EFFECTS OF STANDARDIZED HYDROALCOHOLIC EXTRACTS OF ORTHOSIPHON STAMINEUS ON BEHAVIOUR OF GESTATIONALLY-STRESSED POSTPARTUM RATS

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LIST OF ABBREVIATIONS

BDNF	Brain-derived neurotrophic factor
CMS	Chronic mild stress
CORT	Corticosterone
EOS	95% ethanol extract of OS
ERK 1/2	Extracellular-regulated kinase
EUP	Eupatorin
EWOS	50% ethanol + 50% water extract of OS
FST	Forced swim test
HPTLC	High-performance thin-layer chromatography
IPT	Interpersonal therapy
OB	Olfactory bulbectomized
OFT	Open field test
OS	*Orthosiphon stamineus* (benth.)
OS100	100 mg/kg of OS 50% ethanol extract
OS200	200 mg/kg of OS 50% ethanol extract
PPD	Postpartum depression
RA	Rosmarinic acid
SIN	Sinensetin
TLC	Thin-layer chromatography
TMF	3’-hydroxy-5,6,7,4’-tetramethoxyflavone
TST	Tail suspension test
WOS	Water extract of OS

KESAN EKSTRAK TERPIAWAI HIDROALKOHOLIK ORTHOSIPHON STAMINEUS KE ATAS TINGKAH LAKU TIKUS POSTPARTUM YANG DIARUH STRESS SEMASA GESTASI

ABSTRAK

Orthosiphon stamineus (OS) telah lama digunakan sebagai rawatan tradisional untuk pelbagai penyakit, tetapi bukan untuk penyakit psikiatri. Salah satu sebatian fenolik, iaitu asid rosmarinik, telah terbukti mempunyai kesan positif antimurung terhadap tikus dalam beberapa kajian terdahulu. Objektif utama dalam kajian ini adalah untuk mengenalpasti kesan ekstrak OS etanol terhadap tingkahlaku tikus betina yang hamil dan telah didedahkan dengan stress semasa gestasi, yang merupakan salah satu faktor tingkahlaku kemurungan postpartum bagi tikus. Proses mengekstrak OS dilakukan menggunakan teknik maserasi selama 48 jam. Melalui HPTLC, kewujudan asid rosmarinik, eupatorin, sinensetin, dan TMF telah dipastikan. Hasil ekstrak terbanyak diperoleh daripada pelarut 50% etanol, manakala kepekatan asid rosmarinik paling tinggi diperolehi daripada pelarut 95% etanol. Ekstrak standard etanol OS 50% digunakan untuk kajian *in vivo*. Selepas proses mengawan, tikus-tikus betina Sprague Dawley dibahagikan kepada dua kumpulan – stress dan bukan stress. Kumpulan stress dikenakan prosedur *chronic mild stress* dari selepas proses mengawan sehingga hari melahirkan anak, manakala bagi kumpulan bukan stress pula tidak dikenakan sebarang prosedur. Setelah proses kelahiran, kumpulan stress dibahagikan kepada 4 kumpulan baharu – tanpa rawatan, rawatan amitriptyline 20 mg/kg (Ami), rawatan OS 100 mg/kg (OS100) dan rawatan OS 200 mg/kg (OS200). Rawatan-rawatan diberikan secara oral setiap hari sehingga berakhirnya ujian tingkahlaku. Selepas pemisahan anak dilakukan, tikus-tikus diuji pergerakan lokomotor dan tingkahlaku menyerupai

keresahan dalam ujian *open field*, manakala tingkahlaku menyerupai kemurungan diuji dalam ujian *forced swim*. Melalui ujian *open field*, analisis ANOVA satu arah menunjukkan rawatan dengan amitriptyline dan kedua-dua ekstrak OS memberikan peningkatan ketara dalam pergerakan lokomotor bagi tikus yang menghadapi stress postpartum; keputusan yang sama turut diperoleh daripada kumpulan bukan stress ($p<0.05$). Tiada perbezaan ketara bagi tingkahlaku menyerupai keresahan untuk semua kumpulan. Bagaimanapun, dalam ujian *forced swim* hanya kumpulan Ami dan OS100 mempunyai pengurangan tempoh tanpa pergerakan yang hampir sama dengan kumpulan control ($p<0.0001$). Melalui analisis ELISA pula, paras BDNF daripada kumpulan OS100, OS200, dan Ami lebih rendah daripada yang dijangkakan ($p<0.05$). Kesimpulannya, ekstrak OS etanol terbukti mempunyai kebolehan untuk memulihkan kesan buruk daripada stress gestasi terhadap tikus. Oleh itu, berpotensi sebagai penawar untuk mengatasi kemurungan postpartum. Walau bagaimanapun, mekanismenya bukan diperantarakan oleh neurogenesis oleh BDNF dan memerlukan lebih penyelidikan.

