HPETE	12–hydroperoxyeicosatetraenoic
5–HT	Serotonin
AC	Adenylyl cyclase
ACh	Acetylcholine
Ado	Adenosine
AMPA	Alpha–amino–3–hydroxy–5–methyl–4–isoxazolepropionic acid
ANOVA	One–way analysis of variance
AQCN	*Clinacanthus nutans* aqueous extract
ARs	Adrenergic receptors
ASA	Acetylsalicylic acid
ATP	Adenosine–5'–triphosphate
BK	Bradykinin
BW	Body weight
cAMP	cyclic adenosine monophosphate
CAPZ	Capsazepine
CB1	Cannabinoid type 1
cGMP	cyclic Guanosine Monophosphate
CGRP	Calcitonin gene–related peptide
CNS	Central nervous system
COX	Cyclooxygenase
DA	Dopamine
DAG	Diacylglycerol
dH_20	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
DPPH	1,1–diphenyl–2–picrylhydrazyl
DZP	Diazepam
EACN	*Clinacanthus nutans* ethyl acetate extract
FMHS	Faculty of Medicine and Health Sciences
FRAP	Ferric reducing antioxidant power
FRIM	Forest Research Institute of Malaysia

FVM	Faculty of Veterinary Medicine
GABA	Gamma–aminobutyric acid
GC–MS	Gas Chromatography–Mass Spectrometry
GHS	Globally Harmonised System of Classification and Labelling of Chemicals
Glu	Glutamate
GPCRs	G–protein–coupled receptors
HPLC	High Performance Liquid Chromatography
HSV	Herpes simplex virus
i.p.	Intraperitoneal
i.pl.	Intraplantar
IACUC	Institutional Animal Care and Use Committee
IASP	International Association for the Study of Pain
IBS	Institute of Bioscience
ID_{50}	median infective dose
iGluRs	Ionotropic glutamate receptors
IP3	Inositol 1,4,5–trisphosphate
IUPHAR	International Union of Basic and Clinical Pharmacology
LD_{50}	Median lethal dose
L–Glu	L–glutamic acid
L–NAME	N^{ω}–nitro– L–arginine methyl ester hydrochloride
LOX	Lipoxygenase
mAChRs	Metabotropic muscarinic receptors
MARDI	Malaysian Agricultural Research and Development Institute
MECN	*Clinacanthus nutans* leaf methanolic extract
mGluRs	Metabotropic glutamate receptors
NADA	*N*–arachidonoyl dopamine
NE	Norepinephrine
NIST	National Institute Standard and Technology
NMDA	*N*–methyl–D–aspartate
NO	Nitric Oxide
NOAELs	No–observed–adverse–effect levels
NOS	Nitric oxide synthase
NSAIDs	Non–steroidal anti–inflammatory drugs

ODQ	1H–[1,2,4]oxadiazole[4,3–a]quinoxaline–1–one
OECD	Organisation for Economic Cooperation and Development
ORs	Opioid receptors
p.o.	*Per os*
PAFs	Primary afferent fibres
PAG	Periaqueductal grey matter
PECN	*Clinacanthus nutans* petroleum ether extract
PGs	Prostaglandins
PIP2	Phosphatidylinositol–4,5–bisphosphate
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PNS	Peripheral nervous system
RAS	Recurrent aphthous stomatitis
ROW	Relative organ weight
RTX	Resiniferatoxin
sGC	soluble guanylyl cyclase
SNO	*S*–nitrosothiol
SP	Substance P
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid type 1
UHPLC–ESI	Ultra High Performance Liquid Chromatography–Electrospray Ionisation
UPM	Universiti Putra Malaysia
VC	Vehicle control
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Pain is an essential sensation that plays a vital role as a body natural defence system by alerting us to possible tissue injury. The International Association for the Study of Pain (IASP) defines pain as, "an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in terms of such damage", while nociception is described as "the neural processes of encoding and processing noxious stimuli" that usually leads to pain (Zakaria *et al.*, 2014; Loeser and Treede, 2008).

The process mentioned above is initiated by specialised peripheral sensory neurons (nociceptors) that are activated by noxious stimuli, i.e., mechanical, thermal, and chemical stimuli, due to tissue injury and damage (Pathak *et al.*, 2014), and these nociceptors are usually found in cutaneous tissues, bone, muscle, connective tissues, vessels and viscera (Bomba *et al.*, 2015). These stimuli are transduced into electrical impulses (action potentials) that are transmitted predominantly through Aδ– and C–fibre nociceptors (primary afferent neurons) into the dorsal horn of the spinal cord (Origoni *et al.*, 2014). A variety of excitatory neurotransmitters are released by the primary afferent neurons, such as excitatory amino acids, protons, peptides, lipids and cytokines, and others, which act on their specific receptors and ion channels, to activate the second order neurons of the spinal dorsal horn (Ossipov, 2012; Pavin *et al.*, 2011).

Once activated, the action potentials are then ascended to the thalamus and cerebral cortex through spinothalamic or other tracts that lead to perception of pain (George and Prithishkumar, 2011). In this regard, any substances that are able to block these signalling pathways, both at central and peripheral levels will play an important role in the relief of pain (Meotti *et al.*, 2007).

