ANTIDEPRESSANT EFFECT OF *ANDROGRAPHIS PANICULATA (BURM.F.) WALL. EX NEES* METHANOLIC EXTRACT AND ANDROGRAPHOLIDE ON CHRONIC UNPREDICTABLE STRESS (CUS) ZEBRAFISH MODEL VIA METABOLOMICS APPROACH

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2023

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

July 2023

ACKNOWLEDGEMENT

Completing a Master's degree is an arduous endeavor that demands resolute dedication, perseverance, and the invaluable support and guidance of numerous individuals. I am privileged to express my sincere appreciation to my primary supervisor, Dr. Fauziahanim Zakaria, whose guidance exceeded my expectations and left an indelible impact on my life. Furthermore, I extend my deepest gratitude to esteemed co-supervisors, Associate Professor Dr. Salizawati Muhamad Salhimi and Dr. Raghdaa al Zarzour, whose expertise and involvement significantly influenced my academic journey as an international student in Malaysia.

I am equally indebted to the faculty members of the School of Pharmaceutical Sciences, Universiti Sains Malaysia, whose unwavering assistance and support were always readily available to cater to the needs of the students.

Additionally, I would like to express my heartfelt thanks to my friends here in Malaysia, as well as my cherished family and loved ones back home in Syria, for their unwavering belief in my abilities, their constant motivation during challenging times, their enduring love, and their reminders to embrace the joys of life.

To all those mentioned above and countless others who have played a part in my academic journey, I extend my deepest gratitude. Your unwavering support has been instrumental in my accomplishments, and I am truly grateful.

Zenab Aldurrah.

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%RSD	relative standard deviation
1H NMR	Proton Nuclear Magnetic Resonance spectroscopy
5-HT	5-hydroxytryptamine
A. paniculata	*Andrographis paniculata*
Ac-CoA	acetyl coenzyme A
ACN	Acetonitrile
ATP	adenosine triphosphate
BBB	the Blood-Brain Barrier
BCAAs	branched-chain amino acids
CNS	central nervous system
CoV	coefficient of variation
CPMG	Carr-Purcell-Meinboom-Gill
CUMS	chronic unpredictable mild stress
CUS	chronic unpredictable stress
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	half maximal effective concentration
FID	flame ionization detector
FST	forced swimming test
GABA	γ-Aminobutyric acid
GAD	glutamate decarboxylase

GAS	gas anti-solvent
HMDB	Human Metabolome Database
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High-performance liquid chromatography
HPLC-ESI-MS/MS	high pressure liquid chromatography electrospray ionization tandem mass spectroscopy
i.p injection	intraperitoneal injection
ICH	International Conference on Harmonization
IR	infrared spectroscopy.
LC_{50}	half maximal lethal concentration
LCMS/MS	Liquid Chromatography with tandem mass spectrometry
LDT	Light dark test
LOD	Limit of detection
LOQ	Limit of quantitation
MAOIs	Monoamine Oxidase Inhibitors
MDD	Major depressive disorder
MRM	multiple reaction monitoring
MS	mass spectrometry
MS222	tricaine methane sulfonate
mTor	mammalian target of rapamycin
MW	Molecular weight
NAA	N-acetyl aspartate

NaSSAs	Noradrenergic and Specific Serotonergic Antidepressants
NLRP3	nucleotide-binding domain (NOD)-like receptor protein 3
NMDA	N-methyl-D-aspartate
NMR	high-resolution Nuclear Magnetic Resonance spectroscopy
OECD	The organization for economic cooperation and development
OFT	Open field test
PBS	Phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PDA	photometric diode array
RDoC	the Research Domain Criteria
RNA	Ribonucleic acid
ROC	receiver operating characteristic curve
RSM	response surface methodology
SFE	Supercritical fluid extraction
SIT	Social interaction test
SNRIs	Serotonin and Norepinephrine Re-uptake Inhibitors
SSRIs	Selective Serotonin Re-uptake Inhibitors

TCA Cycle	the citric acid cycle
TCAs	Tricyclic Antidepressants
TCM	traditional Chinese medicine
TEA	triethylamine
TRD	treatment-resistant depression
UHPLC	Ultra-High-Performance Liquid Chromatography
UPM	Universiti Putra Malaysia
USM	Universiti Sains Malaysia
UV	Ultra-violet
VFA	volatile fatty acid
WHO	World health organization
XBXT	Xiao buxin-Tang

KESAN ANTIDEPRESAN EKSTRAK METANOL *ANDROGRAPHIS PANICULATA (BURM.F.) WALL. EX NEES* DAN ANDROGRAFOLIDA PADA MODEL ZEBRAFISH TEKANAN TIDAK DAPAT DIRAMAL (CUS) KRONIK MELALUI PENDEKATAN METABOLOMIK

