EFFECTS OF *CLINACANTHUS NUTANS* LINDAU EXTRACTS ON HYPERURICEMIC MICE

BY

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ABSTRACT

Clinacanthus nutans or Sabah Snake Grass (Acanthaceae) has been commercialised locally as an anti-gout, and anti-hyperuricemia. However, the scientific evidences that supports its efficacy in treating these illnesses is still insufficient. The study aims to investigate the effect of different extracts and doses of *C. nutans* leaf in the normalisation of uric acid levels in hyperuricemic mice, conduct a metabolite fingerprinting on the mice serum through an LC-MS-based metabolomics approach, and to evaluate the acute toxicity effect of the *C. nutans* extract on kidney and liver samples of the mice of the selected doses. The dried leaves were extracted by soaking in different solvents with different concentrations of ethanol in water (0, 20, 50, 70, 100%, v/v). These extracts were tested *in vivo* using a potassium oxonate-induced hyperuricemia mice model. The results showed that the administration of the aqueous and 50% ethanolic extract with the dose 800 mg/kg b.w. for 4 weeks significantly ($P<0.05$) reduced and normalised the serum uric acid level of the mice starting from week 3. The same effect was also noticed for allopurinol whereby uric acid levels were potently normalised throughout the experiment (week 1 to 4). However, the data obtained from the LC-MS based fingerprinting showed that the metabolite profile of the mice serum treated with allopurinol was affected negatively in comparison with the healthy group starting from week 2. Interestingly, the metabolite profile of the mice treated with the *C. nutans* aqueous extracts at 800 mg/kg b.w. dose was better than that of allopurinol whereby they normalised the metabolic profile of the mice in week 4. The treatment with allopurinol, *C. nutans* aqueous and 50% ethanol extracts did not cause any histopathological or behavioural side effects to the mice with zero mortality up to 4 weeks of treatment. Moreover, the identification of the serum metabolites involved in the treatment is necessary to understand the mechanism of action of the *C. nutans* extracts. This study concludes that the *C. nutans* leaf extract has a potential in treating hyperuricemia and gout.

خلاصة البحث

تسوق نبتة كليناكانتوس نوتانس (*Clinacanthus nutans*) أو عشبة الأفعى الصباحية (فصيلة الأقنتيات) محليا كدواء لعلاج داء النقرس وفرط حمض اليوريك في الدم. ولكن الأدلة العلمية التي تثبت فعاليتها في معالجة هذه الأمراض غير كافية. هدفت الدراسة للتحقيق في أثر الجرعات المختلفة لمستخلصات نبتة الكليناكانتوس نوتانس في علاج فرط حمض اليوريك في الدم في الفئران المصابة بفرط في حمض اليوريك، وإجراء التبصيم الأيضي لمصل دماء الفئران باتباع طريقة الميتابولوميات المعتمدة على الطيف الكتلي-الاستشراب السائل(LC-MS) ، ولتقييم التأثير السمي الحاد لجرعات مختارة من المستخلصات. تم استخلاص الأوراق المجففة لهذه النبتة بالنقع في محاليل الإيثانول المائي المختلفة التركيز (0، 20، 50، 70، 100%، v/v). تم اختبار هذه المستخلصات في النموذج الحيواني لفرط حمض يوريك المحدث بمركب أوكسونيك البوتاسيوم. أظهرت النتائج أن إعطاء المستخلصات المائية ومستخلصات 50% إيثانول بجرعة 800 مغ/كج من وزن الفأر لمدة 4 أسابيع خفض بشكل كبير ($p<0.05$) مستويات حمض اليوريك المرتفعة في الدم إلى مستواها الطبيعي بدءا من الأسبوع الثالث. تم ملاحظة نفس الأثر مع الألوبيورينول حيث خفض بشكل فعال مستويات حمض اليوريك المرتفعة في الدم طيلة مدة العلاج (من الأسبوع الأول إلى الرابع). على الرغم من ذلك، أظهرت بيانات الميتابولوميات المعطاة من التبصيم المعتمد على الـ LC-MS أن التوصيف الأيضي لدماء الفئران المعالجة بالألوبيورينول قد تأثر بشكل سلبي بدءا من الأسبوع الثاني مقارنة بالمجموعة الضابطة. كان من المثير للاهتمام أن التوصيف الأيضي للفئران المعالجة بالمستخلصات على جرعة 800 مغ/كج من وزن الفأر كان أفضل من تلك المعالجة بالألوبيورينول حيث قامت بارجاع التوصيف الأيضي لدماء الفئران في الأسبوع الرابع. لم يحدث العلاج بالألوبيورينول، والمستخلصات المائية، مستخلصات 50% إيثانول أي حالة موت أو أثر جانبي من الناحية السلوكية أو الهيستولوجية لغاية 4 أسابيع من العلاج. بالإضافة إلى ذلك، من الواجب القيام بعملية التعرف على المستقلبات التى لها دور في العلاج وذلك لفهم آلية عمل المستخلصات. تستنتج هذه الدراسة أن مستخلصات الكليناكانتوس نوتانس علاج واعد للاستعمال في مداواة الفرط في مستوى حمض اليوريك في الدم.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry).