Opioids (central analgesic), such as morphine, and nonsteroidal anti–inflammatory drugs (NSAIDs; peripheral analgesic), such as acetylsalicylic acid, are universally used for the treatment of pain. Although these treatments for pain have seen rapid progression the field of analgesic drug development, their clinical efficacy and tolerability are often surpassed by adverse effects (Sofidiya *et al.*, 2014). Therefore, there is a need to look for an alternative approach to treat pain that has fewer or, possibly no side effects. Natural product–based medications, particularly plant–derived, are believed to be a valuable source of chemical substances that promise to have a good potential therapeutic applicability (Anser and Najam, 2015).

One of the medicinal plants that has recently received attention from the researchers is *Clinacanthus nutans* (*C. nutans*) Lindau. The herb or a small shrub known as "belalai gajah" in Malaysia, is a member of the Acanthaceae family that can be found in tropical Southeast Asian countries. The plant is traditionally used by the local communities in Indonesia, Malaysia, and Thailand for the treatment of burns, diabetes, diarrhea, dysentery, fever, herpes skin infections, insect and snake bites, mental stress, rheumatoid arthritis, scalds, skin rashes (Shim *et al.*, 2013) and to relieve pain *(Tan et al.*, 2016).

The extracts of *C. nutans* have been shown to exert antibacterial (Yang *et al.*, 2013), anticholinesterase (Lau *et al.*, 2014), anti–dengue (Tu *et al.*, 2014), antidiabetes (Nurulita *et al.*, 2008), antiherpes (Kunsorn *et al.*, 2013), anti–inflammatory (Mai *et al.*, 2016), antimutagenic (Rathnasamy et al., 2013), antioxidant (Che Sulaiman *et al.*, 2015), antiproliferative (Yong *et al.*, 2013), antitumor (Huang *et al.*, 2015), anti–varicella–zoster (Charuwichitratna *et al.*, 1996), and cytotoxic (Liew *et al.*, 2012) activities.

Various chemical compounds have been isolated and identified from *C. nutans*, i.e., stigmasterol, lupeol, *β*–sitosterol, betulin, vitexin, isovitexin, schaftoside, isomollupentin–7–O–*β*–glucopyranoside, orientin, isoorientin, sulphur–containing glycosides, glycoglycerolipids, and monoacylmonogalactosylglycero (Shim *et al.*, 2013). In addition, a variety of phytochemical constituents have been detected in the *C. nutans*, such as flavonoids, phenolic acid compounds (Ghasemzadeh *et al.*, 2014; Peng *et al.*, 2014), diterpenes, phytosterols, saponins (Yang *et al.*, 2013), steroids, i.e., β–sitosterol and stigmasterol, chlorophyll and its various derivatives (Sarega *et al.*, 2016; Sakdarat *et al*., 2009; 2008).

There are various reports on pharmacological activity of this plant, however, to the best of our knowledge, there has been no study on antinociceptive activity of *C. nutans*. The antinociceptive study is suggested to be attributed to anti–inflammatory properties (Wanikiat *et al*., 2008) and phytoconstituents contents of the plant associated with the antinociceptive activity (Yang *et al*., 2013).

1.2 Problem Statement

Pain is associated with morbidity and mortality as well as affecting the quality of life. There are various therapeutic drugs available in the markets that are used to relieve pain, however, these drugs may lead to several adverse effects. For example, NSAIDs are widely known to cause adverse effects, such as gastrointestinal irritation and/or bleeding, decreased platelet aggregation, kidney damage, edema, bone marrow suppression, rashes, as well as anorexia (Cameron *et al*., 2013). Opioid analgesics, lead to adverse effects, such as constipation, dizziness, nausea, respiratory depression, sedation and vomiting (Matava, 2016).

1.3 Justification of the Study

The discovery of natural product–based medications, particularly plant–derived, with good potential therapeutic efficacy is important to replace conventional NSAIDs or opioid drugs for pain management (Anser and Najam, 2015). Although *C. nutans* has been used for the treatment of various diseases including pain relief, there is no scientific evidence to validate the usage of the plant for these purposes. The current study was thus designed to determine the antinociceptive activity and mechanism of action of *C. nutans* methanolic leaf extract and its partitions.

1.4 Hypothesis

We hypothesise that the *C. nutans* leaf methanolic extract (MECN) and its partitions are expected to exhibit significant antinociceptive activity in the mice nociception model.

1.5 Objective of the Study

The general objectives of the study are to investigate the antinociceptive effect of crude and the most effective partition of MECN and to determine its mechanisms of action. The specific objectives are as follows:

1. To identify the bioactive chemical components of *C. nutans* leaf methanol (MECN) and petroleum ether (PECN) extracts.
2. To determine the safety of MECN in the acute and subchronic toxicity study in mice.
3. To determine the antinociceptive effects of MECN and PECN in the chemical– and heat–induced nociception mice model.
4. To determine the mechanism of antinociception afforded by MECN and PECN in mice model.

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