ABSTRAK

Kemurungan telah menjejaskan sekurang-kurangnya 322 juta orang di seluruh dunia. Rawatan untuk kemurungan telah dikaji sejak sekian lama. Walaubagaimanapun, ubat antidepresi terkini mempunyai banyak masalah seperti memakan masa yang panjang untuk mengatasi gejala dan penyakit berulang. Tumbuhan telah digunakan sebagai sumber utama dalam penemuan dadah selama beberapa dekad. *Andrographis paniculata (A. paniculata)* digunakan secara meluas dalam perubatan tradisional di Asia. Penyelidikan ini mengkaji kesan ekstrak *A. paniculata* dan "andrographolide" ke atas tekanan kronik tidak dapat diramalkan dalam model ikan zebra. Ekstrak metanol A. paniculata diprofil menggunakan HPLC-ESI-MS/MS, dan ujian akut ketoksikan dijalankan sebelum mengkaji potensi antidepresi tumbuhan tersebut. Kajian kelakuan iaitu ujian lapangan terbuka, ujian interaksi, dan ujian cerah dan gelap dijalankan untuk mengkaji perubahan tingkahlaku kumpulan stres kronik tidak dapat diramalkan dibandingkan dengan kumpulan kawalan, kumpulan terawat dengan *A. paniculata* dan kumpulan terawat dengan "fluoxetine". Selepas saringan menggunakan rawatan ekstrak tumbuhan, kajian diteruskan menggunakan salah satu sebatian utama tumbuhan tersebut iaitu "andrographolide". Kajian tingkah laku, kortisol dan metabolomic dijalankan pada semua kumpulan. Penambahbaikan dalam gerak alih ikan zebra didapati selepas dirawat dengan

ekstrak *A. paniculata* dan “andrographolide”. Pengurangan ketara didapati dalam tahap kortisol (t-test, p=0.0003) didapati selepas rawatan “andrographolide” (25 dan 50 mg/kg, i.p.). Kajian ini menunjukkan “andrographolide” (50 mg/kg, i.p) mampu mengurangkan depresi dalam stress kronik tidak dapat diramalkan dengan nilai EC_{50}=26.915 mg/kg dan menurunkan tahap kortisol tergantung dos rawatan. Metabolomik berdasarkan NMR mendapati rawatan menggunakan “andrographolide” (50 mg/kg, i.p) menunjukkan perubahan ketara dalam beberapa metabolit seperti “glutamine”, “epinephrine”, “GABA”, “glutathione”, “acetate”, “leucine”, “serotonin”, “creatine”, “betaine”, “homovallinic acid”, “choline”, dan “valine”. Analisis tapak jalan menjangkakan metabolisme alanin, aspartat dan glutamat terlibat dalam memberikan kesan antidepresi “andrographolide”. Kajian lebih mendalam terhadap sel dan molekul yang terlibat dalam memberikan kesan antidepresi oleh “andrographolide” dalam mamalia amat dicadangkan bagi mengkaji potensi ini dan memahami mekanisme yang terlibat dalam mengurangkan depresi dalam rawatan berdasarkan tumbuhan.

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ABSTRACT

Depression affected at least 322 million people worldwide. Treatment for depression has been studied for a long time. However, the current antidepressants have many associated problems, such as delays in symptom resolution and relapse. Plants have been used as the main source of leads in drug discovery for decades. *Andrographis paniculata* (*A. paniculata*) is a widely used plant in Asian traditional medicine. This research investigates the effect of *A. paniculata* extract and its major compound, andrographolide on the chronic unpredictable stress (CUS) zebrafish model. *A. paniculata* methanol extract's constituents was profiled using HPLC-ESI-MS/MS, and acute toxicity tests were analyzed before antidepressive evaluation. The behavioural study, i.e. open field test (OFT), social interaction test (SIT), and light dark test (LDT), were conducted to evaluate behavioural changes in the CUS group compared to the controls, *A. paniculata* (100 mg/L, immersion)-treated and fluoxetine (0.01 mg/L, immersion)-treated zebrafish. After the extract screening, the experiment was preceded by evaluating the antidepressive potential of andrographolide. Behavioural, cortisol and NMR-based metabolomics studies were evaluated in all groups. The behavioural study revealed a significant improvement in zebrafish locomotion after *A. paniculata* and andrographolide treatments. A significant reduction in cortisol levels (t-test, p=0.0003) were observed after andrographolide (25 and 50 mg/kg, i.p.) acute treatment. This study showed that andrographolide (50 mg/kg, i.p.)

able to reduce cortisol levels in the CUS zebrafish model with EC_{50}=26.915 mg/kg. Metabolomics study revealed that some of the metabolites that were significantly changed in the CUS model, such as GABA, epinephrine, glutathione, leucine and serotonin, were reversed to normal after andrographolide treatment. Alanine, aspartate and glutamate metabolism was predicted as the pathway involved in imparting the antidepressive effect of andrographolide. Further evaluation of the cellular and molecular underpinnings of the antidepressive effect of andrographolide in mammals model is strongly recommended to evaluate this potential and understand the underlying mechanism involved in mitigating depression in the plant-based treatment.

CHAPTER 1 INTRODUCTION

1.1 Background of the study

Many cultures believe that psychiatric disorders are a hoax, and a person suffering from mental health issues lacks faith. Hence, these disorders have a long history of being ignored or misdiagnosed. Depression is the most prevalent, disabling, and common psychiatric disorder worldwide. It affects 10% of the general world population, and the percentage can go as high as 20% in clinical settings (Ferrari et al. 2013; Tolentino & Schmidt. 2018). Depression is complicated; it could have many levels and different forms (Birx et al. 2016). It has affected at least 322 million people (WHO. 2017), which has only grown recently, especially with the forced isolation we live in due to Covid-19 (Bueno-Notivol et al. 2021).

Depression prevalence and cost are a considerable burden to governments; because of the consequences of symptoms such as suicide (800,000/year), leading to loss of workforce, low productivity, low productivity, and high cost of treatment. The total world spending on mental disorders reached between 2.05 to 6.98 trillion euros in 2010. In 2015 these numbers were still accurate (OECD/European Union. 2018). This number is expected to grow exponentially by 2030, making mental health problems the most significant expenditure in health sectors (Chisholm et al. 2016).

The fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-V) recognizes at least 256 unique symptoms that characterize major depressive disorder (MDD) (Birx et al. 2016; Buch & Liston, 2021). The diagnosis of depression requires five or more symptoms in two weeks (Birx et al. 2016; Tolentino & Schmidt, 2018). The main

symptoms of depression include anhedonia (the loss of pleasure), reduced cognitive performance, social withdrawal, altered pain perception, and other debilitating psychiatric symptoms (Neumann et al. 2011).

There are five classes of anti-depressants are currently in use, which are: selective serotonin re-uptake inhibitors (SSRIs), serotonin and norepinephrine re-uptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), noradrenergic and specific serotonergic antidepressants (NaSSAs), monoamine oxidase inhibitors (MAOIs). These medications come with varying side effects such as constipation or diarrhea, loss of appetite and weight, fatigue, anxiety, insomnia, nausea, headaches, sexual dysfunction, as well as prolonged delays in symptom resolution, low rates of complete remissions, substantial residual symptoms post-treatment and high rates of relapse (Si & Yu, 2016). These side effects differ in type and severity because each patient receives a tailored medication plan according to their needs while balancing interactions and side effects; these plans can take years to perfect while the patient suffers.

Due to the limitations of current pharmaceutical options, the hunt for new treatments is ongoing. These limitations pave the way toward new herbal medicine, which has always been the first steppingstone of pharmaceutical research. Green chiretta or Crete, king of bitters, Kalmegh, commonly known in Malaysia as *Hempedu Bumi*, are all different names for the same plant *Andrographis paniculata* (*A. paniculata*) belonging to the family *Acanthacea*e. It has been used in traditional medicine in India (known as Ayurvedic, Siddha, Indian tribal medicine), China, Thailand, and Indonesia. It is recommended to treat fever associated with infectious disease, digestive problems, postpartum-related problems, diabetes, cholera, malaria, dysentery, bronchitis, gonorrhea, cancer, and to purify the blood.

A. paniculata is one of widely pharmacologically tested plants for many diseases. It has healing properties such as choleretic, immunostimulant, hepato-protective, antipyretic, anti-inflammatory, antiplatelet, antioxidant, anthelmintic, antiviral bitter tonic, and valuable aid in the treatment of upper-respiratory infections (Abu Bin Nyeem et al. 2017; Dai et al. 2019). The main component of *A. paniculata*, aptly named andrographolide, was studied for its neuroprotective effect in the central nervous system (CNS) in rats, and found to have the ability to cross the blood-brain barrier (BBB) (Lu et al. 2019).

Previous studies were conducted to investigate the antidepressive activity of *A. paniculata.* Thakur et al. studied the antidepressant-like activity of *A. paniculata* on type-2 diabetic rats. MDD and type-2 diabssetes are considered low-grade chronic inflammations and comorbidities. Thakur et al. concluded that *A. paniculata* could be an alternative to traditional drugs used for MDD treatment (Thakur et al. 2014). Another study by Lu et al. found that *A. paniculata* has prophylactic and therapeutic effects in CNS-related disorders (Lu et al. 2019).

The common animal models used for biomedical research are mice, rats, or non-human primates. Nevertheless, the animal model used for this study is the zebrafish. Even though it has been known since 1980, research using it has only begun to gain traction in recent years (Howe et al. 2013). Zebrafish have many advantages as they show complex affective, social, and cognitive responses similar to those observed in rodents and humans as well as high physiological homology to humans and their organ structure and function are similar to humans. Zebrafish also have high throughput value, genetic tractability, low cost compared to other models, sensitivity to various drugs and agents, short reproductive

cycle, and the availability of both larval and adult fish models (Cachat et al. 2010; Egan et al. 2009; Stewart et al. 2013).

Several researchers used the chronic unpredictable stress (CUS) zebrafish model. Chakravarty and her co-workers reported that exposing zebrafish to 15 days of a CUS protocol with two stressors/day will induce anxiety and related mood disorder phenotype that usually appears in rodents and the chemical biomarkers of anxiety in zebrafish brain tissue (Chakravarty et al. 2013). Meanwhile, Pittman and Piato concluded that social isolation following CUS or as part of CUS would induce depression in zebrafish (Pittman & Piato, 2017). Fulcher has also studied CUS protocol with developmental isolation on zebrafish to induce depression and investigated the neurochemical and behavioural responses following isolation, which showed depressive behaviour and chemical hallmarks of depression: lowered serotonin and dopamine levels in the zebrafish brain (Fulcher et al. 2017).

The search for new natural products as new drugs has drawn on many sources, practices, and new biomedical analysis methods and studies. One such method of analysis is ^{1}H NMR-based metabolomics approach. Metabolomics is a comprehensive analysis of the whole metabolome under a given set of conditions (Fiehn, 2002). One primary goal of metabolomics study is to identify biomarkers and understand the interplay between molecular and cellular components. It also allows for simultaneous identification and quantification of metabolites under certain conditions (Kim et al. 2010) and measurement of any changes to the system that might happen over specific time points (Mushtaq et al. 2014). Many instrumental platforms can be used for metabolomics study, such as high-resolution Nuclear Magnetic Resonance (NMR) spectroscopy, mass spectrometry (MS),

and infrared (IR) spectroscopy. This study's most suitable platform is proton nuclear magnetic resonance (^{1}H NMR). ^{1}H NMR approach's main advantage is its suitability for quickly analyzing primary and secondary metabolites. In this study, the ^{1}H NMR approach was used to evaluate the antidepressant effect of *A. paniculata* on CUS induced zebrafish model and help identify biomarkers in the affected pathways.