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

COPCORD	Community oriented program for the control of rheumatic disease
DDD	Defined daily dose
NSAID	Non-steroidal anti-inflammatory drug
COX-2	Cyclooxygenase-2
WHO	World Health Organisation
b.w.	Body weight
N/A	Not available
cPLA2	Cytosolic phospholipase A2
TLR-4	Toll-like receptors-4
ATP	Adenosine triphosphate
PRPP	5-phosphoribosyl 1-pyrophosphate
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
XO	Xanthine oxidase
XD	Xanthine dehydrogenase
XOR	Xanthine oxidoreductase
NAD+	Nicotinamide adenine dinucleotide
FAD	Flavin adenine dinucleotide
MRP4	Multidrug resistance-associated protein 4
SLC22A6	Solute Carrier Family 22 Member 6.
URAT1	Urate transporter 1
ABCG2	ATP Binding Cassette Subfamily G Member 2
GLUT9	Glucose transporter 9
US FDA	United States Food and Drug Administration
SPF Mice	Specific-pathogen-free mice
ICR Mice	Institute for Cancer Research mice

ddy Mice	Deutschland, Denken, and Yoken mice
^{1}H-NMR	Hydrogen-1 nuclear magnetic resonance
PCA	Principal component analysis
HPLC	High performance liquid chromatography
UPLC-MS	Ultra-performance liquid chromatography, mass spectrometry
LC-MS	Liquid chromatography–mass spectrometry
UV	Ultraviolet
GC-MS	Gas chromatography–mass spectrometry
LC-qTOF-MS	Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry
w/w	Weight per weight
w/v	Weight per volume
v/v	Volume per volume
CNE	*Clinacanthus nutans* extracts
ALP	Allopurinol
ESI	Electrospray ionization
CMC	Carboxymethyl cellulose

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Hyperuricemia, or high uric acid, is clinically a common metabolic abnormality. Around 10% of men and women are recorded with hyperuricemia not less than once in their lives with a reported prevalence between 5% and 30% in the general population globally, and it is elevated in certain ethnicities (Kuo et al., 2015). For example, hyperuricemia is more common in the Maori aborigines (27.1%) than the Europeans (9.4%) in New Zealand, and in the Taiwan aborigines the prevalence was around 41.4% (Reginato et al., 2012). Moreover, it was found that the urban population had a higher prevalence of hyperuricemia than the rural population in Beijing (Li et al., 1997). Hyperuricemia is considered to be more prevalent globally because of the improvements in living standards (Lawrence, 1964), prolonged life span, and the side effects of particular medications in few cases (Demartini, 1965; Scott, 1991). This trend has caused a significant high morbidity and has burdened the health care system (Wertheimer et al., 2013).

