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IN VITRO AND IN VIVO EFFECT OF Clinacanthus nutans (BURM. F.) LINDAU AQUEOUS EXTRACT ON IgE AND IgG-MEDIATED ALLERGY PATHWAYS

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Allergy is a hypersensitive reaction against antigens which could be mediated through immunoglobulin (Ig) -E and IgG. About 30 – 40% of people globally are affected with allergy and it is projected to increase due to urbanisation. It has both personal and economic implications thus, requiring urgent attention. *Clinacanthus nutans* (Burm. f.) Lindau (*C. nutans*) is commonly found in Malaysia, Thailand and Indonesia. Traditionally used to treat snake and insect bites, skin rashes and others, it was evaluated for its anti-viral, anti-inflammatory and anti-cancer properties. Although used to treat skin rashes, its anti-allergy property was not evaluated. Hence, this study evaluated the anti-allergy property of *C. nutans* in *in vitro* and *in vivo* allergy models and its underlying mechanism. Meta-analysis was done to identify the commonly analysed soluble mediators in the less established IgG pathway (compared to IgE). The anti-allergy property via IgE pathway of 100% ethanolic, 70% ethanolic-aqueous, 50% ethanolic-aqueous and aqueous *C. nutans* extracts was assessed through *in vitro* IgE-induced mast cell degranulation model. The most active extract was then evaluated in IgG-induced macrophage activation model. The underlying mechanism was studied by analysing its effect on the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways' proteins by Western blotting. The effect was then validated in rodents. Acute toxicity test that analysed the haematological, biochemical and histological profiles was done to determine its safety and safe doses to be used. The overall effect was analysed in ovalbumin-challenged active systemic anaphylaxis (OVA-ASA), whereby both IgE and IgG pathways were activated. The effect of CNAE on specific targeted pathway was analysed in IgE-

challenged passive systemic anaphylaxis (IgE-PSA) and IgG-PSA models. Soluble mediators were quantified by enzyme-linked immunosorbent assay (ELISA). The commonly studied soluble mediators of IgG pathway identified through meta-analysis were platelet activating factor (PAF), histamine, interleukins (IL)-6, -13 and tumour necrosis factor-α (TNF-α). The most active extract - CNAE significantly reduced histamine and β-hexosaminidase in IgE-induced mast cell degranulation model at 5 mg/mL and above. In the IgG-induced macrophage activation model, significant decrease of IL-6 and TNF-α were recorded at 1.75 mg/mL and this was due to the inhibition of the phosphorylation of ERK1/2 of the MAPK pathway. From the acute toxicity test, 5000 mg/kg of CNAE (single dose) was not toxic to the animals and the doses - 125, 500 and 2000 mg/kg were chosen for subsequent analyses. In OVA-ASA, CNAE (2000 mg/kg) inhibited IgG (89.5%), PAF (171.1%) and IL-6 (92.6%) but not IgE. There was no significant inhibition of histamine, IL-4 and leukotriene C_4 (LTC_4) in IgE-PSA even at 2000 mg/kg. However, at 2000 mg/kg, there was 128.2% reduction of PAF and 124.4% reduction of IL-6 in IgG-PSA. Significant reduction of histamine in *in vitro* IgE-induced mast cell degranulation that was not recorded in IgE-PSA could be due to the overall effect of different cells that were activated in IgE-PSA. In the *in vitro* model, Rat Basophilic Leukaemic (RBL-2H3) cells which mimicked mast cells were specifically induced while in IgE-PSA, other cells with high-affinity IgE receptor (FcεRI) such as mast cells, eosinophils and basophils were challenged giving an overall effect. In conclusion, the anti-allergy property of *C. nutans* was potentially in CNAE which targeted the IgG pathway with significant reductions of PAF, IL-6 and TNF-α by inhibiting ERK1/2 pathway. As anaphylaxis is a systemic allergy, the anti-allergy effect of CNAE could be further evaluated on localised models such as atopic eczema or allergic alveolitis.