EFFECTS OF STANDARDIZED HYDROALCOHOLIC EXTRACTS OF ORTHOSIPHON STAMINEUS ON BEHAVIOUR OF GESTATIONALLY-STRESSED POSTPARTUM RATS

ABSTRACT

Orthosiphon stamineus (OS) has been traditionally used for many ailments but for psychiatric disorders. Rosmarinic acid (RA) is one of the phenolic compounds contained in OS, and it has been shown to have positive antidepressant-like effect on rats in other studies. The objective of this study is to investigate the effects of ethanolic OS extract on the behaviours of rat dams exposed to gestational stress, which is linked to being a factor of postpartum depressive-like behaviours in rodents. Extraction of OS was carried out using the maceration technique for 48 hours. Through HPTLC, the presence of RA, eupatorin, sinensetin, and TMF were confirmed. The highest percentage of extract yield was from 50% ethanol, while the most concentration of RA obtained was from 95% ethanol. For *in vivo* study, 50% ethanolic OS extract was chosen to be administered to the rats. Female Sprague Dawley rats were mated and divided into two groups – stressed and non-stressed. The stressed group was subjected to chronic mild stress procedure after mating until the day of parturition, whereas the non-stressed group was left undisturbed. After parturition, the stressed group was further divided into 4 groups – non-treated, amitriptyline-treated 20mg/kg (Ami), OS-treated 100mg/kg (OS100) and OS-treated 200mg/kg (OS200). Treatments were given to the rat dams via oral once daily until the end of the behavioural tests. After weaning of pups, all rat dams were tested for locomotor activity and anxiety-like behaviour in open field test, whereas depression-like behaviour was assessed in forced swim test (all groups n: 7-10). In the open field test, one-way ANOVA analysis showed that

treatments with amitriptyline and both OS concentrations significantly increased the locomotor activity of gestationally-stressed postpartum rats, similar to non-gestationally stressed postpartum rats ($p<0.05$). No significant anxiety-like behaviour was observed in any groups. In the forced swim test, however, only Ami and OS100 groups had a decrease in the immobility behaviour of the dams, which was similar to the control group ($p<0.0001$). From ELISA analysis, BDNF levels in the hippocampus of OS100, OS200, and Ami groups were unpredictably lower than the non-stressed group ($p<0.05$). As a conclusion, ethanolic OS extract showed the ability to reverse the adverse effect of gestational stress on rats, hence it does have the potential as a remedy for depression at the postpartum period. However, the mechanism is not mediated by neurogenesis by BDNF and needs further investigation.

CHAPTER 1

INTRODUCTION

1.1 Postpartum Depression (PPD)

Childbirth and sudden transition to motherhood can cause a critical mental health issue, which is known as Postpartum Depression (PPD). In DSM-5, PPD is known as "depressive disorder with peripartum onset" which can occur during pregnancy or within 4 weeks after delivery. The diagnosis was made using the same diagnosis of major depressive disorder (MDD) (Sharma *et al.*, 2015). It was reported in the year 2009, about 15% of women in Europe to encounter postpartum blues within 10 days after giving birth. PPD was screened among postnatal mothers after 4 weeks to 6 months of delivery, and PPD was diagnosed when mothers were found to be in depressed mood for 2 weeks and more (Pearlstein *et al.*, 2009). In Malaysia, a study was carried out by Mohd Arifin *et al.* (2018) and it showed that statistics of PPD among new mother (4 – 12 weeks) was ranged from 6.80 – 27.30%. The results reported were higher than earlier studies which stated that PPD only affected around 3.50 – 3.90% of Malaysians.

Postpartum blues or maternal blues are the early symptoms of depressed mood and could lead to PPD. Through the Blues Questionnaire, (Henshaw *et al.*, 2004), found that women in severe blues tend to suffer major or minor depression later on, due to longer duration of depressive episodes compared to women who did not experience blues symptoms. Similar results from (Watanabe *et al.*, 2008), using The

Stein's Blues Scale and stratum-specific likelihood ratio (SSLR) it was shown that higher maternity blues leads to more risk of PPD.

The PPD patients were stated to have appetite loss, insomnia, ill-tempered, dysphoria, anxiety, or even suicidality, which was distinguishable from postpartum psychosis that had symptoms like hallucinations or delusions, and rambling speech (O'Hara *et al.*, 2013). They experienced perceived stress due to unstable hormonal changes and severe birth inflammation, and also parenting stress due to difficulty of interacting and handling of infants, (Dunkel Schetter *et al.*, 2016). The rapid changes of reproductive hormones especially estradiol and progesterone during delivery was believed to stimulate mood dysregulation in hormone-sensitive PPD women (Schiller *et al.*, 2015). Besides that, other causes that contribute are history of depression or previously prescribed antidepressants (Mohammed *et al.*, 2014), lack of social support from partner or family (Jones *et al.*, 2013), and age of the mother as younger mother tends to be more likely affected by PPD range age (Cline *et al.*, 2012).

1.1.1 Current treatment choices for PPD

The management approaches of PPD include psychotherapy and pharmacotherapy interventions. The steps of treatments are similar to MDD, which mild to moderate depression are treated with either pharmacotherapy, psychotherapy, or both, or pharmacotherapy plus electroconvulsive therapy (ECT). For severe depression without psychotic features, all of the available treatment modalities except psychotherapy may be given; meanwhile, for severe depression with psychotic features, antipsychotic medication should be added (Karasu *et al.*, 2000).

