**Antidepressant- like effects of rosmarinic acid through mitogen-activated protein kinase
phosphatase-1 and brain-derived neurotrophic factor modulation**

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ABSTRACT

Rosmarinic acid (RA) is one of the major bioactive compounds of *Rosmarinus officinalis*, a culinary and edible aromatic plant and was demonstrated to have several neuroprotective properties including anti-depressive-like effect. The current study was designed to contribute to understanding the molecular mechanism of RA beneficial effects in tail suspension test (TST)- induced depression in mice with bupropion as positive control, and mice without TST as negative control. Changes in serum corticosterone (CORT) level and cerebrum level of catecholamines: dopamine (DOP), noradrenaline (NAD), and adrenaline (ADR) were investigated. Moreover, brain-derived neutrophic factor (Bdnf), mitogen-activated protein kinase phosphatase-1 (Mkp-1), tyrosine hydroxylase (Th) and pyruvate carboxylase (Pc) gene expression in mice cerebrum were investigated using real-time PCR. RA was demonstrated to show anti-depressant-like effect by significant reduction of immobility time in the TST in mice. This effect was accompanied by a downregulation of Mkp-1 to reach the unstressed group level, an upregulation of Bdnf, Th and Pc in the limbic system. Furthermore, RA significantly decreased serum corticosterone level and increased dopamine level in the limbic system in mice brain. Our study provides the first evidence that RA has anti-depressant activity via downregulation of Mkp-1, upregulation of Bdnf and modulation of dopamine and corticosterone synthesis.

Keywords: Rosmarinic acid; Depression; Corticosterone; Mitogen-activated protein kinase phosphatase-1; Brain-derived neutrophic factor;

Pub Chem CID

Rosmarinic acid CID 5281792 ; Bupropion CID 62884 ; Fluoxetine CID 11576374 ; Amitriptyline CID 11065 ; Mitragynine CID 3034396; Dopamine CID 681 ; Serotonin CID 5202; Corticosterone CID 5753 ; Epinephrine, CID 5816; Norepinephrine CID 439260

1. Introduction

Major depressive disorder (MDD) is becoming a significant threat affecting the quality of life of human being all over the world and it is expected to be the second leading cause of disease-related disability in the next decade (Sasaki et al., 2013). Moreover, this psychiatric problem is imposing severe problem to our society, emotionally and financially, since it is leading to the suicide of 850,000 people every year according to World Health Organization (WHO) and its burden is estimated to \$100 billion annually (Duric et al., 2010).

The neuropathological feature of MDD seems to be very complicated catalyzing mood disorders, central nerve system (CNS) abnormalities at structure, function and molecular signaling levels, and dysfunction of several physiological mechanisms (Drevets, 2001) including hormonal (Whybrow & Prangeand, 1981) immune system functions (Capuron et al., 2011). MDD is thought to be a result of repeated stress episodes and hyperactivity of hypothalamic-pituitary-adrenal (HPA) axis. The body copes with stress by releasing the corticotropin-releasing factor which results in high glucocorticoids in the circulation (Sapolsky et al, 2000). Such adrenal glucocortocoids stimulates several physiological functions that enable the body re-establishment to pre-stress level (Gregus et al., 2005). Repeated exposure to stress in human is believed to induce neuronal inflammation, neuronal degeneration, brain micro-damage (Wagner-Smith, & Markou, 2010), cognitive deficits and perturbation in CNS functions (Dagyte et al., 2011).