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

KESAN *IN VITRO* DAN *IN VIVO* EKSTRAK AKUEUS *Clinacanthus nutans* (BURM. F.) LINDAU PADA LALUAN ALERGI YANG DIMEDIASI OLEH IgE DAN IgG

Oleh

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Alergi adalah tindak balas hipersensitif terhadap antigen. Secara imunologi, alergi boleh dimediasi melalui imunoglobulin (Ig) -E dan IgG. Sebanyak 30-40% populasi dunia terjejas dengan alergi dan ini dianggarkan akan meningkat disebabkan oleh pembangunan. Allergi membawa impak negatif pada seseorang individu dan juga ekonomi negara. Oleh itu, ia memerlukan perhatian segera. *Clinacanthus nutans* (Burm. f.) Lindau (*C. nutans*) atau Belalai Gajah biasanya dijumpai di Malaysia, Thailand dan Indonesia. Secara tradisionalnya, ia digunakan untuk merawat gigitan ular dan serangga, ruam kulit dan lain-lain. Beberapa kajian saintifik telah membuktikan ia bersifat antivirus; antiradang dan antibarah. Walau bagaimanapun, sifat antialerginya belum dikaji walaupun ia digunakan untuk merawat ruam kulit. Oleh itu, kajian ini menilai sifat antialergi *C. nutans* dalam model alergi *in vitro* dan *in vivo* dan mekanismenya. Metaanalisis dilakukan untuk mengenal pasti mediator yang paling kerap dianalisis bagi alergi yang dimediasi oleh IgG; dimana ia kurang dikaji berbanding dengan laluan IgE. Sifat antialergi ekstrak 100% etanol (100% EtOH), 70% etanol-akueus (70% EtOH-Ak), 50% etanol-akueus (EtOH-Ak) dan ekstrak akueus (CNAE) *C. nutans* pertamanya dinilai menggunakan model degranulasi sel mast yang diinduksi oleh IgE *in vitro*. Kesan ekstrak paling aktif - CNAE kemudian dinilai dalam model pengaktifan makrofag yang disebabkan oleh IgG. Mekanisme antialergi CNAE kemudiannya dikaji dengan menganalisis kesannya pada laluan 'mitogen-activated protein kinase' (MAPK) dan fosfoinositida-3-kinase (PI3K) menggunakan kaedah 'Western blotting'. Seterusnya, kesan antialergi CNAE dinilai pada tikus. Kajian ketoksikan akut dilakukan untuk menilai keselamatannya dengan menilai profil hematologi,

biokimia dan histologi dan untuk menentukan dos yang selamat untuk analisis seterusnya. Kesan umum antialergi CNAE kemudian dianalisis dalam anafilaksis sistemik aktif yang dicabar oleh ovalbumin (OVA-ASA) di mana kedua-dua laluan IgE dan IgG diaktifkan. Untuk mengenal pasti laluan sasaran tertentu, kesan antialergi CNAE dianalisis lebih lanjut dalam model anafilaksis pasif IgE-PSA dan IgG-PSA. Mediator yang dihasilkan dinilai dengan pemeriksaan imunosorben berkait enzim (ELISA). Dari kajian metaanalisis, mediator laluan IgG yang paling kerap dikaji adalah faktor pengaktifan platelet (PAF), histamin, interleukin (IL) -6, -13 dan faktor nekrosis tumor-α (TNF-α). Ekstrak paling aktif - CNAE dapat mengurangkan tahap histamin dan β-heksosaminidase secara signifikan pada model degranulasi sel mast yang disebabkan oleh IgE pada kepekatan 5 mg/mL dan ke atas. Sebaliknya, dalam model pengaktifan makrofag yang disebabkan oleh IgG, penurunan IL-6 dan TNF-α yang ketara dicatatkan pada kepekatan 1.75 mg/mL. Pengurangan IL-6 dan TNF-α adalah hasil penghambatan fosforilasi ERK dalam laluan MAPK. Kajian ketoksikan akut menunjukkan bahawa satu dos CNAE pada 5000 mg/kg tidak toksik kepada haiwan. Dalam OVA-ASA, CNAE pada 2000 mg/kg mencatatkan perencatan IgG (89.5%), PAF (171.1%) dan IL-6 (92.6%) tanpa pengurangan tahap IgE. Tidak ada penurunan histamin, IL-4 dan leukotriena C_4 (LTC_4) direkodkan dalam IgE-PSA walaupun pada 2000 mg/kg. Walau bagaimanapun, pada 2000 mg/kg, terdapat penurunan PAF sebanyak 128.2% dan penurunan IL-6 (124.4%) dalam IgG-PSA. Penurunan histamin pada model degranulasi sel mast yang disebabkan oleh IgE tidak direkodkan dalam IgE-PSA berkemungkinan disebabkan perbezaan sel yang diaktifkan dalam kedua-dua model ini. Dalam model *in vitro*, sel basofilik yang menyerupai sel mast diaktifkan sebaliknya sel lain seperti sel mast, sel basofilik dan macrofaj juga diaktifkan dalam model *in vivo*. Secara keseluruhannya, sifat antialergi *C. nutans* berpotensi dijumpai dalam CNAE dan mensasarkan laluan alergi IgG dengan penurunan PAF, IL-6 dan TNF-α yang ketara yang mensasarkan laluan ERK1/2. Anafilaksis adalah model alergi sistemik, oleh itu sifat antialergi CNAE boleh dikaji ke atas model alergi setempat seperti ekzema atopi atau alergi alveolus.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

