Antidepressant-like Effect and the Mechanism of Action of *Lippia Citriodora* Ethanolic Extract and Emulsion, and Verbascoside

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Abstract

Lippia citriodora (Lam.), commonly called lemon verbena or "*Louisa*" (Arabic), is an aromatic and medicinal plant rich in terpens and polyphenols used in folk medicine to cure illnesses such as gastrointestinal disorders, fever and headaches and also for its sedative and relaxant properties. The chemical analysis of verbena extract has been shown to contain different compounds, with verbascoside (Vs) as major compound, a phenypropanoid glycoside. A previous study demonstrated, according to traditional information, the relaxant and hypnotic properties of *L. citriodora* and Vs *in vivo* using rotarod test. However, the antidepressant-like effect of the verbena and Vs were not studied *in vivo* and *in vitro* and their molecular mechanisms are still to be elucidated. In this work, the antidepressant-like effect of verbena ethanolic extract (VEE) and Vs was evaluated using on a tail suspension test (TST) and confirmed the results *in vitro* using human neurotypic SH-SY5Y cells. VEE and Vs regulated expression of genes implicated in production of cAMP (such as *Adenylate cyclase*) and increased intracellular calcium levels, including *Inositol 1,4,5-trisphosphate receptor type 2*.

TST was conducted on mice treated orally with VEE (100 mg /kg), Vs (2.5 and 5 mg/kg), Bupropion (20 mg /kg) and Milli-Q water. VEE-treated mice showed an increase

of immobility time compared to control groups, indicating an induction of relaxation. This effect was found to be induced by regulation of genes playing key roles in calcium homeostasis (calcium channels), cAMP production and energy metabolism. On the other hand, Vs showed antidepressant-like effect and was confirmed by serotonin, noradrenalin, dopamine and BDNF expressions. Finally, VEE and Vs enhanced SH-SY5Y cells viability, mitochondrial activity and calcium uptake *in vitro*. The obtained results showed induction of relaxation and antidepressant-like effects VEE and Vs, respectively, through modulation of cAMP and calcium.

Furthermore, studying the ways of industrialization of VEE and Vs in food area is of a great importance. Production of emulsion based on VEE to enhance storage time and deliverance of Vs was carried out. Formulation of VEE based emulsion including lecithin and oleic acid was found to be stable after storage for more than 2 weeks.

This VEE-emulsion was found to exert antidepressant-like effect *in vivo* by reducing the immobility time of mice in TST. Also it enhanced different depression markers and genes expressions that were affected by VEE solution. Understanding the

molecular mechanism by which Vs and VEE, in solution or emulsion, induce the antidepressant-like effect is essential.

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Abbreviations

Ac: adenylate cyclase

Adora2: adenosine A2a receptor

Cacna1c: calcium channel, voltage-dependent, L type, alpha 1C subunit

Camk2n1: Calcium/ calmodulin-dependent protein kinase II inhibitor 1

Camk4: calcium/ calmodulin-dependent protein kinase IV

Dex : dexamethasone

Drd1: Dopamine receptor 1

Gsn: gelsolin

Hs :hastatoside

Htr4: 5 hydroxytryptamine (serotonin) receptor 4

Itpr2: Inositol 1,4,5-trisphosphate receptor type 2

Mchr1: melanin-concentrating hormone receptor

MDD: Major depressive disorder

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Pkc: Protein kinase c

Pmch: pro-melanin-concentrating hormone

Prkg1: cGMP-dependent protein kinase 1

TST : tail suspension test

Ttr: transthyretin

VEE: verbena ethanolic extract

Vn : verbenalin

Vs: Verbascoside

List of publications

Sabti, M.; Sasaki, K.; Gadhi, C.; Isoda, H. Elucidation of the Molecular Mechanism Underlying Lippia citriodora (Lim .) -Induced Relaxation and Anti-Depression. *International Journal of Molecular Sciences* **2019**, *20:1-19*.

Chapter 1:
General introduction

1. Depression

1.1 General description

Depression is one of the most prevalent forms of mental illness, which is characterized by several physical and emotional symptoms including sleep disturbances, fatigue, energy level dysregulation, loss of interest, etc [1]. It is a disease that causes to the patients a loss of life enjoyment and decreases their ability to accomplish daily duties. Nowadays, it is considered as a major burden worldwide, affecting the lives of 350 million people [2].

Major depressive disorder (MDD) is diagnosed by 2 essential symptoms, dysphoria which is a occurrence of sadness, and anhedonia defined as a loss of interest in pleasurable behaviors previous to the onset of disease. These hallmarks are usually followed up by a loss of appetite, sleep disturbance, fatigue, decrease of self-esteem, recurrence of death or suicidal ideology [3].

Antidepressant drugs have been already prescribed to patients to attenuate their symptoms. These drugs have limitation such as complete remission and in some cases the patients develop resistance to the treatments [3].

1.2 Classification

Depression is defined as a heterogeneous mental disease, present in different forms ranging from mild to psychosis characterized by hallucinations. It is complicated to diagnose depression due to its co-morbidity with other illnesses, including anxiety and phobias [4]. Depression is divided into different classes which are major depression disorder, dysthymic disorder, melancholic depression, seasonal affective disorder, post-partum depression and psychotic depression. Major depressive disorder (MDD) is characterized by typical anhedonia and dysphoria. Dysthymic disorder is a type that fulfills all the symptoms described in case of MDD, with the only difference being the discontinuity of symptoms display. Melancholic depression consists of psychomotor retardation, loss of pleasure, mood declining on mornings, with elder people being the mostly the one affected by this form of depression. Seasonal affective disorder occurs annually on fall or winter, and generally the mood of patients is related to weather conditions, and its symptoms are usually similar to previous depression types previously described, in addition to increase of appetite leading to gain weight [5].Post-partum depression is a type of depression specific to mothers and occurs before or after giving

birth, which might be the trigger for MDD [6]. Psychotic depression is a severe kind of depression that is associated with psychosis, presenting delusions. Efficiency of treatments in case of patients is low [7].

1.3 Physiopathology of depression

Understanding the physiopathology of depression has been extensively studied over the last decades, but even though the mechanism of its induction is still unclear. Determination of the physiopathology behind the onset of depression is essential to develop drugs without side effects.

Several hypotheses were given to explain the physiopathology of depression, and the most important ones are the activation of the neural circuitry of depression, induction of the physiopathology by stress and the heritable character of the disease.

1.3.1 Neural circuitry

It is one of the most studied theories, which revealed changes in regions of the brain regulating the mood and reward aspects. Previous studies focusing on hippocampus and prefrontal cortex showed a decrease of the grey-matte volume and glial density in

patients suffering of depression. Hippocampus is an area that was found to inhibit the activity of hypothalamic-pituitary-adrenal axis (HPA), and its malfunction could explain the increased amount of circulating cortisol in case of depression [8,9]. Also, the nucleus accumbens (NAc) and ventral tegmental area (VTA) are implicated in pleasure response to different stimuli (food, drugs,…) and their dysfunction is believed to induce depression [9].

1.3.2 Stress

Stress is believed to be an important factor for the onset of depression and also its recurrence for patients who had suffered from this illness before. High levels of corticosteroids have been associated with morphological changes in some brain regions. One of them is the amygdala which is regulates emotional reactivity and responses to stress [10,11]. Furthermore, it has been documented that corticosteroids administered chronically induced reduction of hippocampus volume [12]. Stress has been found to alter the expression of endogenous antioxidant enzymes such as, catalase, glutathione, peroxidase, etc. Accordingly, chronic administration of glucocoticoids induced ROS generation, coupled with inhibition of antioxidant enzymes expression leading to the

onset of depression [13,14].

1.3.3 Heritability

The pathogenesis of depression depends, in addition to the environmental factors causing stress, on the genetic vulnerability of the patient. This vulnerability is not dependent to polymorphism to a specific gene polymorphism. Different genes are responsible for the patient vulnerability to stress [15]. One of the genes that have been extensively studied is serotonin transporter (5-HTT). Its promoter is the polymorphic region, resulting in the expression of 2 different variants characterized with different lengths. The short variant presents a low activity compared to the long protein, which leads to increase of patient's vulnerability and reduces the response to antidepressants [16,17].

2. Antidepressant drugs

Depressive symptoms are results of neurotransmitters imbalance in brain, and antidepressant drugs are used to correct these anomalies. Antidepressants are chemicals acting mainly on serotonin and norepinephrine release in brains. These remedies are

prescribed also to cure anxiety, eating and obsessive disorders. In some cases, combination of drugs can be prescribed [18].

2.1. Antidepressants classification

At first the antidepressant drugs were classified according to their chemical structure. Drug discovery led to identification of antidepressant molecules that do not match the previous classification. Nowadays, antidepressants are classified according to their functions. The functional categorization is composed of three classes: 1) monoamine oxidase inhibitors (e.g. phenelzine), 2) norepinephrine (e.g. amoxapine), serotonin (e.g. amitriptyline) and dopamine (e.g. bupropion) reuptake blockers, and 3) serotonin type 2A receptor blockers [19].

2.2. Antidepressants side effects

Tricyclic antidepressants were the first drugs used to cure depression, but due to their side effects, they were replaced by selective serotonin reuptake inhibitors in order to reduce side effects. Later studies showed that even these drugs presented side effects.

Tolerability is one of the serious drugs side effects. It consists of patients dropping out treatments before remission, and generally due to suffering from side effects. Although some of newly discovered drugs present fewer side effects, the dropping out rate is still significant rendering the establishment of innovative protocols imminent [20].

Bleeding is a side effect of antidepressant drugs targeting serotonin reuptake. Serotonin is involved in platelet aggregation, and serotonin reuptake inhibitors block this complex to form leading to abnormal bleeding. It has been found that newer drugs, having stronger inhibitory effect, cause more hemostatic abnormalities [20–23].

Several studies have demonstrated the induction of hepatotoxicity by antidepressants. Tricyclic antidepressants were found to have higher risk of liver injury compared to new generation of drugs. Hepatoxicity is considered as one of the major reasons of treatment drop out [24–26].

Antidepressants affect sleep in patient by increasing and/or reducing sleeping time. Tricyclic antidepressants are known to induce sleep in patients, a reason behind using them also as treatment for insomnia. On the other hand, some drugs induce

insomnia, including bupropion which is prescribed to cure attention deficit hyperactivity disorder and to patients suffering from hypersomnia [27–29].

3. *Lippia citriodora* (Lim.)

Verbenaceae family plants are commonly called verbena or vervain plants. *Verbenaceae* is composed of 35 genera containing around 1200 species [30]. They have been used for centuries as medicinal plants due to their beneficial effects to cure several ailments. One of the most important genera is *Lippia*, regrouping 200 species exerting interesting biological activities [31]. *Lippia citriodora* (Lam.), also referred to as *Aloysa triphylla* (L'Herit.), is commonly named lemon verbena, verbena, vervain or Louisa (Arabic). This species is growing naturally in Latin America and cultivated in several European and North African countries, but mainly in Morocco (Carnat *et al.,* 1999). The herbal tea made of this plant was known in folk medicine for its sedative, relaxant, anti-inflammatory, antioxidant, antispasmodic effects and also used as a remedy for gastrointestinal disorders [31]. Recent studies have confirmed the antioxidant and spasmolytic activities of the infusion prepared of lemon verbena [33,34]. Verbena aqueous extract given to rats has proven the hypnotic effect of the plant by promoting

their sleep [35]. Polyphenols extracted from Verbena reduced the obesity burden and restored the mitochondrial activity through AMPK-dependent pathways [36].

4. Objectives

As mentioned previously, antidepressant drugs available until now present serious side effects with limited efficacy. Identification of new treatments with higher efficacy and low health risks is of great importance. Plants are natural resources with high potential of containing molecules efficient in depression therapy with no or less side effects [37]. Lemon verbena and Vs were found to have anxiolytic and hypnotic effects. Since commercial antidepressants can be prescribed in case of anxiety and sleep disorder, verbena and Vs were studied here for their potential as antidepressants. The present work was conducted to evaluate the effect of VEE and Vs *in vivo* and *in vitro*, and to elucidate their mechanisms of action. Furthermore, this work aimed to produce stable emulsions using VEE in order to increase Vs conservation time and biological activity. VEE-emulsion was evaluated for its antidepressant-like effect.

In Chapter 2, the relaxant effect of VEE treatment administered to mice was observed and elucidated the molecular mechanism responsible. In Chapter 3, the

antidepressant-like effect of Vs using TST was determined and confirmed the *in vivo* results by evaluating anti-depression markers. In Chapter 4, VEE-emulsion was formulated in order to potentially increase Vs bioavailability, and the antidepressant-like effect of the emulsion was evaluated *in vivo*.

Chapter 2:

Elucidation of the molecular mechanism underlying *Lippia citriodora* (Lim.)-induced relaxation

1. Introduction

In this Chapter, the effect of VEE at 100 mg/kg of body weight was evaluated and observed an induction of relaxation. The transcriptomic analysis confirmed the regulation of genes involved in relaxation activity.

The Verbenaceae, a family commonly known as verbena or vervain family, is composed of 35 genera containing around 1200 species [30]. They have been used for centuries as medicinal plants due to their beneficial effects to cure several ailments. One of the most important genera is *Lippia*, regrouping 200 species exerting interesting biological activities [31]. *Lippia citriodora* K.*,* also referred to as *Aloysia triphylla* (L'Herit.), is commonly named lemon verbena, vervain or Louisa (Arabic). This species is native to South America and has been cultivated in Europe and North Africa mainly in Morocco [32]. All over Morocco, the plant is used as relaxant and sedative [38]. The herbal tea is traditionally used to alleviate insomnia and restlessness in adults as well as babies [39]. Furthermore, it has been used for its anti-inflammatory, antioxidant, antispasmodic effects and also used as a remedy for gastrointestinal disorders [31]. Recent studies have confirmed the antioxidant and spasmolytic activities of the infusion prepared of lemon verbena [33,34]. Verbena aqueous extract given to rats has proven the

hypnotic effect of the plant by promoting their sleep [35]. Polyphenols extracted from lemon verbena reduced the obesity burden and restored the mitochondrial activity through AMPK-dependent pathways [36].

Verbascoside (Vs), a major phenypropanoid glycoside, is the most abundant polyphenol in lemon verbena tea and its yield is reported to be around 3.94% (w/w dry weight of leaves) [40]. Vs, isolated from *Buddlejia davidii* and *Lippia multiflora,* has already been proven to possess an antioxidant activity [41,42]. Vs has also shown an anti-inflammatory effect *in vitro* on macrophages and THP-1 cells [43,44]. Furthermore, Vs has been reported to exert an antimicrobial activity against *Staphylococcus aureus* and a neuroprotective effect, *in vitro*, on 1-methyl-4-phenylpyridinum ion-induced toxicity using PC12 cells [45,46]. Interestingly, intraperitoneal administration of Vs and lemon verbena aqueous and ethanolic extracts to mice promoted their sleep and induced muscle relaxation, alongside with alleviation of anxiety [47]. In addition to Vs, hastatoside (Hs) and verbenalin (Vn) are two abundant iridoids in verbena extract and have been proved to possess sleep-promoting effect [48]. To date, very little is known about the molecular

mechanism by which lemon verbena or its compounds induce relaxation and act as anti-anxiety remedy.

