UNIVERSITI PUTRA MALAYSIA

TOXICITY AND THERAPEUTIC EFFECTS OF ZERUMBONE EXTRACT ON COMPLETE FREUND'S ADJUVANT-INDUCED RHEUMATOID ARTHRITIS RAT MODEL

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By

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October 2016

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The present study was conducted to investigate the antinociceptive and anti-arthritic effects of zerumbone in Complete Freund's adjuvant induced Rheumatoid Arthritis model in rats. Zerumbone is a sesquiterpene isolated and purified from the rhizomes of *Zingiber zerumbet* plants, locally known as 'lempoyang'. *Zingiber zerumbet* is one of the most important species of cultivated ginger, used in food flavouring. Zerumbone was obtained from the rhizomes through a sequence of isolation processes involving hydro-distillation, rotary evaporation of excessive solvents, column chromatography, and finally followed by recrystallization. The compound was subjected to thin layer chromatography (TLC), gas chromatography mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) to identify its chemical structure and purity. Zerumbone was optimally isolated using the hexane and ethyl acetate solvent system at a ratio of 8:2 with a total yield of 1.2%. In the present study, it was shown that intraperitoneal administration of zerumbone at doses of 10 mg/kg did not show any signs of toxicity in terms of behavioural changes, body weight, liver and kidneys, hematological and liver function parameters. The findings were further supported by histopathological observations of the liver and kidney that demonstrated normal histological architecture. Rheumatoid arthritis was induced by intraplantar injection of CFA on the right hind paw of each rat at day 0. Anti-inflammatory activity of zerumbone on the RA-induced rats was evaluated using plethysmometer test and paw swelling was assessed by measuring the thickness of the hind paw with a digital caliper. The determination of the antinociceptive profile of zerumbone on rheumatoid arthritis-induced model in rats was studied through thermal and mechanical threshold which consists of the Hargreaves plantar test, Von Frey test, and Randall-Selitto analgesiometer

with slight modifications. During the experimental period, the body weight was recorded using a digital weighing balance every 3 days consecutively after CFA injection. With slight modifications, the visual arthritis scoring was used to assess behavioural changes through the ability to stance, mobility and flexion pain test. The clinical assessment of arthritis had been studied through cytokine concentrations and hematological parameters. As a conclusion, the study strongly confirms the antinociceptive and anti-inflammatory activities of zerumbone as well as elucidated the possible mechanism of action through which it exerts its effects. In addition to that, the toxicity studies demonstrated the safety margin of zerumbone in mice, thus scientifically justifying the traditional use of this species of ginger and setting the path for future studies.

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

KESAN KETOKSIKAN DAN TERAPEUTIK ESTRAK ZERUMBONE PADA MODEL TIKUS TERARUH ARTRITIS REUMATOID

Oleh

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Kajian ini telah dijalankan untuk menentukan kesan antinosiseptif dan anti-artritis zerumbone dalam *Complete Freund's adjuvant*(CFA) yang menyebabkan artritis reumatoid (RA) dalam model haiwan. Zerumbone merupakan kompaun seskuiterpena yang dipisahkan dan ditulenkan dari rizom tumbuhan *Zingiber zerumbet*, atau lebih dikenali sebagai lempoyang. *Zingiber zerumbet* adalah salah satu spesis halia yang banyak ditanam dan digunakan untuk peningkatan rasa makanan serta sering digunakan dalam perubatan tradisional untuk mengubat sakit perut, sakit gigi, keseliuhan otot dan juga untuk rawatan lebam dan luka-luka ringan. Zerumbone diperolehi dari rizom melalui proses penyulingan-hidro diikuti oleh proses pengewapan rotari untuk mengeluarkan pelarut dan dipisahkan serta ditulenkan melalui proses kromatografi dan penghabluran semula. Sebatian zerumbone kemudiannya dianalisis menggunakan kromatografi lapisan nipis (TLC), kromatografi gas-spektroskopi jisim (GC-MS) dan kromatografi cecair prestasi tinggi (HPLC) bagi menentukan tahap ketulenan. Zerumbone dipisahkan secara optimum mengunakan sistem pelarut hexane dan etil asetat pada nisbah 8:2 dengan kadar hasil sebanyak 1.245%. Analisis ketoksikan menunjukkan bahawa pemberian zerumbone pada dos 10 mg/kg secara intraperitonial tidak menunjukkan sebarang kesan toksik di mana tiada kesan sampingan dalam kelakuan haiwan, tiada perubahan berat badan, hepar dan ginjal serta tiada perubahan yang signifikan dalam bacaan hematologi dan fungsi hepar. Kesan ketoksikan ini disokong melalui kajian histopatologi di mana histologi hepar dan ginjal berada dalam keadaan normal. Induksi artritis reumatoid dilakukan melaui suntikan CFA secara intraplantar pada tapak kaki belakang kanan tikus pada hari 0. Aktiviti anti-inflamasi zerumbone keatas model RA pada tikus dinilai dengan menggunakan ujian plethysmometer bagi menentukan isipadu tapak kaki dan ketebalan tapak kaki diukur

