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A Medicinal herb, *Melissa officinalis* L. ameliorates depressive-like behavior of rats in the forced swimming test via regulating the serotonergic neurotransmitter

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Abstract

Ethnopharmacological relevance: Depression is a serious psychological disorder that causes extreme economic loss and social problems. However, the conventional medications typically cause side effects that result in patients opting to out of therapy. Lemon balm (*Melissa officinalis* L., MO) is an old and particularly reliable medicinal herb for relieving feelings of melancholy, depression and anxiety. The present study aims to investigate the antidepressant-like activity of water extract of MO (WMO) by evaluating its influence on the behaviors and the relevant neurotransmitters of rats performed to forced swimming test.

Materials and methods: Two phases of the experiment were conducted. In the acute model, rats were administered ultrapure water (control), fluoxetine, WMO, or the indicated active compound (rosmarinic acid, RA) three times in one day. In the sub-acute model, rats were respectively administered ultrapure water (control), fluoxetine, or three dosages of WMO once a day for 10 days. Locomotor activity and depression-like behavior were examined using the open field test and the forced swimming test, respectively. The levels of relevant neurotransmitters and their metabolites in the frontal cortex, amygdala, hippocampus, and striatum were analyzed by high performance liquid chromatography.

Results: In the acute model, WMO and RA significantly reduced depressive-like behavior but the type of related neurotransmitter could not be determined. The results indicated that the effect of WMO administration on the reduction of immobility time was associated with an increase in swimming time of the rats, indicative of serotonergic neurotransmission modulation. Chromatography data validated that the activity of WMO was associated with a reduction in the serotonin turnover rate.

Conclusion: The present study shows the serotonergic antidepressant-like activity of WMO. Hence, WMO may offer a serotonergic antidepressant activity to prevent depression and to assist in conventional therapies.

KEYWORDS: *Melissa officinalis* L., antidepressant, neurotransmitter, forced swimming test

1. Introduction

The World Health Organization (WHO) reported that major depressive disorder (MDD) will be the leading cause of disability and have the highest burden of disease by the year 2030 (Mathers et al., 2008). One of the major pathological hypotheses of depression is neurotransmitter dysregulation (Castren, 2005). Clinical research has indicated that patients with depression have low concentration of monoamines like serotonin (5-HT) in the central nervous system (Stockmeier, 1997; Fava, 2003). Hence, several antidepressants including selective serotonin reuptake inhibitors (SSRIs), nonselective monoamine reuptake inhibitors (tricyclic antidepressants, TCAs), and monoamine oxidase inhibitors (MAOIs) were developed to improve the retention of monoamines in the brain. Though episodes of depression are ameliorated by these medications, undesirable side effects frequently occur and cause patients to opt out of medication. Low acceptability of antidepressants by patients with MDD has been reported (Lin et al., 1995; Gonzalez et al., 2005). In contrast, research has indicated that patients with MDD show interest in complementary and alternative medicines (CAMs), including herbal remedies (Alderman and Kiepfer, 2003; Hsu et al., 2010). Moreover, people in the pre-disease state of depression cannot be

prescribed antidepressants until they are properly diagnosed by a medical doctor. Thus, CAMs offer a feasible way of preventing or complementing conventional MDD therapies.

Lemon balm (*Melissa officinalis* L., MO), an herbal material with a "generally recognized as safe" (GRAS) status, is traditionally used for improving sleep disorders and for de-stressing. Chillemi and Chillemi indicated that MO is a traditional and particularly reliable treatment for relieving feelings of melancholy and depression (Chillemi and Chillemi, 2011). Previous research has identified that MO is composed of phenolic and flavonoid compounds, specifically rosmarinic acid (RA) (Fecka and Turek, 2007). Numerous bioactivities of MO, such as antioxidant (Mimica-Dukic et al., 2004), anti-cancer cell proliferation (Encalada et al., 2011), anti-obesity (Lee et al., 2008) and anti-anxiety (Kennedy et al., 2006) effects, have been reported. For antidepressant-like activity, Taiwo et al. demonstrated that a 10-day administration of MO ethanol extract decreased the immobile time of rats during the FST (Taiwo et al., 2012). Similarly, acute administration of the essential oil or aqueous extract of MO by the intraperitoneal route also decrease the immobility time of rats in the FST (Emamghoreishi and Talebianpour, 2009). Lopez et al. showed that both the ethanol and water extracts of MO (WMO) could inhibit the activity of monoamine oxidase A (MAO_A), the major metabolic enzyme of monoaminergic neurotransmitters (Lopez et