In the present study, the relaxant effect of lemon verbena in mice was investigated and elucidated the molecular mechanisms underlying its effect in brain. Interestingly, the transcriptomic analysis *in vivo* showed regulation of genes implicated in activation of the mitochondrial function. To confirm this finding, the effect of VEE and Vs on cellular ATP production was studied using SH-SY5Y, a human neurotypic cell line. Also, the neuroprotective effect of VEE, Vs, Hs, and Vn on dexamethasone (Dex) induced-neurotoxicity was assessed *in vitro*.

2. Materials and methods

2.1. Extract preparation of Lippia citriodora (Lim.)

The leaves of *Lippia citriodora* were collected in July 2016 from Marrakech Region (Morocco). The species was authenticated by Prof. Ahmed Ouhammou from Cadi Ayyad University, Faculty of Sciences Semlalia, Department of Biology, Marrakech, Morocco. A voucher specimen of plant material (MARK-11186) was deposited in the Herbarium of the same institution. After air drying, the plant material was crushed by a mortar and extracted with ethanol 70%, with a ratio plant material/ solvent of 10% (w/v). The extraction was carried out in the dark for 2 weeks and vigorously shacked twice a day. The extract was centrifuged and the supernatant filtered through 0.22 µm Millipore (Mark Millipore, Ireland) and solvent was evaporated by a rotary evaporator. The yield of VEE was 13.3 %.

2.2. Chemicals

Vs, Hs, and Vn were purchased from Sigma Aldrich (USA). Dulbecco's Modified Eagle Medium (DMEM) / F-12 and Opti-MEM were obtained from Gibco

(USA). Fetal bovine serum was from Gibco (South America). Penicillin - Streptomycin were purchased from Biowhittaker (USA). Non-essential amino acids were from Cosmo Bio Co, LTD(Japan). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and dexamethasone were from Dojindo (Japan). Bupropion was from Wako(Japan). ATP bioluminescence kit was from TOYO Ink (Japan). ISOGEN kit was purchased from Nippon Gene(Japan). RIPA lysis buffer was from Santa Cruz Biotechnology(USA). 2-D Quant was purchased from GE Healthcare Life Sciences, USA. Calcium Kit II-Fluo 4 was from Dojindo (Japan).

2.3. SH-SY5Y cells culture

The *in vitro* experiments were conducted on SH-SY5Y cells. This neurotypic cell line was obtained from America Type Culture Collection (Manassas, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) / F-12, supplemented with 15% fetal bovine serum, 1% Penicillin (5000 µg/ml)- Streptomycin (5000 U/mL) and 1% of non-essential amino acids. The culture was incubated at 37°C in a humidified

atmosphere of 5% CO_2 incubator. Opti-MEM, a reduced serum medium, was used to culture cells for the evaluation of cell viability and intracellular ATP.

2.4. Determination of cytotoxicity and neuroprotection of VEE and pure compounds

MTT assay was used to assess cell viability of SH-SY5Y cells. Briefly, the cells were seeded in a 96-well plate (fibronectin-coated plate) (BD BioCoat, United States) with a density of 2×10^4 cell /well. After 24 h, the culture medium was removed and replaced to fresh media containing VEE (0.5, 1, 2.5 and 5 µg/mL), Vs, Hs or Vn (3.1, 31.2 and 62.4 µg/ml). For the evaluation of the neuroprotective effects, the cells were co-treated with Dex (50 µM). After 72 h incubation period, 10 µL MTT (5 mg/mL) mixed with 100 µL of Opti-MEM was added to each well and the plate was incubated for further 6 h. The formazan crystals formed by the mitochondrial activity were solubilized by adding 100 µL of 10% SDS (w / v). The absorbance was measured at 570 nm using a microtiter plate reader (Dainippon Sumitomo Pharma Co., Ltd., Japan). The results were expressed in percentage of relative cell viability.

2.5. Animals

Male ICR mice, 3 weeks old, weighting between 20 and 30 g were purchased from Charles River laboratories (Tokyo, Japan). Mice were housed individually and had access to food and water *ad libitum*, in a controlled environment (56% humidity, 23°C temperature, 12/12 h light/dark cycle). Before starting the oral administration and the tail suspension test, the mice were allowed to acclimatize for a week. All experiments were performed in strict accordance with NIH guidelines and were approved by the Animal Ethics Committee of the University of Tsukuba, Japan. The ethical approval code is 16-042.

2.6. Tail suspension test

The animals were divided into 3 groups. A negative control group receiving Milli-Q water (10 mL/kg; n = 6), a positive control group treated with 20 mg/kg of Bupropion (n = 7) and VEE-treated group (n = 7) which received a dose of 100 mg/kg. The samples were administrated orally everyday for 7 days.

The tail suspension test (TST) is a widely used technique to screen the antidepressant effects of drugs. The methodology was described in previous literature [49]. Briefly, the TST was performed 60 min after the administration of treatments. The duration of the test was 6 min and the immobility time was measured on the last 4 min of the test. A mouse was considered immobile only when it is hanged passively, showing no resistance to the stress applied by the test. The experiment was recorded using a camera and scored by observing the videos. After completion of the behavioral test, mice were sacrificed by cerebrospinal dislocation, then the whole brains were collected for the subsequent analysis.

2.7. DNA microarray analysis

The total RNA was extracted from the brain tissues previously collected using ISOGEN kit and quantified by Nanodrop 2000 spectrophotometer (Thermo Fisher scientific, USA).

To elucidate the molecular mechanism underlying the effect of VEE on neuronal activities, the total gene expression of brain tissues was evaluated by performing

microarray on RNAs previously extracted. The experiment was conducted according to the Affymetrix Genechip 3' IVT PLUS reagent kit user's guide. Briefly, the RNAs were reverse transcripted to generate double stranded DNA. The latter used as a template to synthesize the Biotin-labeled cRNA. After fragmentation of the labeled cRNA, the mixture was hybridized to the Affymetrix mouse 430 PM array strips (Affymetrix) for 16 h at 45°C in the hybridization station. In Geneatlas Fluidics station, the hybridized arrays were washed and stained, then scanned using Geneatlas imaging station. The total number of genes analyzed by this method is 39396 genes. All brain samples were analyzed by microarray. The data obtained were analyzed by Expression Console and Pathway Studio softwares and DAVID and Consensus Path databases.

2.8. Real time polymerase chain reaction (qRT-PCR) of Gsn, Ttr, Camk2n1, and Itpr2

RNA extracts obtained from mice brains were used as templates to validate the microarray results through evaluation of the expression level of some relevant genes regulated by Verbena treatment. First, a reverse transcription was performed, using the Superscript IV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) following the

manufacturer's protocol. Briefly, a mixture of RNA samples (0.2 µg/µl) and Oligo(dT)$_{12-18}$ /dNTP (0.5 µg/µL; 10 mM) was incubated for 5 min at 65°C, and then place 1 min on ice. The Reverse transcriptase solution was added and incubated the samples at 42°C for 60 min and then 10 min at 60°C. The cDNA produced is used to evaluate the expression of 3 genes: *Gelsolin "Gsn"* (Mm00456679_m1), *Transthyretin "Ttr"* (Mm00443267_m1),*Calcium/ calmodulin-dependent protein kinase II inhibitor 1 "Camk2n1"* (Mm01718432_s1) and *Inositol 1,4,5-trisphosphate receptor type 2 "Itpr2"* (Mm00444937_m1). This experiment was conducted using TaqMan Universal PCR mix and TaqMan Probes and the amplifications were performed in a 7500 Fast Real-time PCR (Applied Biosystems, USA) with the following conditions: 50°C for 2 min, followed by 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min.

2.9. Statistical analysis

Results are expressed as means ± SD, and statistical analyses were performed using a ANOVA 2 using IBM SPSS Statistics 23 software. Differences were determined statistically significant at a *P*-value of less than 0.05.

3. Results

3.1. Effect of VEE and its compounds on SH-SY5Y cells viability

MTT assay was used to assess the effect of VEE on cell viability. Cells were treated with different concentrations of the extract which are 0.5, 1, 2.5 and 5 µg/mL of VEE. As shown in Figure 1A, all VEE concentrations increased significantly the cell viability in a dose-dependent manner, with a higher value of 126.68 ± 7.81% at 2.5 µg/mL. The chemical analysis of various Verbenaceae plants, including *Lippia citriodora* and *Verbena officinalis*, showed a high abundance in Vs, also called acteoside, which is a phenylpropanoid glycoside [50–55]. In this study, cell viability of SH-SY5Y cells treated with 5, 50 and 100 µM of Vs, Hs and Vn was evaluated. The results in Figure 1C show an increase of viable cells in a dose-dependent manner attaining 134.8 ± 3.8% at 100 µM in case of Vs. Since MTT assay is a test based on mitochondrial activity, a cell viability higher than 100% can mean either induction of cell proliferation or over-activation of mitochondria. On the other hand, Hs and Vn decreased the cell viability significantly (Figure 1C). From these results, Vs was selected to be evaluated for its neuroprotective and energy metabolism effects.

Cells co-treated with Vs and Dex showed an enhancement of cell viability by more than 30% compared to Dex cells. These data indicate a neuroprotective effect exerted by Vs.

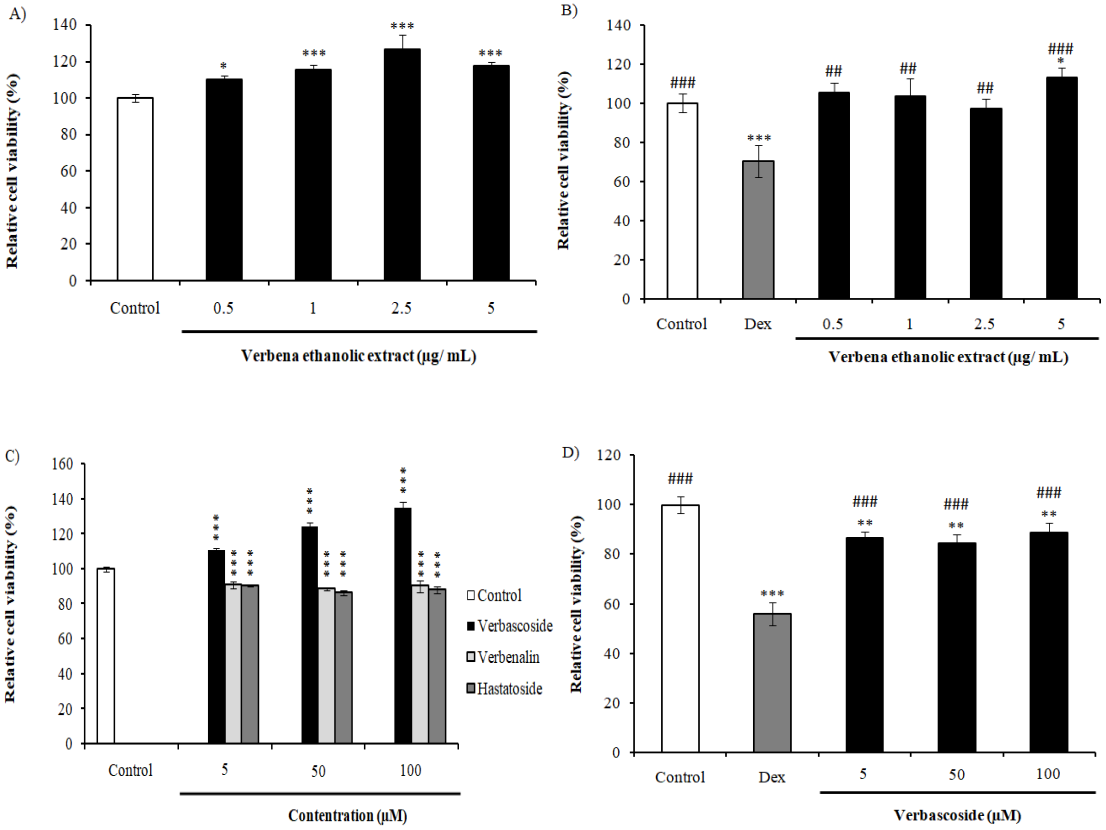

Figure 1. Relative cell viability of SH-SY5Y cells (A) treated with VEE at doses of 0.5, 1, 2.5, and 5 µg/mL, (B) co-treated with VEE and Dex (50 µM), (C) treated with Vs, Hs, and Vn (5, 50 and 100 µM) and (D) co-treated with Vs and Dex (50 µM). Results were expressed in mean of cell viability ± SD.* $P<0.05$; ** $P< 0.001$; *** $P<$ 0.0001 compared with negative control group. # $P<0.05$; ## $P< 0.001$; ### $P< 0.0001$ compared to Dex-treated group.

3.2. Effect of VEE on the immobility time of mice

The tail suspension test was used to assess the antidepressant-like effect of VEE 100 mg/kg compared to the control groups. Normally, drugs having an antidepressant effect decrease the immobility time of mice. In the present study, bupropion was used as positive control, known for its antidepressant property. Bupropion-treated mice showed a decrease of immobility time on the 4th day of TST to 39.37 ± 9.38 seconds (s) compared to the initial test performed on the 1st day with a value of 42.52 ± 25.94s, resulting of the drug's effect (Figure 2). As for the negative control group, the mice were fed with Milli-Q water and showed a gradual increase of immobility time to day 7 with 114.4 ± 22.5 s compared to the initial test with a time of 35.48 ± 26.94 s, proving an induction of depression on mice by TST (Figure 2).

Interestingly, 100 mg/kg body weight VEE-treated mice showed a highly significant increase of immobility time compared to negative and positive controls starting from the Day 4 of the test with 202.64 ± 19.13 s, which gradually decreased to attain 177.63 ± 40.77 s on the 7th day (Figure2). The low immobility time of the depressant mice receiving only water compared the VEE-treated mice suggested that the effect observed was not a result of the stress induced by TST.

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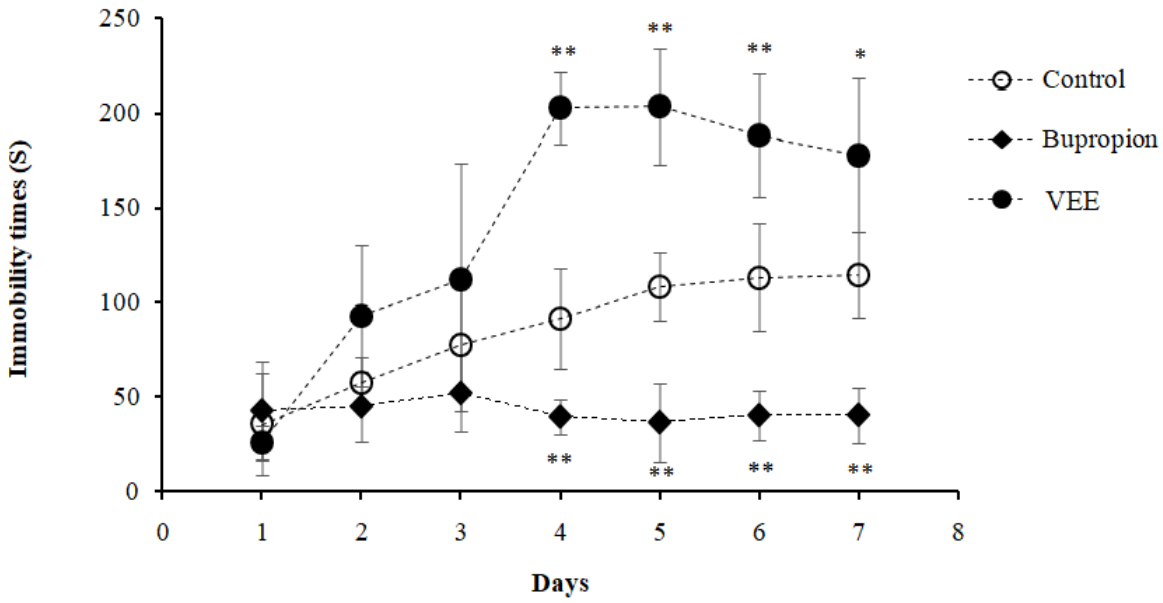


Figure 2. Effect of the oral administration of VEE (100 mg/kg) and bupropion (20 mg/kg) on mice immobility times in tail suspension test compared to the control (water 10 mL/kg, p.o.). Treatments were administered orally to mice and TST conducted 1 h after. The duration of the test was 6 min but immobility time was counted only during the last 4 min. Results were expressed in mean of immobility time ± SD.* $P<0.05$; **$P< 0.001$ compared with Control group.

