INSIGHTS INTO THE ANTIDEPRESSANT EFFECTS OF *CENTELLA ASIATICA*'S TRITERPENOIDS IN UNPREDICTABLE CHRONIC STRESS ZEBRAFISH MODEL: *IN SILICO* AND *IN VIVO* STUDIES

ZAYNAB MANDO

UNIVERSITI SAINS MALAYSIA

2025

INSIGHTS INTO THE ANTIDEPRESSANT EFFECTS OF *CENTELLA ASIATICA*'S TRITERPENOIDS IN UNPREDICTABLE CHRONIC STRESS ZEBRAFISH MODEL: *IN SILICO* AND *IN VIVO* STUDIES

by

ZAYNAB MANDO

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

April 2025

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest praise and gratitude to Allah SWT for his endless grace and blessings throughout my PhD journey. With the courage and patience that He had provided me, I have been able to overcome obstacles and endure setbacks to reach this significant milestone in my life. I would like to extend a very special gratitude and appreciation to my supervisor, Dr. Fauziahanim Zakaria for her exceptional guidance, effort and support which have been instrumental in reaching this goal. A special dedication goes out to Dr. Raghdaa Al-Zarzour for the opportunity she has provided for me. I would also like to acknowledge my co-supervisors, Prof Dr. Khozirah Shaari, AP Dr. Zurina Hassan, and Dr. Adlin Afzan. Many thanks to the committee who significantly enhanced the quality of this work through their insightful feedback. I am deeply grateful to my best friends, Rama Ballouze, Orwa Albitar, Anass Alwade, Mohammad Taleb Agha, for their help and support which made this journey easier. My heartfelt thanks to my wonderful siblings, with a special gratitude to my sister, Dr. Huda for her continuous support and efforts. To my beloved parents, I am profoundly grateful to your countless love and support. Your prayers have lifted me up during time of despair and fear and illuminated my path through the darkest moments in this journey. I owe a great deal of gratitude to my beloved husband. You have been my rock during the toughest time and this achievement would not have been possible without your unwavering love, support and encouragement. Your patience and understanding were invaluable during this journey. To my beloved children, your smiles and words have lifted my spirits and brightened every step of this way. Thank you from my deepest heart for your patience, resilience and understanding which allowed me to complete this work.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

5-HT	5-hydroxy tryptamine, Serotonin
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotrophic hormone
AD	Alzheimer's disease
ANOVA	Analysis of variance
BBB	Blood-brain barrier
BCAAs	Branched-chain amino acids
BDNF	Brain-derived neurotrophic factor
C. asiatica	*Centella asiatica*
CF	Contact frequency
CMS	Chronic mild stress
CNS	Central nervous system
COM	Center of mass
COX	Cyclooxygenase
CRF	Adrenocorticotrophic hormone-releasing factor
CSF	Cerebrospinal fluid
CUMS/ UCMS	Chronic unpredictable mild stress
DMSO	Dimethyl sulfoxide
EPM	Elevated plus maze
FST	Forced swimming test
GABA	γ-amino butyric acid
GAD	Glutamic acid decarboxylase
GC	Gas chromatography
GPx	Glutathione peroxidase
GR	Glucocorticoid receptors
HPA	Hypothalamic-pituitary-adrenal axis
HSQC	Heteronuclear single quantum coherence
IL	Interleukin
i.p.	Intraperitoneal
LC	Liquid chromatography

LPS	Lipopolysaccharide
MAO	Monoamine oxidase
MAOIs	Monoamine oxidase inhibitors
MD	Molecular dynamics
MDA	Malondialdehyde
MDD	Major depressive disorders
MM-PBSA	Molecular mechanics poisson−boltzmann surface area
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OFT	Open field test
PBS	Phosphate buffer saline
PCA	Principal component analysis
PD	Parkinson disease
Rg	Radius of gyration
RMSD	Root mean square deviation
RMSF	Root mean square fluctuations
ROS	Reactive oxygen species
SERT	Serotonin transporter
SIT	Social interaction test
SNRIs	Serotonin norepinephrine reuptake inhibitors
SOD	Superoxide dismutase
SSRIs	Selective serotonin reuptake inhibitors
TCAs	Tricyclic antidepressants
TCM	Traditional Chinese medicine
TNF	Tumor necrosis factor
TSP	Trimethylsilyl propionic acid
TST	Tail suspension test
UCS/ CUS	Unpredictable chronic stress
WHO	World Health Organization

LIST OF APPENDICES

PEMAHAMAN KESAN ANTI-KEMURUNGAN TRITEPENOID *CENTELLA ASIATICA* DALAM MODEL TEKANAN KRONIK TIDAK MENENTU IKAN ZEBRA: KAJIAN *IN SILICO* DAN *IN VIVO*

ABSTRAK

Kemurungan adalah gangguan mental yang melumpuhkan individu dengan kadar prevalens yang tinggi di seluruh dunia. Walaupun antidepresan semasa telah menunjukkan keberkesanan, penggunaannya sering dihadkan oleh pelbagai kesan sampingan, menekankan keperluan untuk pilihan terapeutik baharu. *Centella asiatica* (*C. asiatica*), ialah suatu herba perubatan tradisional, diiktiraf untuk pelbagai manfaat terapeutik neuropsikiatri. Triterpenoid pentasiklik; iaitu asid asiatik dan asid madekassik, serta saponin yang berkaitan; asiatikosida dan madekassosida adalah kandungan utama yang terdapat dalam *C. asiatica*. Beberapa kajian farmakologi tentang aktiviti antimurung sebatian penanda biologi triterpenoid *C.asiatica* dapat diperolehi, dimana mekanisma yang tersembunyi kekal sukar difahami. Kajian ini bertujuan untuk menyiasat potensi kesan antimurung triterpenoid dan hubungkaitnya dengan sistem serotonegis, paksi HPA, tindakbalas imun dan metabolit endogen otak. Kesan antimurung sebatian penanda biologi *C. asiatica* pada sistem serotonergic diuji melalui pendekatan *in silico*. Kajian ini juga meninjau kesan menyerupai antimurung sebatian-sebatian ini terhadap model kemurungan pada ikan zebra yang diaruh oleh tekanan kronik yang tidak menentu (UCS). Tindak balas tingkah laku dalam model ikan zebra UCS dinilai selepas rawatan dengan triterpenoid pada tiga dos (1.25, 2.5 dan 5 mg/kg, i.p.). Saringan aktiviti antimurung diteruskan dengan madekassosida dan asiatikosida melalui penilaian terhadap kesan potensinya pada tahap kortisol dan IL-1β dalam badan ikan zebra UCS menggunakan ELISA dan metabolit otak berubah