Psychotherapy is a sufficient treatment for mild depressed new mothers with Edinburgh Postnatal Depression Scale (EPDS) >10, in handling emotions while saving their babies from the effects of medications through breastfeeding. Interpersonal therapy (IPT) is the most popular intervention therapy which basically targets the issues causing perinatal depression such as stress and deficient of social support and involving few sessions with pregnant mothers within a certain period of time (Lenze *et al.*, 2017). In 2016, Zlotnick and team did an IPT-based intervention, it was called 'Reach Out, Stand Strong, Essentials for new mothers' (ROSE) which consists of some 90-minutes group sessions within 4-weeks period of time. The program proved to be effective in preventing PPD to be developed especially on new mothers after 6 months of giving birth (Zlotnick *et al.*, 2016). Another study in 2016 did a new program called Massachusetts Child Psychiatry Access Project (MCPAP) for Moms, which includes telephonic access in real-time with perinatal psychiatrists and care coordinators according to regions of MCPAP for Moms, support groups, and individual psychotherapy. This program succeeded in making effective treatments of psychotherapy become more accessible and with lower costs (Byatt *et al.*, 2016).

For mothers with moderate to severe cases which psychotherapy alone is not sufficient to control the depressive symptoms, pharmacotherapy is the treatment of choice. The choices of drugs include antidepressants known as Selective Serotonin Reuptake-Inhibitors (SSRIs) for example fluoxetine, sertraline, escitalopram; Serotonin-Norepinephrine Reuptake Inhibitor (SNRI) for example venlafaxine and duloxetine, as well as tricyclic antidepressants, for example, amitriptyline and clomipramine (Jiang *et al.*, 2016).

In randomized and placebo-controlled, double-blind trial of sertraline on PPD patients that were investigated by Hantsoo *et al.* (2014), it was proven that sertraline gave better response rate in Hamilton Anxiety Rating Scale (HAM-D) at 59% than placebo at 26% within 6 weeks duration of treatment. Sertraline also had higher remission rate of 53% compared to placebo with 21%. This showed that antidepressant treatment was better than no treatment as it managed to reduce PPD symptoms especially on early stage of PPD. Another clinical trial was done to compare the effectiveness of cognitive behavioural therapy (CBT), sertraline mono-therapy and combination therapy. This study had shown that many patients quitted taking medications earlier (10.5 weeks) and attended fewer CBT sessions (7.5 sessions) in combination therapy, rather than in sertraline mono-therapy (12.9 weeks) and CBT mono-therapy (10.6 sessions). This might be due to difficulty to comply with both therapies than single therapy. Sertraline mono-therapy proved to be effective in shorter (12 weeks) or longer-term (24 weeks) of treatment. While CBT mono-therapy was shown to be the most superior as it managed to help obtaining the 'minimal' range of depression in the BDI-II score after treatment of 24 weeks (Milgrom *et al.*, 2015).

1.1.2 Effect of conventional drugs on infants

Taking antidepressants worries nursing postpartum depressed mothers as the penetration profile of most of the drugs into the breastmilk are not clearly characterized. This uncertainty makes the mothers reluctant to be treated as they are afraid that their offspring may be adversely affected by the drug. Hence, some researches were done to identify the toxicity limit of

antidepressants in breast milk. A study by Salazar *et al.* (2016) using human milk sample from mothers who took fluoxetine, sertraline, and paroxetine discovered that only low concentrations of antidepressant are present which are considered safe for administration as it does not exceed 3% of relative infant doses. For fluoxetine, the concentrations found in samples were ranged from 42.40 to 65.70 ng/mL, for sertraline it ranged from 24.20 to 81.70 ng/mL while for paroxetine it did not exceed the lower limit of quantification. Another research on penetration of mirtazapine into breast milk, it was observed that the mean of total relative infant dose was only 1.90% of the weight-adjusted maternal dose, for mirtazapine and its metabolites, desmetylmirtazapine. This is generally considered safe as it was lower than 10%. Furthermore, only one out of four infant's plasma had a very low concentration of mirtazapine of 1.50 µg/L (Kristensen *et al.*, 2007). Despite the low concentrations of drugs detected in the breast milk, it still raises uncertainties on the effect of the drugs to the offspring after prolonged exposure.

Serious adverse effects on infants are more commonly observed when antidepressants were taken during pregnancies. Through a review of few studies, paroxetine consumed during the pregnancy period increased the rate of congenital anomalies, as well as put the infants at higher risk of cardiovascular defects and malformations. In addition to that, taking sertraline during pregnancy had a higher risk of septal defects, and bupropion administration at early pregnancy caused low risk of congenital heart defects (Diav-Citrin *et al.*, 2012). Antidepressant exposure during second and third trimesters also associated with preterm birth, and low birth weight compared to healthy unexposed to antidepressant group (Becker *et al.*, 2016).

In animal studies, early life exposure to antidepressants, especially SSRI, had caused long term adverse effects on the offspring which were observed at adulthood. Fluoxetine administered to dam rats from day 2 until day 23 postpartum increased anxiety-like behaviour and impaired the hypothalamic-pituitary-adrenal axis negative feedback in adult male offspring (Gobinath *et al.*, 2016). Reproductive system of adult male rats that were exposed to fluoxetine at prenatal and continued for days after birth also shown to affect their reproductive system (Leivas Vieira *et al.*, 2012).

1.2 Stress-induced animal model of depression

Animal models are useful to address methodological problems and challenges faced by clinical studies. In depression studies, chronic mild stress (CMS) is one of the existing valid animal models of depression. CMS mimics the minor irritations of daily life. Also called 'chronic unpredictable mild stress', 'unpredictable sub-chronic mild stress', and other names, it involves a variety of micro-stressors planned in unpredictable sequences within several weeks (Willner, 2005). The stressors that have been applied in previous studies include food and water deprivation, overcrowding, immersion in cold water, isolation, noise, immobilization, foreign object (López-López *et al.*, 2016), wet wood shavings, box house tilting (45º), inescapable shock at 0.70 mA (Moretti *et al.*, 2012), tail pinch, overnight illumination, swimming (Kumar *et al.*, 2011), and many others.