3.3. Elucidation of the genes regulated by VEE treatment

To determine the molecular mechanism underlying the effect of VEE on immobility time, mice brains were analyzed using DNA microarray to detect the transcriptomic changes. The analysis of the data revealed the up-regulation of 62 genes with a fold-change higher than 1.2, while 256 others were down-regulated below 0.65 fold-change. After annotating the genes, they were clustered in order to study their interactions and the pathways they are implicated in. Bupropion and VEE affected interesting pathways controlling the neuronal proliferation, spatial learning and memory, long-term potentiation and depression, inflammation and reactive oxygen species (ROS) production (Table 1). Interestingly, VEE treatment regulated genes such as *Adenylate cyclase (Ac)* implicated in the production of cyclic-adenosine monophosphate (cAMP). It up-regulated the expression of genes implicated in calcium signaling including *Inositol 1,4,5-trisphosphate receptor type 2 (Itpr2), Protein kinase C (Pkc)* and *Calcium channel voltage-dependent L type alpha 1C subunit (Cacna1c)* [56,57].VEE treatment increased the expression of *Calcium/ calmodulin dependent protein kinase IV (CamkIV),* one of the genes stimulating mitochondrial biogenesis [58]. The expression of *cGMP-dependent*

protein kinase (Prkg1) was affected by verbena treatment, which results in the induction of muscle relaxation [59]. Also, *5 hydroxytryptamine (serotonin) receptor 4 (Htr4)* involved in neurotransmitters production was enhanced, alongside with *AdenosineA2a receptor (Adora2)*, responsible of the development of several neurodegenerative diseases [60–62]. VEE enhanced the expression of *Dopamine receptor D1 (Drd1),* implicated in activation of *Ac* [63].

As shown in the table. 1, out of the all set of genes three were highly expressed in case of VEE-treated mice, which are *Gelsolin (Gsn), Transthyretin (Ttr)* and *Calcium/ calmodulin-dependent protein kinase 2 inhibitor 1 (Camk2n1).* Their expressions were increased 5.26, 3.72 and 2.19 fold-change respectively. Recent studies showed a positive correlation between mitochondrial activity and expression of *Ttr* and *Gsn* [64,65]. As for the *Camk2n1*, it has been shown to possess a role in controlling cell proliferation [66].

VEE treatment decreased the expression of *melanin-concentrating hormone receptor 1 (Mchr1)* to a fold-change equal to 0.55, while bupropion did not affect its transcription level. The down-regulation of this gene was found to enhance the

metabolism [67], which implicates an activation of mitochondria. Also, *Mchr1* antagonist exerted an anti-depressant effect [68].

The *pro-melatonin-concentrating hormone (Pmch)* was drastically down-regulated (Table. 1). It has been shown previously to exert a role in energy metabolism [69].

Table 1. Genes regulated by VEE involved in induction of relaxation and the activation of energy metabolism. The ratios were calculated using the data of mice receiving water as reference

Gene ID	Gene name	Verbena ratio	Bupropion ratio	Function
Gsn	*Gelsolin*	5.26	1.54	Inhibition of amyloid beta peptides aggregation [64,70]
Ttr	*Transthyretin*	3.72	3.91	
Camk2n1	*Calcium/calmodulin-dependent protein kinase 2 inhibitor 1*	2.19	1.03	Tumor suppressor [66]
CaMK4	*calcium/calmodulin-dependent protein kinase IV*	1.46	1.20	Long-term memory [71]
Cacna1c	*Calcium channel, voltage-dependent, L type, alpha 1C subunit*	1.45	1.07	Cytosolic calcium content [57]
Pkc	*Protein kinase c*	1.45	0.98	*Adenylate cyclase* activation [63,72]
Drd1	*Dopamine receptor 1*	1.43	1.07	
Adora2	*Adenosine A2a receptor*	1.34	1.1	cAMP production [73]
Htr4	*5 hydroxytryptamine (serotonin) receptor 4*	1.34	1.25	Modulation of neurotransmitter release [60]
Itpr2	*Inositol 1,4,5-trisphosphate receptor type 2*	1.30	1.22	Intracellular calcium release [56]
Ac	*Adenylate cyclase*	1.28	0.85	cAMP production [74]
Prkg1	*cGMP-dependent protein kinase 1*	1.25	1.32	Induction of relaxation [59]
Mchr1	*melanin-concentrating hormone receptor*	0.55	1.01	Inhibition of cAMP accumulation [75]
Pmch	*pro-melanin-concentrating hormone*	0.12	0.12	Melanin-concentrating hormone activity [76]

3.4. Validation of expressions of Gsn, Ttr, Camk2n1 and Itpr2.

The microarray analysis of brains collected from mice treated with 100 mg/kg of VEE showed up-regulation of genes implicated in mitochondrial activity, with fold-changes higher than 2. These genes are Gsn*, Ttr* and *Camk2n1.* Their up-regulations were confirmed and represented in relative gene expression, with the negative control expression as reference. Expressions of *Gsn*, *Ttr* and *Camk2n1* were increased in case of VEE- treated mice by 305% (relative gene expression), 115% and 110%, respectively (Figure 3A, B and C). The *Camk2n1* is an inhibitor that alters the transportation of Ca^{2+}, responsible of the control of the intracellular amount of this ion to avoid its side effects.

Itpr2 is responsible of intracellular calcium release. This gene was up-regulated by VEE treatment. Its expression was confirmed and showed an enhancement of 160 % in VEE-treated mice compared to control group. Effect of bupropion was not significant compared to VEE, with an increase of 19% (Figure 3D).

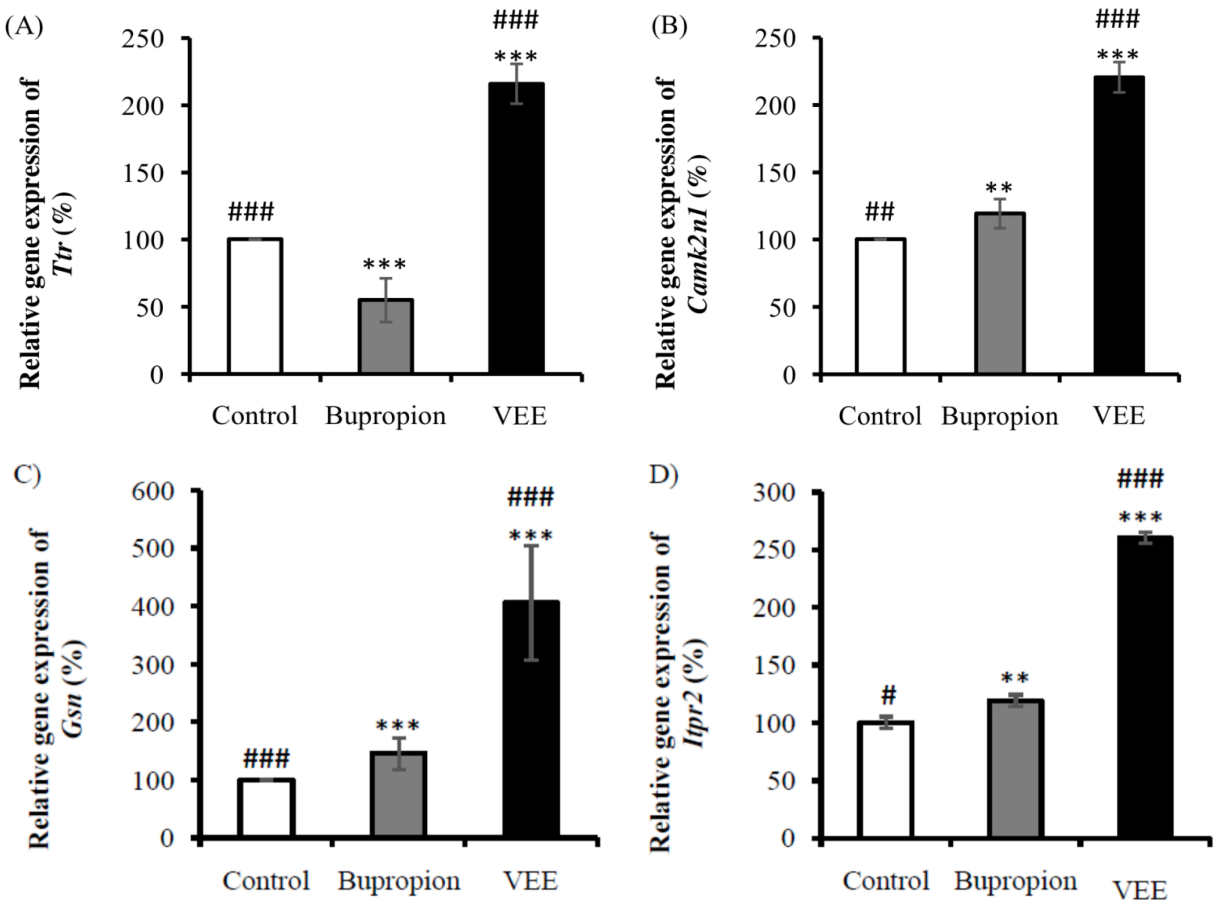


Figure 3. Validation of the expression of genes regulated by VEE treatment (100 mg/kg) which are (A) *Ttr*, (B) *Camk2n1*, (C) *Gsn,* and(D) *Itpr2*. Results were expressed in relative gene expression ± SD.* $P<0.05$; ** $P< 0.001$; *** $P< 0.0001$ compared with negative control group. # $P<0.05$; ## $P< 0.001$; ### $P< 0.0001$ compared to bupropion-treated group.

4. Discussion

Lemon verbena is a medicinal plant exerting important biological activities such as antidepressant, antioxidant, sleep-promoting, and analgesic effects [55,77–79]. The molecular mechanisms underlying these effects are still unknown.

The *in vitro* study showed an increase of cell viability of VEE-treated cells compared to the control, indicating an activation of cellular functionalities. Co-treatment of VEE and Dex significantly enhanced the cell viability compared to the negative control. To determine the compound responsible of the effect observed, cells were treated with the three most abundant compounds in the extract, Vs, Hs, and Vn. The viability was enhanced by Vs in comparison to the control, while Hs and Vn significantly decreased the viability. The effect observed in case of the extract is probably due to Vs. Also, Vs was tested for its neuroprotective effect and was found to alleviate Dex toxicity by more than 30%, suggesting that VEE and Vs have neuroprotective effect.

Furthrmore, the effect of VEE on mice at the molecular level was studied by analyzing the transcriptome using microarray. TST was used to induce psychological

stress in mice. The TST results showed increase of immobility time of VEE-treated mice compared to both control groups. In 2017, Razavi *et al.* reported the anti-anxiety and muscle relaxant effects of VEE and Vs *in vivo* by measuring the motor coordination and balance using rotarod test [47]. Another study showed, using traction test, induction of relaxation in mice and rats treated with essential oil extracted from the aerial part of verbena [78]. Accordingly, aqueous extract of this plant was found to have a sedative effect in rats at high doses (700 and 1000 mg/kg body weight of extract) [35]. Then, the increase of immobility time observed in this study may suggest the relaxant and sedative effects of VEE. Relaxation is defined as physiological and psychological response opposite to stress and/or flight-or-fight responses [80]. Flight is the action of running away of a danger as a way of survival. On the other hand, fight response is the ability of an animal to defend itself against a predator.

The evaluation of the transcriptome in the collected brains showed an enhancement of expression of genes implicated in the production of cAMP in case of mice treated with VEE. *Drd1* expression was increased by VEE, while it remained stable in case of bupropion-treated mice. Previously, the enzymatic activity of *Ac* was found to

be tightly regulated by *Drd1* through *Gβα* [63]. Also, VEE increased the expression of *Ac* in mice brains, while bupropion decreased it. Over-expression and activation of *Ac* by VEE implies an increase of cAMP generation, which has been associated to the induction of relaxation effect [81]. Accordingly, the use of apomorphine, a *Drd1* agonist, was proved to induce relaxation [82]. Moreover, treatment with the plant extract increased *Prkg1* expression, a gene that has been associated with induction of relaxation [59].

VEE affected the expression of genes modulating calcium homeostasis. *Itpr2* is one of the intracellular Ca^{2+} release channels, located in the membranes of endoplasmic and sarcoplasmic reticula. Endoplasmic and sarcoplasmic reticula are organelles rich in Ca^{2+} ion [56]. VEE up-regulated the expression of *Itpr2*, implicating an elevation of the ion in the cytosolic compartment. Ca^{2+}-cytosolic content depends also on channels facilitating the transport of ion from the extracellular compartment [80]. One of these channels is *Cacna1c* which was up-regulated in VEE-treated mice. A previous research evaluated the transcriptomic changes induced by relaxation in humans and found that *Cacna1c*level was increased [80]. The treatment of VEE increased Ca^{2+} levels and up-regulated the expression of *Pkc* which activates *Ac* inducing an over-production of

cAMP through Ca^{2+} signaling[72,83]. On the other hand, calcium homeostasis has been already proved to play an important role in muscle movement and walking behavior in humans. At the brain level, the calcium signaling regulates different functions, including signal transmission and the learning and memory [84–88]. When accumulated in cytoplasm, the calcium is transported into mitochondria, inducing the activation of enzymes implicated in generation of ATP, such as ATP synthase and $NADH^{+}$ dehydrogenase [89]. The inhibition of the calcium uptake by the mitochondria was found to increase the time needed for relaxation [90]. Accordingly, an increase of Ca^{2+} content has been proved to induce ATP production through cAMP generation [91]. These results suggest that VEE has a relaxant effect on mice through the generation of cAMP, which in addition to high intracellular Ca^{2+} level induce activation of mitochondria.

In VEE-treated mice, *Gsn*, *Ttr,* and *Camk2n1* showed the highest expression levels compared to the set of genes analyzed by microarray, and the increase was more than 2 fold-changes, while mice receiving Bupropion showed a decrease of *Ttr* expression, whereas *Gsn* and *Camk2n1* expressions were slightly increased (less than 1.5 fold-change). Mutant mice over-expressing *Gsn* revealed an enhancement of respiratory

chain activity [64]. Several studies have demonstrated the neuroprotective role of *Ttr* [92–96], and its positive correlation to mitochondrial function [65]. These findings proved an increase of mitochondrial activity, implying an over-production of ATP. VEE-treated group presented high level of *Camk2n1* expression compared to control group, which implicates a controlled cell proliferation. Previously, a study demonstrated the tumor suppressive effect of *Camk2n1* [66].

