UNIVERSITI PUTRA MALAYSIA

POTENTIAL INHIBITION OF PRO-INFLAMMATORY MEDIATORS BY Ficus deltoidea Jack LEAVES AQUEOUS EXTRACT IN LIPOPOLYSACCHARIDE-INDUCED MICROGLIAL CELLS

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FPSK(m) 2022 22

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

November 2021

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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To date, Alzheimer's disease (AD) is responsible for the majority of dementia cases among the elderly population around the globe. Neuroinflammation and oxidative stress are among the fundamental factors that lead to the progression of the disease. Recently, there has been a resurgence of interest in implementing natural products to slow the progression of AD due to their enhanced therapeutic benefits when compared to available synthetic drugs. *Ficus deltoidea* (FD), also known as "Mas cotek", is widely consumed in traditional medicine as a treatment to various ailments in Malaysia. Among the many types of bioactive compounds, vitexin and isovitexin are abundantly found in the leaves of FD that possessed many pharmacological properties including neuroprotection. Nonetheless, its effects on key events in neuroinflammation are unknown. In this study, FD aqueous extract was investigated for its potential anti-neuroinflammatory and antioxidant properties on lipopolysaccharide-induced microglial cells by assessing the level of pro-inflammatory and cytotoxic factors. FD aqueous extract was prepared and freeze dried prior to use in *in vitro* study. Quantification of vitexin and isovitexin in the extract was carried out using high performance liquid chromatography (HPLC). In silico test was performed to predict the effectiveness of the ligand molecules of both vitexin and isovitexin to penetrate through the blood-brain barrier (BBB). The extract was evaluated for its cytotoxicity activity via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Pre-treatment with the extract on lipopolysaccharide (LPS)-induced microglial cells was done to determine its anti-neuroinflammatory and antioxidant properties by measuring the level of reactive oxygen species (ROS), nitric oxide (NO), tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) via 2′-7′-dichlorofluorescin diacetate (DCFDA) assay, Griess assay and Western blot respectively. The result showed that the extract at tested concentrations (0.1, 1, 10, 100 µg/mL) were not cytotoxic as the percentage cell viability were above ~80%. At the highest concentration (100 µg/mL), the extract significantly reduced the production of ROS, NO, TNF-α, IL-

1β and IL-6 in microglial cells induced by LPS. From this study, the extract demonstrated neuroprotective effects by attenuating the production of pro-inflammatory and cytotoxic factors in LPS-induced microglial cells, possibly by mediating the nuclear factor-kappa B (NF-κB) signalling pathway.

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

POTENSI EKSTRAK AIR DAUN *Ficus deltoidea Jack* UNTUK MENGHALANG PENGHASILAN AGEN PRORADANG KE ATAS SEL MIKROGLIA TERARUH LIPOPOLISAKARIDA

Oleh

SITI ZAIDATHUL IMAN BINTI ZOLKIFFLY

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Kini, penyakit Alzheimer merupakan penyebab utama bagi kebanyakan kes demensia dalam kalangan warga tua di seluruh dunia. Radang saraf dan tekanan oksidatif adalah antara faktor utama yang membawa kepada perkembangan penyakit ini. Sejak kebelakangan ini, terdapat fokus baharu dalam merencatkan perkembangan penyakit ini iaitu penerapan produk semula jadi yang kaya dengan pelbagai manfaat berbanding dengan ubat sintetik di pasaran. *Ficus deltoidea* (FD), atau dikenali sebagai "Mas cotek", banyak digunakan dalam perubatan tradisional sebagai rawatan untuk pelbagai penyakit di Malaysia. Vitexin dan isovitexin adalah antara sebatian bioaktif yang terdapat di dalam daun FD dan mempunyai sifat farmakologi seperti perlindungan saraf. Namun begitu, kesan FD kepada fasa penting dalam proses radang saraf masih tidak diketahui. Dalam kajian ini, potensi ekstrak air FD sebagai antiradang saraf dan antioksidan kepada sel mikroglia teraruh lipopolisakarida dinilai berdasarkan kadar produksi faktor-faktor proradang dan sitotoksik. Ekstrak air FD disediakan dan dikeringbekukan sebelum digunakan dalam kajian *in vitro*. Proses kuantitatif vitexin dan isovitexin dalam ekstrak tersebut dinilai menggunakan kromatografi cecair prestasi tinggi. Ujian *in silico* dijalankan bagi menguji keupayaan molekul ligan vitexin dan isovitexin untuk menembusi sawar darah otak. Seterusnya, penilaian kesitotoksikan ekstrak dilakukan melalui ujian 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT). Bagi mengenal pasti tahap spesies oksigen reaktif (ROS), nitrik oksida (NO), faktor nekrosis tumor-α (TNF-α), interleukin-1β (IL-1β) dan interleukin-6 (IL-6), pra-rawatan dengan ekstrak ke atas sel mikroglia teraruh lipopolisakarida telah dijalankan melalui ujian 2′-7′-dichlorofluorescin diacetate (DCFDA), ujian Griess dan blot barat. Hasil kajian menunjukkan bahawa ekstrak pada kepekatan yang diuji (0.1, 1, 10, 100 µg/mL) tidak sitotoksik kerana peratusan daya hidup sel berada dalam lingkungan ~ 80%. Pada kepekatan tertinggi (100 µg/ mL), ekstrak tersebut berjaya menurunkan jumlah ROS, NO, TNF-α, IL-1β dan IL-6 dalam sel mikroglia teraruh

lipopolisakarida. Justeru, ekstrak air FD menunjukkan kesan positif dalam perlindungan saraf melalui pengurangan faktor-faktor proradang dan sitotoksik dalam sel mikroglia teraruh lipopolisakarida. Proses ini diyakini berlaku melalui laluan isyarat faktor nuklear-kappa B.