menggunakan metabolomik berasaskan ^{1}H NMR. Pengedokan molekul mendedahkan kesan menghalang triterpenoid pada pengambilan semula serotonin melalui pengikatan dengan pengangkut serotonin. Simulasi dinamik molekul mengesahkan kestabilan kompleks madekassosida dan asiatikosida dengan protein. Kajian tingkah laku mendedahkan peningkatan aktiviti pergerakan ikan yang tertekan dalam ujian lapangan terbuka selepas rawatan dengan madecassoside (1.25, 2.5 dan 5 mg/kg), asiaticoside dan asid asiatik (5 mg/kg). Tiada perubahan ketara dalam ujian interaksi sosial diperhatikan antara kumpulan. Madekassosida pada semua dos yang diuji dan asiatikosida pada dos 2.5 dan 5 mg/kg secara signifikan menurunkan tahap kortisol berbanding kumpulan UCS. Pengurangan yang signifikan dalam kepekatan IL-1β juga diperhatikan selepas rawatan akut dengan madekassosida dan asiatikosida pada dos 1.25 dan 5 mg/kg, masing-masing. Hasil kajian metabolomik menunjukkan UCS yang diaplikasikan mengaruh perubahan signifikan dalam 25 metabolit otak, di mana 18 daripadanya dapat dibalas oleh rawatan dengan asiatikosida dan 13 daripadanya oleh madekassosida. Metabolisma alanina, aspartat dan glutamat merupakan laluan utama yang diramal terganggu dalam model UCS dan dibetulkan selepas rawatan. Kajian ini memberi pencerahan tentang potensi berbilang sasaran madekassosida dan asiatikosida dalam mengurangkan gejala kemurungan melalui pengawalaturan beberapa sistem yang terganggu. Penilaian lanjut diperlukan untuk mengesahkan potensi sebatian-sebatian ini sebagai rawatan kemurungan.

INSIGHTS INTO THE ANTIDEPRESSANT EFFECTS OF *CENTELLA ASIATICA*'S TRITERPENOIDS IN UNPREDICTABLE CHRONIC STRESS ZEBRAFISH MODEL: *IN SILICO* AND *IN VIVO* STUDIES

ABSTRACT

Depression is a debilitating mental disorder with high prevalence around the world. While current antidepressants have shown efficacy, they are limited by side effects, highlighting the need for novel therapies. *Centella asiatica* is a traditional medicinal herb recognised for its various neuropsychiatric benefits. The pentacyclic triterpenoids; asiatic acid and madecassic acid, and their related saponins; asiaticoside and madecasosside are the major constituents of *C. asiatica.* Few pharmacological reports on the antidepressant activity of *C. asiatica*'s triterpenoids biomarker are available, whereby the underlying mechanisms remain poorly understood. This work aims to investigate the potential antidepressant effects of the triterpenoids and their correlation with the serotoninergic system, hypothalamic-pituitary-adrenal (HPA) axis, immune response and brain endogenous metabolites. The antidepressant effect of *C. asiatica*'s biomarkers on the serotoninergic system was evaluated via *in silico* approach. This study also explored the antidepressant-like effects of these compounds on the unpredictable chronic stress (UCS)-induced depression zebrafish model. The behavioural responses in the UCS zebrafish model were assessed after treatment with the triterpenoids at three doses (1.25, 2.5, and 5 mg/kg, i.p.). Screening of the antidepressant activity was continued with madecassoside and asiaticoside by evaluating their effects on cortisol and IL-1β levels in UCS zebrafish' bodies via ELISA, and brain metabolites changes using ^{1}H NMR-based metabolomics. Molecular docking disclosed the impeding impact of the triterpenoids on serotonin reuptake

through binding with serotonin transporter. Molecular dynamics simulation confirmed the stability of madecassoside and asiaticoside complexes with protein. Behavioural study revealed the activation of stressed fish locomotion in the open field test after treatment with madecassoside (1.25, 2.5, and 5 mg/kg), asiaticoside and asiatic acid (5 mg/kg). No significant differences in the social interaction test were observed between groups. Madecassoside at all doses and asiaticoside at 2.5 and 5 mg/kg significantly decreased cortisol levels compared to the UCS group. IL-1β concentrations was also significantly reduced after treatment with madecassoside (1.25 mg/kg) and asiaticoside (5 mg/kg). The results of the metabolomics study manifested that the applied UCS induced significant changes in 25 metabolites where, 18 of them were influenced by treatment with asiaticoside and 13 by madecassoside. Alanine, aspartate and glutamate metabolism was the main predicted pathway to be perturbed in the UCS model and altered after treatment. The current study highlights madecassoside and asiaticoside as potential multi-target antidepressants that alleviate depressive symptoms by mediating various disrupted systems. Further research is needed to confirm them as promising leads for depression treatment.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Depression is a debilitating psychiatric disorder that affects approximately 280 million people of different ages worldwide, which accounts for 3.8% of the world's population, as reported by the World Health Organization (WHO), with the expectation to be the leading cause of the global burden of disease by 2030 (Bains & Abdijadid, 2023; Liu et al., 2020; World Health Organization, 2023). In Malaysia, one in four adolescents suffered from depression in 2022, according to the National Health and Morbidity Survey, with an increment of 5% compared to 2019 (Azmi, 2023). A total of 1081 suicide attempts were recorded in 2020, and by 2030, it is expected that mental health issues will influence the Malaysian economy by 6 trillion dollars (Amir, 2022).