1.2 Problem statement/Originality of research

A previous study using mice as an animal model and applying Chronic unpredictable mild stress protocol by Geng et al. (2019) failed to determine andrographolide EC50 as they only tested two concentrations of andrographolide which is not sufficient to plot and calculate EC50. Similarly, another study by Thakur, Soni et al. (2014) studied the effects of andrographolide as well as A. paniculata extract on stress induced pathologies in rats and used only two doses of andrographolide. They simply observed the lowest effective daily dose to produce results but again, failed to calculate it precisely. This exhibits the existing gap of knowledge regarding EC50 of andrographolide.

Metabolomics represents a rapidly expanding field of study that centers around the comprehensive analysis of small molecules, known as metabolites, present in biological samples. This discipline presents various advantages that contribute to its significance in diverse research areas and applications. These advantages include providing a holistic view of cellular metabolism, offering insights into functional phenotypes, facilitating drug discovery and development processes, enabling a systems biology perspective, and aiding in the comprehension of the metabolic effects of drugs, as well as the assessment of their

efficacy and toxicity. Additionally, metabolomics has the capacity to identify metabolic pathways influenced by drugs, thereby leading to the discovery of new drug targets or biomarkers.

While previous metabolomics studies have been conducted on andrographolide, these investigations have primarily focused on different illnesses and body areas. For instance, Luo et al. (2021) concentrated their research on the effects of andrographolide against lung cancer, utilizing urine as the targeted sample. Furthermore, Alapid et al. (2021) directed their study towards blood samples, examining the effects of andrographolide on uninfected red blood cells (uRBCs). Consequently, a knowledge gap exists concerning the metabolomics of andrographolide, particularly with respect to its effects on the brain or depression. Furthermore, the pathways implicated in its potential impact on the brain have yet to be investigated.

1.3 Research questions

Some of the research questions in the study are:

1. How does *A. paniculata* extract show antidepressant activity in the zebrafish model via behavioural analysis?
2. What hallmark biochemical alterations in the metabolome of CUS-induced zebrafish after treatment with andrographolide could be linked to the plant extract's antidepressant action?

1.4 Research hypotheses

A. paniculata is a well-known and widely used plant in Asian traditional medicine (Jiao et al. 2019) that has many aspects that qualify it as a potential antidepressant. If it does have activity as an antidepressant, then its crude extract and principal component, andrographolide, will instigate a positive change in the CUS zebrafish model when administered.

1.5 Research aim and objectives

1.5.1 General Objective

The main objective of this research was to investigate the effect of *A. paniculata* extract and its major compound, andrographolide on the chronic unpredictable stress zebrafish model and to compare its potential as an antidepressant versus the currently most prescribed antidepressant, i.e. fluoxetine toward gaining a better idea of plant use in alleviating depression.

1.5.2 Specific Objectives

1. To profile chemical constituents in *A. paniculata* methanolic extract by using the HPLC-ESI-MS/MS method.

2. To evaluate the behavioural effects of *A. paniculata* extract on the chronic unpredictable stress zebrafish model.

3. To study behavioral changes, cortisol levels and brain's biochemical profile of chronic unpredictable stress-induced zebrafish model before and after exposure to andrographolide.

CHAPTER 2 LITERATURE REVIEW

2.1 Depression

Depression, an extensively studied psychiatric disorder, exhibits diverse manifestations across multiple dimensions. Among its various forms, Major Depressive Disorder (MDD) stands out as the most common and prevalent condition requiring prolonged treatment. MDD significantly impacts both the productivity and the overall quality of life of affected individuals (Birx et al. 2016; Kennedy et al. 2006).

As a complex and heterogeneous syndrome, depression presents a multitude of etiological factors (Buch & Liston, 2021; Krishnan & Nestler, 2008). The Diagnostic and Statistical Manual of Mental Disorders, a contemporary diagnostic resource, recognizes an extensive array of 256 distinct symptoms that characterize MDD (Birx et al. 2016; Buch & Liston, 2021). To meet the criteria for depression diagnosis, it is required that five or more symptoms persist for a duration of at least two weeks (Birx et al. 2016; Tolentino & Schmidt, 2018).

Prominent symptoms associated with depression include anhedonia (loss of pleasure), sleep disturbances, social withdrawal, changes in body weight, psychomotor agitation or retardation, fatigue or loss of energy, impaired cognitive function, feelings of worthlessness or excessive guilt, and suicidal ideation. These symptoms are assessed in a binary manner, denoting their presence (1) or absence (0), irrespective of their severity (Birx et al. 2016; Kennedy et al. 2006; Nguyen et al. 2014; Tolentino & Schmidt, 2018). Notably, individuals diagnosed with depression also exhibit an increased prevalence of comorbid conditions, such as cardiovascular morbidity with a 40% higher likelihood

compared to the general population (Dhar & Barton, 2016), as well as diabetes, peptic ulcers, cognitive impairment, and compromised immune function (Brown et al. 2004).

Regarding the underlying causes of depression, the prevailing theory posits two primary types: psychogenic and organic (Beck & Alford, 2009). Organic depression can be attributed to various factors, including endocrine disorders, certain types of cancers and tumors, and medication side effects (e.g. internal isotretinoin for acne). On the other hand, psychogenic depression stems from psychological factors such as negative self-perception, sensitivity to rejection, neuroticism, rumination, and negative emotional states (Remes et al. 2021). Stress also emerges as a significant contributing factor in the development of depression (Kalueff & Tuohimaa, 2004).