CMS was proven able to prompt anhedonia, which is a core symptom of depression, and decreased social interactions among male rats, as the preference of sucrose solution lessen while the immobility time taken during forced swim test (FST),

increased (Kompagne *et al.*, 2008). CMS was compared with another stress protocol which is chronic restraint stress (CRS), and the mice subjected to CMS displayed more depressive-like behaviour through higher increased of immobility time in tail suspension test (TST) and FST, and also depleting preference towards sucrose solution which indicates despair behaviour. Results from open field test (OFT) and elevated plus maze test were almost equal between CMS and CRS mice. Both showed lesser time spent in the inner zone and increased in the total distance moved when compared with the controls which interpreted as anxiety-like behaviours (Zhu *et al.*, 2014). Another research by Ji *et al.* (2014), also showed almost similar results. CMS induced mouse had proven to have anxiety-like behaviours showed from the significant increased of immobility time in FST and TST, and reduced the number of crossings, rearings, and groomings in OFT when compared to control group.

CMS induced rats in a different study also significantly decreased in the number of crossings and rearings in OFT when compared to non-stressed control group. Additionally, a BDNF analysis in the amygdala was done and revealed that the levels of BDNF were reduced as compared to the non-stressed control group (Luo *et al.*, 2013). Gestating rats subjected to CMS also displayed change in maternal care by decreasing the nursing time by 11% and increasing the time away from pups by 35%. Plasma corticosterone levels were also affected in these rats, where CMS animals had higher corticosterone level compared to the controls (Bourke *et al.*, 2013). CMS procedure was chosen as a stressor in this study because the procedures are varied, affordable and simpler to be implemented than CRS, as well as reflect the actual life more similarly. Besides that, from previous studies, CMS showed better depressive and anxiety-like effect too.

1.3 Herbs with anti-depressant like effect

Herbs are mainly used in traditional medicine for centuries especially in Asian and Africans. Due to many side effects of most commercial drugs, some people change into a so-called greener alternative which is claimed to be safer. As stated by Zhang *et al.* (2015), herbal medicine need be tested on the level of toxicity, safe dose range, and safety window of effective dose in order to guarantee the safety and potency.

A group of researchers in China has proved the antidepressant-like effect of sesquiterpenoids in ginseng root (Ge *et al.*, 2017). At the dose of 1.0 mg/kg, the immobility time taken by mice in forced swimming test (FST) significantly dropped without affecting the locomotor activity of mice. It also managed to slightly increase the level of dopamine in the mice's brain. *Hypericum polyanthemum*, a plant found in Brazil contains Uliginosin B was proven to have antidepressant-like effect as at the dose of 10 mg/kg (once for acute, while once daily during 3 days for sub-acute). It reduced the immobility time of rats in TST and FST after 1h of acute administration but not after 3h, while sub-acute administration showed reduced immobility time after 1 and 3h. Also, it intensified Na^+, K^+-ATPase activity in cerebral cortex by 18% after 1h of acute administration and 20% after 1h and 3h of sub-acute administration (Stein *et al.*, 2016). Curcumin from turmeric was found to deplete the learning deficit of olfactory bulbectomized (OB) rats in passive-avoidance test, and reduced the hyperactivity of OB rats in OFT, as compared to normal male albino rats. Also, it raised the serotonin, dopamine and noradrenaline in hippocampus and frontal cortex to control group's levels. It also decreased the 3,4-dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid in the hippocampus and frontal cortex of male albino rats (Chang *et al.*, 2016).

Rosemary or *Rosmarinus officinalis* a native plant of the Mediterranean region has been commonly studied for its antidepressant effect. In 2012, a study on *Rosmarinus officinalis* hydroalcoholic extracts (ROHE) on bulbectomized mice revealed that ROHE decreased the hyperactivity of olfactory bulbectomized (OB) mice in novel object test and number of rearings, in locomotor activity and exploratory behaviour in novel cage test as well, and managed to obliterate anhedonic-like behaviour in time spent grooming of splash test. Besides, ROHE treatment was capable to restore serum glucose level to normal, and reduce the activity of acetylcholinesterase in hippocampal, but not frontal cortex (MacHado *et al.*, 2012). In another study, the *Rosmarinus officinalis* ethanol extract (EERO) managed to upregulate the expression of tyrosine hydrolase and pyruvate carboxylase mRNA, and significantly elevated the norepinephrine, dopamine, serotonin, total choline, and acetylcholine in mice brain. 50 and 100 mg/kg of EERO also capable to reduce the immobility time in mice during TST. One of the plant's main compounds, rosmarinic acid (RA) is said to be the contributing factor to the antidepressant-like effect of the plant (Sasaki *et al.*, 2013).