menggunakan angkup digital. Penentuan kesan antinosiseptif zerumbone dikaji melalui ambang terma dan mekanikal yang terdiri daripada ujian plantar Hargreaves, ujian von Frey dan ujian Randall-Selitto analgesiometer bagi menilai ambang terma kesakitan. Sepanjang tempoh eksperimen, berat badan tikus direkodkan menggunakan alat penimbang berat digital setiap 3 hari berturut-turut. Skala skor artritis secara visual dilaksanakan bagi mengukur perubahan tingkah laku melalui keupayaan untuk berdiri, bergerak dan ujian kesakitan fleksi seperti yang dinyatakan sebelum ini dengan sedikit pengubahsuaian. Penilaian klinikal artritis telah dikaji melalui analisis kepekatan sitokin dan parameter hematologi. Analisis eksperimen yang telah digunakan adalah analisis dua hala varians (ANOVA) diikuti dengan ujian post-hoc Bonferroni, dimana $p \leq 0.05$ telah diterima sebagai signifikan. Secara kesimpulannya, kajian ini mengesahkan aktiviti antinosiseptif dan anti-inflamasi zerumbone dan juga menjelaskan mekanisme yang berkebarangkalian terlibat. Tambahan pula, tahap keselamatan penggunaan zerumbone pada tikus melalui kajian ketoksinan turut menjustifikasikan penggunaan tradisional spesis halia ini secara saintifik dan membuka ruang untuk kajian pada masa hadapan.

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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LIST OF ABBREVIATIONS

AFM	Arthritis Foundation Malaysia
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
CAM	Complementary alternative medicine
CAMs	Cell adhesion molecules
CFA	Complete Freund's adjuvant
CNS	Central nervous system
COX-2	Cyclooxgenase-2
DIP	Distal interphalangeal
DMARDs	Disease-modifying anti-rheumatic drugs
DMSO	Dimethyl Sulfoxide
GC-MS	Gas chromatography-mass spectrometry
Hb	Hemoglobin
HPLC	High performance liquid chromatography
Ht	Hematocrit
i.p.	Intraperitoneal
IASP	The International Association for the Study of Pain
IBS	Institute of Bioscience
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-6	Interleukin 6
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCP	Metacarpophalangeal
MCV	Mean corpuscular volume
MMPs	Matrix metalloproteinases
MTP	Metatarsalphalangeal
NK	Natural killer

NSAIDs	Non-steroidal anti-inflammatory drugs
PGE2	Prostaglandin E2
PID	Post-inoculation day
PIP	Proximal interphalangeal
RA	Rheumatoid Arthritis
RBC	Red Blood Cell
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor alpha
tR	Retention time
VEGF	Vascular endothelial growth factor
WBC	White Blood Cell

CHAPTER 1

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that typically affects the small joints in hands and feet. Unlike the wear-and-tear damage of osteoarthritis, RA affects the lining of joints, causing painful swellings that eventually result in bone erosion and joint deformity. It is a long-term autoimmune disorder which has been found as one of the most common chronic inflammatory diseases worldwide. RA occurs when the body immune system mistakenly attacks and eventually destroys the healthy joints and tissues. The etiology of this disease still remains unknown until today.

The onset of disease can occur at any age, but the peak incidence occurs within the fourth and fifth decades of life. Apparently, females are 2.5 times more likely to be affected than males (Tehlirian & Bathon, 2008). It is known to affect approximately 0.5% to 1% of the adult population. In Malaysia, it affects about five in 1000 people (AFM, 2009-2010).

In the early stage of RA, pain can be felt when there are some minor movements. However, spontaneous pain during rest can occur later. The primary focus of the inflammation is in the synovium, which is the lining tissue of the joint. Inflammatory chemicals released by the immune cells may cause swelling and damage to cartilage and bone. The diagnosis of RA is based on the symptoms described and physical examination findings such as warmth, swelling, pain in the joints, and presence of rheumatoid nodules (Alyce, Oliver, & William, 2008).