Dealing with MDD, several synthetic drugs were developed in the last decades including the

tricyclic antidepressant (TCAs), monoamine oxidase inhibitors (MAOIs), selective serotonin
 reuptake inhibitors (SSRIs), selective serotonin-noradrenaline reuptake inhibitors (SNRIs),
 and melatonergic anti-depressant drugs like agomelatine. Although these drugs alleviated
 patient suffering, they failed to treat all symptoms of MDD and their molecular and
 therapeutic mechanisms are not fully understood (McGrath et al., 2006; Sasaki et al., 2013). In
 this respect, development of new anti-depressant therapeutic agent without or at least with few
 side effects will be of high interest. At present, several researches are focusing on the anti-
 depressant like effect of polyphenols and plant extracts including green tea polyphenols (Zhu
 et al., 2012), *Ginkgo biloba*, *Valeriana officinalis*, and St. John's wort herb, among others
 (Beaubrun & Gray, 2000). Polyphenols are a large group of phytochemicals. Their dietary
 intake is known to exert several health benefits including antioxidative, neuroprotective,
 cognitive properties, anti-depressive, anti-anxiety, improvement of hippocampal long term
 potentiation, improvement of cognitive deficits, and promotion of adult hippocampal
 neurogenesis (Van Praag et al., 2007; Duffy et al., 2008; Rossi et al., 2008; Bouayed , 2010;
 Mandel et al., 2011; Queen et al., 2010; El Omri et al., 2010; Dias et al., 2012; Spencer et al.,
 2012).
 It has been demonstrated that *Rosmarinus officinalis* may exert an anti-depressant like effect
 (Machado et al., 2009; Machado et al., 2013; Sasaki et al., 2013). Moreover, we reported that
 rosmarinic acid (RA) is one of the active compounds endowed with antidepressant-like effect.
 RA is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Fig. 1). RA is one of major

polyphenolic ingredients of *Rosmarinus officinalis* (El Omri et al., 2010) and it is available in Lamiaceae group plants and mainly in culinary herbs and medicinal plants. A recent screening of RA-containing plants showed that RA is distributed in 26 families from land plants like hornworts up to highly evolved species of the monocotyledonous and dicotyledonous plants (Petersen et al., 2009). Recent research demonstrated that RA is endowed with multiple biological and pharmacological activities, including antioxidant, anti-allergic and anti-inflammatory effects (Ito et al., 2008). Furthermore, RA was reported by our team and others to have several neuronal benefit insults like anti-amyotrophic lateral sclerosis (ALS) (Shimojo et al., 2010), anti-Alzheimer's disease (AD) (Alkam et al., 2007), antioxidant and anti-excitotoxicity in experimental ischemia-model (Fallarini et al., 2009), improving effect on experimental Parkinson's disease model (Wang et al., 2012), nootropic effect, and anti-depressive (Ito et al., 2008; Sasaki et al., 2013).

Although reported previously that RA has anti-depressant effect in rodents, the current study was based on our previous findings that *Rosmarinus officinalis* has anti-depressant effect through monoamenergic, and cholinergic functions modulation (Sasaki et al., 2012).

The current research was designed to contribute to the understanding of the molecular mechanism behind the anti-depressant effect of RA in mice with a focus on catecholamine and corticosterone modulation, brain-derived neurotrophic factor (Bdnf), mitogen-activated protein kinase phosphatase-1 (Mkp-1), tyrosine hydroxylase (Th) and pyruvate carboxylase (Pc) gene expression in mice brain.

2. Materials and methods

2.1. Animals

Male ICR mice (Charles River laboratories Japan Inc., Yokohama, Kanagawa, Japan), 3 weeks old, weighing 25-30 g, were received in our animal experiment facility. All mice were lodged individually and maintained with free access to water and food under a 12:12 h light/dark cycle and animals were acclimatized to laboratory conditions for 1 week. All manipulations were carried out between 9:00 and 16:00. All procedures in this study were approved by the Ethics Animal Care and Use Committee of the University of Tsukuba, Japan under the number 12-309 valid from 2012-6-1 to 2013-5-31.

2.2. Sample treatment

After the acclimatization to the laboratory conditions, All mice were divided into 5 groups and administered distilled water (DW), or RA (MP Biomedicals, Santa Ana, CA, USA): Control group (DW without TST), Model group (DW + TST), Bupropion (Wako, Osaka, Osaka, Japan) administrated group (Bup: 20mg/kg·day + TST) and RA administered group (RA: 5 and 10 mg/kg·day + TST). RA treated groups were fed with RA for 7 days using oral administration route. RA was dissolved in drinking water and orally administered to mice at 200 microlitre / dose/ mice/ day containing the right amount of RA daily 3 h before TST.