Depression is characterised by the presence of specific somatic and cognitive abnormalities, including three core symptoms (anhedonia, depressed mood, and low energy levels) as described by the International Classification of Diseases (ICD-10), additional symptoms (sleep disturbances, apathy, appetite/weight alterations, feelings of worthlessness or guilt and psychomotor agitation or retardation) which persist for more than two weeks, and other comorbid conditions such as anxiety and social withdrawal, which can lead to thoughts of self-harm or even suicidal attempts in some cases (Becker et al., 2021; Kaltenboeck & Harmer, 2018; Leite-Almeida et al., 2022; Penn & Tracy, 2012; Planchez et al., 2019).

Treatment options for depression, such as psychotherapy, drug treatment, and neuromodulation, depend on the patient's medical condition (Chand & Arif, 2023).

Tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs) and serotonin-dopamine activity modulators are the available prescribed medications for depression treatment (Chand & Arif, 2023). However, depression is often overlooked and undertreated in primary care, resulting in poor treatment outcomes (Bandopadhyay et al., 2023). Additionally, currently available antidepressant drugs have undesirable side effects, low efficacy in mild cases, delayed onset, symptom recurrence and treatment resistant (Penn & Tracy, 2012). Moreover, the development of new antidepressant drugs has not shown success in the last decades. By the end of 2019, only 292 out of the 828 antidepressants under development were effective, according to Cortellis database, and many of them have been discontinued or have not advanced (Li et al., 2021). Consequently, the development of new alternative and complementary antidepressants is in high demand.

For a long time, medicinal plants have been utilised to discover potential novel or lead nutraceuticals for the treatment of various diseases. The global use of herbal plants for treating depression and other psychiatric ailments has risen (Martins & Brijesh, 2018). *Centella asiatica* (L.) Urb (*C. asiatica*), known as pegaga in Malaysia, is a worthy ethnomedicinal herb used for its various therapeutic properties. Due to its numerous benefits in boosting the nervous system function and improving various neuropsychiatric ailments, *C. asiatica* has been renowned as “brain tonic”(Alfarra & Omar, 2013; Belwal et al., 2019). The term "Centelloids" refers to the main compounds or terpenoids found in *C. asiatica*. The most prevalent and biologically active constituents, including the pentacyclic triterpenoids saponins madecassoside and asiaticoside, and their related aglycones, madecassic acid and asiatic acid have been regarded as biomarker components of *C. asiatica* (Alfarra & Omar, 2013; Hashim et

al., 2011; Orhan, 2012; Roy et al., 2018). These triterpenes are widely distributed in the brain as well as other organs in the body (Sun et al., 2020). Hence, the main biological activity of *C. asaitica* is believed to be attributed to the presence of these compounds.

The antidepressant activity of *C. asiatica* has been reported for the first time with its total triterpenes by Chen and his colleagues, as evidenced by a reduction in the immobility time of the forced swimming mice acute model of depression (Chen et al., 2003). The mechanisms involved have been related to ameliorating the imbalance of brain levels of amino acids and the function of the hypothalamic-pituitary-adrenal (HPA) axis (Chen et al., 2003, 2005). The reduction in the immobility time was further supported by Selvi and co-workers, as well as an increase in the exploratory behaviour of mice after treatment with the ethanolic extract of *C. asiatica* (Selvi et al., 2012). Another group of researchers has studied the antidepressant effect of the leaf extract of *C. asiatica* in the olfactory bulbectomy chronic stress depression rat model. The hyperactivity in the elevated plus maze (EPM) and open field paradigm was reversed by treatment with the leaves extract, as well as other physiological parameters such as the reduction of body weight and temperature and heart rate (Kalshetty et al., 2012). A recent study has indicated that *C. asiatica* prevented morphological aberrations induced in rat's hippocampus by chronic unpredictable mild stress (CUMS) via modulation of oxidative stress markers (Jagadeesan et al., 2022). A reserpine-induced zebrafish stress-like model has been developed, and treatment with *C. asiatica*'s extract exhibited a significant effect on the behaviour of the stressed fish, and a metabolic pathway alteration has been identified (Zakaria et al., 2023).

The discovery process of novel drugs has gained a great benefit by combining computational and experimental approaches (Ferreira et al., 2015). Molecular docking

has become the most widely employed tool for *in silico* drug screening (Meng et al., 2012). It is a procedure of positioning a small molecule (ligand) into the binding site of a macromolecule (target protein) in a variety of orientations and positions to predict the conformation that fits the active site-pocket both energetically and geometrically (Azam & Abbasi, 2013; Jakhar et al., 2020). Molecular dynamics (MD) simulation is a powerful computational technique contributing to drug discovery which imitates the actual structure of a biological molecule over time, disclosing full atomic details about the interactions between a potential drug candidate and its targeted protein and addressing comprehensive information about the stability of their complexes (Badar et al., 2022). Compared to traditional methods of drug discovery, *in silico* strategies have proven their efficiency in the process of drug discovery and development, enabling rapid, cheap, robust and extensive screening (Vincent et al., 2020).

Animal models have significantly advanced the field of psychiatry studies. Rodent-based models are well-established and extensively used for stress and depression studies (de Abreu et al., 2018a; Demin et al., 2019). Recently, the zebrafish (*Danio rerio*) has rapidly become a worthy complementing model species for translational neuropsychiatric areas, including depression (de Abreu et al., 2018a; Fonseka et al., 2016). Besides its small size, high fertility rate, low cost of husbandry and rapid development, the remarkable homology to mammals both genetically and physiologically has posed zebrafish as typical for developing reliable experimental models of depression and finding new treatments (Fonseka et al., 2016; Lachowicz et al., 2021). Moreover, zebrafish display highly robust phenotypes that are ideal for identifying depression-like indices by several behavioural testing approaches based on various fields, which are parallel to those used in rodents (Fonseka et al., 2016).