Currently, treatments for RA focus on controlling symptoms and preventing joint damage. Among the common medications are disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs) as well as corticosteroid. Usually, the combinations of different drugs are used to achieve the desired effects (Marsland & Kapoor, 2004). Medications are often prescribed to slow down or stop joint damage, relieve pain, and decrease inflammation.

Undisputedly, conventional drug treatments are available to reduce the inflammation completely or put on halt the destruction of the joints. The accompanying side effects caused by the steroids, however, result in gastrointestinal injuries like peptic ulcer and gastrointestinal bleeding in both healthy and RA patients (Marsland & Kapoor, 2004). This can lead to the discontinuation of the treatments by the patients. Indeed, it is crucial to develop new classes of medication which are equally or more effective than the existing medicines, specifically without side effects and able to cure the disease.

Throughout medicinal evolution, the importance of natural products for medicine and health has been enormous. The history of modern medicine could be traced back to centuries ago; the earliest known written document is a 4000-year old Sumerian clay tablet that records remedies for various illnesses (Kong, Goh, Chia, & Chia, 2003). Similar to the Chinese ancient civilisation, Indians and North Africans too provide written evidence for the use of natural products in curing various diseases (Phillipson, 2001). For instance, turmeric possesses blood clotting properties while raw garlic was prescribed for circulatory disorders and mandrake for pain relief. Some of these natural sources are still being used in several countries as alternative medicines.

However, it was not until the 19th century that scientists isolated active components from natural ingredients that were known to have beneficial effects. The sources of these ingredients had been studied in depth and comprehensively. In the past decades, a variety of active ingredients was being extracted through purification processes of natural sources. They were recorded and classified according to their structure and mechanism of action (McCurdy & Scully, 2005).

Despite centuries of folkloric usage, numerous successes and even well-documented treatment of diseases, many scholars have often dismissed the effectiveness of natural products (Riddle, 1985) in medicine. Sadly, in today's society, those who control the economy of drugs and therapeutic agents prefer to showcase the superiority of modern medicine and portray herbal medicine as well as traditional healing as unscientific.

The use of ginger is so widespread that its usage can be found in various traditional systems of medicine in the world. The cultivation of ginger could be traced back to centuries ago at the University of Maryland Medical Centre where ginger was written to be used in China for over 2,000 years (Steven, 2010) and as medicine from the Vedic period in India, called "*Maha aushadhi*" meaning the great medicine (Palatty, Haniadka, Valder, Arora, & Baliga, 2013). In Sanskrit, ginger is known as Sringavera. It is speculated that this term may have given way to Zingiberi in Greek and then to Latin Zingiber (Vasala, 2004). The botanic name of ginger is *Zingiber officinale* Rosc. which belongs to the family of Zingiberaceace.

Zingiberaceae species are among the most prolific plants in tropical rainforest. There are about 160 species from 18 genera found in Peninsular Malaysia, mostly growing in damps, lowlands, and hill slopes as scattered plants or thickets (Larsen, Ibrahim, & Wong, 1999). Collectively, in terms of its widespread traditional use and availability, there are myriads of reasons for scientifically evaluating the use of ginger to fully explore its potentials.

1.1 Problem statement

The unwanted side effects of common treatment have contributed negatively to the management of inflammation as well as pain in rheumatoid arthritis patients. Therefore, the search for safer antinociceptive and anti-inflammatory agents for treatment of RA is regarded necessary.

1.2 Hypothesis

1. Zerumbone extract has no significant difference compared to control group.
2. Zerumbone extract has positive therapeutic effects in CFA induced rheumatoid arthritis rat model.

1.3 Objectives

1.3.1 General Objectives

1. To investigate the toxicity effects of zerumbone extract.
2. To determine zerumbone extract therapeutic effects in CFA induced rheumatoid arthritis rat model.

1.3.2 Specific Objectives

1. Evaluate the toxicity effects of zerumbone extract in rats.
2. Evaluate the effects of zerumbone on the paw thickness and paw volume in CFA-induced RA model in rats.
3. Investigate the effects of zerumbone on the mechanical allodynia and hyperalgesia in CFA- induced RA model in rats.
4. Investigate the effects of zerumbone on the thermal hyperalgesia in CFA- induced RA model in rats.

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