1H-NMR	Proton nuclear magnetic resonance
AAPH	2,2'-azobis (2-amidinopropane) dihydrochloride
ANOVA	One-way analysis of variance
Btk	Bruton's tyrosine kinase
Ca2+	Calcium
C. nutans	*Clinacanthus nutans*
CEFs	Chick embryo fibroblasts
cGMP	Cyclic guanosine monophosphate
CNAE	100% aqueous Clinacanthus nutans
cPLA2	Cytoplasmic phospholipase A2
DAG	Diacylglycerol
DGDG	Digalactosyl diglyceride
DNA	Deoxyribonucleic acid
DNP-BSA	2,4-dinitrophenol bovine serum albumin
DPPH	1,1-diphenyl-2-picrylhydrazyl
DV2	Dengue virus serotype 2
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanolic
EtOH-Aq	Ethanolic-aqueous
EPP	Ethyl phenylpropiolate
ERK	Extracellular signal-regulated kinases
FcεRI	High-affinity IgE receptor

FcγR	Low-affinity receptor
FRAP	Ferric reducing antioxidant power
fMLP/CB	N-formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B
GAB2	GRB2-associated-binding protein 2
GDP	Guanosine diphosphate
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HSV	Herpes Simplex virus
IC50	Half maximal inhibitory concentration
IFN	Interferon
Ig	Immunoglobulin
IgE-PSA	Immunoglobulin-E-challenged passive systemic anaphylaxis
IgG-PSA	Immunoglobulin-G-challenged passive systemic anaphylaxis
ITAMs	Immunoreceptor tyrosine-based activation motifs
IL	Interleukin
JNK	c-Jun N-terminal kinase
LAT	Linker for activation of T cells
LTC4	Leukotriene C4
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEK	MAP/ERK kinase
MGDG	Monogalactosyl diglyceride
MIP-1α	Macrophage inflammatory protein-1α
MPO	Myeloperoxidase

NO	Nitric oxide
NTAL	Non-T-cell activation linker
OVA	Ovalbumin
OVA-ASA	Ovalbumin-challenged active systemic anaphylaxis
PA	Phosphatidic acid
PAF	Platelet activating factor
PDK1	PI3K-dependent protein kinase 1
PH	Pleckstrin homology
PI3K	Phosphoinositide-3-kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PLD	Phospholipase D
PLS	Partial least-square
PMA	Phorbol myristate acetate
Ras	Guanine nucleotide-binding protein
RBL-2H3	Rat Basophilic Leukaemic cells
RBP4	Retinol binding protein 4
RPM	Revolutions per minute
SOD	Superoxide dismutase
SOS	Son of Sevenless
TCM	Traditional and complementary medicine
TCR	T cell antigen receptor

TKBM	Traditional knowledge-based medicine
TNF-α	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor
VZV	Varicella-Zoster virus
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Background

Allergy is a hypersensitivity reaction caused immunologically and it can be presented in many forms such as anaphylaxis, urticaria, asthma, atopic dermatitis and many others (Ring, 2014). The prevalence of allergic diseases has been on the rise in the last decade. It was estimated that globally, 30 – 40% of people will be affected by one form or another of allergy and the most vulnerable group comes from the younger population (Pawankar *et al.*, 2011). The rise of allergy prevalence puts an economic burden to the world and affected individuals usually have decreased quality of life as they experienced limitation to their activity and function (Mendis, 2014). Hence, it is a matter that requires urgent attention.