1.4 Antidepressant-like effect of rosmarinic acid

RA has been tested and proven to have antidepressive-like effect on rats and mice. Jin *et al.* (2013), had tested the effect of RA on chronic unpredictable stressed (CUS) rats. In this study, they have observed that treatment of RA elevated the hippocampal BDNF and phospho-ERK 1/2 levels that were reduced by chronic unpredictable stress, although there were no changes in the analysis of FST, OFT and Morris water maze. Interestingly, the effects on the neuroproteins levels were in a dose-dependent manner, which increased of levels were observed in rats treated with

10mg/kg RA but not with 5mg/kg RA. It was also shown that administration of RA with U0126 (an ERK 1/2 phosphorylation inhibition) on CUS rats, did not have any improvement which meant U0126 inhibited the cell proliferation that was promoted by RA. Almost similar research by Nie *et al.* (2014), had confirmed that RA of 10 mg/kg dose established significance difference in elevated plus-maze test compared to RA of 5 mg/kg. RA 10 mg/kg also were found to have improved hippocampal cell proliferation in enhanced single prolonged stress rats by lifting the number of BrdU-positive cells and increased hippocampal phospo-ERK 1/2 levels. The U0126 was proven to inhibit cell proliferation as well.

Another study had shown the ability of RA to reduce anxiety-like behaviour of hippocampal tat-injected (HIV-1 protein) group and induced repetitive restraint stress group. It was proven that RA managed to increase levels of glucocorticoid receptors and mineralocorticoid receptors protein, as well as BDNF levels until similar to the control groups. RA was also said to be neuroprotective with respect to tat-treated toxicity (Makhathini *et al.*, 2018). A research by Kondo *et al.* (2015) using mice with TST as stressors, had shown that RA managed to suppress the immobility time of mice in TST, lower the level of corticosterone in blood serum to almost the same level as bupropion, and had a slight increase in the levels of dopamine, noradrenaline, and adrenaline in mice brain. Furthermore, RA significantly decreased Mkp-1 mRNA expressions compared to TST-induced stress and raised BDNF mRNA expressions as well.

1.5 BDNF regulation in animal studies of depression

Brain-derived neurotrophic factor (BDNF) is a critical neurotrophic factor, that assists the survival of neurons whether for growth, differentiation, and also maintenance (Gene, 2018). BDNF protein is also needed in regulating synaptic plasticity, which is essential for learning and memory (Lee *et al.*, 2010). Through previous studies, (Björkholm *et al.*, 2016) concluded that BDNF-TrkB signalling was involved in mediating the antidepressant effects of conventional drugs, and hippocampus is the important site for BDNF to perform antidepressant effects.

While depression is associated with reduced level of BDNF, not all antidepressant treatments could increase BDNF expressions in hippocampus or prefrontal cortex of depressive-like behaviour animals. A study showed that single or repeated injections of antidepressants given within 14 days had different outcomes. Single or repeated injections of desipramine and maprotiline (noradrenaline uptake inhibitors) did not increase the levels of BDNF when measured 24 hours after the last injection. For other antidepressants like SSRI's (fluoxetine, paroxetine, and sertraline) and monoamine oxidase inhibitor (tranylcypromine), single injection caused no effect on BDNF expressions, while repeated injections showed reduction of BDNF expressions by 26-30% when measured 24 hours after last injection (Coppell *et al.*, 2003). Another research by Balu *et al.* (2008), presented that acute administration (once daily of drug for 1 day) of desipramine (10 mg/kg), fluoxetine (10 mg/kg), and phenelzine (10 mg/kg) were unable to increase BDNF levels in the hippocampus, frontal cortex, amygdala or olfactory bulb after 24 hours of administration. However, chronic administration (once daily of the drug for 21 days) showed a significant

increase of BDNF levels for all except phenelzine as it had 10% decreased of BDNF levels.

Besides antidepressant, fish-oil treated rodents did show some positive increase of BDNF levels. Pudell *et al.* (2014) did look into the effect of fish oil on BDNF levels in an olfactory bulbectomy model of depression. Male offspring of fish oil-treated rats were divided into 4 groups – control (C), olfactory bulbectomy (OB), fish-oil treated OB (OBFO), and sham fish oil (FO). FO had higher BDNF expressions in the hippocampus compared to the control group, while OBFO proved to have increased BDNF level when compared to the OB group. The study concluded that OB cause hyperactivity, low BDNF and had depressive-like effects, but treatment with fish-oil managed to abolish the effects. Curcumin was also proven to alter corticosterone-induced depressive-like behaviour through increasing of the BDNF levels, done by (Huang *et al.*, 2011). CORT injections on rats managed to reduce sucrose consumption in rats, higher immobility time in FST, and lower BDNF protein levels in the hippocampus and frontal cortex compared to control group. Curcumin treatment of 20 mg/kg on rats showed the total opposite results with increasing sucrose consumption (46%, $p<0.01$), significantly reduced immobility time in FST (27%, $p<0.01$), and significantly increased BDNF levels in both hippocampus and frontal cortex (68%, $p<0.01$).

Another natural remedy that had improved levels of BDNF in mice was ginseng extracts. As done by Boonlert *et al.* (2017), ginseng effect was dose-dependent as 100 and 800 mg/kg of G115 had better antidepressant effects on ethanol-induced depression rats than 200 and 400 mg/kg. In FST, ginseng 100, 200, and 800 mg/kg had shorter immobility time and higher swimming time. Ethanol administration lowered

the BDNF levels in both hippocampal and prefrontal cortex, while ginseng 200, 400, and 800 mg/kg managed to increase BDNF levels in hippocampus and prefrontal cortex when compared to ethanol-induced group ($p<0.05$).