al., 2009). In fact, a pharmacokinetic study showed that RA could be detected in the plasma and brains of rats after gavage and intraperitoneal injection (Fale et al., 2011). Previous research indicated that the immobile behavior of mice was reduced by the administration of RA and its metabolite, caffeic acid (Takeda et al, 2002). Moreover, depressive-like behavior was also improved by the administration of RA, which was shown in a chronic animal study for depression (Jin et al., 2013). These results suggest that RA can directly influence the central nervous system and is implicated in the bioactivity of MO in the brain. In this study, we investigated the antidepressant-like effect of WMO in the forced swimming test (FST) and the metabolism of related neurotransmitters.

2. Materials and Methods

2.1. Plant Material

Leaves of *Melissa officinalis* L. were purchased from the Hualien District Agricultural Research and Extension Station in Hualien, Taiwan. The taxonomy was confirmed by Prof. T. L. Chang in the Department of Horticulture and Landscape Architecture at National Taiwan University. The voucher specimens (voucher number: 388449) have been kept at the Herbarium of Taiwan Forestry Research Institute.

2.2. Extraction and Composition Determination of WMO

To obtain the WMO, the freeze-dried MO leaves were crushed and blended into a powder using a blender. The MO powder (1 kg) was stirred with 2 L of ultrapure water at 80°C for 2 h. The insoluble materials were subsequently removed through centrifugation at 10,000 g for 30 min. Finally, the resulting supernatant was filtered and freeze-dried. Fluoxetine (FLX) was obtained from Eli Lilly Company (Taiwan). RA (PubChem CID: 5281792) was purchased from Sigma (St. Louis, MO, USA).

LC-NET II/ADC (JASCO, Easton, MD, USA), a high performance liquid chromatography (HPLC) system consisting of a pump (PU-2089-PLUS) and a UV detector (UV-2075-PLUS), with a Phenomenex Luna C18 column (250 × 4.6 mm, 5 μm, Torrance, CA, USA) and the Chrompass™ program was used for analyzing the RA content in WMO. The mobile phase consisted of 2% acetic acid (A) and 7/3 2% acetic acid/acetonitrile (B), and a linear gradient from 90 to 40% A in 40 min was applied for elution. The flow rate was 1 mL/min and the column effluent was monitored by UV detection at 320 nm.

2.3. Animals and Treatments

Male Sprague-Dawley rats (6-week-olds) were purchased from BioLasco Taiwan Company (Taipei, Taiwan) and housed individually with controlled day/night cycle (12 h light/dark), temperature (23 ± 2 °C), and humidity (50 ± 10%). Food and water

were provided and could be freely accessed. Two experimental protocols were designed in this study: i) sub-acute administration (0, 19 and 24 prior to the test) and ii) sub-chronic administration (10 days). The habituation for each experiment was one week. For the acute experiment, the rats were separated into 4 groups: control (CTL, ultrapure water), WMO (300 mg/kg body weight), RA (36 mg/kg body weight), and FLX (18 mg/kg body weight). The dosage of RA was equal to its content in WMO. In the acute experiment, after the first FST for 15 min, oral administration occurred at three time points, 0, 19 and 24 h. For the sub-acute model, the rats were separated into 5 groups: CTL (ultrapure water), low dosage WMO (L-WMO, 30 mg/kg body weight), medium dosage WMO (M-WMO, 100 mg/kg body weight), high dosage WMO (H-WMO, 300 mg/kg body weight), and FLX (18 mg/kg body weight). The dosages of WMO usd in the sub-acute model were based on the results in the acute model and on previous reports by Taiwo et al. (Taiwo et al., 2012) and Emamghoreishi and Talebianpour (Emamghoreishi and Talebianpour, 2009). Oral administration in the sub-acute model was initiated after habituation and proceeded for 10 days once a day. For each model and treatment, there were six to eight per group. The protocol complied with the guidelines described in the “Animal Protection Law”, which was amended on June 29, 2011 to Hua-Zong- (1)-Yi-Tzi-10000136211 by the Council of Agriculture, Executive Yuan, Taiwan.

2.4. Open Field Test (OFT)

The autonomous behavior of rats was evaluated by the OFT after 10 days of administration. The open field apparatus was divided into central and peripheral areas. The rats were placed individually in the central area of the open field (76 × 57 × 35 cm) for 5 min and videotaped. The bouts (the number of central and peripheral squares entered), duration, distance, and velocity were analyzed using the TOP SCAN v2.0 software (Clever Sys Inc., Reston, VA, USA).