The use of natural products in the form of plant to address medical needs is a common practice among the locals of a certain country. It is usually a practice that was passed down from previous generations. Historical documents showed that medicinal plants have been in used in the past 60,000 years in Iraq and elsewhere (Pan *et al.*, 2014). Under the wide umbrella of traditional medicine, plant-based medicines have been gaining more attention lately. This is due to the side effects of modern medicine; the limitation of modern medicines in treating the root of the disease; microbial resistance and unparalleled spending on pharmaceutical research and development of modern medicine (Pan *et al.*, 2014). Undeniably there are many beneficial plants that are yet to be discovered in our backyard. However, the use of traditional plant-based medicine is usually shunned upon as it lacks proper scientific evaluation and proper regulation. Furthermore, there is no good documentation that could support the beneficial usage of these plants. Hence, it is of importance to scientifically evaluate and document the traditional medicinal claims of these plants so that they could be use in complement to modern medicine practice for a better outcome.

Clinacanthus nutans Burm. f. (Lindau) (*C. nutans*) is a perennial plant found mainly in Southeast Asia especially in Malaysia, Thailand and Indonesia. Known by the locals by many names – *'Belalai Gajah'*, *'You Dun Cao'*, *'Payayor'* and *'Daun Dandang Gendis'* - this shrub was used to treat many conditions in these countries (Khoo *et al.*, 2018b). These include snake and insect bites, skin rashes, eczema, Varicella Zoster lesions, diabetes, haematoma, gastrointestinal problems to name a few (Yahaya *et al.*, 2015; Zulkipli *et al.*, 2017). Based on these traditional claims, numerous pharmacological studies have been conducted such as anti-viral activities against Herpes Simplex virus (HSV), Varicella-Zoster virus (VZV) and dengue; anti-inflammatory activity, anti-venom, anti-nociceptive, anti-bacterial and anti-

fungal, anti-cancer, anti-diabetic, anti-oxidant, immunomodulating property and neuronal protection (Khoo *et al.*, 2018b). *C. nutans* have been used as a traditional remedy for skin rashes - a characteristic of an allergic reaction but it has not been pharmacologically evaluated for its anti-allergy property. Thus, this study was aimed to identify the potential anti-allergy property of this plant. This study will also expand the pharmacological documentation of *C. nutans* in the Malaysian Herbal Monograph (MHM) and it could also be beneficial to consumers and the economy of one country.

1.2 Problem Statement

The number of allergy cases worldwide is projected to rise as a result of urbanisation and the burden of allergy is not limited to only the affected individual. It is thus a matter of concern which requires urgent attention. *C. nutans* is a perennial shrub that has been used traditionally among locals of Southeast Asian countries to treat skin rashes, a symptom of allergy. However, there are no pharmacological studies thus far that has evaluated the anti-allergy property of this plant. Hence, in this study the anti-allergy property of *C. nutans* was evaluated both *in vitro* using cells and *in vivo* using rodents. Additionally, the mode of action was also studied.

1.3 Direction of Study

In this study, the anti-allergy property of *C. nutans* was analysed in both *in vitro* and *in vivo* models of allergy. Allergy can be mediated through two pathways known as the IgE-mediated pathway (classical) and IgG-mediated pathway (alternative). In comparison to the IgE-mediated pathway which is more commonly studied, the IgG-mediated pathway is relatively new and less studied. Until now there was no consensus on the commonly studied pro-inflammatory soluble mediators for this pathway unlike in the IgE-mediated pathway. Therefore, a meta-analysis study was conducted to identify the pro-inflammatory soluble mediators that were commonly evaluated in an IgG-mediated pathway of allergy. Meta-analysis is a statistical analysis of numerous individual studies with the aim to consolidate the outcomes. Results from previous studies were systematically assessed and quantitated in order to draw conclusions about the study (Haidich, 2010). Hence, in this meta-analysis, the most common pro-inflammatory soluble mediators that are released upon induction by IgG were being identified from a collection of previous studies conducted. Subsequently, these identified soluble mediators were evaluated in the following assays to determine the anti-allergy property of *C. nutans* in the IgG-mediated pathway.

