UNIVERSITI PUTRA MALAYSIA

UNTARGETED TANDEM MASS SPECTROMETRY-BASED MOLECULAR NETWORK AND ZEBRAFISH EMBRYOTOXICITY AND TERATOGENIC EFFECTS OF Christia vespertilionis (L. F.) Bakh. F. LEAF EXTRACT

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By

ANIS IRFAN BINTI NORAZHAR

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

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UNTARGETED TANDEM MASS SPECTROMETRY-BASED MOLECULAR NETWORK AND ZEBRAFISH EMBRYOTOXICITY AND TERATOGENIC EFFECTS OF *Christia vespertilionis* (L. F.) Bakh. F. LEAF EXTRACT

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Chairman : Professor Khozirah Shaari, PhD
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Christia vespertilionis (L.f.) Bakh. f., is a non-climbing ornamental plant with unique butterfly-shaped leaves, hence its vernacular name 'butterfly wing' or 'pokok rerama' in Malay. In Malaysia, the green-leafed variety gained popularity in recent years due to testimonial reports by local users and distributors for its medicinal uses, which included among others, as a cure for cancer. Despite these popular uses, there is very limited information on the chemical constituents of the species, presenting a significant gap in the cheminformatics of the species. Additionally, more information on the safety profile of the plant is required, towards safeguarding consumer safety. Therefore, the primary objectives of the present study were to establish the chemical profile of the green-leafed variety of the plant, specifically the leaf extract and to evaluate the extract for potential toxicity effects. The metabolite profile of the leaf methanolic extract was established by deploying untargeted tandem mass spectrometry-based molecular networking approach. The toxic effects of the extract involving mortality rate, heartbeat rate, hatchability rate, and spontaneous tail coliling as well as the teratogenic effects were determined on zebrafish (*Danio rerio*) embryos, as the *in-vivo* assay model. Zebrafish embryos at 5 hour post-fertilization (hpf) were exposed to 50, 100, 200, 400, and 800 µg/mL of the extract up to 120 hpf. The multi-informative molecular map generated for the leaf metabolome permitted the putative identification of 62 metabolites, comprising 10 *C*-glycosylflavones, 2 mono- and 2 di-hydroxyflavones, 1 flavone-*C,O*-diglycoside, 3 flavonol-3-*O*-glycosides, 15 phenolic acids, 1 phenethyl glycoside and 1 its derivative, 4 hydroxyjasmonic acid derivatives, 4 carotenoids, 2 chlorophylls, 3 monoacylglycerols, 1 sphingolipids, 4 amino acids, 1 nucleoside, 3 organic acids, 1 coumarin derivative and 4 fatty acid amides. As a means of structural validation, two unknown chemical constituents were targeted for isolation which subsequently resulted in their characterization as apigenin-6-*C*-*β*-D-glucoside 4′-O-*α*-D-apiofuranoside **[2]** and apigenin-6-*C*-*β*-D-[(4″,6″-*O*-dimalonyl)-glucoside] 4′-*O*-*α*-D-apiofuranoside **[10]**, which were newly reported in the plant kingdom as the

new derivatives of apigenin-6-*C*-*β*-D-glucoside. In the toxicity assay, the median lethal concentration (LC_{50}) value of the extract was determined to be 419.84 µg/mL, which was classified as safe. Nevertheless, from evaluation of possible teratogenic effects on zebrafish embryos, it was further revealed that the extract was toxic at higher concentrations starting from 200 µg/mL onwards with multiple signs of developmental defects. The magnitude of these defects was observed to be concentration-dependent. Moreover, no hatching and spontaneous movement of tail coiling were observed at concentrations of 400 and 800 µg/mL due to the delayed growth and early mortality, respectively. A significant reduction in heartbeat rate was also recorded in all surviving embryos at 400 µg/mL. The present study has provided some important insights on the plant's chemical and biological characteristics which are a pre-requisite to further research towards its valorization as a candidate in phytotherapy. Further extensive studies of the leaf extract using other animal models as well as the *in-vi*tro assay are required for the establishment of its safe dose for human consumption.

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

SPEKTROMETRI JISIM TANDEM TAK BERTUMPU BERASASKAN RANGKAIAN MOLEKULAR DAN KESAN TOKSIK DAN TERATOGENIK EMBRIO ZEBRAFISH EKSTRAK DAUN *Christia vespertilionis* (L. F.) Bakh. F.