2.2 Current antidepressant drugs and their limitations

Five classes of anti-depressants are commonly prescribed to patients which are: selective serotonin re-uptake inhibitors (SSRIs), serotonin and norepinephrine re-uptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), noradrenergic and specific serotonergic antidepressants (NaSSAs), monoamine oxidase inhibitors (MAOIs). These medications come with varying side effects such as constipation or diarrhea, loss of appetite and weight, fatigue, anxiety, insomnia, nausea, headaches, sexual dysfunction, as well as prolonged delays in symptom resolution, low rates of full remissions, substantial residual symptoms post-treatment and high rates of relapse (Si & Yu, 2016). These side effects differ between patients in terms of type and severity because each patient receives a tailored medication plan according to their needs while still balancing interactions and side effects; these plans can take years to perfect while the patient suffers.

The effectiveness of current antidepressants is under scrutiny because only 11% of patients receiving treatment have gone into full remission (Kennedy et al. 2006; Mendlewicz, 2008). Furthermore, 30% of diagnosed patients have failed to respond to treatment with current antidepressants (Geng et al. 2019). Moreover, 85% of patients who go into remission tend to relapse within a decade, while 50% or more relapse within six months of apparent clinical remission if they discontinue treatment (Sim et al. 2016). Currently, SNRIs and TCAs are first line chronic medication choices for MDD. Remission rates are, however, poor following a 2- to 3-month treatment period, ranging from 20 to 40% in naturalistic studies to 40 to 60% in randomized trials. Additionally, despite taking many chronic antidepressants, some individuals may not achieve remission; this condition is known as treatment-resistant depression (TRD).

2.3 Plants used as antidepressants

The knowledge of utilising plants for medicinal purposes began to be developed thousands of years ago. More than 20,000 species of plants in the world have been estimated to be utilised as traditional medicine, which is a huge reservoir for potential discovery of new drugs.Even in this new era of largely allopathic medicine, researchers worldwide are focusing on herbal medicines to complement contemporary medications and as sources of novel drug leads since medicinal plants have historically demonstrated their worth as sources of molecules with therapeutic potential (Rocchetti et al. 2019).

An et al. (2015) studied the traditional Chinese medicine (TCM) *Xiao buxin-Tang (XBXT*) for antidepressive effect on mice. XBXT comprises four TCM herbs, one of which (*Folium phyllostachydis henonis*) has known anti-inflammatory characteristics. XBXT

was found to significantly affect treating depression compared to duloxetine (An et al. 2015). Another plant studied for its effect on depression is *Camellia sinensis* or green tea. The polyphenols in green tea were investigated by Zhu et al. (2012) on mice depression model in behavioral test and in serum corticosterone levels. Green tea polyphenols were found to have an anti-depressant like effect in behavioral tests and to have reduced serum corticosterone levels in forced swim test mice and hypothesized that the mechanism may involve inhibition of the hypothalamic–pituitary–adrenal axis. A following study by Liu et al. (2013) investigated the antidepressant effects of polyphenols on CUMS mice and concluded that the effect was mediated through both monoaminergic pathways (serotonin and noradrenaline) as well as antioxidant defenses. *Morinda officinalis* or Indian mulberry was postulated to have antidepressive and anti-inflammatory effects. (Zhang et al. 2018). Zhang et al. (2002) studied the antidepressive quality of Indian mulberry in both rats and mice via the DRL 72-s schedule and forced swimming test (FST) respectively. The plant extract managed to produce an effect in rats similar to that of the comparison drug desipramine, while in FST on mice, the extract exhibited a significant reduction in immobility duration similarly to desipramine, thus proving it has antidepressive activity.

Another study by Liao et al. (2020) looked into the potential anti-depressant properties of curcumin, the primary secondary metabolite of turmeric (*Curcuma longa*) in CUMS rats via behavioral tests, serum corticosterone levels, PCR analysis, antioxidant activity and several blots and stains. Liao and colleagues found that curcumin can prevent depression in CUMS rats by reversing changes in oxidative stress, the Nrf2-ARE signaling pathway, and the synaptic and neural plasticity. Curcumin has been studied clinically in several trials on human, mostly randomized double blind trials, where curcumin

demonstrated positive effects on mood and behavior, with reduction of depression symptoms, as well as better results than placebo. (Moragrega & Ríos, 2022)

Crocus sativus, or saffron, from family *Iridaceae* is also a commonly used traditional herb with extensive studies on its various properties. The main component of saffron is safranal. Saffron extract has been proven to inhibits reuptake of monoamines (dopamine, noradrenaline, serotonin) and also exhibits NMDA receptor antagonism and $GABA_A$ agonism. These traits appear to bear responsibility for saffron`s antidepressant-like and anxiolytic effects demonstrated in animal models in both elevated plus maze and open field test (Hosseinzadeh & Sadeghnia, 2007; Khazdair et al. 2015; Lechtenberg et al. 2008). Several clinical studies were performed using saffron to assess its antidepressive quality, mostly randomized double blind trials. It was tested against postpartum depression, MDD and anxiety associated with type-2 diabetes, MDD associated with menopause, as well as generalized and severe depression. Saffron exhibited positive effects in most cases with a moderating effect on postmenopausal hot flashes as well. (Moragrega & Ríos, 2022).