Pmch and *Mchr1* were significantly down-regulated by VEE. *Pmch*-deficient mice, as well as *Mchr1*-deficient mice, were found to be more active than wild type mice, and showing an increase in metabolic rate [67,69]. A specific *Mchr1* antagonist has showed antidepressant and anxiolytic effect [68]. The increase of immobility time of VEE-treated mice is due to the relaxant effect of the plant extract, and the molecular analysis proved its antidepressant effect.

Taking all these findings together, VEE administered to mice showed induction of relaxation through modulation of genes implicated in cAMP and Ca^{2+} production. VEE

and Vs showed a neuroprotective effect *in vitro* against Dex which suggests a protection of mitochondrial integrity. This effect could be related to *Gsn* and *Ttr* up-regulation.

Since in Chapter 2 a relaxant effect of VEE 100 mg/kg administered to mice, was observed, in Chapter 3 the effect of Vs *in* vivo was evaluated and elucidated the molecular mechanisms behind it.

Chapter 3:

Elucidation of the molecular mechanism underlying Vs-induced antidepressant-like effect

1. Introduction

In this Chapter the antidepressant-like effect of Vs administered to mice was observed. Depression markers were assessed and confirmed the results obtained *in vivo*.

Verbena, vervain or verbenaceae family is composed of 35 genera, with *Lippia* being one of the most important genera grouping 200 species presenting interesting biological activities [30,31]. *Lippia citriodora* K.*,* also referred to as *Aloysia triphylla* (L'Herit.), is commonly named lemon verbena, vervain or Louisa (Arabic). This plant has been used traditionally in Morocco as relaxant and sedative [38]. Also it enhances insomnia and restlessness in adults and babies [39]. Furthermore, it has been used for its anti-inflammatory, antioxidant, antispasmodic effects and also used as a remedy for gastrointestinal disorders [31]. Lemon verbena infusion was showed to have antioxidant and spasmolytic activities [33,34]. Also, the hypnotic effect of verbena aqueous extract has been proven [35]. The previous Chapter showed that VEE 100 mg/kg induced relaxation and regulated genes implicated in the process [97].

Verbascoside (Vs), a major phenypropanoid glycoside in lemon verbena, has been shown to exert antioxidant, anti-inflammatory and antimicrobial activities [40–43,45,98]. Furthermore, Vs has been reported to have a neuroprotective effect, *in vitro*, on 1-methyl-4-phenylpyridinum ion-induced toxicity using PC12 cells [46]. Interestingly, intraperitoneal administration of Vs and lemon verbena aqueous and ethanolic extracts to mice promoted their sleep and induced muscle relaxation, alongside with alleviation of anxiety [47]. In Chapter 2, Vs and VEE showed a neuroprotective effect on SH-SY5Y cells against Dex [97]. However, the antidepressant-like effect Vs and its mechanism of action has not been studied yet.

In the present study, the antidepressant-like effect of Vs in mice and its molecular mechanisms were studied. Interestingly, the transcriptomic analysis *in vivo* showed regulation of genes implicated in activation of the mitochondrial function through cAMP production and enhancement of calcium uptake. The intracellular calcium levels enhancement by Vs and VEE was confirmed *in vitro*. This effect was correlated with activation of mitochondria.

2. Materials and methods

2.1. Chemicals

Vs, Hs and Vn were purchased from Sigma Aldrich, USA. Dulbecco's Modified Eagle Medium (DMEM) / F-12 and Opti-MEM were obtained from Gibco, USA. Fetal bovine serum was from Gibco, South America. Penicillin - Streptomycin were purchased from Biowhittaker, USA. Non-essential amino acids were from Cosmo Bio Co, LTD, Japan. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Dex were from Dojindo, Japan. Bupropion was from Wako, Japan. ATP bioluminescence kit was from TOYO Ink, Japan. ISOGEN kit was purchased from Nippon Gene, Japan. RIPA lysis buffer was from (Santa Cruz Biotechnology, USA). 2-D Quant was purchased from GE Healthcare Life Sciences, USA. Calcium Kit II-Fluo 4 was from Dojindo, Japan.

2.2. Cell culture

The *in vitro* experiments were conducted on SH-SY5Y cells. This neurotypic cell line was obtained from America Type Culture Collection, Manassas, USA. Cells were

maintained in Dulbecco's Modified Eagle Medium (DMEM) / F-12, supplemented with 15% fetal bovine serum, 1% Penicillin (5000 μg/ml)- Streptomycin (5000 U/mL) and 1% of non-essential amino acids. The culture was incubated at 37°C in a humidified atmosphere of 5% CO_2 incubator. Opti-MEM, a reduced serum medium, was used to culture cells for the evaluation of cell viability and intracellular ATP.

2.3. Animals

Male ICR mice, 3 weeks old, weighting between 20 and 30 g were purchased from Charles River laboratories (Tokyo, Japan). Mice were housed individually and had access to food and water *ad libitum*, in a controlled environment (56% humidity, 23°C temperature, 12/12 h light/dark cycle). Before starting the oral administration and the tail suspension test, the mice were allowed to acclimatize for a week. All experiments were performed in strict accordance with NIH guidelines and were approved by the Animal Ethics Committee of the University of Tsukuba, Japan. the ethical approval code is 16-042.

2.4. Tail suspension test

A second TST was conducted as described previously to evaluate the effect of Vs 2.5 mg/kg on mice (n = 6), representing the content of this bioactive compound in 100 mg/ Kg of VEE used in first *in vivo* experiment. Another group was treated with Vs 5 mg/ Kg body weight (n = 7) on mice. HPLC analysis showed that VEE contains 2.5% of Vs (data obtained by Dr. Sasaki, AIST). Other control groups (Milli-Q, n = 6; Bupropion 20 mg/ mL, n = 7) were used for the second test. TST was performed according to the protocol previously described.

2.5. DNA microarray analysis.

The total RNA was extracted from the brain tissues previously collected using ISOGEN kit and quantified by Nanodrop 2000 spectrophotometer (Thermo Fisher scientific, USA).

To elucidate the molecular mechanism underlying the effect of Vs on neuronal activities, the total gene expression of brain tissues was evaluated by performing microarray on RNAs previously extracted. The experiment was conducted according to the Affymetrix Genechip 3' IVT PLUS reagent kit user's guide. Briefly, the RNAs were

reverse transcripted to generate double stranded DNA. The latter used as a template to synthesize the Biotin-labeled cRNA. After fragmentation of the labeled cRNA, the mixture was hybridized to the Affymetrix mouse 430 PM array strips (Affymetrix) for 16 h at 45°C in the hybridization station. In Geneatlas Fluidics station, the hybridized arrays were washed and stained, then scanned using Geneatlas imaging station. The total number of genes analyzed by this method is 39396 genes. All brain samples were analyzed by microarray. The data obtained were analyzed by Expression Console and Pathway Studio softwares and DAVID and Consensus Path databases.

2.6. Quantification of neurotransmitters and BDNF

To confirm the antidepressant effect Vs, the levels of serotonin (Sert), noradrenaline (NA), dopamine and BDNF in brains were quantified. The proteins were measured in frontal cortex. First, 100 mg of tissue was homogenized in 1 ml of RIPA buffer. The homogenate was centrifuged for 5 min at 10000 G and 4˚C. The supernatant was collected and stored at -80˚C. The dopamine, Sert and NA were quantified using ELISA kits (Immusmol SAS, France). BDNF was measured by colorimetric sandwich ELISA kit (Proteintech, USA). The experiments were conducted following the

manufacturer's instructions. The results of each treatment group were corrected by their respective total protein content determined using 2-D Quanti kit.

2.7. Measurement of mitochondrial activity

Mitochondrial function was measured using rhodamine 123, a fluorescent dye. The protocol was as described previously by Matsukawa et al., 2017 [99]. Briefly, treated SH-SY5Y were incubated for 20 min at 37˚C after addition of rhodamine 123 (10 µg/ml). Cells were lysed by 1% Triton X-100 and the fluorescence intensity of rhodamine (excitation/emission 485/528 nm) was measured.

2.8. Measurement of the intracellular ATP

The mitochondrial activity was assessed by measuring the intracellular ATP content of cells using ATP bioluminescence kit. Cells were cultured (2×10^5 cells/mL) in a fibronectin-coated 96-well plate and treated with different concentrations of VEE and Vs for 6, 12, 24, 48, and 72 h. The cells were lysed and the ATP content measured by adding 100 µL of luciferin-luciferase solution. The luminescence was measured using the microtiter plate reader (Dainippon Sumitomo Pharma Co., Ltd., Japan).

2.9. Measurement of intracellular calcium level

Calcium Kit II-Fluo 4 was used to measure intracellular calcium levels of SH-SY5Y. The measurement was conducted according to the manufacturer's protocol. Briefly, SH-SY5Y cells were seeded in black clear-bottom 96 well plates (Corning, NY,USA) and then treated with loading buffer (5% Pluronic F-127, 250-mmol/l Probenecid and 1-μ g/μ l Fluo 4 AM in Hanks'–HEPES Buffer) for 1 h. The supernatant was removed and cells were washed with PBS. Cells were treated with VEE and Vs as described previously. Fluorescence intensity (excitation/ emission 485/528 nm) was measured every 30 min using a Powerscan HT plate reader.

2.10. Statistical analysis.

Results are expressed as means ± SD, and statistical analyses were performed using a ANOVA 2 using IBM SPSS Statistics 23 software. Differences were determined statistically significant at a *P*-value of less than 0.05.

3. Results

3.1. Antidepressant effect of Vs

Control group showed higher immobility time compared to other treatments for 7 day of test (Figure 4A). The immobility recorded on the first day was 63.81 ± 21.88 s for the control, which increased to reach 84.33 ± 34.22 s on day 7. This increase proved induction of depression in mice. Bupropion treated mice scored an immobility time of 16.96 ± 3.34 s on the first day and decreased to 1.56 ± 0.94 s on the last day of the test, proving the antidepressant effect of bupropion. Results obtained on first day showed a significant difference between control group and Vs groups. On the second day, Vs treatments decreased the immobility time and the scores were statistically comparable to bupropion treated group, while the difference was highly significant compared to the control. Similar results were observed for the rest of the test.

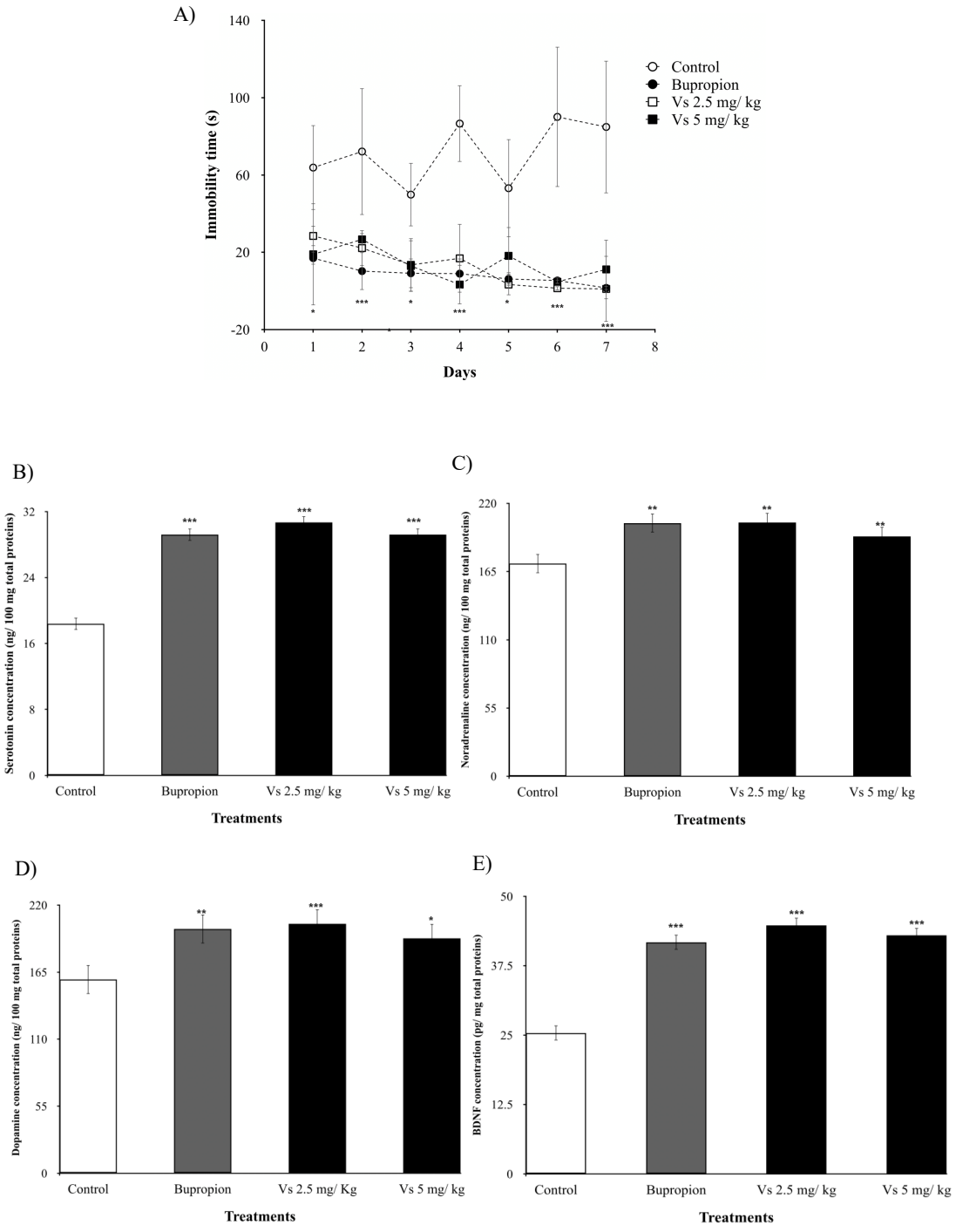


Figure 4. Effect of the oral administration of Vs (2.5 and 5 mg/kg) and bupropion (20 mg/kg) on (A) mice immobility times in tail suspension test compared to the control (water 10 mL/kg, p.o.) and their respective expression levels of (B) serotonin, (C) noradrenaline (D) dopamine and (E) BDNF. Results were expressed in mean of immobility time (s) and protein level ± SD.* P<0.05; **P< 0.001 and **P< 0.0001 compared with Control group.

For decades, depression has been associated with levels of monoamines and catecholamines in the system [100]. Depressive patients have been found to present Sert and NA (norepinephrine) deficiency [8,101]. To confirm the antidepressant effect of the treatments on mice, amounts of Sert and NA in mice brains were quantified. The results showed a low concentration of Sert and NA for control group with an amount of 18 and 171 ng/ 100 mg total proteins, respectively (Figure 4B and C). Bupropion increased significantly Sert level by 61% compared to control group. Similar effect was observed in case of mice treated with Vs 2.5 and 5 mg/kg showing improvement of 69.19 and 61.04% total proteins, respectively. An enhancement of 19% was observed in NA level in case of bupropion treated mice. Also, the treatments increased NA concentration with a higher rate of 19.35% for Vs 2.5 mg/kg treated group.