Repeated exposure to stress factors is a major cause of neuropsychiatric diseases, mainly anxiety and depression (Chakravarty et al., 2013). The first unpredictable chronic stress (UCS) model, validated in rodents, has resulted in the identification of several behavioural, endocrine, and neural changes that resemble those found in individuals suffering from major depressive disorders (MDD), which could help lead to better treatments for depression (Nollet, 2021). The response of zebrafish to the UCS paradigm has been reported to be similar to that in humans and rodents. An increase in corticotrophin-releasing factor and cortisol levels, a hallmark of anxiety and mood-related disorders, as well as a decrease in glucocorticoid receptors (GR) expression, have been reported (Piato et al., 2011). The exposure to UCS also induced reduced dopamine and serotonin metabolite levels (Fulcher et al., 2017), increased norepinephrine concentrations in the whole brain of zebrafish (Demin et al., 2021), and decreased neurogenesis (Chakravarty et al., 2013). Additionally, it has been demonstrated that the gene expression of pro-inflammatory markers; interleukin-6 (IL-6) and cyclooxegenase-2 (COX-2) in zebrafish brain increased after the exposure to the UCS, whereas treatment with anxiolytic and antidepressant drugs prevent such biochemical and other molecular and behavioural perturbations (Marcon et al., 2016). These data indicate the potential of the UCS model in zebrafish to be utilised in stress-related pathologies research and evaluating new drugs.

Metabolomics is a comprehensive analysis that aims to identify and quantify endogenous molecules from a biospecimen which represent the final outcome of biological processes in a particular biofluid, cell, tissue or organism (Clish, 2015; Wang et al., 2010). Therefore, the precise characterisation of a metabolic phenotype is affordable by metabolomics studies which can characterise metabolic disturbances that underlie disease, find novel targets for treatment, and identify biomarkers that can be

used to diagnose or monitor effectiveness of therapeutic interventions (Clish, 2015). Lipids, amino acids, organic acids, peptides and nucleic acids represent a diverse group of low-molecular weight structures collectively known as metabolites. The vast collection of diverse metabolites makes comprehensive analysis a difficult challenge (Wang et al., 2010). Thus, the availability of proper analytical platforms to identify the wide range of chemically varied metabolites in intricate biological samples is crucial for metabolomics research (Macedo et al., 2021). Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the major employed analytical methods in the metabolomics field (Emwas, 2015). The rapid development of analytical platforms enabled separation, characterisation, detection and quantification of diverse metabolites (Wang et al., 2010).

In the present study, the potential benefits of *C. asiatica*'s candidate molecules in alleviating depressive symptoms were assessed via an *in silico* study to investigate the possible effects on the serotoninergic system through interactions with serotonin transporter (SERT), combined with various *in vivo* experiments in the UCS zebrafish model of depression to evaluate the involvement of the HPA axis, immune system and brain metabolites, highlighting their multi-target antidepressant activity.

1.2 Problem statement/ Originality of research

The pentacyclic triterpenoids saponins madecassoside and asiaticoside, and their related aglycones, madecassic acid and asiatic acid represent the most abundant and vital constituents of *C. asiatica* and have been regarded as the core provenance of its therapeutic activity. The favourable safety profiles and pharmacokinetic properties of these triterpenoids are well-documented. They are widely distributed in the brain, which is crucial for the activity on the central nervous system.

Numerous studies have shown that *C. asiatica* and its triterpenoids components can boost the nervous system's performance and potentially improve various neuropsychiatric ailments. However, the antidepressant properties have not been extensively researched compared to other neurological activities. Most of these studies were conducted on the plant extract and focused on behavioural assessments (Ceremuga et al., 2015; Chen et al., 2003; Jagadeesan et al., 2019; Kalshetty et al., 2012; Liang et al., 2008; Selvi et al., 2012; Wei-wei & Bo, 2008). Only a few studies have investigated the possible mechanisms underlying the antidepressant action of *C. asiatica*'s pure triterpenoids (Girish & Sanjay, 2020; Liu et al., 2004; Luo et al., 2015; Wang et al., 2020). Still, further elucidation of the corresponding mechanisms associated with the antidepressant-like effects of these compounds is still in great demand.

Although several studies have suggested that the antidepressant effect of *C. asiatica* and its triterpenoid compounds is mediated through the monoaminergic system (Chen et al., 2005; Girish & Sanjay, 2020; Liu et al., 2004; Wang et al., 2020), the involved antidepressant mechanisms remain to be further studied. However, according to existing research, no previous investigations of the potential interaction of these triterpenoids molecules with SERT, neither on the orthosteric nor on the allosteric sites, involved in depression have been conducted.

Dysregulation of the HPA axis has been established as a hallmark of MDD. The effect of *C. asiatica*'s extract on cortisol levels has been previously evaluated (Chen et al., 2005; Jagadeesan et al., 2019; Zakaria et al., 2023). However, the effect of the pure biomarkers of *C. asiatica* on cortisol levels has not been evaluated yet.

The sustained activation of the immune system or chronic inflammation is probably one of the mechanisms involved in the pathogenesis of depression

(Nemeroff, 2020). A sole study has reported that treatment with asiaticoside downregulated the levels of the main proinflammatory cytokines in the hippocampus of chronic unpredictable mild stress mice model of depression (Wang et al., 2020). Such an effect has not yet been explored with the other biomarkers of *C. asiatica*.

Metabolomics represents a field of study that elucidates the pathophysiology mechanistic of a disease and possible therapeutic targets by identifying novel biomarkers and facilitates drug discovery and monitoring treatment activity (Clish, 2015; Nagana Gowda & Raftery, 2021). To date, no metabolomics studies have been conducted to investigate the effects of *C. asiatica*'s biomarkers against depression.

1.3 Research questions

1. Do *C. asiatica*'s triterpenoids biomarkers (asiaticoside, madecassoside, asiatic acid, and madecassic acid) show any *in silico* interactions with serotonin transporter?
2. Do *C. asiatica*'s triterpenoids biomarkers show any antidepressant effect in the UCS-induced depression zebrafish model? If yes, what is the effective dose?
3. How do *C. asiatica*'s triterpenoid compounds exert their antidepressant action in the UCS zebrafish model?
4. Does treatment with *C. asiatica*'s triterpenoids molecules exhibit any effect on the endocrine system and immune system of the UCS-induced depression zebrafish model?
5. What are the significant alterations in the brain metabolome of UCS-induced depression zebrafish upon treatment with *C. asiatica*'s triterpenoids compounds that could be attributed to their antidepressant activity?