ACKNOWLEDGEMENTS

All glory is due to Allah, the Almighty, whose Grace and Mercy have been with me throughout the duration of my research project entitled “Potential Inhibition of Pro-Inflammatory Mediators by *Ficus deltoidea* Jack Leaves Aqueous Extract in Lipopolysaccharide-Induced Microglial Cells.”

I would like to extend my gratitude and appreciation to my supervisor, Dr. Muhammad Zulfadli bin Mehat and co-supervisor, Prof. Johnson Stanslas for their guidance and assistance throughout the project. With their support and cooperation, I was able to complete my research project successfully.

To all the postgraduate students from both Pharmacotherapeutic Laboratory and Human Anatomy Laboratory in Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, I offer my gratitude for your endless guidance in helping me to complete my research project. Their insightful comments and suggestions had helped me a great deal.

My gratitude goes to my beloved parents, Zolkiffly bin Ab. Aziz and Zaharah binti Hasan, my husband as well as family members for their constant prayers, understanding and endurance while away.

Lastly, to my best friend, Nashreeyn binti Mohamed Naeemmudeen, I am grateful to have you by my side in this journey. A good friend is like a four-leaf clover: hard to find and lucky to have.

This thesis was 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 were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research and the writing of this thesis were done under our supervision;
- supervisory responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2015-2016) are adhered to.

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LIST OF ABBREVIATIONS

°C	Degree of Celsius
AChEIs	Acetyl cholinesterase inhibitors
AD	Alzheimer's disease
AICD	APP intracellular domain
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
Arg-1	Arginase-1
Aβ	Amyloid beta protein
BBB	Blood-brain barrier
BSA	Bovine serum albumin
BV-2	Microglial mouse cell line
Ca^{2+}	Calcium ions
CNS	Central nervous system
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DCF	2',7'-dichlorofluorescein
DCFDA	2',7'- dichlorofluorescin diacetate
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylydrazyl
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FD	*Ficus deltoidea*

FDA	Food and Drug Administration
FRAP	Ferric reducing antioxidant potential
FT-IR	Fourier transform infrared
FTC	Ferric thiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDO	Global Dementia Observatory
H_2O_2	Hydrogen peroxide
HD	Huntington's disease
HPLC	High performance liquid chromatography
IFNs	Interferons
IKK	IκB kinase
IL-10	Interleukin-10
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IκB	Inhibitory protein
LOD	Limit of detection
LOQ	Limit of quantitation
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MS	Multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide
MyD88	Myeloid differentiation factor 88

NaCl	Sodium chloride
NCE	Novel chemicals entities
NED	N-1-napthylthylenediamine dihydrochloride
NF-κB	Nuclear factor-kappa B
NMDA	N-Methyl-D-aspartic acid
NO	Nitric oxide
P/S	Penicillin-streptomycin
PBS	Phosphate buffered saline
PD	Parkinson's disease
PDA	Photodiode array
PGE2	Prostaglandin E2
psi	Pounds per square inch
QPlogBB	Brain and blood partition co-efficient
QPlogPo/w	Hydrophobicity
QPlogS	Aqueous solubility
r^2	Regression
RNS	Reactive nitrogen species
RO5	Rule of Five
ROS	Reactive oxygen species
RSD	Relative standard deviation
RT	Retention time
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STZ	Streptozotocin

TBA	Thiobarbituric acid
TBS-T	TBS containing 0.1% of Tween 20
TEMED	Tetramethylethylenediamine
TLR-4	Toll-like receptor 4
TNF-α	Tumour necrosis factor-α
TPC	Total phenolic content
TREM2	Triggering receptor in myeloid cells-2
Tris-HCl	Tris hydrochloride
v/v/v	Volume/volume/volume
w/v	Weight/volume
w/w	Weight/weight
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background of study

Among several types of neurodegenerative brain disorders, Alzheimer's disease (AD) is known to be the most common one. AD is an irreversible pathological condition that is responsible for the majority of dementia cases among the elderly population around the globe (Kumar et al., 2015; Godyn et al., 2016). In addition to neurodegeneration, AD is characterized by impairment in cognition, abnormal behaviour and personality changes that ultimately lead to full dementia. Previous studies have shown that AD is histopathologically characterized by the presence of extracellular senile plaques around reactive microglia, abundant intracellular neurofibrillary tangles and progressive loss of neurons in the brain (Li et al., 2012; Moon et al., 2012). The extracellular senile plaques and intracellular neurofibrillary tangles are composed of the accumulation of amyloid beta protein (Aβ) and hyperphosphorylated tau protein respectively (Cheignon et al., 2018). Currently, the worldwide number of AD cases is more than 46 million and it is expected to increase to 131.5 million by the year 2050, with the majority are from low- and middle-income countries (Alzheimer's Disease International, 2015). In Malaysia, the prevalence of dementia is estimated to be at 0.126% and 0.454% in 2020 and 2050 respectively (Tey et al., 2016).