One of the important targets of antidepressants is the dopaminergic system. The effect of the treatments on dopamine levels in mice brains were evaluated. Bupropion showed an increase of dopamine content by 26% (Figure 4D). The lowest dopamine enhancement (21.21%) was obtained for mice treated with 5 mg /kg of Vs.

Furthermore, the concentrations of BDNF, one of the depression markers, were measured in brains. The findings showed an increase of BDNF levels in all treatments. Bupropion enhanced BDNF expression by 64.34% (Figure 4E). Interestingly, Vs at 2.5 and 5 mg/kg were found to exert more substantial effect regarding BDNF level with an enhancement of 76.36 and 69.26%, respectively.

3.2. Genes regulated in mice brains by Vs treatments

A transciptomic analysis of Vs treated mice was conducted in order to understand the mechanism behind its antidepressant-like effect. The analysis showed that Vs 2.5 mg/kg up-regulated 119 genes with a fold-change higher than 2 FC, while Vs 5 mg/kg enhanced the expression of 124 genes. Both treatments, Vs 2.5 and 5 mg/kg down-regulated the expression of 76 and 106 genes below 0.5 FC, respectively. Several of the highly regulated genes are implicated in interesting processes, such as cAMP production, learning and memory, neuron differentiation and depression (Table 2). The top up-regulated gene was *G protein-coupled receptor 6 (Gpr6)* with 7.44 and 9.07 FC in mice brains treated with Vs at 2.5 and 5 mg/kg, respectively. Over-expression of *Gpr6* has been found to increase intracellular cAMP level in neurons, inducing neurite

outgrowth and axonal regeneration [102]. Also, *Gpr6* has been shown to mediate the intra-cellular calcium levels and to activate *Ac* [103]. *Ac* was up-regulated to a higher extent in case of low dose of Vs (2.28 FC FC) compared to 100 mg/kg of VEE (1.28 FC), indicating a negative correlation between cAMP production and Vs concentration. Vs 2.5 and 5 mg/kg increased *Drd1* expression by 5.15 and 6.49 FC, respectively. Furthermore, Vs treatments increased the expression of *solute carrier family 35, member D3 (Slc35d3)*, involved in dopamine signaling [104]. *RASD family, member 2 (Rasd2)* is a gene implicated in dopamine signaling, associated to the onset of Schizophrenia-like behavior [105]. Interestingly, *LIM homeobox protein 8 (Lhx8)*,a key regulator of cholinergic pathway implicated in neurodegenerative diseases, was over-expressed by Vs treatments by over 4 FC [106].Vs enhanced the expression of *G-protein coupled receptor 88 (Gpr88)* implicated in motor behavior and learning aptitude [107]. *Protein phosphatase 1, regulatory (inhibitor) subunit 1B (Ppp1r1b)*, a gene marker associated to neuronal loss, was highly up-regulated by Vs treatments [108]. Vs increased the expression of *Adora2a* higher than VEE 100 mg/kg.

On the other hand, Vs highly down-regulated *pro-opiomelanocortin-alpha* which has been found to be over-expressed in patients suffering of sleep syndrome apnea [109]. Also, Vs decreased the expression of *Pmch*. *Glyoxalase (Glo1)*is a gene implicated in the physiopathology of depression, and Vs down-regulated its expression in mice brains [110]. Expression of *indoleamine 2,3-dioxygenase 1(Ido1)*has been associated to depression and its development, which was down-regulated by Vs [111].

Table 2. Genes regulated by Vs at 2.5 and 5 mg/kg involved in the antidepressant-like effect. The FC were calculated using the data of control water mice as reference.

Gene ID	Gene name	Vs 2.5 mg/kg	Vs5 mg/kg	Bupropion	Function
Gpr6	*G protein-coupled receptor 6*	7.44	9.07	0.98	cAMP production and
Ppp1r1b	*Protein phosphatase 1, regulatory (inhibitor)*	6.68	8.03	1.26	Neurogenesis [108]
Gpr88	*G-protein coupled receptor 88*	5.85	6.82	0.99	Neuropsychiatric disorders
Drd1	*Dopamine receptor D1*	5.15	6.49	0.92	*Ac* activation [63,72]
Slc35d3	*Solute carrier family 35, member D3*	4.33	5.87	1.21	Dopamine signaling [104,105]
Rasd2	*RASD family, member 2*	5	5.5	2.14	
Lhx8	*LIM homeobox protein 8*	4.21	4.85	2.47	Acetylcholine production[106]
Adora2	*Adenosine A2a receptor*	3.05	2.59	1.28	cAMP production [73]
Ac	*Adenylate cyclase*	2.28	2.22	1.09	Production of cAMP [74]
Itpr2	*Inositol 1,4,5-trisphosphate receptor type 2*	1.18	1.15	1.25	Intracellular calcium release
Inpp4a	*Inositol polyphosphate-4-phosphatase, type I*	1.82	1.37	1.40	Neuron anti-apoptosis [112]
Cacna2d2	*calcium channel, voltage-dependent, alpha*	1.14	1.17	1.05	Epilepsy [113]
Fbxo32	*F-box protein 32*	0.59	0.67	0.87	Muscle atrophy [114]
Trp53	*Transformation-related protein 53*	0.79	0.71	0.80	Apoptosis [115,116]
Siah1a	*seven in absentia 1A*	0.93	0.91	1.04	
Cdkn1a	*cyclin-dependent kinase inhibitor 1A (P21)*	0.45	0.49	0.62	Cell cycle arrest [117]
Glo1	*Glyoxalase 1*	0.27	0.24	0.47	GABAergic signaling [110]
Pde10a	*Phosphodiesterase 10A*	0.27	0.23	0.84	cAMP signaling [118]
Ido1	*Indoleamine 2,3-dioxygenase 1*	0.21	0.18	0.75	Tryptophan metabolism [111]
Slc6a3	*solute carrier family 6 (neurotransmitter*	0.6	0.1	1.02	Mood changes [119–121]
Pomc	*pro-opiomelanocortin-α*	0.51	0.01	1.04	Depression [109]

3.3. Evaluation of the mitochondrial activity of cells treated with VEE and Vs

In order to measure the mitochondrial activity, the rhodamine 123 assay was used to mark the active mitochondria specifically. Both VEE and Vs induced mitochondrial activation of SH-SY5Y cells in a dose-dependent manner, with higher effect at lower concentrations. VEE at 0.5 μg/ml increased mitochondrial activity by 17% and its effect decreased to reach 9.37% for cells treated by 5 μg/ml (Figure 5A). Mitochondrial activity of cells treated with 5 μM of Vs was 115% compared to control, while the higher concentration enhanced the function only by 3% (Figure 5B). These results implicated a stimulation of energy production of VEE and Vs treatments.

The same concentrations of VEE and Vs were evaluated for their effect on energy generation by quantifying ATP level. As the Figure 5C shows, VEE treatments were not effective on energy metabolism at 6 h, but they show a highly significant increase after 12h, with a maximum of 129.71 ± 2.73%. The ATP content decreased in a time and dose-dependent manner to reach energy homeostasis after 72 h. Treating the cells with Vs increased ATP production significantly after 12 h (Figure 5D), which decreased gradually to attain the normal status at 72h. These results proved the stimulation mitochondria by VEE and Vs.

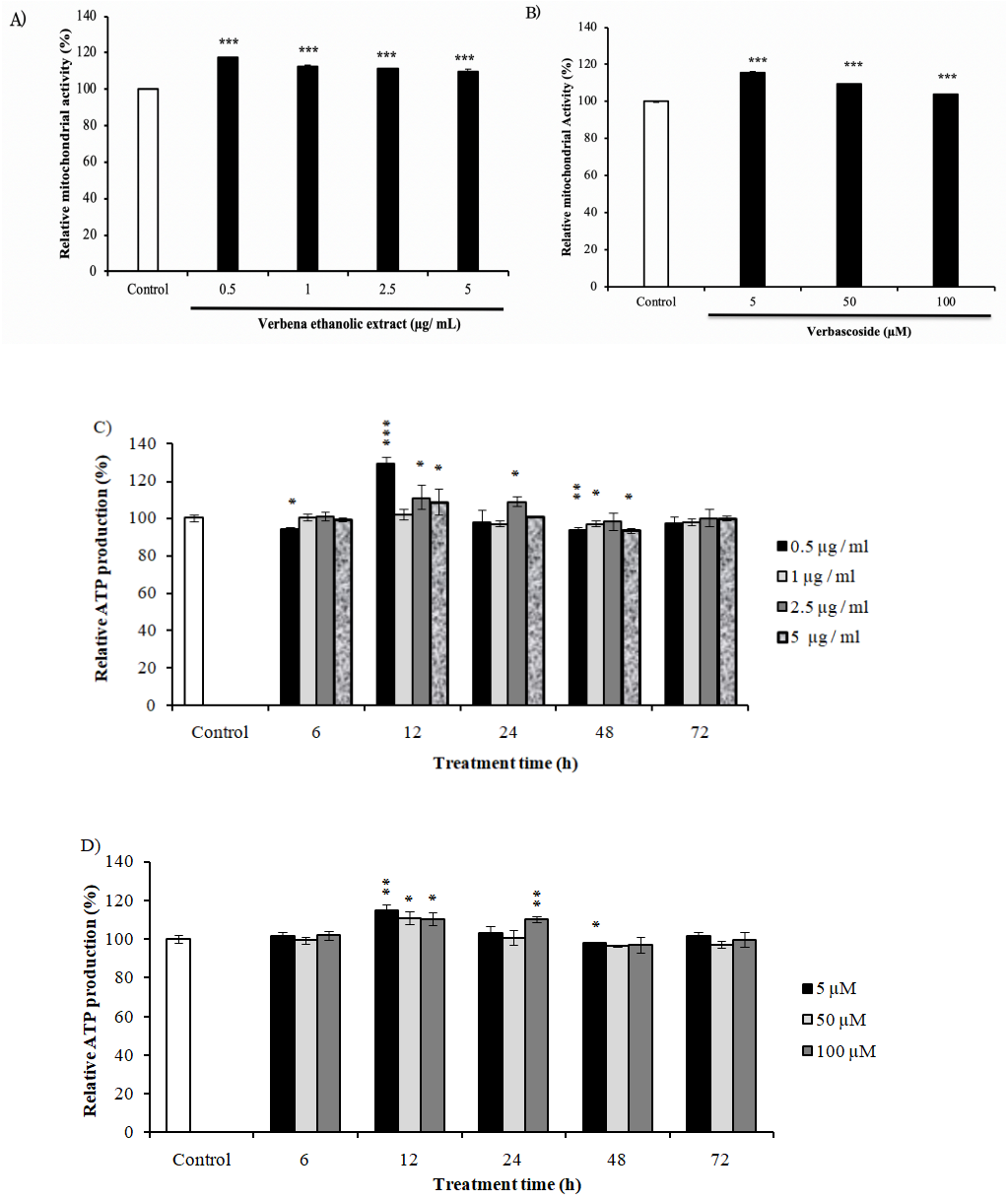


Figure 5. Evaluation of mitochondrial activity of SH-SY5Y cells treated with different concentrations of (A) VEE and (B) Vs. The intracellular ATP production of was assessed *in vitro* using the same concentrations of (C) VEE and (D) Vs at 6, 12, 24, 48, and 72 h. Results were expressed in mean of relative mitochondrial activity or ATP production (%) ± SD.* $P<0.05$; ** $P< 0.001$; *** $P< 0.0001$ compared with control cells treated with Opti-MEM.

3.4. Effect of VEE and Vs on intracellular calcium levels

Studies have shown a correlation between intracellular calcium uptake and mitochondrial activation. Transcriptomic analysis showed regulation of genes involved in Ca^{2+} in case of mice treated with VEE. Here, the effects of VEE and Vs on Ca^{2+} levels on SH-SY5Y were evaluated. VEE increased Ca^{2+} uptake after 30 min of treatment in concentration and time-dependent manner, with higher effect at lower concentrations (Figure 6 A). Accordingly, Vs showed similar effect on Ca^{2+} with higher activity at lower doses (Figure 6 B). These results proved the implication of Ca^{2+} in the observed activities, with Vs being responsible of VEE effects.

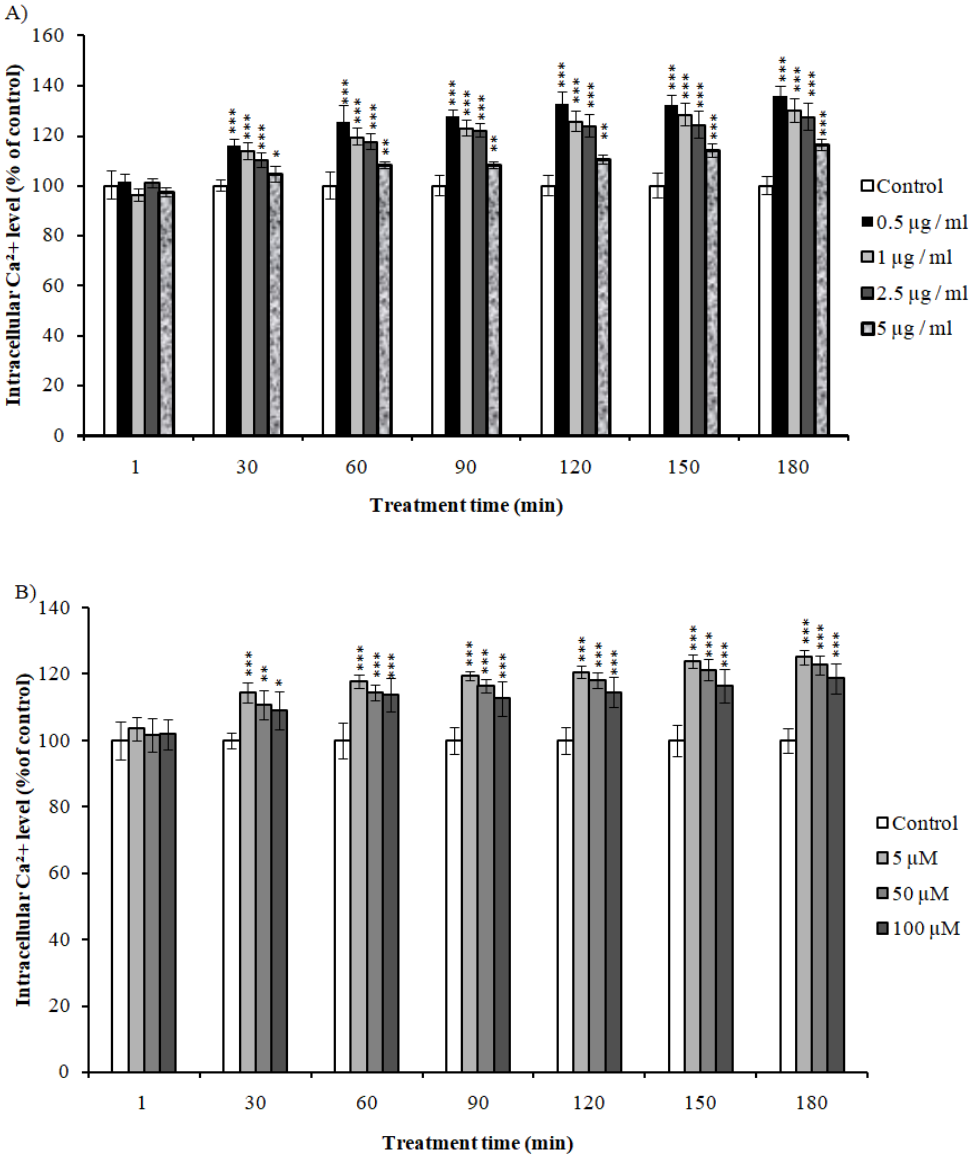


Figure 6. Evaluation of intracellular calcium levels of SH-Sy5Y cells treated with different concentrations of (A) VEE and (B) Vs for 1-180 min. Results were expressed as percentage of control cells treated with Opti-MEM ± SD.* $P<0.05$; ** $P< 0.001$; *** $P< 0.0001$ compared with control cells.