1.4 Research hypotheses

C. asiatica is a valuable herb well-known for its rejuvenating, neuroprotective and psychoactive medicinal properties. The antidepressant activity of the plant extract has been reported and the mechanisms involved have been related to ameliorating the dysregulated levels of brain neurotransmitters and function of the HPA axis. Additionally, treatment with *C. asiatica*'s extract has been revealed to exhibit a significant effect on the brain metabolome and a metabolic pathway alteration has been identified. Moreover, the anti-inflammatory properties *C. asiatica* have been documented. Consequently, the most abundant biomarker compounds of *C. asiatica* (asiaticoside, madecassoside, asiatic acid, and madecassic acid) represent promising potential antidepressant agents which can modulate the HPA axis, immune system and brain metabolites and neurotransmitters linked to depression.

1.5 Research aim and objectives

The present study aims to improve the understanding of the antidepressant therapeutic efficacy of *C. asiatica*'s pure triterpenoids biomarker and provide insights into the involved mechanisms contributed to the serotoninergic system, HPA axis, immune response and changes in brain endogenous metabolites.

1.5.1 Specific objectives

1. To predict the inhibitory impact of *C. asiatica*'s triterpenoids compounds on serotonin reuptake, based on *in silico* molecular docking and molecular dynamics simulation studies.
2. To evaluate the behavioural changes in the UCS zebrafish after treatment with asiaticoside, madecassoside, asiatic acid, and madecassic acid.

3. To evaluate the effects of the triterpenoids on cortisol and IL-1β levels in UCS zebrafish.
4. To evaluate the effects of the triterpenoids on the metabolic profile of the brain of UCS zebrafish.

CHAPTER 2

LITERATURE REVIEW

2.1 Pathophysiology and aetiology of depression

Depression, also referred to as MDD, clinical depression and melancholia, is a great contributor to disability and economic burden worldwide (Li et al., 2021; Wang et al., 2021). It has quietly become a global disease that threatens hundreds of millions of people, with an approximate lifetime propagation of 15% and an increase of about 18% over the past ten years (Wang et al., 2021).

Depression is a multifactorial disorder caused by the interaction of different internal and external factors (Chand & Arif, 2023). Therefore, no specific theory can account for the pathogenesis of depression on its own (Li et al., 2021). Genetic factors perform a significant role in early-onset depression, while biological risk factors and physical illnesses such as neurodegenerative disorders, cancers, autoimmune disorders, stroke, cardiovascular diseases and chronic pain have been recognised to increase the risk of depression in the elderly (Chand & Arif, 2023). Lately, the impact of environmental factors on the incidence of depression episodes has drawn a growing emphasis among researchers (Wang et al., 2021). Adverse childhood experiences are a prevalent route to depression in adults and a potential cause of anxiety and depression in adolescence (Wang et al., 2021). Life stressors such as traumatic episodes, loss of social support, poor interpersonal relationships, low self-esteem, conflicts and financial issues can trigger depression (Chand & Arif, 2023; Wang et al., 2021).

2.1.1 Monoamine neurotransmitters

The multiple potential aetiologies have posed depression as a heterogeneous disorder with complex linked pathophysiological mechanisms underlying its signs and symptoms. Despite the growing knowledge of the pathophysiology of depression due

to ongoing research, the exact mechanisms by which depression develops remain incomplete (Jesulola et al., 2018). The monoamine-deficiency hypothesis is the first and clinically most relevant theory of depression that postulates the diminution of monoamine neurotransmitters levels in the central nervous system (CNS), centres on serotonin, dopamine, and norepinephrine as the leading cause underlying symptoms of depressive disorder (Hasler, 2010). However, progress in molecular psychiatry has reassessed the dysregulation of monoamine neurotransmitters as a secondary downstream of other primary abnormalities, including several neural, endocrine and metabolic components (Chávez-Castillo et al., 2019).

2.1.2 Excitatory and inhibitory neurotransmission

In the past few decades, compelling evidence from preclinical and clinical studies has demonstrated the involvement of the excitatory neurons as well as inhibitory interneurons and the functional consequences of their deficits in the biological mechanisms underlying the pathophysiology of depression (Khoodoruth et al., 2022) . The maintenance of homeostasis between glutamate, the main excitatory neurotransmitter which is eminently involved in synaptic plasticity, learning, and memory, and γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter that is accountable for overall regulating of excitatory transmission, is critical for proper neural activity (Chávez-Castillo et al., 2019; Duman et al., 2019). Structural and functional alterations in both major neuronal types have been reported in chronic stress models and depressed patients, including atrophy of neurons, altered connectivity and degradation of signal integrity in the prefrontal cortex and hippocampus brain regions implicated in depression (Duman et al., 2019). Treatment strategies targeting the glutamate and GABA pathways offer superior rapid-acting interventions against depressive disorder (Duman et al., 2019).

2.1.3 Brain-derived neurotrophic factor

BDNF, one of the most distributed neurotrophins in the mammalian brain, has drawn considerable attention. BDNF plays an important role in neurogenesis, neuroplasticity, neuronal differentiation as well as serotonergic and dopaminergic neurotransmission (Colucci-D'amato et al., 2020). The reduced neurotrophic growth indicated by the low levels of brain BDNF in depressed patients has been supported as a third important potential pathophysiological mechanism of depression (Verduijn et al., 2015).

2.1.4 Hypothalamic-pituitary-adrenal axis

Biological systems, including the HPA axis and the inflammatory response system, are believed to play a prominent role in the genesis of depression and clarify how it is associated with stress and physical health (Iob et al., 2020). Maintaining body homeostasis is mainly dependent on the HPA axis which its activation is a hallmark of the body's response to stress (Iob et al., 2020; Mikulska et al., 2021). Exposure to any significant influence can trigger the hormonal cascade of the HPA axis, which consists of stimulating forward and feedback inhibition loops and begins with the secretion of adrenocorticotrophic hormone-releasing factor (CRF) and vasopressin from the hypothalamus. These secretions induce the release of adrenocorticotrophic hormone (ACTH) from the pituitary. ACTH, in turn, activates the release of glucocorticoids from the adrenal cortex. Glucocorticoids then interact with their receptors in multiple target tissues, responsible for the feedback inhibition loop (Baumeister et al., 2016; Druzhkova et al., 2022; Keller et al., 2017). Hyperactivity of the HPA axis in depression results from impaired negative feedback mediated by endogenous glucocorticoids (Baumeister et al., 2016; Pariante & Lightman, 2008). An abundance of experimental and clinical data has strongly supported the dysregulation of the HPA axis as one of the

most consistent biological findings in depressive disorder (Baumeister et al., 2016; Pariante & Lightman, 2008; Varghese & Brown, 2001). Numerous reports have documented hypercortisolemia in patients with major depression (Iob et al., 2020; Keller et al., 2017). Higher cortisol concentrations in plasma were found in individuals with depression who attempt suicide (Mendoza et al., 2022). In chronic stress models, elevated corticosterone levels have been observed in response to stress, and inhibition of corticosterone synthesis or adrenalectomy has been associated with suppression of depression-like behaviour (de Andrade et al., 2013; Kvarta et al., 2015; Nandam et al., 2020). The regulation of the HPA axis overactivity in depressed patients may evidence to be an effectual target of pharmacotherapy, and a comprehensive understanding of the mechanisms underlying their synergistic action may come up with the development of new therapeutic approaches for depressive disorders (Menke, 2019; Mikulska et al., 2021).