Additionally, another natural remedy proven to regulate BDNF levels from depressive-like behaviour, named *Gyejibokryeong-hwan* (GBH) which is a traditional Korean medicine consisting of few herbs including *Ramulus Cinnamomi cassia, Scierotium Poriae cocos, Radix Albus paeoniae Lactiflorae, Cortex Radicis moutan, and Semen Pruni persicae*. The study portrayed that reserpine-induced depression mice, managed to overcome depressive-like behaviour when given GBH of 100, 300, and 500 mg/kg as the treatment manage to reduce immobility time in FST and TST, while only GBH 100 had higher level of BDNF when compared to non-treated reserpine-induced depression group ($p<0.01$) (Park *et al.*, 2018).

1.6 Orthosiphon stamineus (OS)

Orthosiphon stamineus or also known as 'misai kucing'/ 'Java Tea' is most commonly available in Southeast Asia and Europe, usually used for medicinal purpose and ornamental display (Tnah *et al.,* 2014). The taxonomic classification of OS is as in Table 1.1. OS plant is a perennial herb and only has a height of around 0.6 to 1.0 m. It has simple green leaves with the leaf apice of acuminate type and has dentate leaves edges (Figure 1.1). The leaves are of 3 sizes depending on maturity, small (5 cm), medium (11 cm) and large (25 cm). The leaf stalk is short about 0.3 cm and is reddish-purple coloured. While for the flowers, OS has bell-shaped, pale purple coloured with long fine stamens that resemble the cat's whiskers (Almatar *et al.,* 2018).

Table 1.1 Taxonomic classification of *Orthosiphon stamineus*

Local Name	Misai Kucing
Kingdom	Plantae
Sub- kingdom	Tacheobionta (Vascular plants)
Order	Lamiales
Family	Lamiaceae
Genus	*Orthosiphon*
Species	*aristatus, labiatus, grandiflorum, spicatus, stamineus*

Figure 1.1 *Orthosiphon stamineus* (benth.)

1.6.1 Active compounds isolated from OS

The active compounds obtained from OS as stated in previous studies were tabulated in Table 1.2. Flavonoid is one of the most common and high abundance constituents present in OS consisting of sinensetin, eupatorin, and others. Besides that OS also contains polyphenol, dipeptide, diterpene, and triterpene groups. Alkyl aldehyde, alkyl alcohol, alkyl epoxide, alkyl ketone and alkyl ester groups were also found in OS. Furthermore, some essential oils were also present and mainly from monoterpene and sesquiterpene groups (Asmawi *et al.*, 2012; Adnyana *et al.*, 2013).

Table 1.2 Active compounds isolated from OS

Group	Active compound
Flavonoids	sinensetin eupatorin 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF) tetrametylscutellarein salvegenin pillion ladancin vomifoliolm 7,3',4'-tri-*O*-methylluteolin scutellarein tetramethylether (5,6,7,4'-tetramethoxyflavone) 5-hydroxyl-6,7,3',4'-tetramethoxyflavone 6-hydroxy -5,7,4'-trimethoxyflavone 5,6-dihydroxy-7,4'-dimethoxyflavone
Polyphenols	caffeic acid rosmarinic acid cichoric acid 2,3-dicaffeoyltartaric acid
Dipeptide	aurantiamide acetate
Diterpene	orthosiphol A to Z 6-hydroxyorthosiphol B 7-*O*-deacetylorthosiphol B 3-*O*-deacetylorthosiphol I 2-*O*-deactylorthosiphol J

	14-deoxo-14-*O*-acetylorthosiphol Y staminol A to D orthosiphonone A to D 2-*O*-deactylorthosiphonone A staminolactone A and B secoorthosiphol A to C noroorthosiphonolied A norstaminolactone A norstaminol A to C norstaminone A neoorthosiphonone A neoorthosiphol A and B siphonol A to E orthochromene A
Triterpene	ursolic acid oleonolic acid betulinic acid hydroxybetulinic acid maslinic acid α-amyrin β-amyrin oleonolic acid
Alkyl aldehyde	hexanal *trans*-2-hexanal 1-octen-3-ol, 3-octanol Heptanal 4-heptenal *trans, trans*-deca-2,4-dienal β-cyclocitral safranal *cis*-2-octenal decanal
Alkyl alcohol	cis-3-hexen-1-ol hexan-1-ol trans-2-cis-(6)-nonadienale
Alkyl peroxide	2-pentenyl furan 2-amylfuran perillen
Alkyl ketone	acetophenone cis-linalool oxide 2,6,6-trimethyl-2-cyclohexe-1,4-dione *trans, trans*-octa-3,5-die-2-one *trans, cis*-octa-3,5-die-2-one
Alkyl ester	isobornylacetate

Essential oils (monoterpene and sesquiterpene)	campor menthone δ-terpineol isomenthone borneol cittoneallol carvone geranyl acetone damascenone trans-linalool oxide linalool bornyl acetate limonene 1,8-cineol p-cymene camphene α-pinene, β-pinene α-copaene β-bourbonene β-elemene cis-caryophyllene β-carryophyllene α-cubebene γ-elemene α-humulene germacrene B and D α-muuiolene δ-cadinene caryophyllene oxide hexahydrofamesyl acetone

1.6.2 Pharmacological activities of OS

OS has been proven to have potential in treating some diseases through animal research, one of them is antihypertensive. Manshor *et al.* (2013) has found that water (WOS) and methanol-water extracts (WMOS) of OS had antihypertensive effect by blunting the rising of blood pressure in rats, and might have a role in decreasing vasoconstriction similar to losartan (AT_1 receptor blocker). WOS and WMOS also had a vasorelaxant effect which plausibly due to the release of endothelium-derived nitric oxide. Almost similarly, chloroform fraction of 50% methanolic extract of OS (CF) managed to show greater vasodilation effect and induced vasorelaxation on the endothelium-intact aortic ring compared to endothelium-denuded aortic ring. This showed that CF influenced in multiple mechanisms whether as directly (endothelium-independent) or indirectly (endothelium-dependent). The mechanisms included the NO/cGMP pathway, calcium and potassium channels, and muscarinic and beta-adrenergic receptors (Yam *et al.*, 2016).