4. Discussion

In order to evaluate the effect of Vs content in VEE, a second TST was conducted. The treatments used were 2.5 and 5 mg/kg of Vs. Interestingly, the results showed a decrease in immobility time compared to control group, and scores were statistically comparable to bupropion treated mice. These findings suggest that Vs at both doses have antidepressant-like effect. In accordance with the transcriptomic analysis conducted here above, Vs might induce mitochondrial activation through accumulation of cAMP and Ca^{2+}, to a lesser extent than VEE 100 mg/kg, resulting in agitation of mice rather than their relaxation. To prove the antidepressant-like effect observed *in vivo*, the levels of different depression markers were measured. Sert and NA implication in depression has been documented and are considered as targets of antidepressants [8,101]. In this study, Vs was found to enhance Sert and NA levels demonstrating an antidepressant-like effect of the treatments on mice.

Previous studies found that antidepressants targeting the expressions of Sert and NA only present limitations. Patients might show movement retardation, lack of

concentration or even persistence of anhedonia [122]. Accordingly, drugs acting on dopaminergic system have been developed. Dopamine is a catecholamine responsible of expression of emotions such as pleasure and motivation, and stimulates concentration [122]. Hence, dopamine levels in brains were assessed. The results showed a highly significant increase of dopamine expression by Vs compared to control group. These findings prove the antidepressant-like activity of Vs by stimulating dopamine signaling pathway.

It has been documented that antidepressants acting on serotonergic and norepinephric mechanisms lead to enhancement of BDNF levels in rodents [123,124]. Effect of Vs treatments on BDNF in brains were evaluated. The results showed a highly significant increase of BDNF by Vs treatments. Also, it has been documented that Ca^{2+} and cAMP levels regulate BDNF expression through CREB (cAMP response element-binding protein) [125].

In order to understand the difference of responses depending on the dose administered, the transciptome of mice brains treated with Vs was analayzed.

Interestingly, the analysis showed highly regulated set of genes implicated in cAMP production and dopamine signaling. For instance, *Gpr6* was up-regulated by more than 7 and 9 FC in case of 2.5 and 5 mg/kg treatments, respectively. Previous study has shown that over-expression of *Gpr6* induced increase in intracellular cAMP through activation of *Ac*, leading to stimulation of neurite outgrowth and axonal regeneration [102,103]. *Drd1* and *Adora2a* expressions, genes responsible of cAMP production, were higher in Vs-treated mice brains compared to 100 mg/kg VEE treatments, suggesting a more significant effect of the bioactive compound [63,73]. Hence, Vs increased the expression of *Ac* expression proving the activation of cAMP signaling pathway. Levels of cAMP have been found to be decreased in patient suffering of depression, which have been elevated by antidepressant treatments [126]. These findings prove that Vs-induced antidepressant-like effect is regulated by cAMP production. Moreover, up-regulation of *Lhx8* was found to promote neural stem cells through stimulation of acetylcholine production [127–129]. Vs increased *Lhx8* expression suggesting an overproduction of acetylcholine inducing neurogenesis, a biological process that has been correlated with antidepressant-like and anxiety-like effects of drugs [130]. Accordingly, *Ppp1r1b* is a

gene involved in neurogenesis was up regulated by Vs treatment [108]. Previous works have shown that *Glo1* and *Ido1* are over-expressed in case of depression, which the inhibition alleviated the disease burden [110,111]. This analysis demonstrated down-regulation of *Glo1* and *Ido1* proving the antidepressant-like effect of Vs. Taken these results together, Vs showed an induction of antidepressant-like effect through stimulation of cAMP production, resulting in increase of BDNF level. Calcium signaling enhancement was less significant than VEE. A ration between cAMP and calcium content might be the key behind the activity difference.

The results obtained *in vivo* revealed the activation of mechanisms responsible of the increase of cytosolic Ca^{2+} and cAMP generation, messengers inducing the mitochondrial activity. To confirm this hypothesis, the effect of VEE and Vs on mitochondrial activity was studied. The results showed enhancement of mitochondrial function in a concentration-dependent manner. Accordingly, Vs increased mitochondrial function in a similar tendency as VEE. ATP production *in vitro* was evaluated to confirm the effect of VEE and Vs mitochondrial activity. Human neurotypic SH-SY5Y cells treated with VEE showed a significant increase of ATP content in a dose-dependent

manner after12h treatment. Energy metabolism gradually decreased to regain the initial state. Vs is one of the most important compounds contained in VEE, and has been proven to induce muscle relaxation in mice [47]. Next, the effect of Vs on mitochondrial activity was evaluated. Vs-treatment showed an increase in ATP production at 12h, which restored to its original condition progressively. In 2013, Bhasin *et al.* evaluated the transcriptomic changes in humans in response to relaxation condition and showed regulation of genes activating energy metabolism [80]. ATP increase has been found to be regulated positively by activation of mitochondrial calcium uptake, as result of different stimuli such as alimentation, hormones and neurotransmitters [89,131–134]. The *in vitro* study showed that VEE and Vs enhanced intracellular calcium levels in a concentration and time-dependent manner with similar tendency as mitochondrial activation. These results proved the increase of calcium and energy metabolism related genes regulated by the treatments *in vivo*.

Taken together, these findings suggested that Vs induce antidepressant-like effect, related to the levels of Ca^{2+} and cAMP generated by treatment. These messengers

are transported to mitochondria and activate energy metabolism producing more ATP for cell survival. The effect of VEE observed on ATP production is due mostly to Vs.

In this Chapter, Vs was found to induce antidepressant-like effect *in vivo*, which might be considered as a potential treatment for depression. For this reason, in Chapter 4 a stable emulsion was produced allowing protection of the bioactive compound from degradation and potentially enhance Vs bioavailability. Also, the antidepressant-like effect of VEE-emulsion was evaluated in mice.

Chapter 4:

Formulation of stable VEE-based emulsion and its depressant-like effect

1. Introduction

In Chapter 4, stable VEE-emulsion protecting Vs from degradation was produced and determined its antidepressant-like effect on mice.

Emulsion is a term used, in pharmaceutical field, to refer to preparations to be ingested (oral administration). The mixtures destined to be applied on skin are called cream [135]. In general, an emulsion is a biphasic system consisting of a uniform spreading of droplets of the dispersed phase in a continuous phase [136]. The stability of this system is limited in time because it is thermodynamically unstable, which results in reverting to the immiscibility state due to different physicochemical factors like the gravitational and phase separations, flocculation, coalescence and particle coalescence [136]. Addition of an emulsifier agent to the system stabilizes the emulsion by forming a layer around the droplets of the dispersed face, preventing their break down [137,138].

There are two types of emulsions, oil in water (O/W) and water in oil (W/O). In O/W emulsion, the oil is the dispersed phase in the aqueous phase, and usually used for oral administration of target molecules. While the W/O is more used for cosmetic because

of their viscosity and consists of dispersion of an aqueous solution in a hydrophobic continuous phase [136–138]. The choice of the emulsion type depends on the polarity of the molecule to be delivered. Emulsion's main attribute is to insure a higher bioavailability of bioactive compounds by reducing their degradation in the gastrointestinal system [139].

A previous study showed that Vs is completely degraded at pH 7 after 7 days of storage, showing a high interest to encapsulate this molecule for a cosmetic use [42]. Also, they performed a preliminary test to evaluate its stability in O/W system under accelerated degradation condition (40°C) after 150 days. The degradation was significantly decreased.

Since in the present work the treatment was destined to be administrated orally, the type of emulsification used was O/W emulsion. The objective of this study was to produce stable VEE–rich emulsions. Also, the antidepressant-like effect of VEE-based emulsion was assessed *in vivo*.

2. Materials and methods

2.1. Extract preparation of Lippia citriodora (Lim.)

The leaves of *Lippia citriodora* were collected in July 2016 from Marrakech Region (Morocco). The species was authenticated by Prof. Ahmed Ouhammou from Cadi Ayyad University, Faculty of Sciences Semlalia, Department of Biology, Marrakech, Morocco. A voucher specimen of plant material (MARK-11186) was deposited in the Herbarium of o the same institution. After air drying, the plant material was crushed by a mortar and extracted with ethanol 70%, with a ratio plant material/ solvent of 5% (w/v). The extraction was carried out in the dark for 1 week under continuous agitation. The extract was filtered using Whatman paper (11 µm) under vacuum. The filtrate was filtered further through 0.22 µm Millipore (Mark Millipore, Ireland) and solvent evaporated by a rotary evaporator. The extract was dissolved in water, kept overnight at – 20˚C and then freeze-dried for 4 days. The yield of VEE was 17.6%.

2.2. Interfacial tension measurement

Prior to production of emulsion, the emulsifying potential of VEE and Vs was

determined by measuring their effect on the interfacial tension at the interface between the oil and water.

An automatic tensiometer (PD-W, Kyowa Interface Science Co., Ltd., Saitama, Japan) was used to evaluate the parameter by the pendant method. Briefly, a syringe loaded with different concentrations of VEE (0.0025, 0.125, 0.25, 0.5, 1, and 2% w/w) and Vs (0.025 and 0.05% w/w). The needle was placed inside a glass cell containing the oil (Oleic acid or soybean oil). Drops were made in it until they reached their maximum volumes. Images of the drops were taken by a high-resolution camera, then the software estimated the interfacial tension according to the shape and size of the drop, and the density between the solution and the oil.

2.3. Emulsification

Emulsions were obtained by mixing aqueous solution of VEE at 1% (w/w water) with the oil phase at 5 % of total preparation. The oil phase consisted either of soybean oil or oleic acid containing 1% (w/w) of lecithin. To evaluate the effect of the minerals contained in VEE on emulsion stability, EDTA (chelator) was added to the preparations at

different concentrations (0.001, 0.005, and 0.01 mg/mL).

The biphasic structure was ruptured using a rotor-stator homogenizer (Polytron, PT-3000 Kinematica-AG, Littace, Switzerland) at 10000 rotations/min for 5 min. Afterward, the homogenate was passed through a high-pressure homogenizer (NanoVater, NV200,Yoshida Kikai, Nagoya, Japan) at 100 MPa for 8 cycles.

2.4. Emulsion stability

The stability of the emulsion was evaluated under different storage conditions (5 and 25°C). The stability was determined by measuring the particle size relative to droplets. A laser diffraction particle size analyzer was used in this experiment (LS 13,320, Beckman Coulter, Brea, USA).

2.5. Animals

Male ICR mice, 3 weeks old, weighting between 20 and 30 g were purchased from Charles River laboratories (Tokyo, Japan). Mice were housed individually and had access to food and water *ad libitum*, in a controlled environment (56% humidity, 23°C

temperature, 12/12 h light/dark cycle). Before starting the oral administration and the tail suspension test, the mice were allowed to acclimatize for a week. All experiments were performed in strict accordance with NIH guidelines and were approved by the Animal Ethics Committee of the University of Tsukuba, Japan.

2.6. Tail suspension test

The animals were divided into 3 groups. A negative control group receiving Milli-Q water (10 mL/kg), a positive control group treated with 20 mg/kg of Bupropion and mice treated with VEE, at a dose of 10 mg/kg, in emulsion. The samples were administrated orally everyday for 7 days. The Tail Suspension Test was conducted as explained in Chapter 2, section 2.6.

2.7. Quantification of dopamine and noradrenaline

To confirm the antidepressant-like effect of VEE-emulsion, the levels of noradrenalin (NA) and dopamine were quantified. The proteins were measured in frontal cortex. First 100 mg of tissue was homogenized in 1 ml of RIPA buffer. The homogenate was centrifuged for 5 min at 10000x g and 4˚C. The supernatant was collected and stored

at -80˚C. The dopamine and NA were quantified using ELISA kits (Immusmol SAS, France). The experiments were conducted following the manufacturer's instructions. The results of each treatment group were corrected by their respective total protein content determined using 2-D Quanti kit.

2.8. Real time polymerase chain reaction (qRT-PCR) of Gsn, Ttr, Camk2n1, and Itpr2

RNA extracts obtained from mice brains were used as templates to validate the microarray results through evaluation of the expression level of some relevant genes regulated by VEE-emulsion treatment. First, a reverse transcription was performed, using the Superscript IV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, a mixture of RNA samples (0.2 µg/µl) and Oligo(dT)$_{12-18}$ /dNTP (0.5 µg/µL; 10 mM) was incubated for 5 min at 65°C, and then place 1 min on ice. The Reverse transcriptase solution was added and incubated the samples at 42°C for 60 min and then 10 min at 60°C. The cDNA produced is used to evaluate the expression of 3 genes: *Gelsolin "Gsn"* (Mm00456679_m1), *Transthyretin "Ttr"* (Mm00443267_m1), *Calcium/ calmodulin-dependent protein kinase II inhibitor 1 "Camk2n1"* (Mm01718432_s1) and *Inositol 1,4,5-trisphosphate receptor type 2 "Itpr2"*

(Mm00444937_m1). This experiment was conducted using TaqMan Universal PCR mix and TaqMan Probes and the amplifications were performed in a 7500 Fast Real-time PCR (Applied Biosystems, USA) with the following conditions: 50°C for 2 min, followed by 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min.

3. Results

3.1. Effect of VEE on interfacial tension

Interfacial tension is a parameter used to determine the emulsifying potential of a solution or a compound. The lower the interfacial tension is the greater the emulsifying potential. The interfacial tension of VEE was measured at different concentrations. The values decreased in a concentration–dependent manner, from 24.22 to 6.74 mN/m at concentration of 0.0025 and 2% of VEE (w/w), respectively (Figure 7A). These results suggest that VEE has a significant emulsifying potential.

Furthermore, the interfacial tension of Vs was measured to check if the later effect is related to its activity. The concentrations used are 250 and 500 µg/mL and their interfacial tension were 20.15 and 18.86 mN/m (Figure 7B). These results suggest that Vs is surface active.