2.1.5 Chronic inflammation

The inflammatory theory of depression has been developed based on a cumulative build of evidence linking depression with inflammation (Hashmi et al., 2013). The significance of several pro-inflammatory cytokines in the pathogenesis of depression and antidepressant response have been emphasised in various studies, both peripherally and centrally, wherein the three pro-inflammatory cytokines IL-1β, tumour necrosis factor-alpha (TNF-α) and IL-6 have been stated to display the most prominent roles in the pathophysiology of depressive illness (Farooq et al., 2017; Ferentinos et al., 2021). Elevated levels of inflammatory biomarkers such as IL-1, IL-6, TNF-α, C-reactive protein and interferon-gamma and their gene expressions have been consistently reported in patients with depression, suggesting the chronic inflammation or sustained activation of the immune system as one of the potential mechanisms

involved in the pathogenesis of depression (Nemeroff, 2020; Radu V. Saveanu, 2012). Psychological stress may trigger depression via its effects on the immune system (Munshi et al., 2020). Activation of the CNS cytokines network, as well as the peripheral inflammatory factors lead to cascade of effects that influence brain's functioning and may contributed to the pathogenesis of depression through the modulation of serotonergic and glutamatergic transmission, affecting oxidative stress and reducing neurogenesis, neural plasticity and neurotrophic factors (Berthold-Losleben & Himmerich, 2008; Ferentinos et al., 2021; Hashmi et al., 2013; Suneson et al., 2021; Ting et al., 2020). Additionally, impaired regulation of the HPA axis and inflammation could be involved in the same pathophysiological process of depression. The ineffectiveness of glucocorticoid hormone on target tissues, which resulted from glucocorticoid resistance, could trigger immune activation. Conversely, inflammation may directly impact the brain through the action of cytokines or by developing glucocorticoid resistance, further stimulating the HPA axis activity (Baumeister et al., 2016).

Cytokines represent a class of small diverse peptides that influence the proliferation and differentiation of cells as well as inflammation (Harsanyi et al., 2023). The role of cytokines in depression, anxiety and stress has been extensively studied. In depression, different cytokines are released in the body and transported to the brain, where they impact the neurotransmitter systems (Harsanyi et al., 2023). During stress, an immune response may be elicited through the danger-associated molecular patterns (DAMPs) that are released on the cellular level and stimulate the NLRP3 inflammasome, which in turn is involved in the processing of IL-1β into its mature releasable form. The production of other inflammatory cytokines is then induced by the increase in IL-1β release (Felger & Lotrich, 2013; Xia et al., 2023). Interestingly, in

response to both infections and stress, IL-1β is produced at an early stage and activates the expression of IL-6, while TNF-α and IL-6 act to potentiate the IL-1β effects (de Abreu et al., 2018b; Kirsten et al., 2018). Accordingly, clinical trials employing anti-inflammatory treatments (Selective COX-2 inhibitor; celecoxib) in depressed individuals have been carried out, and findings were promising in certain cases (Kaltenboeck & Harmer, 2018; Köhler et al., 2014). The use of an anti-inflammatory agent adjacent to an antidepressant medication significantly reduced depression scores in treatment-resistant depressive subjects, supporting the inflammatory system as a valuable target for future treatment of depression (Baumeister et al., 2016).

2.1.5(a) Interleukine-1beta (IL-1β)

IL-1β is a crucial mediator of inflammatory response and is strongly implied in the pathogenesis of depression, primarily within the framework of the inflammasome hypothesis (Ferentinos et al., 2021). Additionally, IL-1β can cause a functional resistance of glucocorticoid receptors by activating the HPA axis. Furthermore, it can alter tryptophan availability and upregulate enzymes involved in the neurotoxicity of the kynurenine pathway, thus reducing neurogenesis in the hippocampus (Ferentinos et al., 2021). Finally, IL-1β can increase the uptake of serotonin, which is the mainly neurotransmitter involved in the aetiology of MDD, by acutely activating the serotonin transporter, thereby decreasing its abundance in synapses and affecting serotonergic neurotransmission (Farooq et al., 2017). IL-1β was found to be elevated in 38.5% and 75.6% of studies in reaction to acute or chronic stress, respectively (Harsanyi et al., 2023). The haematopoiesis of zebrafish exhibits a morphological and molecular complexity close to that of mammals, with a wide array of cytokine subfamilies conserved between zebrafish, humans and rodents (Nguyen et al., 2014), which emphasises the utility of zebrafish in psychoneuroimmunology research (de Abreu et

al., 2018b). In zebrafish, the IL-1β expression and activity have undergone comprehensive characterisation (de Abreu et al., 2018b). Taken together, IL-1β was chosen to be evaluated in this study.

Inconsistent dysfunctions have been observed in the pathophysiology of individuals with depression, and changes in the biological markers were widely relevant to the progression of clinical disease. Therefore, to date, no biomarker has been verified to be extensively clinically employed as a diagnostic tool (de Menezes Galvão et al., 2021).