According to an article by Kuo et al. (2015) in Nature Reviews, gout is reportedly rare in Malaysia. Moreover, Malaysia was categorised by the COPCORD (Community Oriented Program for the Control of Rheumatic Diseases) as having low gout prevalence (<0.5%). However, despite that claim, the use of anti-gout preparations in Malaysia, measured by DDD, the assumed average maintenance dose per day for a drug used for its main indication in adults (WHO, 2004), was ranked second (1.5554 DDD/1000 population/day) in 2010, and the three most used anti-gout preparations were allopurinol, colchicine, and probenecid (Ministry of Health, 2014).

In the same report, anti-inflammatory and anti-rheumatic products, which are also prescribed in cases of gout (to relief inflamed joints during attacks due to crystal deposition), ranked first (11.7605 DDD/1000 population/day) in 2010.

Gout was known centuries ago, and a better comprehension about its pathophysiology is already developed (Pai, Raslan, and Schlesinger, 2015). The metabolic disorder results from the deposition of monosodium urate or uric acid crystals in joint structures and in other sites in the form of tophi. Impaired renal uric acid excretion alone, or in combination with genetic malfunctions that regulate uric acid could lead to gout. Elevated serum uric acid or hyperuricemia is a prerequisite for the formation of the crystals. The distinct resembling features of gout are acute attacks of joint inflammation, which often occur at the first metatarsophalangeal joint (the big joint of the big toe), although their incidence in other joints and in bursae is also very common (Pascual and Sivera, 2007). Gout is the most common type of inflammatory arthritis in adults. Gout affects 8.3 million Americans (Zhu, Pandya, and Choi, 2011) and 5% of US veterans (Singh, Hodges, Toscano, and Asch, 2007). It leads to frequent emergency room visits (Garg et al., 2013) and costs 20 billion dollars annually (Wertheimer et al., 2013).

According to a survey, in Malaysia, anti-inflammatory agents are the drugs that are mostly used for the alleviation of acute and chronic gout, with a low prescription of corticosteroid (Yeap et al., 2009). In that study, for the treatment of acute gout, 68.0% of doctors use non-selective non-steroidal anti-inflammatory drugs (NSAIDs), 53.9% use selective COX-2 inhibitors, 66.4% use colchicine and 10.2% use allopurinol (Yeap et al., 2009). In the treatment of chronic gout, 36.7% use NSAIDs, 44.5% use COX-2 inhibitors, 19.5% use colchicine and 93% use allopurinol. In both acute and chronic gout, corticosteroids are not used by over 90% of respondent

doctors. Xanthine oxidase inhibitors lower the uric acid concentration in blood by inhibiting the xanthine oxidase enzyme which is the cause for the generation of uric acid from the purines. As a result of the inhibition, uric acid concentrations in blood and urine are lowered, and there will be a concurrent rise in the elimination of xanthine and hypoxanthine that are the more soluble precursors of uric acid (Angstadt, 1997). Patients who are characterised with persistent elevation of uric acid or those with insufficient renal excretion are best prescribed with xanthine oxidase inhibitors such as allopurinol (Wright and Pinto, 2003).

Traditional remedies are consumed in the primary health care by about 75 to 80 % globally, particularly in developing countries. The consumption of herbal products is also popular in some developed nations such as Germany, United States of America, and France (Ekor, 2014). The WHO has estimated the demand for medicinal plants is approximately $14 billion per annum (2006) and the demand is growing at the rate of 15–25% annually. The WHO estimates that by 2050 the trade will be up to US$ 5 trillion (Zhang et al., 2012). The herbs and herbal products sales in the United States of America and the European Union are estimated to be worth more than US $ 8 billion and 20 billion annually, respectively, while the worldwide herbal medicine market is appraised to be from 30 to 60 billion dollars annually (Vunnava et al., 2014). Previously, therapeutic plants were the source for a number of clinically significant drugs such as morphine, digoxin and atropine, and in the search for new drugs these plants are excellent sources of lead compounds.