Bupropion (dopamine and noradrenaline reuptake inhibitor; DNRI), which is an

antidepressant selectively increasing dopamine and noradrenaline levels in synaptic cleft in brain was used as positive control.

2.3. Tail suspension test (TST)

The TST was conducted as previously described with some modifications 60 min after oral administration of RA. Briefly, mice were individually suspended from tail with a clamp (10 mm from the tail tip in a white box (30 x 15 x 50 cm (L x W x H)), with minimal background noise, with the head 30 cm from the bottom. Each mouse was suspended for a total session of 6 min, and the duration of immobility was recorded during the final 4 min interval of the test. Mice were considered immobile only when they hung passively and completely motionless. Immobility time displayed in TST has been hypothesized to reflect depressive disorders in human.

Following the last TST, each mouse was sacrificed by cervical spine dislocation blood was collected and Brains were isolated, washed in PBS (-) and immediately immersed in liquid nitrogen, then kept at -80°C until use.

2.4. Corticosterone level in mice serum

CORT levels in the serum were measured by Assay Max Corticosterone ELISA kit (Assaypro, Saint Charles, MO, USA). Blood serum was collected by centrifugation at 2000xg for 10 min then diluted at 1/200 into EIA diluent, and immediately stored at -20°C until use. Twenty five microlitre of different serum samples or standard solution were used for

corticosterone determination according to manufacturer's instructions. When optimal blue colour density developed the reaction was stopped and the absorbance was recorded at 450 nm. The level of CORT was calculated using standard curve.

2.5. Dopamine (DOP), noradrenaline (NAD) and adrenaline (ADR) levels in mice brain

DOP, NAD and ADR levels in the mice brains were measured by 3-CAT Research ELISA kit (LDN, Nordhorn, Niedersachsen, Germany). Briefly, 100 mg of limbic area isolated from brain tissue, and homogenized in MilliQ. Sixty microlitre of sample or standards were used for catecholamine extraction, acylation and determination according to manufacturer's instructions. After optimal colour development, the reaction was stopped and the absorbance at 450 nm was recorded.

2.6. RT-PCR analysis of mRNA expression in mice brain

To investigate the molecular mechanism of RA anti-depressant like-effect, real time-PCR was used to investigate the mRNA expressions of different biomarkers considered in this study.

Total RNA was extracted from 100 mg limbic system, using Isogen kit then quantified and quality assessed with Thermo scientific nanodrop 2000 (Thermo, Waltham, MA, USA).

Reverse transcription reactions were carried out with the superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) using 1 µg of total RNA as reported in our previous study (Sasaki et al., 2013). All primer sets and TaqMan probes for experimental genes were from Applied Biosystems (Carlsbad, CA, USA) :mouse tyrosine hydroxylase (Th)

(Mm00447557_m1), mouse pyruvate carboxylase (Pc) (Mm00500992_m1), mouse MAPK phosphatase (Mkp-1) (Mm00457274_g1), mouse brain-derived neurotrophic factor (Bdnf) (Mm04230607_s1) and mouse GAPDH (Mm99999915_g1). For the quantification of mRNA, TaqMan Real Time-PCR amplification reactions were carried out in an AB 7500 fast real-time system (Applied Biosystems, Carlsbad, CA, USA). Amplifications were performed using 70 ng cDNA template in TaqMan Universal PCR Master Mix UNG (Applied Biosystems, Carlsbad, CA, USA), mixed with the corresponding primer/probe mix as reported by (Sasaki et al., 2013). Cycling conditions were: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min.