St. John’s wort, or *Hypericum perforatum,* has been traditionally used in Europe as a melancholy herbal remedy (Dauncey et al. 2019; Sarris, 2018). St. John's wort has a high affinity for adenosine, GABA-A, GABA-B, and glutamate receptors and may be an inhibitor of MAO-A and MAO-B activity as well as the neuronal reuptake of serotonin, dopamine, and noradrenaline. Many of the plants pharmaco-activity can be accredited to main components hypericin, hyperforin and existing flavonoids (Butterweck, 2003). This plant was studied clinically, where several placebo controlled trials showed the plant has no effect, while other proved the opposite. Several review studies analyzed the trials and

concluded that St. John's wort is more effective than placebo in mild to moderately severe depression, has better results than placebo in MDD while nearing the results of SSRIs only with fewer side effects. These results indicate that standardized extracts of St. John's wort have the impact of standard anti-depressant without most of the side effects and thus represent a valid alternative. (Moragrega & Ríos, 2022)

2.4 *A. paniculata* chemical profile

A comprehensive exploration of *A. paniculata* has involved the systematic analysis of various plant parts, each exhibiting distinct chemical compositions. These investigations encompassed phytochemical analyses and compound isolation, employing diverse methods for solvent fractionation, harvest conditions, and chromatographic techniques. Notably, thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), liquid chromatography, micellar electrokinetic capillary chromatography (MECC), high-speed counter-current chromatography (HSCCC), Silica Gel Chromatography (SGC), flow injection spectrophotometry (FIS), and proton nuclear magnetic resonance (^{1}H NMR) have been employed for the analysis of *A. paniculata* (Hossain et al. 2021; Tajidin et al. 2019). As of the present study, a total of 142 distinct compounds have been identified from *A. paniculata*, among which 50-60% have demonstrated therapeutic efficacy. These secondary metabolites primarily belong to the lactones, diterpenes, flavonoids, quinic acid, xanthones, noriridoids, and other compound classes.

Among the identified compounds, the largest proportion (82) falls within the terpenoid class, with andrographolide ($C_{20}H_{30}O_5$) being the most abundant secondary metabolite in A. paniculata. This labdane diterpenoid is present throughout the entire

plant, with higher concentrations isolated from the roots and leaves, and exhibits diverse medicinal activities. Notably, andrographolide attains its peak concentration during the vegetative stage, shortly before flowering, typically occurring around 130 days after the initial cultivation (Intharuksa et al. 2022; Mussard et al. 2019). Other terpenoids present in A. paniculata include neoandrographolide ($C_{26}H_{40}O_8$), andrographiside, and glucopyranosylandrographolide. Flavonoids, comprising the second most abundant compound group (42), include apigenin 7-O-glucoronide and its derivatives. While flavonoids were detected in the aerial parts of the plant, their primary source of isolation and understanding lies within the roots. Additionally, xanthones such as 1,2-dihydroxy-6,8-dimethoxy xanthone, quinic acid derivatives like 3,4-dicaffeoylquinic acid, rare noriridoids such as andrographidoids A, B, C, D, and E, as well as sitosterols and other compounds, constitute the less abundant components (Aminah et al. 2021; Chao & Lin, 2010; Dua et al. 2004; Hossain et al. 2014).

2.5 Pharmacological activity of *A. paniculata* and its constituents

A. paniculata has been found to have healing properties such as choleretic, immunostimulating, hepato-protective, antipyretic, anti-inflammatory, antiplatelet, antioxidant, anthelmintic, antiviral bitter tonic, and valuable aid in the treatment of upper-respiratory infections (Abu Bin Nyeem et al. 2017). Andrographolide has already proven to have anti-oxidative, anti-inflammatory, neuroprotective, neurotherapeutic, anti-malaria, anti-bacterial, hepato and cardiovascular protective effects (Dai et al. 2019).

2.6 *A. paniculata* potential as antidepressant

Previous studies were conducted to investigate the antidepressive activity of *A. paniculata*, but they were only done on specific case scale and not on general depression cases such as major depression disorder (MDD). Thakur et al. studied the antidepressant-like activity of *A. paniculata* on type-2 diabetic rats. Both MDD and type-2 diabetes are considered as low-grade chronic inflammations as well as comorbidities. Studies have shown a high comorbidity rate between depression and insulin resistance in the brain suggesting a possible interplay of biological substrates. One such substrate was revealed to be P2X7-mediated activation of NLRP3 inflammasome which plays a valued role at the onset and progression of both MDD and type-2 diabetes (Wang et al. 2020). Thakur et al. concluded that *A. paniculata* could be a phyto-alternative to traditional drugs used for this type of depression (Thakur et al. 2014). Sani et al. studied the antidepressive activity on lipopolysaccharide-induced rats (Sani et al. 2019; Zhang et al. 2019). Another study done by Lu et al. found that *A. paniculata* has prophylactic and therapeutic effects in CNS related disorders (Lu et al. 2019). These results give hope and a strong chance of *A. paniculata* affecting more general and non-disease related depression such as MDD, which is the main form of clinically diagnosed depression. Recently, andrographolide's (2.5 and 5 mg/kg) antidepressive effect in chronic unpredictable mild stress (CUMS) was also studied in mice (Geng et al. 2019).

2.7 Animal model in drug discovery

The standard models to study depression and antidepressant activity are rodents, non-human primates, and, most recently, zebrafish (Berton et al. 2012). These models

have their benefits and limitations. Rodents have been the traditional translational study model for depression research since 1977-1978 (Porsolt et al. 1978).

In MDD, the current understanding of pathogenic mechanisms related to affective (emotion), cognitive, and homeostatic abnormalities remains insufficient. (Berton et al. 2012). As a result, selecting an animal model is an essential aspect of experimental design. The chosen model must meet specific requirements to understand the disease's origins and pathophysiology. Despite the lack of success in producing new and better drugs and treatments for psychiatric illnesses, research should continue. In vivo models are vital in bridging the gap between preclinical discoveries and clinical development (Bouwknecht, 2015).