2.2 Clinically used antidepressants and their limitations

2.2.1 First-generation antidepressants

Nowadays, pharmacological treatments and non-pharmacological interventions for short-term treatment of acute depression are relatively well established with at least moderate efficacy (Sim et al., 2016). First-generation antidepressants including TCAs and MAOIs, have a long record of efficacious treatment of depression (Chand & Arif, 2023; Penn & Tracy, 2012). MAOIs function by preventing the breakdown of serotonin, dopamine and norepinephrine through the inhibition of the oxidative deamination activity of MAO enzymes, while TCAs act by nonselective inhibiting the reuptake of monoamine neurotransmitters into presynaptic storage vesicles in the brain, hence elevating their levels (Ferguson, 2001; Santarsieri & Schwartz, 2015). However, the anticholinergic side effects, such as constipation, blurry vision, urinary retention and dry mouth induced by antagonising muscarinic acetylcholine (ACh) receptors, as well as drowsiness and notable weight gain often limit the utility of TCAs (Santarsieri & Schwartz, 2015). In addition, TCAs have a narrow therapeutic index, and at high doses, they can cause seizures as well as abrupt cardiac death (Ferguson, 2001; Santarsieri &

Schwartz, 2015). The use of MAOIs, like the TCAs, is also limited due to adverse events. Weight gain, fatigue, hypotension, as well as lethal interactions with food and other medications are promoted by MAOIs and fatal serotonin syndrome or hypertensive crisis may develop by inappropriate use of these agents (Santarsieri & Schwartz, 2015). Consequently, the early mainstay of depression treatment is often the last pharmacologic choice after other options have failed to yield remission (Santarsieri & Schwartz, 2015).

2.2.2 Second-generation antidepressants

Currently, the second-generation antidepressants SSRIs and, to a lesser extent, SNRIs are first-line pharmacotherapy for patients with depressive disorders (Braund et al., 2021; Strawn et al., 2023). Adrenergic alpha-2 receptor antagonists, selective noradrenaline reuptake inhibitors, selective noradrenaline-dopamine reuptake inhibitors, melatonin receptor agonists, serotonin-dopamine activity modulators, serotonin 5-HT2C receptor antagonists and atypical antidepressants are prescribed less often (Chand & Arif, 2023; National Library of Medicine, 2020).

The SSRIs are comprised of fluoxetine, citalopram, escitalopram, paroxetine, sertraline, fluvoxamine, vilazodone and vortioxetine. The SNRIs include duloxetine, venlafaxine, desvenlafaxine, and levomilnacipran (Chand & Arif, 2023; Santarsieri & Schwartz, 2015). The high selectivity reuptake inhibition property avoids many of cholinergic and cardiac side effects of the classical antidepressants and offers superior safety and tolerability profiles (Penn & Tracy, 2012; Santarsieri & Schwartz, 2015). However, a growing body of recent research suggested that newer generation of antidepressants are no less safe than the initial ones, and concerns about side effects which are more specific to noradrenaline or serotonin selectivity have been raised, such as higher risks of bleeding and hyponatremia (Wang et al., 2018). Broad-based

experience with SSRIs has demonstrated a higher frequency and type of side effects compared to data from clinical trials (Ferguson, 2001). For instance, post-marketing trials have reported higher rates of sexual dysfunction up to 75% compared to 1.9% in the original clinical trials. Hyponatremia is now known to occur in elderly patients with SSRIs medicaments even though it was not reported in the original clinical trials (Ferguson, 2001). A wide range of side effects is also associated with SSRIs treatment including fatigue, nausea/vomiting, weight loss, dizziness, headaches, sleep disturbance, and increased risk of suicidal thoughts and cardiovascular and cerebrovascular events (Braund et al., 2021)**.** Side effects can impact the prognosis of successful treatment outcome resulting in treatment discontinuation in up to 43% of individuals with depression (Wang et al., 2018).

Despite the evidence of efficacy of antidepressants, significant problems persist, such as the slow onset of action, limited efficacy in mild states, and resistance to treatment (Penn & Tracy, 2012). About 50% of the patients may not initially respond. Complete remission is not common, and many patients need combination of treatments to hold the symptoms (Chand & Arif, 2023). The case has frequent relapses. Many patients experience early relapses with an average of 50% within six months or later recurrences over 85% within a decade following initial short-term improvement or remission (Sim et al., 2016), resulting in an inferior quality of life of individuals with depressive disorders. Hence, the search for agents with improved efficiency, safety and tolerability continues.

2.3 Traditional medicine

Traditional medicine, as outlined by the WHO, refers to “the sum total of the knowledge, skill, and practices based on theories, beliefs and experiences indigenous to

diverse cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness" (World Health Organization, 2012). The traditional medicine systems of China (TCM), India, and Africa are currently the most popular used (Che et al., 2017). While some traditional remedies are supported by vast numbers of literatures, others are passed down generations through oral education (Che et al., 2017). The terms "complementary medicine" or "alternative medicine" are often used in place of traditional medicine in many countries, where herbal therapies are the most popular form of traditional medicine (World Health Organization, 2017). Plants have been utilised worldwide for medicinal purposes from time immemorial. According to the estimations of WHO, more than 80% of the population in underdeveloped nations rely on plants as a primary source for their therapeutic demands which is mainly attributed to their ease of access and low cost (Kihagi et al., 2019). The use of herbal therapies has also gained widespread acceptance in developed countries as complementary and alternative medicines (Ekor, 2014). About 11% out of the total drugs in the essential medicine list of WHO are of plant origin (Wachtel-Galor & Benzie, 2011). The emerged medicinal herbalism science and others have allowed the expanded usage of medicinal plants (Maldonado Miranda, 2021). Traditional medicine has become an essential component of the healthcare system, which overcomes conventional medicine in some respects (Pan et al., 2014). Thus, the employment of traditional medicine needs to be optimised based on a research and development strategy (Pan et al., 2014).