Another potential of OS is as diuretics. In a study, caffeic acid and flavonoid derivatives in methanol-water OS extract were capable to increase output of urinary and electrolytes excretion. This helped to reduce the chance of crystals to aggregate or grow. Uric acid production was also lowered, thus might help to reduce the formation of calcium oxalate stone, anti-gout effect, and other uric-acid related diseases (Arafat *et al.*, 2008). A study by Adam *et al.* (2009) also supported the diuretic potential of OS water extract. At the dose of 5 and 10 mg/kg, OS water extract managed to increase output of urine almost similar to furosemide (diuretic drug) with diuretic action of 15.51. It

increased the urine potassium every hour but did not affect sodium and chloride levels. The diuretic activity of OS extracts was said to possibly be moderated through the changes in potassium transport, by inhibiting potassium absorption, or stimulate secretion of potassium.

Besides that, antiangiogenic and antiproliferation of OS extracts were also proven by some studies. A study by Hussain *et al.* (2012), presented that hexane fraction (HF) of OS extract had 100% antiangiogenic activity, while chloroform and ethyl acetate fraction showed no antiangiogenic activity. The antiangiogenic activity was said to be due to high contents of betulinic, oleanolic and ursolic acids in HF. Additionally, as the given dose was lower than the cytotoxicity level hence antiangiogenic activity was not due to cell death. A more detailed antiangiogenic study was described by Majid *et al.* (2011). It showed that ethanol OS extract had proven to significantly inhibit important steps of angiogenesis. RA was found to inhibit proliferation, migration, adhesion, and formation of endothelial cells, whereas eupatorin inhibits in-vitro proliferation of cancer cells, and sinensetin induces antiproliferation moderated by apoptosis and CYP1 in human cancer cells.

1.6.3 Toxicity studies of OS

OS extracts were found to be relatively safe as reported by Yam *et al*. (2013) that acute and subchronic study of 50% methanolic extract had no noticeable signs of toxicity nor mortality when they were administered to tested rodents in doses up to 5000 mg/kg. While in another study, OS extracts administered orally up to 2000 mg/kg per day to pregnant female rats were proven to not cause any maternal toxicity to the mother or foetus (Muhammad *et al*., 2013).

1.6.4 The extraction process of OS

Extraction of OS can be carried out in many ways to obtain the active compounds. Table 1.3 summarizes the different extraction methods on OS and the findings of the active compounds such as RA, EUP, SEN, and TMF. The chemical structures of the active compounds are as depicted in Figure 1.2.