2.5. Forced Swimming Test (FST)

The FST was developed by Porsolt et al. (Porsolt et al., 1979). This well-established model being used in the present study was described previously (Lin et al., 2014). Following the standard protocol, the rats were forced swim for 15 min on the final day of administration. After 24 h, the rats were forced swim again for 5 min, and they were videotaped for further behavioral analyses such as calculating the duration of immobility, swimming, and struggling by the ForcedSwimScan™ software (CleverSys, Reston, VA, USA). The definition of these different behaviors is based on the activity of the four limbs and the ratio of body area that was under or above the water surface.

2.6. Sacrifice and Monoamine Analysis

After the FST, the rats were suffocated by CO_2 and then decapitated immediately. The frontal cortex, striatum, hippocampus, and amygdala were dissected from the brain and frozen at −80 °C until further analysis. The monoamine analysis used in the present study has been described previously (Lin et al., 2013). The concentrations of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were measured by HPLC with an LC-4C electrochemical detector (ECD, West Lafayette, IN, USA) set at a 10 nA range and equipped with a 1 Hz filter, a 0.510 V AppE cell, and an autosampler (CMA 200 refrigerated microsampler, Stockholm, Sweden). The mobile phase contained 0.17 M NaH_2PO_4, 0.63 mM ethylenediaminetetraacetic acid, 0.60 mM octane-1-sulfonic acid sodium salt, 2.0 mM KCl, and 20% methanol that was adjusted to pH 3.11 with 85% H_3PO_4. The flow rate was 0.5 mL/min. The reference standards of the monoamines used in the HPLC-ECD, 5-HT and its metabolite 5-HIAA, were purchased from Sigma (USA).

2.7. Statistical Analysis

All results are represented as the mean ± the standard deviation. All data were analyzed by one-way analysis of variance with Duncan's multiple range post-hoc test when appropriate. Any differences were considered significantly different when $p < 0.05$.

3. Results

3.1. The RA content in WMO

We determined that RA is the major active component by HPLC. Figure 1 shows the chromatogram of RA in WMO. Matching the retention time of the standard (Fig. 1A), the peak of RA in WMO appeared approximately 12 min after injection (Fig. 1B). According to the calibration curve of the standard, the RA content in WMO and in the dried leaves were 12.0% and 5.0%, respectively.

3.2. Effects of WMO on body weight, daily intake, and behaviors during the OFT

During the 10-day experimental period, only FLX decreased the food intake of rats and further hindered their weight gain (see Supplementary Fig. 1)**.** After 10 days of administration, there was no significant difference in the autonomous behaviors between the CTL, FLX and WMO groups (data not shown).

3.3. Effects of WMO on the depressive-like, swimming, and struggling behaviors of rats in the FST

Figures 2 and 3, respectively show the behaviors of rats subjected to the FST in the acute and sub-acute models. In the acute model, the immobility duration was significantly reduced ($F(3, 20)=6.153$, $p = 0.004$) in the FLX, WMO and RA groups (Fig. 2A). However, the swimming ($F(3, 20)=6.153$, $p = 0.261$) and struggling ($F(3, 20)=6.153$, $p = 0.219$) behaviors were not significantly affected by each treatment

(Fig. 2B-2C). In the sub-acute model, the immobility time of rats in the FST significantly decreased in all three WMO dosages and the FLX group ($F(4, 29) = 3.119$, $p = 0.03$) (Fig. 3A). Furthermore, the duration of swimming ($F(4, 28) = 4.141$, $p = 0.009$) but not struggling ($F(4, 31) = 0.78$, $p = 0.546$) increased after each WMO and FLX administration (Fig. 3B-3C).

3.4. Effect of WMO on the serotonergic neurotransmitters of rats in the acute model

Figure 4 indicates the turnover rate of 5-HIAA to 5-HT in the acute model. FLX significantly reduced the metabolic rate of 5-HT in all four brain regions of the rats ($p < 0.05$). However, WMO and RA only significantly ($F(3, 20) = 25.524$, $p < 0.001$) reduced the metabolic rate of 5-HT in the hippocampus, (Fig. 4D). The contents of 5-HT and 5-HIAA in four brain regions of rats in the acute model was shown in Supplementary Table 1.