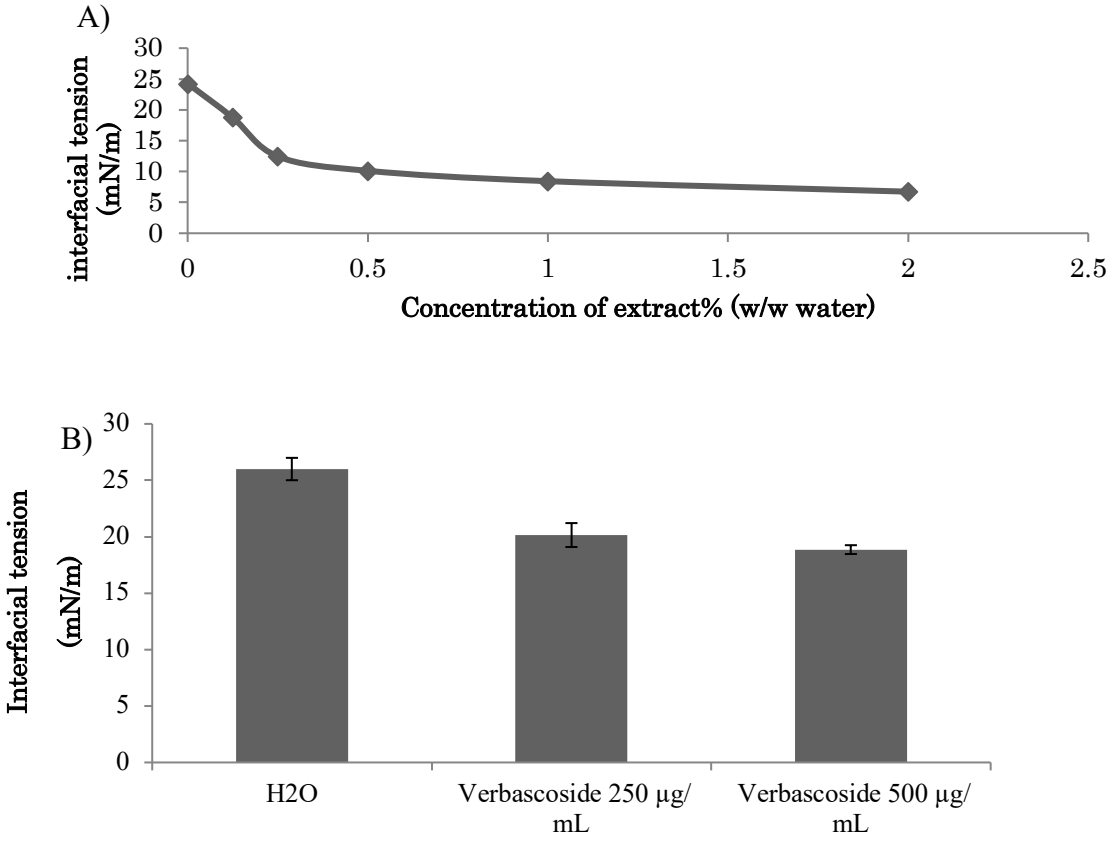


Figure 7. Measurement of interfacial tension of (A) VEE and (B) Vs. Results were expressed in mean of interfacial tension ± SD.

3.2. Emulsion stability

Emulsion prepared of VEE and soybean oil showed a droplet size was 0.198 μm on day 0. The droplets increased in size significantly to reach 7.43 μm, resulting of destabilization of the emulsion (Figure 8). One week later, the preparation started to show a separation of oil and water phases, called oiling-off.

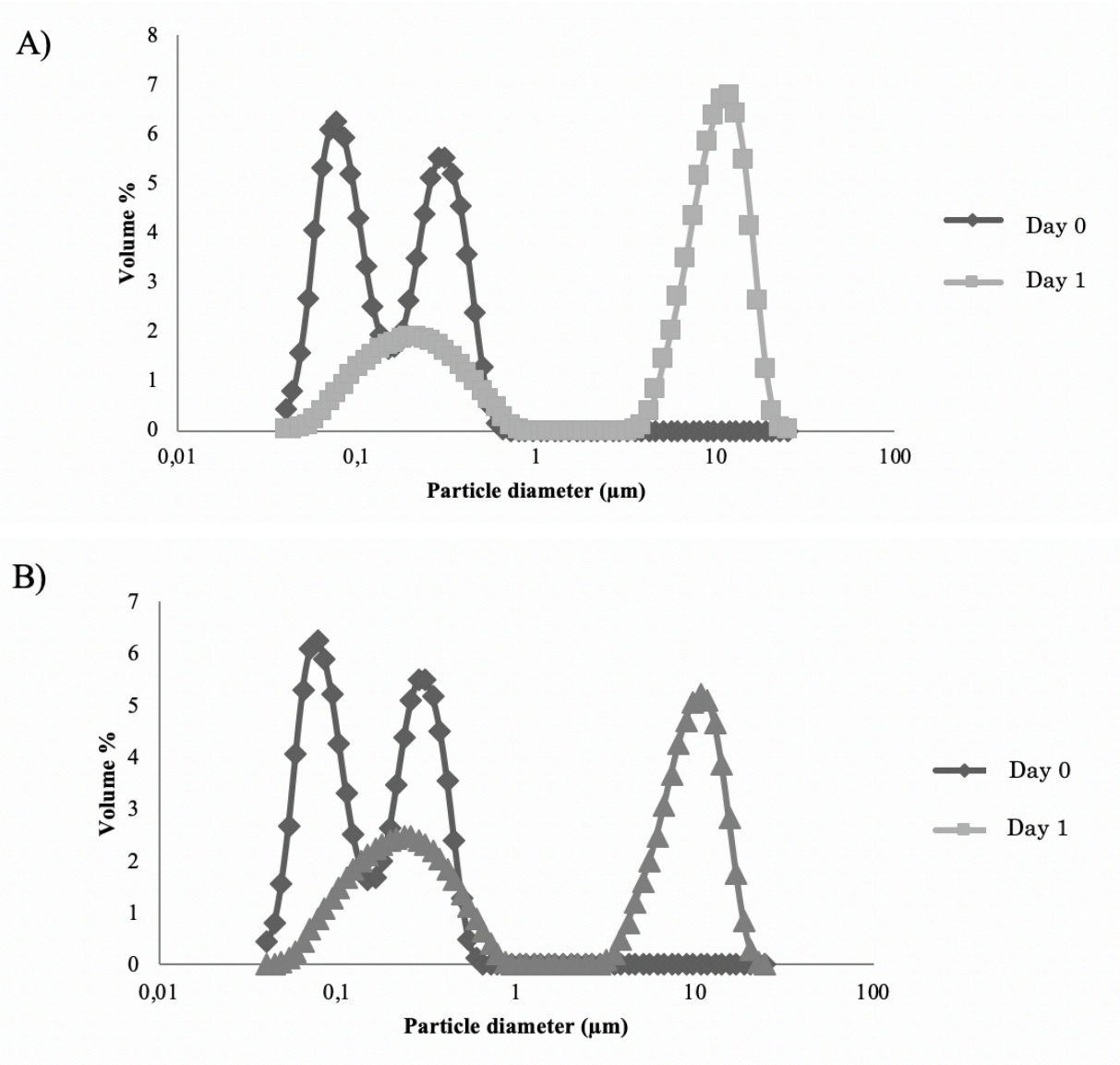


Figure 8. Measurement of droplet size of emulsion after incubation of 1 day stored at (A) 5°C and (B) 25°C. Results were expressed in mean of volume % ± SD.

The instability could be caused by the presence of minerals in VEE. To evaluate the effect of minerals on emulsion, EDTA was used at different concentrations. EDTA is a chelator that binds to ions. Droplet size was 0.440 μm at day 0 in presence of 0.001 mg/mL of EDTA, which increased to 6.79 μm after 7 days of incubation at 5°C (Figure 9A). Other EDTA concentrations showed similar results (Figure 9B and C). These findings imply that minerals contained in VEE did not affect emulsion stability.

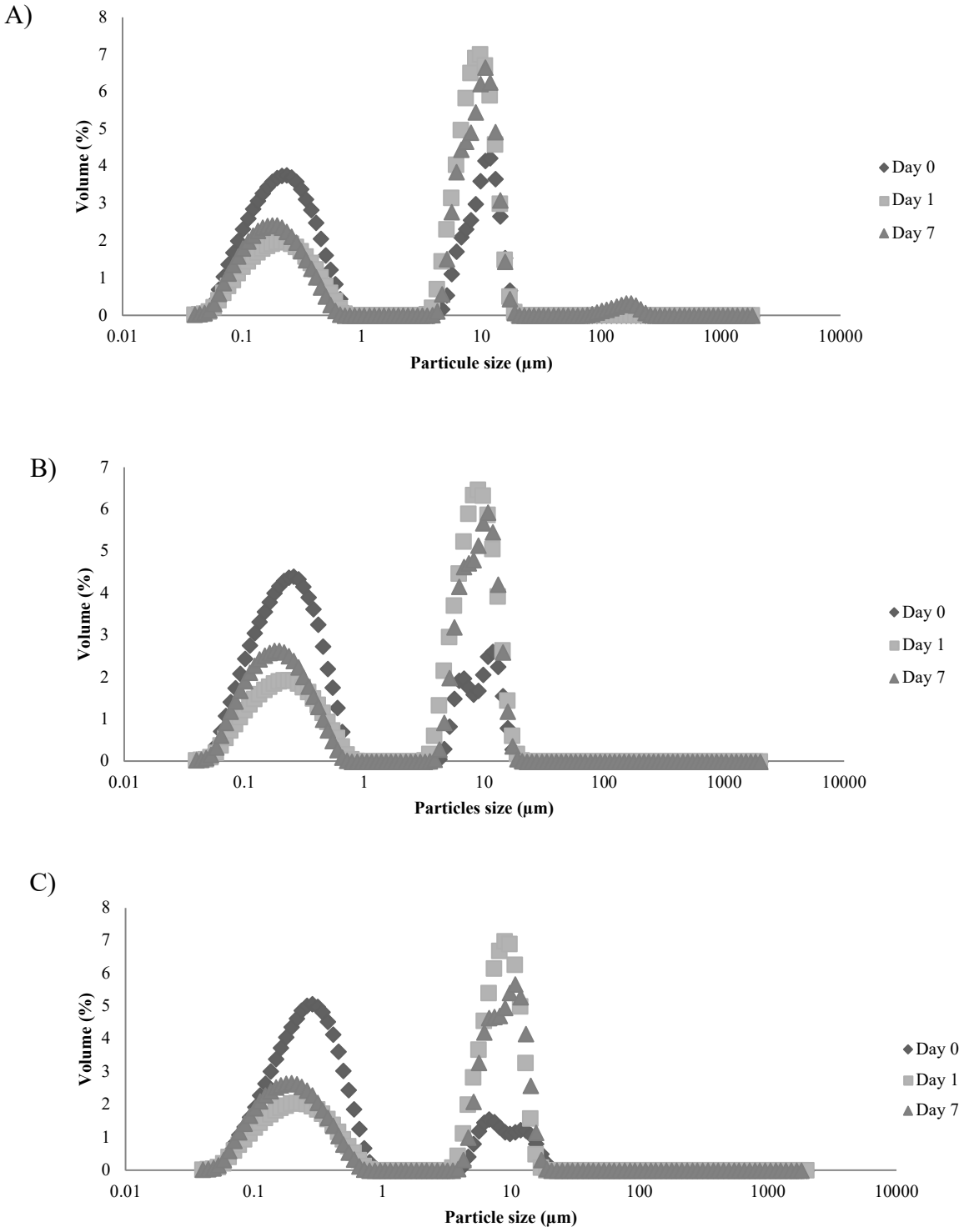


Figure 9. Measurement of droplet size of emulsion after incubation of 0, 1, and 7 days containing different concentrations of EDTA. (A) 0.001 mg/ mL, (B) 0.005 mg/ mL and (C) 0.01mg/ mL. Results were expressed in mean of volume % ± SD.

In order to increase the emulsion stability at storage conditions, the oleic acid was used as oil phase instead of soybean oil. The oleic acid contained 1% of lecithin, a natural emulsifier. The emulsion showed high stability even after 15 days of storage at 5°C, and had a droplet size of 0.440 μm (Figure 10). These results suggest that oleic acid-lecithin 1% confers more stability to VEE based emulsion.

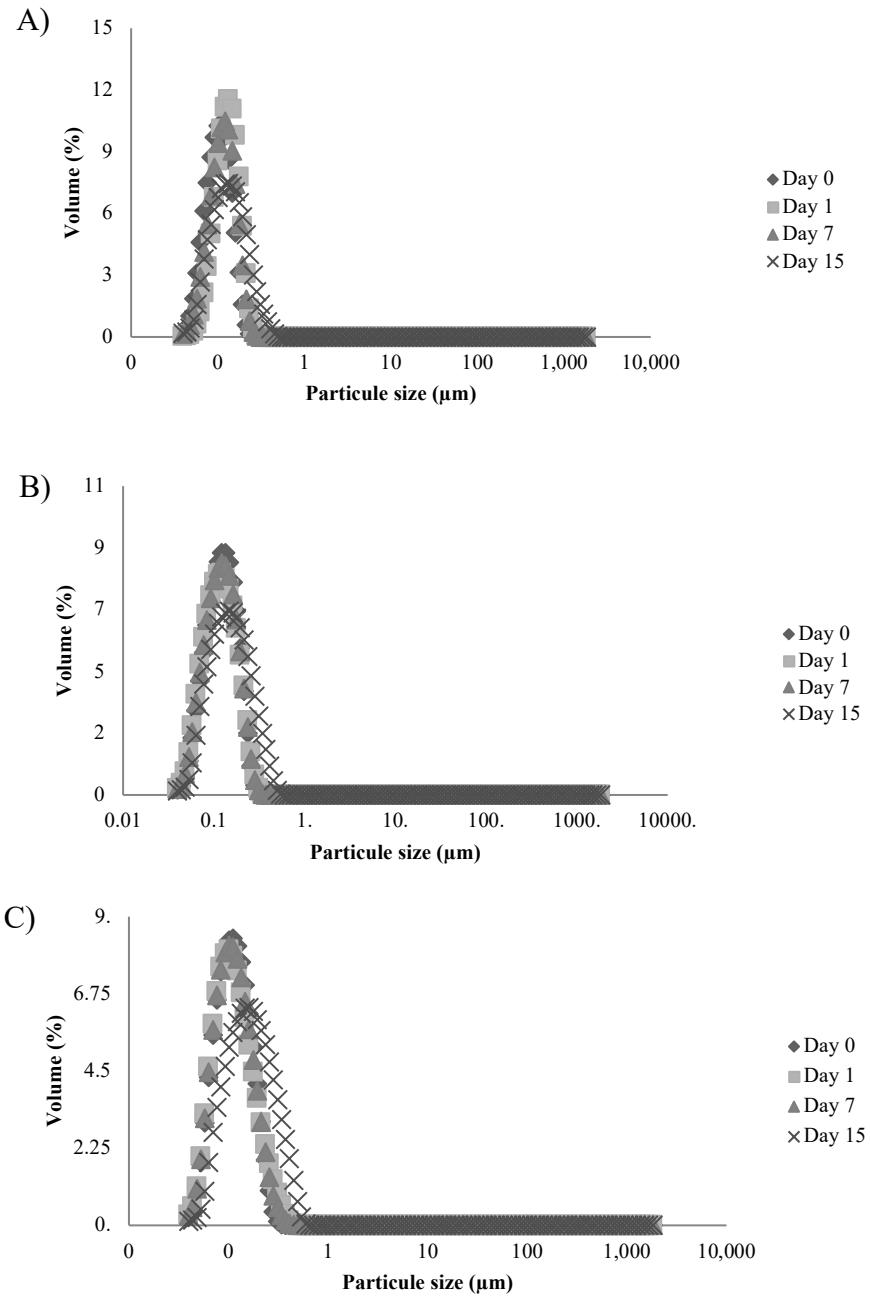


Figure 10. Measurement of droplet size of emulsion after incubation of 0, 1,7 and 15 days containing different amounts of VEE (A) 0%, (B) 0.3% and (C) 0.6%. Results were expressed in mean of volume % ± SD.