Oleh

ANIS IRFAN BINTI NORAZHAR

Ogos 2020

Pengerusi : Profesor Khozirah Shaari, PhD
Institut : Biosains

Christia vespertilionis (L. f.) Bakh. f., adalah tanaman hiasan yang tidak memanjat dengan daun berbentuk rama-rama yang unik, oleh itu nama vernakularnya 'sayap rama-rama' atau 'pokok rerama' dalam bahasa Melayu. Di Malaysia, varieti daun hijau memperoleh popularity dalam beberapa tahun kebelakangan ini kerana laporan testimoni oleh pengguna dan pengedar tempatan mengenai kegunaan perubatannya, yang antara lainya termasuklah sebagai penawar penyakit barah. Walaupun penggunaannya popular, terdapat maklumat yang sangat terhad tentang unsur kimia spesies ini, yang menunjukkan jurang yang ketara. Sebagai tambahan, lebih banyak maklumat mengenai profil keselamatan pokok ini diperlukan, untuk menjamin keselamatan pengguna. Oleh itu, objektif utama kajian ini adalah untuk menentukan profil sebatian kimia dari varieti daun hijau, khususnya ekstrak daun dan untuk menilai kemungkinan kesan toksik ekstrak ini. Profil sebatian kimia ekstrak metanol daun ditentukan dengan menggunakan pendekatan rangkaian molekul berasaskan spektrometri jisim tandem tak bertumpu. Kesan-kesan toksik dari ekstrak yang melibatkan kadar kematian, degupan jantung, kadar penetasan, dan gelungan spontan ekor serta kesan teratogenik ditentukan ke atas embrio zebrafish (*Danio rerio*), sebagai model uji *in-vivo*. Embrio zebrafish pada 5 jam pasca pensenyawaan (hpf) didedahkan kepada 50, 100, 200, 400, dan 800 µg/mL ekstrak sehingga 120 hpf. Peta molekul pelbagai maklumat yang dihasilkan daripada metabolom daun membolehkan pengenalpastian sebanyak 62 sebatian kimia yang terdiri daripada 10 *C*-glikosilflavon, 2 mono- dan 2 di-hidroksiflavon, 1 flavon *C*,*O*-glikosida, 3 flavonol-3-*O*-glikosida, 15 fenolik asid, 1 fenil glikosida dan 1 deravatifnya, 4 deravatif asid hidroksijasmonik, 4 karotenoid, 2 klorofil, 3 monoacilgliserol, 1 sphingolipid, 4 asid amino, 1 nukleosida, 3 asid organik, 1 derivatif kumarin dan 4 asid lemak amida. Untuk pengesahan struktur kimia, dua komponen kimia yang tidak diketahui telah disasarkan untuk pengasingan yang kemudiannya dicirikan sebagai apigenin-6-*C*-*β*-D-glukosida

4′-O-*α*-D-apiofuranosida **[2]** and apigenin-6-*C*-*β*-D-[(4″,6″-*O*-dimalonil)-glukosida] 4′-*O*-*α*-D-apiofuranosida **[10]**. Dalam uijian toksik, nilai kepakatan median maut (LC_{50}) ekstrak ditentukan sebagai 419.84 μg/mL, yang diklasifikasikan sebagai selamat. Walaupun begitu, berdasarkan penilaian kemungkinan kesan teratogenik ke atas embrio zebrafish, didapati bahawa ekstrak ini sangat toksik pada kepekatan yang tinggi bermula dari 200 μg/mL dan seterusnya dengan pelbagai tanda kecacatan perkembangan. Magnitud kecacatan ini dilihat bergantung kepada kepekatan. Selain itu, tiada penetasan dan pergerakan spontan gegelung ekor diperhatikan pada kepekatan 400 dan 800 μg/mL kerana pertumbuhan yang terbantut dan kematian awal, masing-masing. Kajian ini telah memberikan beberapa pandangan penting mengenai ciri-ciri kimia dan biologi pokok ini yang menjadi prasyarat untuk penyelidikan selanjutnya ke arah membangunkannya sebagai calon fitoterapi. Kajian luas yang lebih lanjut mengenai ekstrak daun ini menggunakan model haiwan lain dan juga ujian *in-vitro* diperlukan untuk menentukan dos yang selamat untuk penggunaan manusia.

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This thesis was submitted to the Senate of the 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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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

1D	One-Dimensional
2D	Two-Dimensional
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C-NMR	Carbon -13 Nuclear Magnetic Resonance
ACN	Acetonitrile
CD_3OD	Deuterated Methanol
CID	Collision-Induced Dissociation
COSY	Correlation Spectroscopy
d	Doublet
DAD	Diode Array Detector
dd	Doublet of Doublet
ESI	Electrospray Ionization
FA	Formic Acid
HESI	Heated Electrospray Ionization
HMBC	Heteronuclear Multiple Bond Coherence
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
J	Coupling Constant
LC-MS	Liquid Chromatography Mass Spectrometry
mg	Milligram
µg	Microgram
mL	Milliliter
min	Minute
m/z	Mass-to-charge ratio
nm	Nanometer
ppm	Parts per million

Prep-HPLC	Preparative High Performance Liquid Chromatography
PTFE	Polytetrafluoroethylene
RT	Retention time
s	Singlet
t	Triplet
TIC	Total Ion Chromatogram
TMS	Tetramethylsilane
UPLC-MS/MS	Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background