3.5. Effect of WMO on the serotonergic neurotransmitter of rats in sub-acute model

After the 10-day experimental period, the metabolic rates of 5-HT in the four brain regions are shown in Figure 5. Low and medium dosages of WMO down-regulated the metabolic rate of 5-HT in the frontal cortex ($F(4, 26) = 14.837$, $p < 0.001$), amygdala ($F(4, 27) = 13.951$, $p < 0.001$) and striatum ($F(4, 28) = 11.027$, $p < 0.001$), while the high dosage of WMO only had an effect on the metabolic rate of 5-HT in the striatum. Moreover, the metabolic rate of 5-HT in the hippocampus was

not affected by the three WMO dosages. Additionally, FLX reduced the metabolic rate of 5-HT in each region ($p < 0.05$). The 5-HT and 5-HIAA contents in the four brain regions of rats in the sub-acute model was shown in Supplementary Table 2.

4. Discussion

Because it contains two groups of active components, essential oil and a phenylpropanoid derivative, MO is a widely used traditional herbal medicine. Previous research indicated several bioactivities of RA, the major active phenylpropanoid compound in the water extract of MO. RA was found in all organs of the MO plant and approached 6% in dried leaves (Parnham and Kesserlring, 1985; Weitzel and Petersen, 2011). Compared to commercial tea bags, which only contained approximately 2% RA (Fecka and Turek, 2007), we found 5% in the leaves that were freshly obtained from a farm. Shekarchi also showed that there is approximately 3.6% RA content in MO extract (80% aqueous solvent) (Shekearchi et al., 2012). However, commercial tea bags typically contain not only tea leaves but stalks. Therefore, fresh crushed leaves might be the better source to obtain RA from MO.

The FST, a well-established behavioral model to evaluate the activity of antidepressants in mice and rats (Slattery and Cryan, 2012), is sensitive to both acute and chronic administration with antidepressant materials. However, the FST has some

drawbacks, including the possibility of obtaining false positives or negatives. Drugs enhancing motor activity may give a "false" positive effect in this test, whereas drugs decreasing locomotion may give a 'false' negative result. However, the open field test can be used to abrogate this locomotor component in the FST analysis. After 10 days of FLX and WMO administration, the locomotor activity of rats, including bouts, distance, and velocity parameters, was not significantly influenced. This phenomenon indicated that the reduction in immobility time of rats treated with WMO in the FST was not caused by unspecific effects of WMO.

During the FST, the major behaviors of the rodents could be categorized into active (swimming and struggling) and passive behaviors (immobility), the latter of which is typically regarded as depressive-like behavior (Slattery and Cryan, 2012). The active behaviors in the FST, such as struggling and swimming, are associated with neurotransmission modulation specifically of the noradrenergic and serotonergic systems, respectively. Hence, the FST is a great model to assess potential serotonergic or noradrenergic antidepressants via observing changes in swimming, struggling and immobility. In the present study, although acute oral administration of FLX, WMO or RA significantly reduced immobility time, the swimming and struggling behavior times were not altered compared to the CTL group (Fig. 2). These results indicated that the acute administration of WMO and RA produces antidepressant-like effects,

but the specific neurotransmitter systems modulated could not be determined. In contrast, animals administered sub-chronic doses had a decreased duration of immobility and increased swimming behavior time, but struggling time was not affected. Therefore, we hypothesize that WMO may be associated with modulation of the serotonergic system.

The circuit of serotonergic neurons originates from the raphe nucleus and projects to other regions including the frontal cortex, amygdala, striatum, and hippocampus. Previous studies indicated that patients with MDD present with low concentrations of 5-HT in the brain (Stockmeier, 1997; Fava, 2003). High 5-HT turnover rates in the brains of patients with MDD were also reported (Barton et al., 2008). Moreover, rodents subjected to unpredictable chronic stress present depressive-like behaviors, which were associated with abnormal serotonergic metabolism (Van den Hove et al., 2014). To clarify the relationship between the WMO/RA and the impact on serotonergic neurotransmitters and behaviors, we further analyzed both the 5-HT content and its metabolic rate in the frontal cortex, amygdala, striatum, and hippocampus. The results of neurotransmitter analysis indicated that in the acute model, the 5-HT turnover rate was generally not influenced by WMO and RA (Fig. 4). This observation is deserves from the swimming time results in the FST (Fig. 2). In contrast, the administration of FLX and different dosages of WMO to rats

for 10 days reduced serotonin turnover rate, especially with the medium WMO dosages and in the amygdala and striatum (Fig. 5). Thus, WMO could reduce serotonin turnover in the brain and further promote swimming behavior.