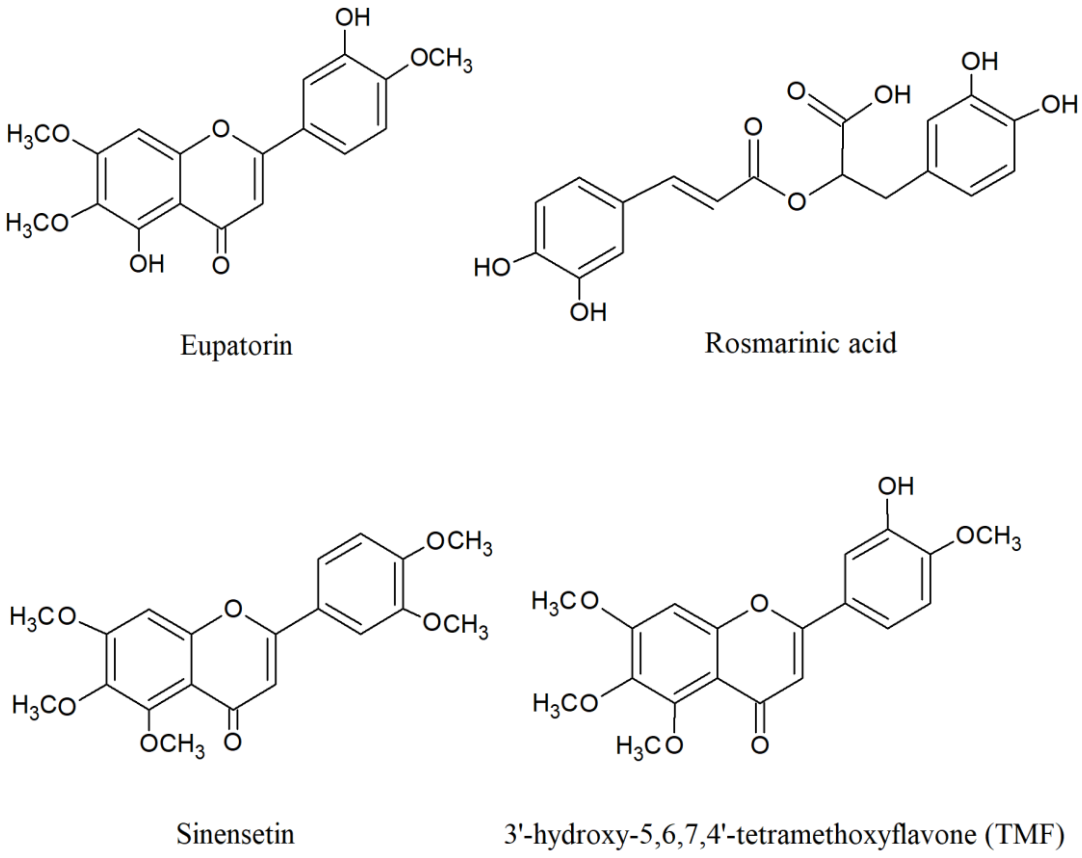

Figure 1.2 Major compounds found in *Orthosiphon stamineus* extracts

Amzad Hossain *et al*. (2012) used 50% methanol, combined with powdered leaves of OS and used ultrasonication for about 30 mins. Later, 1 g of dry methanol-extract was mixed in 70% acetone-water before heated at 40^{o}C with ultrasonication for another 30 mins. The extracts obtained were then scanned using HPTLC, mostly consists of sinensetin with percentage of 0.32, 0.19, 0.16, 0.13, and 0.15 for 70% acetone extract, 50% acetone extract, 100% acetone extract, 100% methanol extract, and 50% methanol extract, respectively (Amzad Hossain *et al*., 2012). Few other studies also used methanol as solvent, such as by Lim *et al.* (2013) which used 1 g of OS powder soaked in 20 mL of methanol for 24 h and then was centrifuged at 3000 rpm.

The extracts obtained had the most phenols in *in vitro* plantlets (0.50 mg/mL) compared to field-grown mature plants (0.22 mg/mL), and cell suspension cultures (0.35 mg/mL). Some studies did comparison between concentrations of methanol, one of them was by Ho *et al.* (2010) which used 50 g of OS powdered leaves macerated in different concentrations of methanol (25%, 50%, 75%, 100%) and water for 8 h, transferred to an incubator shaker for 16 h before being filtered, evaporated and dried at 40^{o}C. The concentration of RA gained were 33.90 ± 1.25 mg/L, 31.20 ± 0.76 mg/L, 26.70 ± 0.64 mg/L, 11.70 ± 1.43 mg/L and 1.11 ± 0.02 mg/L for 50% methanol, 75% methanol, 100% methanol, 25% methanol, and water, respectively.

Similarly done by Hashim *et al.* (2016), extraction of OS dry powder was done using different concentration of methanol such as 0%, 25% 50% and 100% while the mixture was shaken at 150 rpm by incubator shaker (40^{o}C) at different time of 2, 4, and 8 hours. Results presented that 50% methanol contained the highest RA concentration and total phenolic content with approximately 4.50 mg/g at 2h, 4h, and 8h. They were followed by 100% methanol with RA of 3.50 mg/g at 2h and 4h, and 4.00 mg/g at 8h. For 25% methanol, RA obtained were 2.60 mg/g at 2h and 3.50 mg/g at 4h and 8h. Lastly for 0% methanol with 2.50 mg/g at 2h and 4h and 3.00 mg/g at 8h. While for the most concentration of TMF and SEN, was highly found at 8h of extraction in 100% methanol, 50% methanol, 25% methanol, and 0% methanol accordingly. In a study that was executed by Kamarudin *et al*. (2016), 10 g of leaves of OS was soaked in 200 mL of water, ethanol, and 50% ethanol for three days in cold maceration method. While for Soxhlet method, the samples were put in with the time span set to 6 hours. Later, the samples were filtered

and concentrated using vacuum rotary evaporator and dried using freeze dryer. The results presented that for cold maceration, the highest total phenolic compound obtained was from 50% ethanol (17.41 g/g), then ethanol (7.54 g/g), and water (2.61 g/g). Concurrently for Soxhlet extraction, the phenols collected were almost the same for ethanol (14.32 g/g) and 50% ethanol (14.21 g/g), while water had the least (6.53 g/g).

In 2012, Dolečková *et al.* (2012) and the team did extraction of dried leaves of OS, mixed with chloroform and was centrifuged for 10 mins at 20,000 g before being filtered and evaporated in a rotary vacuum evaporator to obtain chloroform extracts consisting of eupatorin with concentration of 0.53 ± 0.08 mg/g. Akowuah *et al.* (2005) had carried out a study to compare the effects of different solvents on varying phenolic extracts of OS. Each 10 g of leaves powder of OS was extracted with 100 mL of solvents such as chloroform, water, methanol, 50% methanol, and 70% acetone for 2, 4 and 8h at 40^{o}C. Then, the extracts were analysed using HPLC and the results of percentage weight were, for RA the highest amount was obtained from 50% methanol with 0.97% (4h), for EUP and SEN the most were from chloroform with 0.14% (4h) and 0.43% (4h), respectively. While for TMF, there was not much difference in the amount obtained from 70% acetone, methanol and chloroform, which was at about 0.01%. Study done in 2013, also used chloroform as solvent in extracting dried leaves of OS by using Soxhlet and maceration method. The Cf2 fraction of the chloroform extract was found to have the most active hyperglycaemic principles and contained 1.48% of sinensetin, 2.26% of eupatorin and 0.58% of 3’-hydroxy-5,6,7,4’-tetramethoxyflavone (Mohamed *et al.*, 2013).