2.7. Data analysis

All the results were expressed as mean $\pm$ standard error deviation, and the statistical evaluation was performed using the Student's *t*-test between Controls versus Vehicle group, then One way ANOVA followed by Tukey HSD 95 % was used for multicomparison between groups subjected to TST. When statistical rules are violated all groups were considered for multi-comparison study as indicated in figure legend. Statistical analyses were performed using STATGRAPHICS ®. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of rosmarinic acid on the immobility time in the tail suspension test in mice

TST is a widely used behaviour animal model for the investigation of depression and its neuro-pharmaco-biological mechanisms (Steru et al., 1985; Bourin et al., 2005; Cryan et al., 2005; Hao et al., 2013; Dhingra, & Bansal, 2014). The anti-depressant like activity of RA was investigated in TST-induced stress in mice. RA at 5 and 10 mg/kg (p.o) in mice for 7 consecutive days did neither cause the death of any animal, nor change the mice hair colour, nor result in a significant body weight change. As shown in Fig. 2, the immobility time remained unchanged for all treatment groups, until the 4th day. However, Immobility time in vehicle (TST stress) groups increased from Day 5 to Day 7 (D5, 122.0 ± 16.1 sec; D6, 149.7 ± 31.7 sec; D7, 158.6 ± 19.0 sec, respectively). On the other hands, RA treatment significantly suppressed the increase of the immobility time to 67.8 ± 13.2 and 42.2 ± 11.3 sec at 5 and 10 mg/kg (p.o) in comparison with bupropion 28.0 ± 10.6 sec used as positive control and control mice (water treated group) 158.6 ± 19.0sec. RA at 10 mg/kg almost reduced the immobility time by almost 4 fold and it was closely similar to bupropion treatment.

3.2.Effects of RA on serum corticosterone levels in mice'blood serum

TST- induced stress in mice significantly increased ($P < 0.05$) serum corticosterone levels of vehicle group to 1118.7± 67.3 ng/ml as compared to mice not subjected to TST (585.9± 89.7 ng/ml). RA treatment at 5 and 10 mg/kg elicited a significant reduction ($P < 0.05$) in TST-induced increase of serum corticosterone levels in mice to 284.9± 49.8 and 362.2 ± 114.3 ng/ml. This effect was similar to bupropion treatment (273.5±117.8 ng/ml ($P < 0.05$ vs vehicle

group)) (Fig.3).

3.3.Effects of RA on catecholamine neurotransmitter levels in the limbic system of mice exposed to tail suspension test

As shown in Fig.4, TST-induced stress in mice, on the one hand, significantly decreased DOP and ADR levels in the limbic system by 3 and 2 folds of control, respectively ($P < 0.051$, t' test Vehicle versus Control) (Fig.4A and C). On the other hand NAD level was decreased to 58.6 ± 24.7 in vehicle group versus control group 86.8 ± 8.8 ng/ 100 mg tissue without a statistically significant difference (Fig.4 B). The oral administration of RA (5 and 10 mg/kg) significantly elevated the contents of DOP (985.0 ± 157.9 and 2050.8 ± 248.8 ng/100 mg tissue, respectively). Bupropion treatment (1188.6 ± 118.3 ng/100 mg tissue) showed similar effect to 5mg/kg RA and was less effective than 10 mg/kg RA (Fig.4A).

For NAD level in the limbic system, only bupropion treatment (101.1 ± 10.5 ng /100 mg tissue) showed a significant effect versus vehicle group (58.6 ± 24.7 ng /100 mg tissue). RA treatment showed a positive effect 94.4 and 90.6 ng /100 mg tissue, respectively for 5 and 10 mg/kg increase. However, this effect was not significant when compared to vehicle and bupropion groups (Fig 4 B).

Similarly, for adrenalin only bupropion treatment showed a significant effect. RA treatment showed a slight non-significant increase when compared to vehicle group, and a statistically significant difference when compared to bupropion treated group (Fig.4 C). These results

demonstrated a clear difference between bupropion and RA treatments in TST-induced depression in mice.