The complexity of the pathogenesis of AD is multifactorial. This includes genetic factors, traumatic brain injury, inflammation, hyperglycemia, hormonal disturbance and depression (Godyn et al., 2016). Several fundamental cellular and molecular events that cause neurodegeneration are neuroinflammation, oxidative stress, induction of apoptosis, modification of autophagy, deposition of protein aggregates and impaired mitochondrial function (Hoglund and Salter, 2013). To date, there are two classes of approved drugs used to treat AD namely acetyl cholinesterase inhibitors (AChEIs) and N-Methyl-D-aspartic acid (NMDA) receptor antagonist, in which 80% of AD patients receive therapy with these drugs (Johnell et al., 2013). However, published reports have shown that the drugs caused adverse effects such as gastrointestinal problems (nausea, vomiting and diarrhoea) and cardiovascular effects (syncope, bradycardia and torsades de pointes ventricular tachycardia) (Howes, 2014). Not only that, none of the available therapies manage to halt the progressive loss of neurons that later deteriorate the cognitive faculties.

1.2 Significance of study

Therefore, this necessitates the crucial need to explore a more effective therapy with less side effects to provide neuroprotection for AD patients. One of such strategy is the implementation of natural products in drug discovery which have

been the basis for many early medicines such as aspirin, morphine, digitoxin and pilocarpine (Butler, 2004). Hence, this study will investigate in-depth of a specific natural plant product as a new therapeutic approach capable of reducing the production of pro-inflammatory cytokines and oxidative stress mediators involved in neuroinflammation. One significant plant widely known for its potential pharmaceutical importance is *Ficus deltoidea* or traditionally known as Mas Cotek. According to Kementerian Pertanian dan Industri Asas Tani (2011), *Ficus deltoidea* is among 11 medicinal herbs that have been identified with great potential to be developed into high-value products. All the medicinal herbs are actively cultivated for commercial purposes. *Ficus deltoidea* is a native epiphytic shrub abundantly distributed throughout Southeast Asia countries such as Malaysia, Indonesia and Thailand (Dzolin et al., 2012). Previous results showed that the extract of *Ficus deltoidea* possessed pharmacological properties such as neuroprotective (Nurdiana et al., 2018), antioxidant (Omar et al., 2011), anti-diabetic (Choo et al., 2012), anti-inflammatory (Zakaria et al., 2012) and anti-nociceptive (Sulaiman et al., 2008).

For decades, researchers have extracted and identified vitexin and isovitexin as bioactive components that have various biological activities and pharmacological potentials. The leaves of *Ficus deltoidea* is rich in bioactive compounds and one of them is flavonoids in which vitexin showed a great number of pharmacological activities including antioxidant and neuroprotective activities as well as anti-Alzheimer's (Choi et al., 2014; Wang et al., 2015; Mun et al., 2019). According to He et al., (2016) these compounds exerted significant outcomes in treating AD among many other diseases. Thus, this study serves to explore the anti-neuroinflammatory and antioxidant properties of *Ficus deltoidea.* Subsequently, data from this study can be used to complement *in vivo* studies in representing a therapeutic approach for the screening of potential drugs in the treatment of AD in future.

1.3 Research hypotheses

1. *Ficus deltoidea* aqueous extract improves the viability of BV-2 cells.
2. *Ficus deltoidea* aqueous extract lowers the production of the inflammatory mediator, nitric oxide (NO), in LPS-induced BV-2 cells.
3. *Ficus deltoidea* aqueous extract lowers the production of reactive oxygen species (ROS) in LPS-induced BV-2 cells.
4. *Ficus deltoidea* aqueous extract reduces the protein expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in LPS-induced BV-2 cells.

1.4 Research objectives

1.4.1 General objective

The general objective of this study is to investigate the anti-neuroinflammatory and antioxidant properties of *Ficus deltoidea* aqueous extract in *in vitro* models that can potentially uncover future drug candidates for AD.

1.4.2 Specific objectives

1. To develop and validate a HPLC method for the standardization of *Ficus deltoidea* aqueous extract.
2. To predict the pharmaceutical properties of vitexin and isovitexin.
3. To determine the cytotoxicity of *Ficus deltoidea* aqueous extract on BV-2 cells.
4. To investigate the effect of *Ficus deltoidea* aqueous extract on the production of the inflammatory mediator, nitric oxide (NO), in LPS-induced BV-2 cells.
5. To assess the effect of *Ficus deltoidea* aqueous extract on the production of reactive oxygen species (ROS) in LPS-induced BV-2 cells.
6. To determine the effect of *Ficus deltoidea* aqueous extract on the protein expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in LPS-induced BV-2 cells.

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