2.3.1 Plants and phytochemicals as antidepressants

For a long time, herbal plants have revealed their therapeutic benefits in many indications, including the management of mental disorders. They have been utilised to identify potential therapeutic compounds or leads for new drug development

(Bahramsoltani et al., 2015). The wide variety of phytochemical components of medicinal plants provides a vast field of research in antidepressant therapies. The global use of herbal plants for treating depression and other psychiatric ailments has risen (Martins & Brijesh, 2018). More than 100 medicinal plants have been reported to possess *in vivo* antidepressant activity (Martins & Brijesh, 2018). Many plant metabolites, including flavonoids, alkaloids, saponins, terpenoids, amines, polyphenols, carbohydrates, fatty acids and essential oils exhibited anxiolytic and antidepressant effects (Bahramsoltani et al., 2015; Martins & Brijesh, 2018). Volatile oils such as lavender oil and *Acorus tatarinowii* oil are typically used to alleviate depression (Zhang et al., 2021). *Hypericum perforatum* (commonly known as St John's Wort) represents the only herbal alternative to clinical antidepressants used in treating mild to moderate depression (Wurglics & Schubert-Zsilavecz, 2006). The phloroglucinol derivative; hyperforin counts among the main constituents of St John's Wort, which is closely related to its antidepressant effect. Other components, such as hypericin and flavone derivatives may also participate in this activity. The phenolic compound curcumin from *Curcuma longa*, one of the few phytochemicals studied in clinical trials, demonstrated antidepressant effects at doses of 1 g/day (Sanmukhani et al., 2014). *Crocus sativus*, commonly known as saffron, has also found its way into human studies and exhibited equivalent effects to fluoxetine and imipramine at doses of 30 mg/day (Akhondzadeh Basti et al., 2007). Other herbs and their active constituents have also shown positive effects in reducing the symptoms of depression in clinical trials, such as turmeric, chamomile, valerian, *Ginkgo biloba*, *Echium amoenum* and *Rhodiola rosea* L. (Setorki, 2020). Many flavonoids obtained from nature have shown antidepressant-like effects *in vivo* studies, such as liquiritin derived from *Glycyrrhiza uralensis* (Zhao et al., 2008), quercetin (Bhutada et al., 2010), and proanthocyanidins (Xu Ying et al., 2010).

Alkaloids such as the amide alkaloid; laetispicine from *Piper laetispicum* (Yao et al., 2009), mitragynine from *Mitragyna speciosa* (Farah Idayu et al., 2011), piplartine from *Piper tuberculatum* (Cícero Bezerra Felipe et al., 2007), montanine from *Hippeastrum vittatum* (da Silva et al., 2006) and berberine from *Berberis aristata Linn* (Kulkarni & Dhir, 2008) possessed significant effects in animal models making them potentially useful in depression treatment. Coumarins such as scopoletin from *Polygalasa sabulosa* and psoralen from *Psoralea corylifolia* showed the ability to reverse the depression-like behaviour in animal models (Capra et al., 2010; Xu et al., 2008). Polysaccharides and essential oils from Magnolia bark and ginger rhizome showed antidepressant synergistic effects (Yi et al., 2009). Numerous triterpenoids and triterpenoids saponins from different plant sources have possessed antidepressant effects in preclinical studies, such as cucurbitacin IIa (Zhou et al., 2017a), chiisanoside (Bian et al., 2018) xanthoceraside (Guan et al., 2021) and ursolic acid (MacHado et al., 2012). The effectiveness of these various natural compounds on the nervous system and their antidepressant properties have been attributed to different mechanisms, including increasing synaptic monoamine neurotransmitters through the interaction with receptors or through inhibition of MAO enzymes, regulating the HPA axis, increasing BDNF levels, reducing oxidative stress, involvement of GABA, benzodiazepine and opioid receptors and regulating inflammatory mediators (Bahramsoltani et al., 2015; Fathinezhad et al., 2019; Jiang et al., 2019; Martins & Brijesh, 2018).

Herbal medicines are utilised as an alternative treatment for depression. However, most herbal remedies used to treat depression are extracts, either crude or semi-purified, and research involving the active component accountable for antidepressant activities is scarce (Hamid et al., 2017; Munir et al., 2020). Since plant bioactivity may be attributed to a single constituent or a combination of chemicals,

identifying active phytochemicals with both *in vitro* and *in vivo* inspections of mechanisms of action is essential for the proper characterisation of plant-based medications.

2.4 *Centella asiatica*

Centella asiatica Linn. Urban (Synonym *Hydrocotyle asiatica* Linn.) (commonly known as Asiatic Pennywort, Indian Pennywort and Gotu Kola) is a tropical medicinal plant belonging to *Apiaceae (Umbelliferae*) family indigenous to Southeast Asian countries such as Malaysia, India, China, Sri Lanka, and Indonesia, as well as Australia, South Africa, Madagascar, and Eastern South America (Chandrika & Prasad Kumara, 2015; Thakurdesai, 2021; Zahara, 2014). It is a herbaceous, faintly aromatic perennial plant (Fig. 2.1) used as a health tonic consumed as a fresh vegetable in Malaysia and Indonesia and in the preparation of juice, drink and other food products in different regions of the world (Chandrika & Prasad Kumara, 2015; Zahara, 2014). *C. asiatica* has been used as an ethnomedicinal herb for thousands of years with a vast range of therapeutic indications. It has been listed in the *Ayurveda* medicine under the name of *Mandukaparni*, as well as one of the Traditional Chinese Medicine (Alfarra & Omar, 2013).

Figure 2.1 *Centella asiatica* plant, adapted from Gray et al., 2018.

Numerous studies have reported on the various biological activities associated with *C. asiatica* such as; an antiulcer (Husori et al., 2021), an antidiabetic (Fitrianda et al., 2017), a hepatoprotective (Park et al., 2021), a cardioprotective (Bunaim et al., 2021), an immunomodulator (Griana, 2019), an anti-inflammatory (Qureshi et al., 2015), an anti-cancer (Manmuan et al., 2021), an antibacterial (Ramli et al., 2020), an antifungal (Akter et al., 2020), an anticonvulsant (Umamageswari et al., 2021), an antioxidant (Jhansi & Kola, 2019) and for venous insufficiency treatment (Chong & Aziz, 2013). *C. asiatica* is one of the superior herbs that are acting potently in the treatment of dermatological disorders (Park, 2021), wound healing (Azis et al., 2017), neuroprotective activity and revitalising nerves and brain cells, hence primarily known in India as the “Brain food” or "Brain tonic” according to the WHO monographs (Alfarra & Omar, 2013; Belwal et al., 2019; Chandrika & Prasad Kumara, 2015; European Medicines Agency, 2022). Due to its wide range of therapeutic purposes, the use of *C. asiatica* has notably increased in recent years and keeps rising from the primarily Asian market to the American and European markets (Idris & Nadzir, 2021).