3.4.Effects of RA on Mkp-1 and Bdnf mRNA expressions in limbic system of mice exposed to tail suspension test

As shown in (Fig. 5A and B), the TST-induced stress increased the *Mkp-1* mRNA expressions in the limbic system ($140.7 \pm 5.2\%$) ($P < 0.01$) and decreased mRNA expression of *Bdnf* ($45.9 \pm 4.4\%$) ($P < 0.01$, respectively). RA (5 and 10 mg/kg) administration in mice significantly ($P < 0.05$) decreased *Mkp-1* mRNA expressions by 27 and 35% versus vehicle group, respectively (Fig. 5A). Bupropion treatment showed also significant inhibition of *Mkp-1* mRNA by 44% in comparison to control. In addition, RA (5 and 10 mg/kg) administration increased *Bdnf* mRNA expressions (59.9 ± 2.8 and $61.9 \pm 2.7\%$, respectively), in comparison to vehicle group and RA treatments ($P < 0.05$) (Fig. 5B).

3.5.Effects of RA on Th and Pc mRNA expressions in limbic system of mice exposed to tail suspension test

Previously, Sasaki et al., (2013) demonstrated that RA significantly increased *Th* and *Pc* in a proteomics conducted study in PC12 cells. In the current study RT-PCR investigation of *Th* and *Pc* mRNA expression level in the limbic system of RA-treated mice (Fig.5C and Fig.5D) demonstrated that TST significantly decreased *Th* and *Pc* mRNA expression to (39.6 ± 2.2 and

83.1 $\pm$ 2.9% of control, respectively)($P < 0.05$ and $P < 0.01$ respectively). RA (5 mg/kg and 10 mg/kg) significantly improved *Th* mRNA expression to reach (50.6 $\pm$ 1.1 and 75.0 $\pm$ 3.1 %, respectively); and significantly upregulated *Pc* mRNA expression (140.9 $\pm$ 4.2 and 149.1 $\pm$ 4.6 % of control, respectively) in comparison to vehicle group ($P < 0.05$, respectively). Bupropion treatment upregulated *Th* mRNA expressions to reach (131.0 $\pm$ 4.3%) ($P < 0.05$). In contrast, bupropion seems to reduce *Pc* at translational level (71.8 $\pm$ 1.6%) ($P < 0.01$) to inferior level than vehicle group. The investigation of *Th* and *Pc* showed a significant difference in the effect of bupropion and RA.

4. Discussion

Pharmacologists are developing different anti-depressants to alleviate the impact MDD. However, even the newest drugs - although good enough to be selective - may produce multiple side effects like headache, nausea, insomnia, appetite and sex drive disturbance, muscle stiffness, high risk of heart attack, and high risk of seizure among others. For this reason, the management of these side effects is becoming a part of routine clinical practice (Uzun et al., 2009).

In this respect, a significant number of epidemiological and experimental studies demonstrated that modulation of MDD using natural polyphenols could play an important role in the prevention, mitigation, and treatment of MDD (Dias et al., 2012; Sasaki et al., 2013).

Our finding is concordant with previous results reported by Takeda et al. (2002) showing that

RA reduced the duration of immobility in forced swimming test, another well known despair behavioral test similar to TST. In fact, exposure to an acute stressor like TST in mice, is believed to reproduce a condition similar to human depression, and is known to activate the HPA axis, resulting in production and release of corticosterone which leads to a cascade of endocrine events (Huang et al., 2011). Corticosterone release is considered as an adaptive feedback regulation mechanism. However, once stress persists, glucocorticoid hypersecretion reduces glucocorticoid receptor (GR) levels and impairs negative feedback inhibition, subsequently some neuro-damage and micro-injuries may affect the hippocampus and some harmful events including depressive syndromes and cognitive deficits may occur (Juruena et al., 2004; Ago et al., 2008). In the current study, the repeated TST trials significantly increased the serum corticosterone levels. RA treatment significantly reversed this effect, in a similar way to bupropion and previous results obtained using fluoxetine, amitriptyline and mitragynine in mice exposed to TST (Idayu et al., 2011). These data strongly suggested that RA exerted its antidepressant-like activity, at least in part, by regulating serum corticosterone levels, thus normalizing the HPA axis hyperactivity.