Subjective sensations, such as suicidal tendencies, which are difficult to measure and model in animals, are the most conspicuous manifestation of depression. Modelling a depressive symptom that can be translated into behaviour and evaluated in animal models, such as motor responses to stress (more movement when anxious but none when depressed), social withdrawal, and enhanced sensitivity to the light/dark cycle is an alternate technique (Duman, 2010; Golla et al. 2020; Green et al. 2012).

2.8 Zebrafish model to study stress-related disorders

Recent studies have reported a similarity between zebrafish and rodents as depression models with zebrafish requiring less time to exhibit results, making it a reliable model for depression (Zhang et al. 2021). Furthermore, zebrafish are genetically homologous to humans by 70%. A large number of orthologous genes of the serotoninergic, dopaminergic, opioidergic, and GABAergic systems all relevant to stress-related study (Demin et al. 2021). Zebrafish have also been shown to have cognitive maps

and spatial memory (Gerlai, 2016; Howe et al. 2013; Lee et al. 2015). In addition, the short reproductive cycle, high throughput, low cost, genetic tractability, high sensitivity to anti-depressant drugs, availability of both larval and adult fish models, lower sentience, and generally well-defined and sophisticated behavioral responses that can be easily tracked using video monitoring make zebrafish an ideal and attractive model animal for CNS-related study (de Abreu et al. 2018; Dooley & Zon, 2000; Kalueff et al. 2014; Lachowicz et al. 2021; Nguyen et al. 2014). Just like humans, the stress reactions in zebrafish are mediated by cortisol, which is activated by a cascade of hypothalamopituitary hormones activating through glucocorticoid receptors making them highly sensitive to chronic stress and have similar main neurotransmitters to humans (Demin et al. 2021; Marcon et al. 2016)

In addition, the zebrafish contains 25 pairs of chromosomes, which contain around 26,000 protein-coding genes. The significant genetic homology enables easy translation into humans. Zebrafish studies have validated the relationship of specific risk genes in humans that have long been assumed to be linked to the formation of the habenular brain network similar to mammals (Bühler & Carl, 2021; Howe et al. 2013).

2.9 Metabolomics approach in drug discovery study

Metabolites are the end products of cellular regulatory processes, as biological responses to genetic and environmental changes. Metabolomics is a multidisciplinary field of research studying low-molecular-weight (commonly MW<1500 Dalton) metabolites. Hence, metabolomics can be a beneficial tool for understanding the cellular and molecular basis of disease as well as how it is affected by medications, and the accompanying alterations and processes in the CNS providing fresh perspectives on the mechanisms and

pathways shifts behind CNS disorders. Metabolites chemically transform in response to a particular stimulus or biological process leaving indicative traces of what is actually happening on a molecular level, thus furnishing unbiased monitoring of a broad range of changes in brain metabolism. (Ivanisevic & Siuzdak, 2015).

Approaches to metabolomics analysis in use can be categorized as targeted or un-targeted metabolomics, however it is worth of note, that Fiehn and Nicholson (Fiehn, 2002; Nicholson et al. 1999) have suggested a classification system. Targeted metabolomics can be defined as "the quantitative or semi-quantitative measurement of a defined group of metabolites known to be involved in a specific biochemical pathway or metabolic reaction", while untargeted metabolomics is defined as "a global unbiased analysis of all the metabolites present in a biological system, including chemical unknowns, under a set of circumstances" (Naz et al. 2014). Non-targeted metabolomics appear to work best in discovery phase yet due to the considerable amount of data obtained using this approach, it must be coupled with advanced chemometric techniques such as univariate/multivariate data treatment to organize the data and assign importance so we can work with a smaller manageable data set. (Roberts et al. 2012).

Brain tissue metabolomics has been engrossed in studying various animal models with induced brain injury or disorders as the availability of human brain tissue especially a control sample is limited and challenging because death and asphyxia of the brain can introduce a chemical shift in brain metabolites . Nonetheless animal models provide ample evidence for research, but the great complexity of the brain poses a challenge for results analysis and understanding (Gonzalez-Riano et al. 2016).

Metabolomics machination utilizes several analytical platforms such as LCMS/MS, GCMS, and NMR, each with their own advantages. NMR advantages include noninvasive and unbiased detection, great objectivity and reproducibility among samples given the relatively short run time, and simple processing steps that require minimal preparation of samples and little to no separation (Duan & Xie, 2020; Snowden et al. 2012).

Several studies have previously utilized biofluids (the cerebrospinal fluid, plasma, saliva, urine) instead of tissue for analysis as it is easier to obtain especially from human targets, and the metabolic profiling can be reliably assessed using the aforementioned analytical platforms. But, due to the restricted passage of many metabolites over the BBB, it is unclear, though, how much the biofluid's content represents the tissue activity (Ivanisevic & Siuzdak, 2015), therefore brain tissue (from biopsies or post-mortem brain analysis whether human or animal) and brain cell lines provide more reliable results. hence our choice to analyze the whole brain from each animal model we used. Following global metabolite profiling available in databases such as KEGG and HMDB obtained using ^{1}H NMR-based metabolomics has increased the understanding and characterization of CNS disorder signaling and linked these disorders to dysregulated metabolism, altered signal pathways, and variations in central carbon pathways. (Dumas & Davidovic, 2015). While McClay et al. used GCMS and LCMS/MS to understand an antipsychotic drug's reaction and mode of action for the treatment of schizophrenia in C57BL/6 J mice brain tissue and identified the disturbance of sphingolipid metabolism brought on by long-term haloperidol administration as well as the deterioration of the NAAG signaling pathway in haloperidol mechanism of action (McClay et al. 2015). Another study utilized targeted