The dynamics of the serotonergic neurotransmitter affected by the FST was also investigated. Pan et al. indicated that in comparison to mice without stress, the FST elevates the metabolic rate of serotonin in brains of mice (Pan et al., 2005). In the same study, Pan showed that the effect of the FST on the serotonergic neurotransmitters is associated with up-regulating MAO activity, both A and B types. In fact, the weak MAO_A inhibiting ability of WMO was indicated herein. An *in vitro* study demonstrated that the half maximal inhibitory concentration (IC_{50}) of WMO to inhibit MAO_A was 48.2 μg/mL (Lopez et al., 2009). Takeda et al. also showed that RA and its metabolite, caffeic acid, presented antidepressant-like effects. However, the mechanisms could not be clarified, as even the enzymatic results indicated that RA and caffeic acid might have weak MAO_A inhibiting abilities (Takeda, 2002). In addition to the *in vitro* enzymatic study and an *in vivo* study using the FST, a recent study revealed that long-term administration of RA, which ameliorates the depressive-like behavior of rats in chronic mild stress model. (Jin et al., 2013). Certain studies have revealed that RA (Fale, 2011) and caffeic acid (Tsai, 2011) are able to cross the blood-brain barrier. Hence, RA and caffeic acid can be considered

the active compounds that are responsible for the antidepressant activity of WMO. In addition to RA and caffeic acid, certain flavonoid components, such as quercitrin and luteolin derivatives, might have MAOI activity in MO (Lopez, 2009). Thus, in this study, the up-regulating effect on the 5-HT retention in the brain that the antidepressant-like activity of WMO approaches was related to mechanisms in MAO_A inhibition.

5. Conclusion

Using both chromatography and behavioral observations, we demonstrated that WMO has a high RA content and that the antidepressant-like effects are related to the serotonergic system. The results of neurotransmitter analysis indicated that oral WMO administration down-regulated the turnover of 5-HT in the brain regions associated with serotonergic neurotransmission. Previous researches has indicated that the antidepressant effect of MO may due to the inhibition of MAO, but in the present study, a clear mechanism still could not be clarified. Thus, the findings of this study provide a potential pharmacological target on the serotonergic system to prove the antidepressant-like action of *Melissa officinalis* L.

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Conflict of Interest

None

Abbreviations: WMO, water extract of *Melissa officinalis* L.; RA, rosmarinic acid; FLX, fluoxetine; FST, forced swimming test; 5-HT, serotonin; MDD, major depressive disorder; MAO, monoamine oxidase; HPLC, high performance liquid chromatography