Plenty of studies suggest a link between HPA axis activation and monoaminergic system involved in depression. Although there is no consistent evidence of a simple relationship between them (Mokrani et al., 2007), recent studies reported that glucocorticoid hypersecretion suppressed GRs expression and glucocorticoid feedback regulation, then the reduced GRs inhibit tyrosine hydroxylase (TH), and subsequently contributes to the

monoamine deficiencies (Heydendael et al., 2009). Present study suggested that RA exerted antidepressant-like activity by modulation of corticosterone and dopamine, through the upregulation of TH gene expression in the mice brain. The current research supports the hypothesis that RA treatment may normalize HPA axis activation and subsequently increase dopamine levels in the brain and finally improve the anti-depressant state in mice. In contrast, RA treatment in mice failed to improve adrenaline and noradrenaline levels when compared to the positive control and the untreated group. This observation needs further experiments to clarify the relationship between dopamine and corticosterone in RA-treated mice. RA treatment significantly increased PC gene expression in mice brains in a concordant way with our previous findings in a proteomics conducted study in PC12 cells (Sasaki et al., 2013). PC is a catalyzing enzyme involved in the carboxylation of pyruvate to oxaloacetate and has an anaplerotic role for the tricarboxylic acid cycle. It is an important enzyme for *de novo* synthesis of glutamate in astrocytes, supplied to neurons (Sasaki et al., 2013). Thus, it has an important role in energy homeostasis in the brain and in the glutamate – glutamine cycle between astrocyte and neurons. PC catalyzes the conversion of pyruvate to oxaloacetate. Oxaloacetate is 1 of 2 essential substrates needed to produce citrate, the first substrate in gluconeogenesis. A deficiency or loss of PC activity will result in perturbation of glutamate production and dysfunction of citric acid cycle and gluconeogenesis, leading to several metabolic disturbance, depression, arousal deficiency, lack of attention, reduced motor activity

and curiosity (Abdallah et al., 2014; De Souza, 2014). Both energy deficiency and glutamate metabolism are known to be an underlying biomarker of MDD (Abdallah et al., 2014).

Dealing with the etiopathophysiology of MDD, monoamine and endocrine hypothesis do not fully explain the onset of the disease. Postmortem, preclinical studies identified MKP-1 as a new target biomarker for therapeutic interventions in MDD (Duric et al., 2010). In earlier study, Dwivedi et al. (2001) reported that p-ERK1/2 was significantly decreased in frontal cortical areas and the hippocampus of suicide subjects with major depression from eleven case observation (Dwivedi et al., 2001). In current study, RA treatment significantly decreased *Mkp-1* mRNA expression in mice brain. In fact, it is well documented that high glucocorticoids levels increase MKP-1 expression in hippocampus. Such upregulation significantly dephosphorylate p-ERK1/2 expression and subsequently trigger apoptosis and microdamage in hippocampus (Kim et al., 2005). Moreover, from our previous studies and others, RA showed neuroprotective effect against corticosterone in PC12 cells (Sasaki et al., 2013) and significant phosphorylation of ERK1/2 in rodents brain (Jin et al., 2013).

Basal MKP-1 protein in neuronal cells is very low, but its expression is activated by MAPK phosphorylated proteins, so MKP-1 will end up the signal that induced its expression. MKP-1 is rapidly degraded by proteasome, but BDNF synthesis can prolong its half life stability and activation (Jeanneteau et al., 2010) and (Deinhardt and Jeanneteau, 2012). Our study demonstrated that RA significantly increased BDNF gene expression in mice brains. In fact

BDNF is essential for neurogenesis and remodeling of axonal arbors in the brain (Jeanneteau et al., 2010). Furthermore, BDNF supply to neurons was demonstrated to alleviate the neurotoxic insults effect of corticosterone (Nitta et al., 1999). This close relationship between BDNF-MKP-1 and BDNF-glucorticoids explains the important role of BDNF modulation of MDD. Beside our findings, recent publications demonstrated that RA has anti-depressant like effect in mice through promotion of neurogenesis in hippocampus (Ito et al., 2008), and promotion of BDNF in the neurons and astrocytes in rats submitted to chronic unpredicted stress (Jin et al, 2013).