Malaysia is a fast developing country with a population that approaches 31.7 million (Department of Statistics Malaysia, 2015). The prevalence of traditional and complementary medicine usage in Malaysia is moderately high (Aziz and Tey, 2009; Hasan et al., 2009), particularly for adults in their middle-ages who consume these

medicines to improve their well-being, which includes sexual libido (Hassali et al., 2012). Studies in Malaysia have also shown that patients with chronic diseases (Hasan et al., 2009), and cancer (Farooqui et al., 2012) also choose traditional and complementary medicines as an alternative source of treatment. The search for novel anti-hyperuricemic compounds from medicinal plants and herbal products is continuing. Currently, the largest underexplored rainforest for the discovery of novel drugs is in tropical and subtropical provinces of the world (Balick et al., 1996). Malaysia having located in this area is well recognised for its diverse nature and forests. Malaysians also adopt traditional remedies as an alternative choice for the prevention and treatment of diseases, including gout and rheumatism, as indicated by the overserved abundance of herbal supplements in the Malaysian market. However, the validity of most of these claims has not been scientifically proven and therefore, it is of interest to evaluate the anti-hyperuricemic effect of the local plants in Malaysia.

Clinacanthus nutans Lindau is a plant species that is categorised under the Acanthaceae family. It is a small shrub generally found in South East Asia, specifically in Malaysia and Thailand (Tuntiwachwuttikul et al., 2004).

In Malaysia, the dried leaf of *C. nutans*, in a specific amount, is traditionally boiled with water and consumed as a herbal tea. The fresh leaves of *C. nutans* are usually consumed raw, or mixed with other juices, such as apple juice, green tea, or sugarcane juice, and served as a fresh drink or an energising beverage, moreover, there are many products on the market that are based on *C. nutans* products, such as herbal teas, capsules, coffee sachets, tablets, and concentrated plant extracts (N. Shakir, personal communication, June 14, 2016). It has been traditionally used to treat cancer, diabetes, fever, diarrhoea, and dysuria (Chu, 2013). It has also been established that this plant has heat and stasis-reducing effects, and regulation of

menstruation, and liver and gallbladder cleansing, (Shim et al., 2013). Leaves of *C. nutans* has been also widely used as an anti-hepatitis, analgesic, antioxidant, antidiabetic, and anti-herpes agent. In Thailand and Indonesia, it has been used as anti-inflammatory agents for the treatment of insect bites and allergic responses (Yoosook et al., 1999).

Metabolomics resemble a snapshot picture of a particular organism, which displays the compounds available and in what quantities at a certain point of time. Analysing several samples (i.e. several snapshots) allows metabolites marker identification or patterns that are distinguishing for a species, a cultivar, or a particular stage of development (Worley and Powers, 2013). It also enables the identification of conditions, such as stress, disease state, or daily and seasonal changes. In other words, the number of variables in a metabolomics study is almost unlimited. To organise these variables, they can be separated to three types: a genotype's metabolomic characterisation; states of development; and changes due to environmental or external factors (Kim et al., 2011).

Recently, novel methods are developing, which could assist in offering a better understanding of the mechanism of action of medicinal plants, and could guide to the likelihood of getting evidence and assurance of the pharmacological activities over variable raw materials batches. These approaches are based on the holistic consideration of the natural products, i.e. the majority of phytochemical components, instead of concentrating on particular distinct components or a sets of active compounds (Yuliana et al., 2011). On the other hand, the triggered changes of a particular sample on the, and/or the metabolomic patterns, transcriptome, or proteome can be compared with the changes seen after treatment with identified drugs, instead of only observing explicit changes.

1.2 PROBLEM STATEMENT

Conventional medications for the treatment of hyperuricemia, such as allopurinol and probenecid, are mostly used to inhibit the production of uric acid and promote the excretion of uric acid, and they are clinically effective. However, these drugs have significant side effects, such as liver and kidney function damage, bone marrow suppression, gastrointestinal reactions, and relapse after medication withdrawal, thus, long-term administration of these medications is not recommended (Fam et al., 2001; Khoo & Leow, 2000; Pan et al., 2013). Therefore, the development of novel hypouricemic agents with greater efficacy and a broader safety profile is greatly needed. Traditional uses of *C. nutans* leaf suggest that it possesses anti-inflammation and anti-hyperuricemia properties which relate to suppression of gout with low side effects. However, these claims lack scientific evidence which will be explored in this study.