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FIGURES AND CAPTIONS

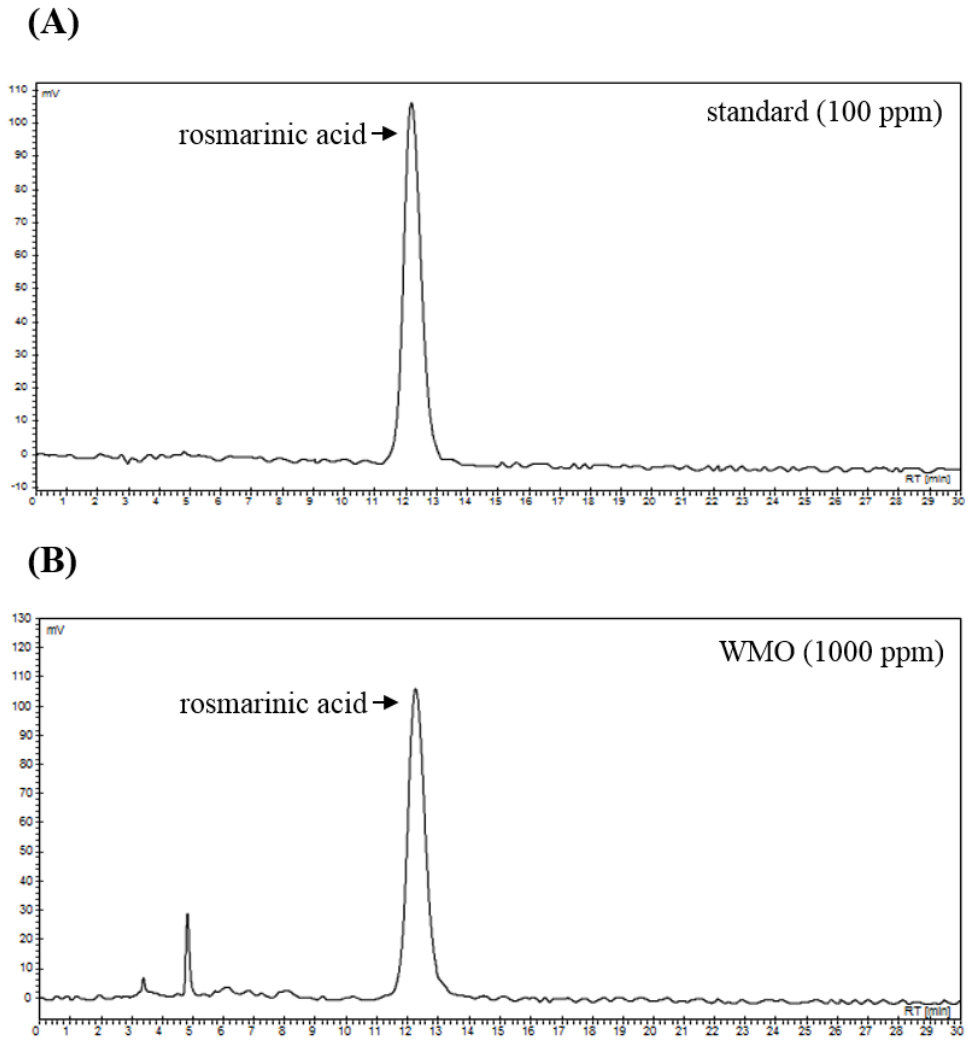

Figure 1. High performance liquid chromatography. Chromatograms of the (A) 100 ppm rosmarinic acid standard and (B) 1000 ppm water extract of fresh leaves of *Melissa officinalis* L.

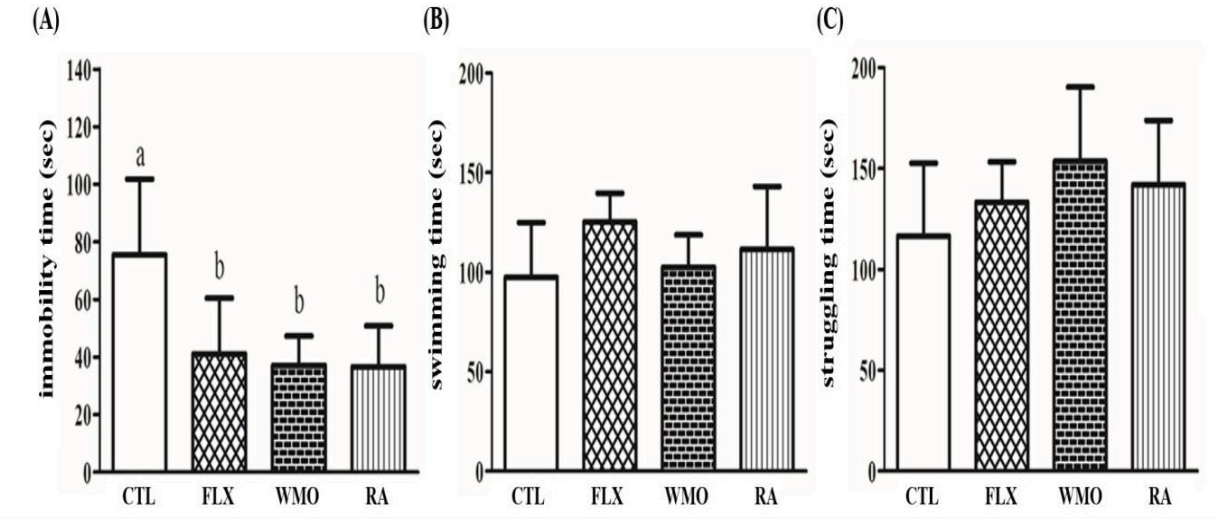

Figure 2. Behaviors of rats treated with *Melissa officinalis* L. (WMO) subjected to the forced swimming test in the acute model. (A) Immobility time, (B) Swimming time, and (C) Struggling time. Rats were administered ultrapure water (control, CTL), fluoxetine (FLX, 18 mg/kg body weight), WMO (300 mg/kg body weight) or rosmarinic acid (RA, 36 mg/kg body weight) three times in one day. All results were subjected to one-way analyses of variance with Duncan's post hoc analysis ($p < 0.05$). $^{a, b}$ indicate significant differences between the different treatments.