Since time immemorial, humans have relied on the plants as a main source of therapeutic agents for the treatment of various kinds of diseases and ailments, as well as for maintaining their health. The precious ethnomedicinal knowledge of the diverse plant resources used by the different cultures around the world has been passed on over many generations. Despite the exponential growth of the utilization of synthetic drugs in modern treatment, the practice of using herbs and herbal formulations as alternative medicine continues to be important among communities across the globe with the belief that natural forms are safer than the synthetic ones. Statistically, it has been estimated that four billion people living in developing countries (80% of the world population) still depend on herbal medicine as their primary healthcare source (Hudson *et al*., 2018). In recent years, multiple product forms derived from plant materials such as supplements, health drinks, tonics, and teas have been marketed in order to meet consumer demands and preferences. Nevertheless, the safety of these particular plant materials as well as their derived products is not guaranteed due to insufficient scientific data concerning their safety and toxicological profiles. This is particularly important since herbal medicines are more often consumed under self-medication without a medical supervision (Pariyani *et al*., 2015). Surprisingly, it has been reported that the consumption of herbals may also cause adverse effects such as nausea, vomiting, weakness, dizziness, hypotension, paraesthesia of the mouth and tongue, arrhythmia, and ventricular fibrillation (Hussin *et al*., 2001). These adverse effects are strongly attributed to the phytochemical composition of the plants, in which some phytochemical constituents could also act as toxin-like substances (Chandra *et al*., 2012). Hence, it is crucial to have proper scientific information regarding the phytochemical composition and toxicological data of the plants in order to prove their safety as an alternative medicine. In the toxicological study, the use of zebrafish (*Danio rerio*) embryos as an alternative animal model to the classical rodent model has gained attention in recent years. The new model is increasingly being applied to evaluate the toxicity profiles of chemical compounds (Jayasinghe & Jayawardena, 2019). The optical transparency of the embryos which allows direct visualization of the internal organs for assessment of toxicity endpoint as well as by having 70% homology with the human's genes have made zebrafish embryos one of the most promising animal model for studying toxicity (Hill *et al*., 2005; Howe *et al*., 2013).

Christia vespertilionis (L. F.) Bakh. F., (Family: Fabaceae) is popularly known as 'butterfly wing', 'mariposa' (meaning butterfly in Spanish) or pokok rerama in the Malay language. Several varieties of the small plant species are commonly grown for ornamental purposes but there has been records of its use in traditional medicine for treating diverse ailments such as tuberculosis, bronchitis, cold, muscle weakness, poor blood circulation and snake bites (Dash, 2016). The health use of the species has recently risen in popularity in Malaysia, following several testimonials from

consumers on the effectiveness of the water decoction of the green-leafed variety as an anticancer herbal remedy. These reports have raised concerns on the safety issues of the plant use and directed interests among researchers with a view to ascertain the health claims made. To date, a number of biological investigations have been carried out and the plant species has been reported to possess antioxidant (Abd Mutalib & Abd Latip, 2019), anti-proliferative (Hofer *et al*., 2013), anti-malarial (Upadhyay *et al*., 2013; Nguyen-Pouplin *et al*., 2007), and cytotoxic properties (Nguyen-pouplin *et al*., 2007; Abd Mutalib & Abd Latip, 2019). However, there still remains a huge gap on the phytochemical composition of the plant, especially on its leaf metabolome. Although initial results from the bioactivity studies indicated that the plant could prove to have therapeutic utility, the safety profile of the plant is still largely unknown. These knowledge and information gaps need to be filled and supplemented for a better understanding of its safety implications and potential applications in healthcare.

1.2 Objectives of the present study

The present study was undertaken with the primary objective of mapping the chemical space within the leaf metabolome of the plant. To achieve this goal, a comprehensive metabolite profiling of the leaf methanolic extract of the green-leafed variety of *C. vespertilionis* was thus performed using ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS)-based molecular networking approach. From the metabolite profile obtained, MS targeted isolation was also conducted in an attempt to validate the proposed structures of the unknown chemical constituents. Furthermore, the toxicity and teratogenic effect of the plant extract also was evaluated through an *in-vivo* study using zebrafish (*Danio rerio*) embryos. The objectives are summarized as follows:

1. To profile the chemical constituents of the leaf methanolic extract of the *C. vespertilionis* (green-leafed variety) via untargeted tandem mass spectrometry-based molecular networking approach.
2. To isolate and elucidate the structures of the targeted compounds using spectroscopic techniques.
3. To evaluate the toxicity and teratogenic effects of the leaf methanolic extract towards zebrafish (*Danio rerio*) embryos.

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Figure 2.2. Some commercial products made from *Christia vespertilionis* available on the Malaysian market. Downloaded from Tok Guru Pondok, by Ustaz Nik Ab Rahim bin Tuan Guru Dato' Nik Abdul Aziz, 2018, retrieved from http://www.tokgurupondok.com.my.

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