C. nutans was extracted using two different solvents, namely ethanol and water in different compositions yielding four different extracts. These extracts – 100% ethanol; 70% ethanol: 30% water; 50% ethanol: 50% water and 100% water

(denoted as 100% ethanol, 70% ethanol-aqueous, 50% ethanol-aqueous and 100% aqueous respectively hereafter) were prepared and the most active extract was identified via the *in vitro* IgE-induced mast cell degranulation model. The most active extract among the four was determined by its ability to reduce the amount of pro-inflammatory soluble mediators released upon treatment. Through this model, the anti-allergy effect of the four extracts were also analysed. As allergy is mediated through two pathways, the anti-allergy property of the most active extract was then evaluated in the IgG-induced macrophage activation model. After which, the mode of action of this most active *C. nutans* extract was explored by evaluating its effect on a number of signaling proteins that governs the degranulation and activation of the immune cells to produce pro-inflammatory soluble mediators. This happened mainly through the activation of MAPK and Akt pathways. In these pathways, signaling proteins such as ERK, JNK and p38 of the MAPK pathway were evaluated while the Akt protein was evaluated in the Akt pathway. Evaluation was done by quantifying the upregulation or down regulation of these signaling proteins by Western blot analysis.

Next, the study of the anti-allergy property of *C. nutans* was conducted *in vivo* in rodents using anaphylaxis models. Anaphylaxis models were chosen as anaphylaxis is the acute form of allergy and it involves the whole body i.e., systemic reaction. The models were ovalbumin-challenged active systemic anaphylaxis (OVA-ASA), IgE-challenged passive systemic anaphylaxis (IgE-PSA) and IgG-challenged passive systemic anaphylaxis (IgG-PSA). Before evaluating the anti-allergy effect of the most active extract in a biological system, the toxicity of the extract was determined. This was to ensure that the extract does not cause any harm to the animals and also to determine the safe doses that could be used for the anti-allergy experiments. An acute toxicity study profile of the most active extract was done with the highest recommended dosage by the Organisation of Economic Co-operation and Development (OECD) i.e., at 5000 mg/kg. The toxicity effect was assessed based on physical and behavioural observations including haematological, biochemical and histopathological analyses.

After determining the safety of the extract, the anti-allergy effect was explored in the OVA-ASA model where both IgE and IgG pathways of allergy could be activated. As this model activates both pathways, it aimed to give a general evaluation on the effect of the extract. To further determine the targeted pathway of the extract, specific IgE and IgG passive systemic anaphylaxis (PSA) models were used next. In the IgE-PSA model, only the IgE-mediated pathway will be activated while only the IgG-mediated pathway will be activated in the IgG-PSA model. The anti-allergy effect of the most active extract was determined through the ability to suppress the amount of the pro-inflammatory soluble mediators that were released by ELISA.

1.4 Research Objectives

1.4.1 General objectives

In this study, the anti-allergy property of *C. nutans* (Burm. f.) Lindau was analysed both *in vitro* and *in vivo*. In addition, the mode of action was studied.

1.4.2 Specific objectives:

I. To identify the common soluble mediators that are important in IgG-mediated allergy by meta-analysis

II. To evaluate the anti-allergy property of *C. nutans* extracts by *in vitro* IgE-induced mast cell degranulation model and most active extract in IgG-induced macrophage activation model and to analyse its anti-allergy mode of action

III. To evaluate the acute toxicity of the most active *C. nutans* extract in rodents

IV. To validate the anti-allergy property of the most active *C. nutans* extract in rodents by OVA-ASA model and to identify the anti-allergy pathways inhibited by the most active *C. nutans* extract by IgE-PSA and IgG-PSA models

1.5 Hypotheses

It was hypothesized that *C. nutans* extract does possess anti-allergy property and it will exert its effect via IgE- or IgG-mediated pathway through regulation of certain signaling molecules in allergy.

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