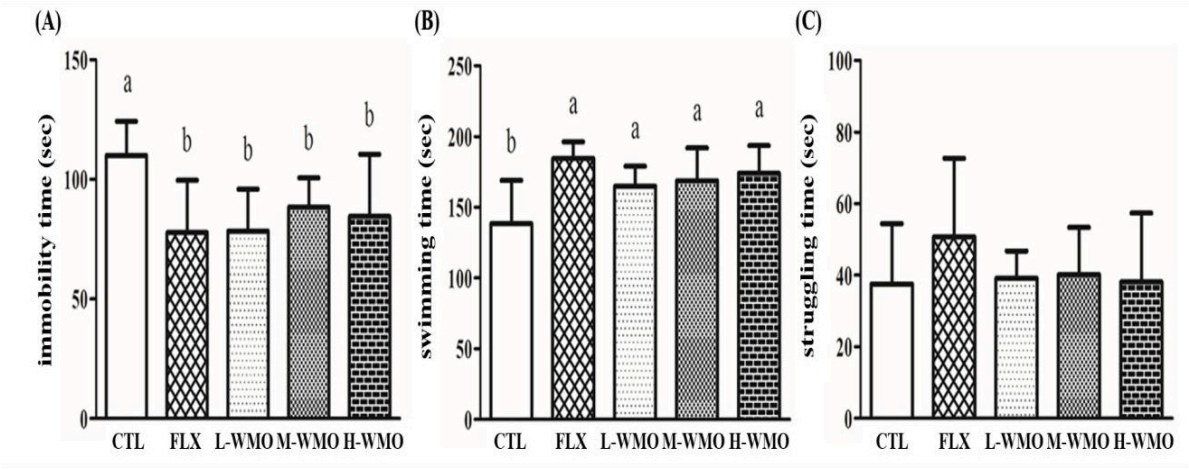

Figure 3. Behaviors of rats treated with *Melissa officinalis* L. (WMO) subjected to the forced swimming test in the sub-acute model. (A) Immobility time, (B) Swimming time, and (C) Struggling time. Rats were administered with ultrapure water (control, CTL), fluoxetine (FLX, 18 mg/kg body weight) or different dosages of WMO (low dose of WMO, L-WMO, 30 mg/kg body weight; medium dose of WMO, M-WMO, 100 mg/kg body weight; high dose of WMO, H-WMO, 300 mg/kg body weight) once a day for 10 days. All of the results were subjected to one-way analyses of variance with Duncan's post hoc analysis ($p < 0.05$). $^{a, b}$ indicate significant differences between the different treatments.