5. Conclusions

Taken together, our data suggest that RA exerts anti-depressant-like effect in animal model of depression. Such properties appear to be mediated by HPA axis activation modulation, MKP-1 downregulation, BDNF upregulation, and an increase in dopamine level in the brain. This newly discovered effect of RA may contribute to understanding of its neuropharmacological properties. The observed effects of RA showed significant differences with bupropion used as positive control in this study, indicating a difference in the molecular mechanism of both compounds. The molecular mechanisms that translate RA extracellular signals into neurogenesis in relationship with BDNF, MAPK , monoamines and glucorticoids signaling is still not understood and it will be the focus of our next research.

Conflict of interest

Authors declare no conflict of interest

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Fig.1 - Structure of rosmarinic acid and bupropion

Fig. 2 -Effects of administration of RA (5 and 10 mg/kg/day) on the immobility time in the TST. Mice were administrated orally with water (Vehicle), bupropion (20 mg/kg/day) or rosmarinic acid (5, 10 mg mg/kg • day) for 7 days and subjected to TST 60 min after drug administration. The immobility time during the final 4 min from 6 min total session were measured. (A) Represents the daily immobility time of each group. (B) Represents the last day TST immobility time.Each group represents the mean $\pm$ SD ($n = 5$). Bars sharing the same letter are statistically significant at 5% One Way ANOVA multi-comparison test followed by Tukey Post hoc test between groups submitted to TST stress

Fig.3 - Effects of administration of RA (5 and 10 mg/kg) on serum corticosterone levels in the mice subjected to TST.

Mice were administrated orally with water (Vehicle), bupropion (20 mg/kg/day) or rosmarinic acid (5, 10 mg mg/kg/day) for 7 days and subjected to TST 60 min after drug administration. Corticosterone levels in mice'blood serum was quantified using ELISA as indicated in Materials and Methods. Each bar represents the mean $\pm$ SD ($n = 3$).).* $P < 0,05$ ** $P < 0.01$ (student t'test) control vs vehicle. Bars sharing the same letter are statistically significant at 5% One Way ANOVA multi-comparison test followed by Tukey Post hoc test between groups submitted to TST stress

Fig.4 - Effect of administration of RA (5 and 10 mg/kg) on the levels of the catecholamine (DOP, NAD and ADR) in mice brain limbic system.

Mice were administrated orally with water (Vehicle), bupropion (20 mg/kg/day) or rosmarinic acid (5, 10 mg mg/kg/day) for 7 days and subjected to TST 60 min after drug administration. Dopamine, adrenaline and noradrenaline in the brain limbic system, were quantified using ELISA as detailed in Materials and Method. Each bar represents the mean $\pm$ SD ($n = 3$).* $P < 0,05$ ** $P < 0.01$ (student t'test) control vs vehicle. Bars sharing the same letter are statistically significant at 5% One Way ANOVA multi-comparison test followed by Tukey Post hoc test between groups submitted to TST stress

Fig.5 -Effect of administration of RA (5 and 10 mg/kg) on the mRNA expressions in the brain limbic system of the mice. Mice were administrated orally with water (Vehicle),

bupropion (20 mg/kg/day) or rosmarinic acid (5, 10 mg mg/kg/day) for 7 days and subjected to TST 60 min after drug administration. Gene expression **A:** *Mkp-1*, **B:** *Bdnf*, **C:** *Th*, **D:** *Pc* normalized to GAPDH and expressed as ratio of control group, were quantified from mice' brain limbic system as indicated in Material and Methods. Each bar represents the mean $\pm$ SD ($n = 3$). * $P < 0,05$ ** $P < 0.01$ (student t'test) control versus vehicle. Bars sharing the same letter are statistically significant at 5% One Way ANOVA multi-comparison test followed by Tukey Post hoc test between groups submitted to TST stress.

Figures

Rosmarinic acid

Bupropion

Fig. 1.

A

Figure 2

B

Figure 2

Figure 3

A

Figure 4

B

Figure 4

C

Figure 4

A

Figure 5

B

Figure 5

C

Figure 5

D

Figure 5