NMR-based metabolomics on Guinea pig cortical brain slices to define the metabolic activity of GABAρ receptors in the brain and how it relates to $GABA_A$ receptors (Rae et al. 2015). The focus of this study is on depression and primarily MDD. MDD has been associated with disturbances in amino acid metabolism, energy metabolism, and lipid metabolism and metabolomics have the potential to discern MDD patients from healthy controls as well as evaluate treatment response. A study by Prabhu et al. explored metabolite changes susceptible to stress in socially defeated mice as a model of depression, and found there were alterations in several metabolism pathways related to amino acids, lipids, and neurotransmitters in several brain areas (Prabhu et al. 2019). Chen et al. also studied the metabolites disturbances in CUMS rats using non-targeted metabolomics and utilizing GCMS analysis. They found significantly altered levels of isoleucine, glycerol, N-acetyl aspartate and beta alanine in CUMS rats prefrontal cortex compared to control rats (Chen et al. 2015). Another study by Wang et al. utilized metabolomics in drug discovery by investigating the effects of St. Johns wort, ginsenoside, and clomipramine on CUMS rats brain tissue using GCMS metabolomics towards gaining better mechanistic insights on their work. The study found that all three have protective effects in CUMS rats, while clomipramine and St. johns wort decrease the changes to monoamine neurotransmitter metabolites, ginsenosides affects both excitatory/inhibitory amino acids and monoamine neurotransmitters with the highest amount of restoration of cerebellar and peripheral metabolites. (Wang et al. 2012). Meanwhile Zakaria et al. used ^{1}H NMR-based metabolomics on reserpine zebrafish brain to detect changes in amino acid metabolism related to depression and found several perturbations (Zakaria et al. 2021).

The aforementioned considerations guide the utilization of non-targeted ^{1}H NMR-based metabolomics analysis of zebrafish brain tissue subjected to chronic unpredictable stress (CUS) for the present study. This approach is chosen to provide comprehensive insights into the potential antidepressive effect of *A. paniculata* extract and its primary constituent, andrographolide. Consequently, the investigation encompasses the assessment of molecular alterations in the zebrafish brain metabolome following treatment with *A. paniculata* extract and andrographolide, employing ^{1}H NMR-based metabolomics. Additionally, concurrent analysis of behavioral changes and cortisol levels is conducted to complement the metabolomics findings. Bioinformatics tools were used to enrich the data and analyze it further reducing the complexity of the data and improving our understanding of it to help identify biomarkers and pathways (Barupal et al. 2018; Marco-Ramell et al. 2018).

CHAPTER 3 METHODOLOGY

3.1 Materials and chemicals

The leaf part *of A. paniculata* was provided by Melor Nursery, Bukit Tinggi, Johor, Malaysia as well as the whole living plant for identification purposes. Fluoxetine hydrochloride (CAS number: 56296-78-7) and Andrographolide (purity:98%) (CAS number:5508-58-7), Chloroform-d (99.8 atom% D), and 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (98 atom% D) were acquired from Sigma Aldrich (St. Louis, Missouri, USA), analytical grade methanol was purchased from Fischer Scientific and HPLC grade acetonitrile and methanol was supplied by Merck, Germany. Ultrapure water with a resistivity greater than 18.2 MΩ, obtained from a certified Milli-Q system (Millipore, Bedford, MA, USA), was used for HPLC in quantitative analysis and LCMS/MS analysis for chemical profiling. Cortisol assay kit catalog number KGE008B was acquired from R&D systems, USA. Deuterated methanol (99.8 atom% D) and deuterium oxide were purchased from ACROS organics, Switzerland. R&M chemicals, Malaysia, supplied Sodium dihydrogen phosphate (NaH_2PO_4), Di-sodium hydrogen phosphate (Na_2HPO_4) and Sodium azide (NaN_3).

3.1.1 Experimental Overview

The research methodology employed in the current study is visually depicted in Figure 3.1. Initially, the plant material was subjected to extraction, followed by lyophilization. Subsequently, it was dissolved and prepared to facilitate the identification and quantification of the major compound, namely andrographolide, using HPLC-ESI-

MS/MS analysis. Next, zebrafish of the species Danio rerio (short fin) were obtained, with a balanced representation of both males and females (50% each) and a total of n=210 individuals. These zebrafish were allowed to acclimatize for a period of two weeks. To determine the lethal concentration that affects 50% of the test organisms (LC50), an acute toxicity test was conducted.

Following the acute toxicity assessment, four groups of zebrafish were established, namely the control group, chronic unpredictable stress (CUS) group, CUS group with *A. paniculata* treatment, and CUS group with fluoxetine treatment. Over a duration of 14 days, these zebrafish groups were subjected to seven stressors to induce a state of depression. Subsequently, immersion was utilized as the mode of administration for the *A. paniculata* extract (at a concentration of 100 mg/L) and fluoxetine (at a concentration of 0.05 mg/L) to treat the zebrafish. The selection of the dose for *A. paniculata* extract was informed by the findings of the toxicity study conducted in the present investigation. Conversely, the dose of fluoxetine was determined based on the work of Marcon et al. (2016). Behavioral testing was then conducted on these treated zebrafish.

The results of the behavioral tests indicated a positive influence of the plant extract on the zebrafish model. Consequently, the major constituent of the plant, andrographolide, was selected for further investigation. Chronic stress was induced in six additional groups of zebrafish, and subsequently, these groups were treated with different doses of andrographolide (5, 25, and 50 mg/kg) and fluoxetine (10 mg/kg) for comparative purposes. The selection of andrographolide doses was guided by the studies conducted by Zhang et al. (2019) and Geng et al. (2019b) . Furthermore, the dose of fluoxetine was