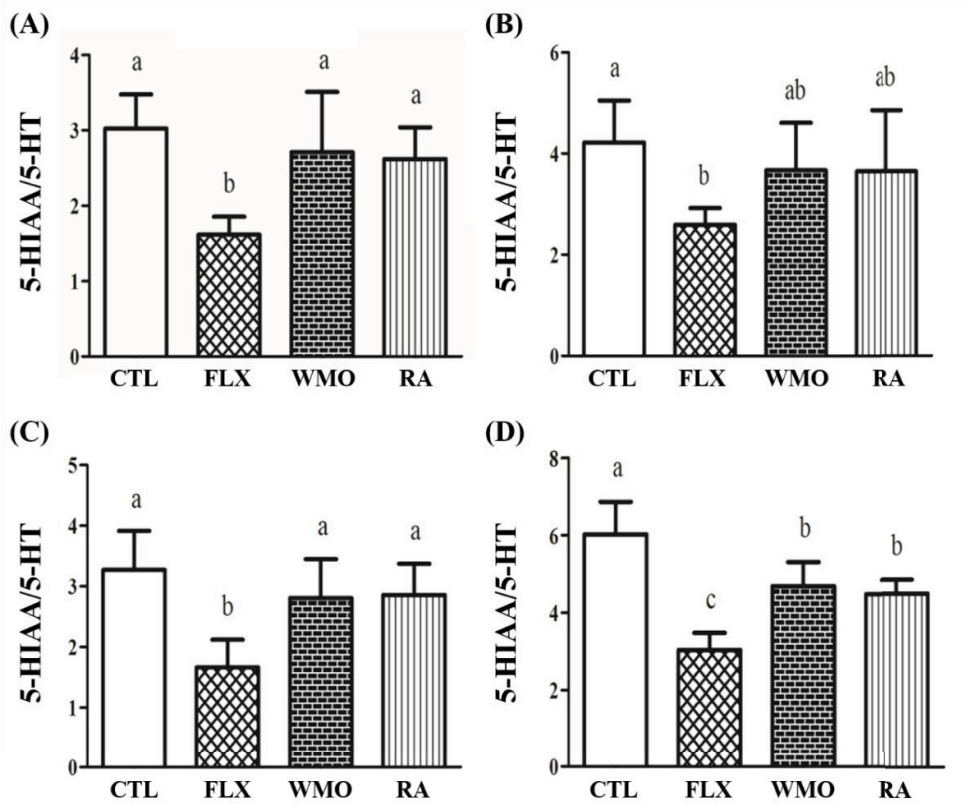

Figure 4. Effect of water extract of *Melissa officinalis* L. (WMO) on serotonin turnover (5-HIAA/5-HT) in the brains of rats in the acute model. (A) Frontal cortex, (B) Amygdala, (C) Striatum, and (D) Hippocampus. Rats were administered with ultrapure water (control, CTL), fluoxetine (FLX, 18 mg/kg body weight), WMO (300 mg/kg body weight), or rosmarinic acid (RA, 36 mg/kg body weight) three times in one day. All results were subjected to one-way analyses of variance with Duncan's post hoc analysis ($p < 0.05$). [a, b, c] indicate significant differences between the different treatments in the same region.

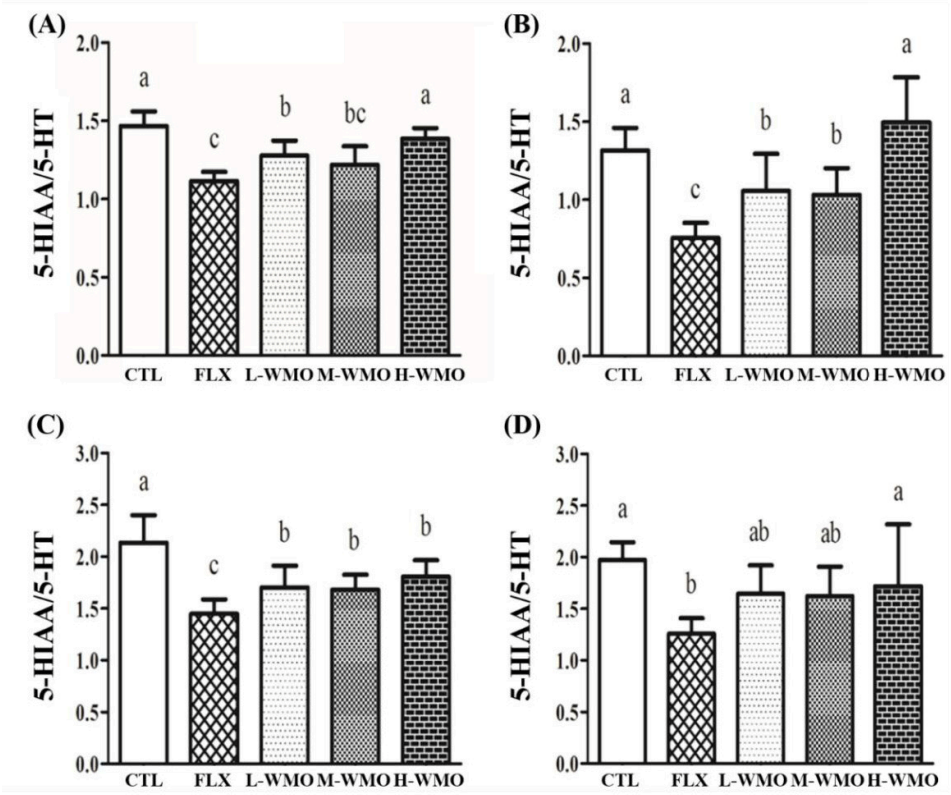

Figure 5. Effect of water extract of *Melissa officinalis* L. (WMO) on serotonin turnover (5-HIAA/5-HT) in the brains of rats in the sub-acute model. (A) Frontal cortex, (B) Amygdala, (C) Striatum, and (D) Hippocampus. Rats were administered ultrapure water (control, CTL), fluoxetine (FLX, 18 mg/kg body weight) or different dosages of WMO (low dose of WMO, L-WMO, 30 mg/kg body weight; medium dose of WMO, M-WMO, 100 mg/kg body weight; high dose of WMO, H-WMO: 300 mg/kg body weight) once a day for 10 days. All results were subjected to one-way analyses of variance with Duncan's post hoc analysis ($p < 0.05$). [a, b, c] indicate significant differences between the different treatments in the same region.

Graphical Abstract (for review)