1.3 RESEARCH OBJECTIVES

This study embarks on the following objectives:

1. To investigate the effect of different extracts and doses of *C. nutans* leaf in the normalisation of uric acid levels in hyperuricemic mice.
2. To investigate the effect of oral administration of *C. nutans* leaf in hyperuricemic mice based on metabolite fingerprinting of their serum following the metabolomics approach.
3. To evaluate the acute toxicity effect of *C. nutans* extract on the kidney and liver at the selected doses.

1.4 RESEARCH QUESTIONS

1. Which extracts and doses of *C. nutans* leaf extracts is effective in normalising uric acid levels in hyperuricemic mice?
2. Will the most effective extract and dose of *C. nutans* leaf be able to normalise hyperuricemia mice based on metabolite fingerprint of its serum?
3. Do the effective doses have toxic effects?

1.5 RESEARCH HYPOTHESIS

C. nutans leaf extract is effective in treating hyperuricemia.

CHAPTER TWO

LITERATURE REVIEW

2.1 *CLINACANTHUS NUTANS* LINDAU

2.1.1 Botanical Aspects and Geographical Distributions

Clinacanthus nutans Lindau (Figure 2.1) is known as Sabah Snake Grass, or *Pokok Belalai Gajah* in Malaysia, *Dandang Gendis* or *Ki Tajam* in Indonesia, twist of flowers or alligator flower in China, and *phaya yo*, *phaya plong thong*, or *saled pangpon tua mea* (saliva of female mongoose) in Thailand (Aslam et al., 2015; Kosai et al., 2016; Nadiah et al., 2016). It belongs to the family of Acanthaceae, and it is a well-known South East Asian medicinal plant. Taxonomically this plant can be classified as the following: Kingdom: Plantae; phylum: Magnoliophyta; class: Magnoliopsida; subclass: Asteridae; order: Lamiales; family: Acanthaceae; genus: Clinacanthus Lindau; and species: *C. nutans* (Burm. f.) Lindau (Wu et al., 2001).

Figure 2.1 *Clinacanthus nutans* farm in Malaysia

This plant is a small shrub that can be found throughout South East Asia and China, and it is primarily indigenous to Thailand, Indonesia and Malaysia. The Acanthaceae family is a principal dicotyledonous flowering group of plants that includes about 2,500 species and 250 genera. The majority of these plants are shrubs, twining vines, or tropical herbs; and a number of them are epiphytes. Species from the Acanthaceae family are mainly distributed in Africa, South and Central America, and South-East Asia. The species of this family can strive in most habitats, including dense or open forests, damp soils and valleys, sea shores, swamps, and mangroves. This family is believed to be among the main origins of therapeutic herbs that yields effective traditional remedies against particular diseases. *C. nutans* is one of the significant species of the Acanthaceae family and it is being utilised extensively in South East Asia. At present, *C. nutans* has been widely studied regionally for its numerously acclaimed medicinal properties (Yahaya et al., 2015). This plant is known among traditional healers in Thailand as an antidote against snake venom. In Thailand, Malaysia, and Indonesia, *C. nutans* is for treating a number of diseases, for instance fever, skin rashes, diabetes mellitus, insect bites, and they work as diuretics (Kongkaew & Chaiyakunapruk, 2011; Shim et al., 2013)

This perennial herb can reach a height of 1 m with the growth of pubescent branches. The leaves are structurally simple, narrowly elliptic-oblong or lanceolate in shape, opposite in direction (3.5–14.0 cm long × 1.0–2.0 cm wide), and pale green to dark green in colour. The stems of these shrubs are cylindrical, striate and glabrescent, which resembles the curve of an elephant's trunk, hence, the name *Pokok Belalai Gajah* (elephant's trunk plant) in Malay. The petioles are 0.5–1.5 cm in length, and they are sulcated, bifarious. The leaf blades are structurally lanceolate-ovate, lanceolate, or linear-lanceolate. The tips of the leaves are acute or acuminate; and with