3.3. Antidepressant-like effect of VEE-emulsion

The results showed a decreased immobility time in case of VEE-emulsion treated mice and scores were significantly different from negative control group. The statistical analysis showed no significant difference between bupropion and VEE emulsion, proving its the antidepressant-like effect. Interestingly, the immobility time of control emulsion group decreased from day 5, which might be related to involvement of lecithin and/or oleic acid in depression process (Figure 11). Molecular pathways regulated by these treatments are still to be studied.

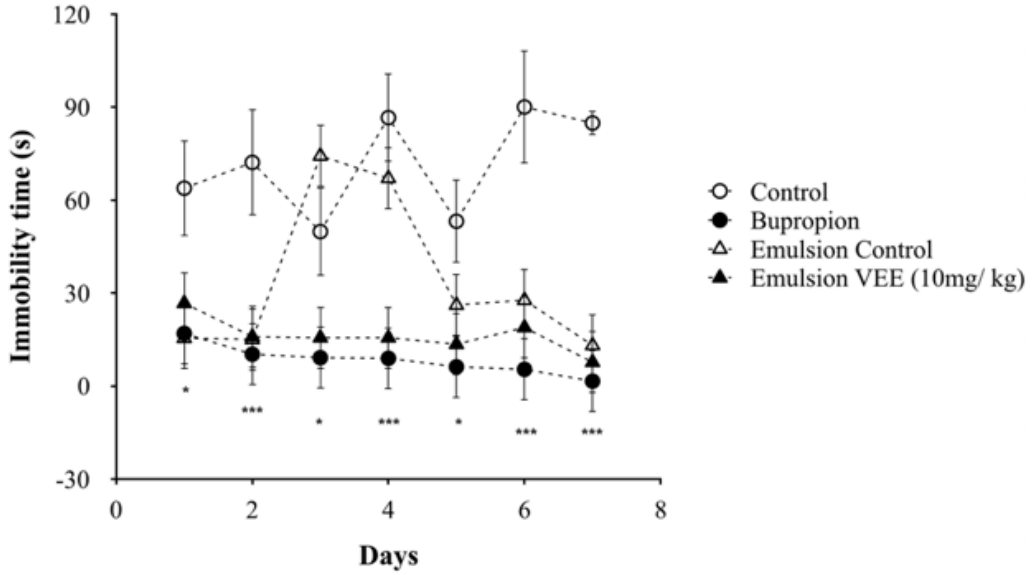


Figure 11. Effect of the oral administration of VEE-emulsion (VEE 10 mg/kg), control emulsion and bupropion (20 mg/kg) on mice immobility times in tail suspension test compared to the control (water 10 mL/kg). Results were expressed in mean of immobility time ± SD.* $P<0.05$; **$P< 0.001$ compared with Control group.

3.4. VEE-emulsion up-regulated dopamine and NA

Brains analysis showed an enhancement of NA and dopamine expression in case of VEE-treatment and bupropion by 34 and 26%, respectively. On the other hand control-emulsion showed no effect on this protein expression.

Also, VEE-emulsion and bupropion increased NA protein expression, by 10 and 19% respectively. Control-emulsion effect on NA production was statistically insignificant.

These results proved the antidepressant-like effect of VEE-emulsion. The decrease of immobility time by control-emulsion was due to factors other than anti-depression, since dopamine and NA were not affected.

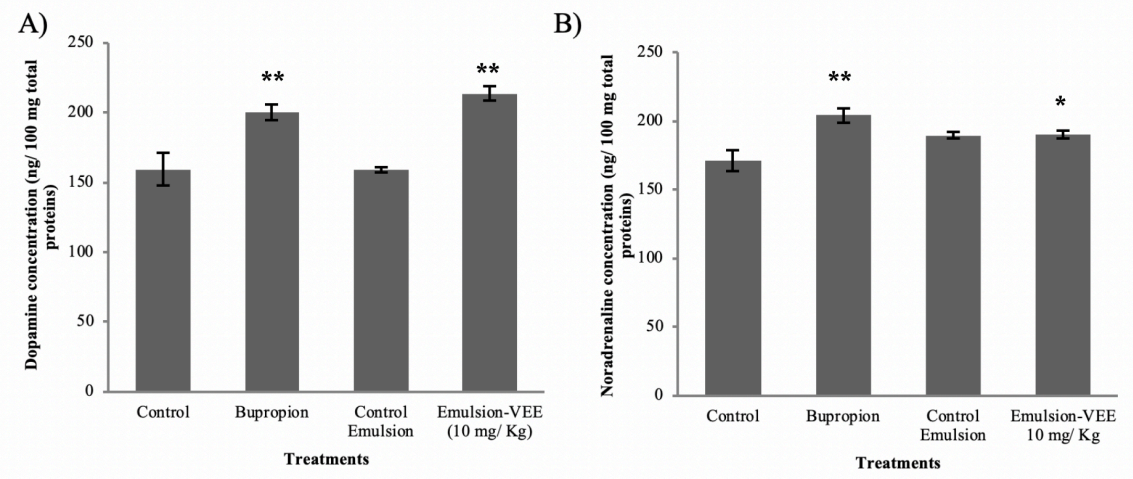


Figure 12. Effect of the oral administration of VEE-emulsion (10 mg/kg), control-emulsion and bupropion (20 mg/kg) on expression levels of (A) dopamine and (B) noradrenaline (NA). Results were expressed in mean of protein level ± SD.* $P<0.05$; **$P< 0.001$ and **$P< 0.0001$ compared with Control group.

3.5. VEE-emulsion regulated genes involved in VEE effect

The microarray analysis of brains collected from mice treated with 100 mg/kg of VEE showed up-regulation of *Gsn*, *Ttr* and *Camk2n1* implicated in mitochondrial activity, with fold-changes higher than 2. The expression of these genes were evaluated in VEE-emulsion treated mice to investigate the existence of similarities between treatments. Their up-regulations were confirmed and represented in relative gene expression, with the negative control expression as reference. Expressions of *Gsn*, *Ttr* and *Camk2n1* were increased in case of VEE-emulsion treated mice by 129.53% (relative gene expression), 129.75% and 150.24%, respectively (Figure 13A, B and C). These effects are similar to the results obtained previously, while control-emulsion and bupropion showed no significant change compared to control group.

Itpr2 is responsible of intracellular calcium release. This gene was up-regulated by VEE 100 mg/kg treatment (Figure 13D). VEE-emulsion treatment did not show any effect on this gene's expression.

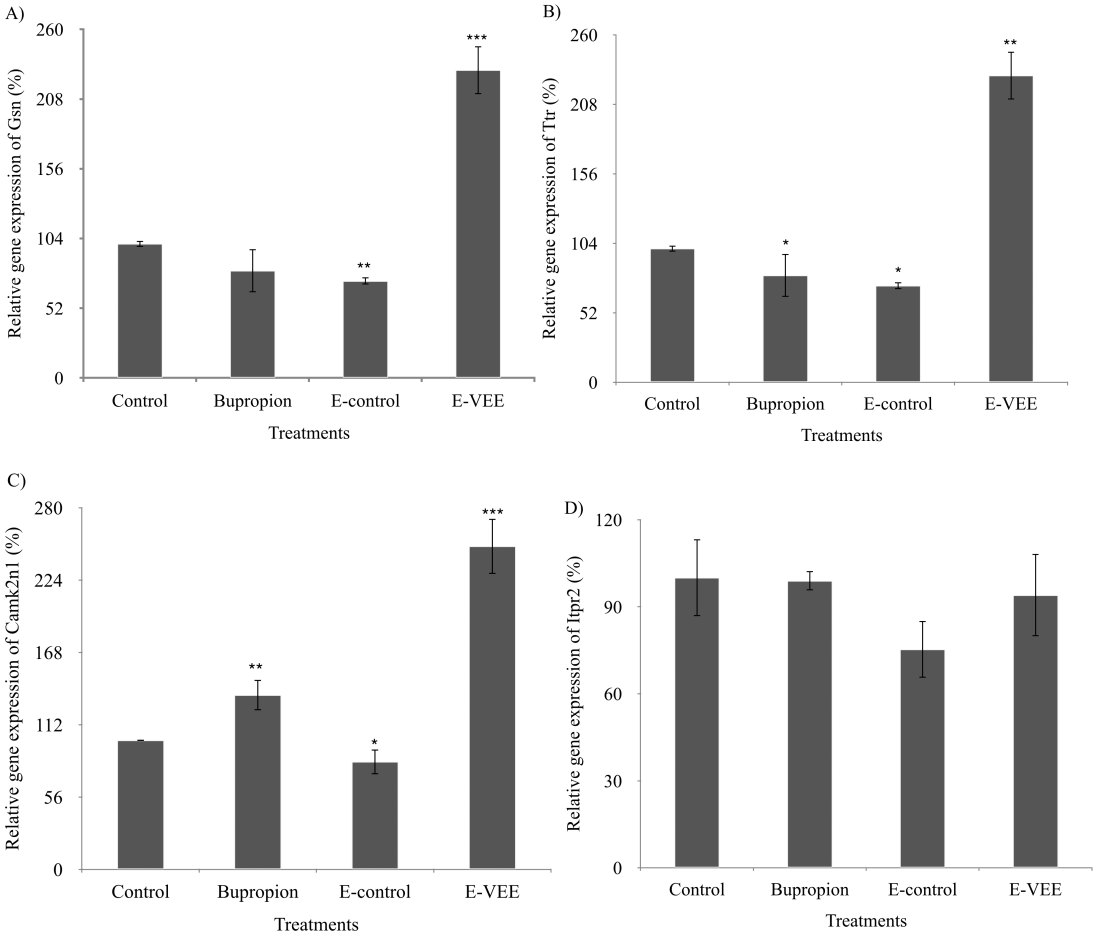


Figure 13. Evaluation of the expression of genes regulated by VEE-emulsion treatment (10 mg/kg) which are (A) *Ttr*, (B) *Camk2n1*, (C) *Gsn,* and (D) *Itpr2*. Results were expressed in relative gene expression ± SD.* $P<0.05$; ** $P< 0.001$; *** $P< 0.0001$ compared with negative control group.

4. Discussion

In this work, stable VEE-emulsion was formulated and evaluated its antidepressant-like effect *in* vivo. VEE was found to exert low interfacial tension, meaning to possess an emulsifier potential. VEE-based emulsion using soybean oil as lipidic phase was unstable after one of day incubation. Presence of ions in the emulsions was found to induce emulsion disruption [140,141]. One of the methods used to counter this factor is chelation by EDTA [142]. In the present work EDTA was used to chelate any ions, originated from VEE, disrupting emulsion structures. The stability of VEE-emulsion was not enhanced by introduction of EDTA in the preparation, suggesting the non-involvement of ions in the instability of the emulsion. Lecithin, a natural compound extract from soybean, was used to enhance VEE-emulsion stability. Lecithin possesses an emulsification property and increase the droplet dispersion in preparation [143]. Oleic acid was also added to the preparation, known to enhance emulsion dispersion [144]. The results showed stabilization of droplet size of VEE-emulsion even after 15 days of incubation.

The antidepressant-like effect of 10 mg/kg of body weight in emulsion was evaluated using TST. The results were compared to bupropion and control group. Interestingly, the results showed decrease of immobility time compared to water-treated mice, and scores were statistically comparable to bupropion-treated mice. This proved the antidepressant-like effect of VEE-emulsion. To prove the antidepressant-like effect of VEE-emulsion different depression markers were measured. NA and dopamine have been studied and linked to anti-depression and expression of emotion such as pleasure and motivation, and stimulation of concentration [8,101,122]. VEE-emulsion was found to increase the expression of these markers confirming the antidepressant-like effect observed *in vivo*.

Furthermore, the expression of genes that were previously regulated by VEE treatment were assessed. *Gsn* and *Ttr* were highly up-regulated by VEE-emulsion treatment, with more than 2 fold-change, while bupropion treatment decreased their expression in mice brains. A previous study showed that mice over-expressing *Gsn* presented increase respiratory chain activity [64]. *Ttr* was demonstrated to possess neuroprotective activity and to be positively correlated to mitochondrial activity

[65,92–96]. These findings proved an increase of mitochondrial activity, implying an over-production of ATP. VEE-emulsion treated mice showed increase of *Camk2n1* expression compared to control group, which implicates a controlled cell proliferation. Previously, a study demonstrated the tumor suppressive effect of *Camk2n1* [66]. These effects were comparable to low doses treatments of VEE on mice previously conducted.

In order to study the implication of calcium effect on the observed mice behavior, the expression of *Itpr2* was evaluated. *Itpr2* is one of the intracellular Ca^{2+} release channels, located in the membranes of endoplasmic and sarcoplasmic reticula. These are organelles are rich in Ca^{2+} ion [56]. The expression of *Itpr2* was not affected by VEE-emulsion, signifying that calcium was not involved in the antidepressant-like effect of VEE-emulsion. This effect could be explained by the enhancement of cAMP production induced by neurotransmitters stimulation.

Taking all these results together, they suggest that oleic acid and lecithin are emulsifiers allowing the increase of VEE-based emulsion stability. This leads to preserve Vs from degradation and to have an antidepressant-like effect on mice.

Chapter 5:
General discussion

Although some studies demonstrated some beneficial effects of lemon verbena such as anxiolytic, antidepressant, and sleep-promoting, but the impact of treatment doses and molecular mechanisms behind the observed effects remain unclear [54, 76-77].

In the present study, VEE at 100 mg/kg induced relaxation in mice, which has been demonstrated by a previous study using an absolute ethanolic extract [46]. Verbena aqueous extract at 700 and 1000 mg/kg body weight administered to rats was found to induce sleep [35].

Transcriptomic analysis of mice treated with VEE revealed the regulation of gene implicated in cAMP and Ca^{2+} modulation, such as *Ac*, *Itpr2*, and *Cacna1c* which are important signaling pathways in relaxation. Interestingly, this treatment regulated also gene markers of mitochondrial activation and depression. These findings prove that increase of immobility time observed *in vivo* is a result of relaxation accompanied with activation of energy production, which are key activities in prevention of depression. Vs induced regulation of Ca^{2+} signaling to a lesser extent compared to dopamine signaling pathway responsible of cAMP production. This messenger was found to be highly

correlation to antidepressant-like effect of drugs which confirm the results obtained. Also, Vs induced Sert, NA, and dopamine up regulation at the protein level triggering signaling pathways producing cAMP. BDNF was also up-regulated, a protein requiring Ca^{2+} and cAMP for its production, confirming the increase of the intracellular levels of two messengers. To confirm the intracellular levels of cAMP, mitochondrial activity was assessed *in vitro*. Low concentrations of VEE and Vs increased mitochondrial activation more significantly compared to higher concentration, proving that small concentrations are more effective on cAMP. Similarly, low concentrations of VEE and Vs induced intracellular Ca^{2+} accumulation. Molecular comparison showed that genes responsible of cAMP production are less expressed in mice brains treated with VEE, leading to suggest the existence of feedback controls. The identification of these control genes is to be considered to have a better understanding of difference of action between VEE and Vs.

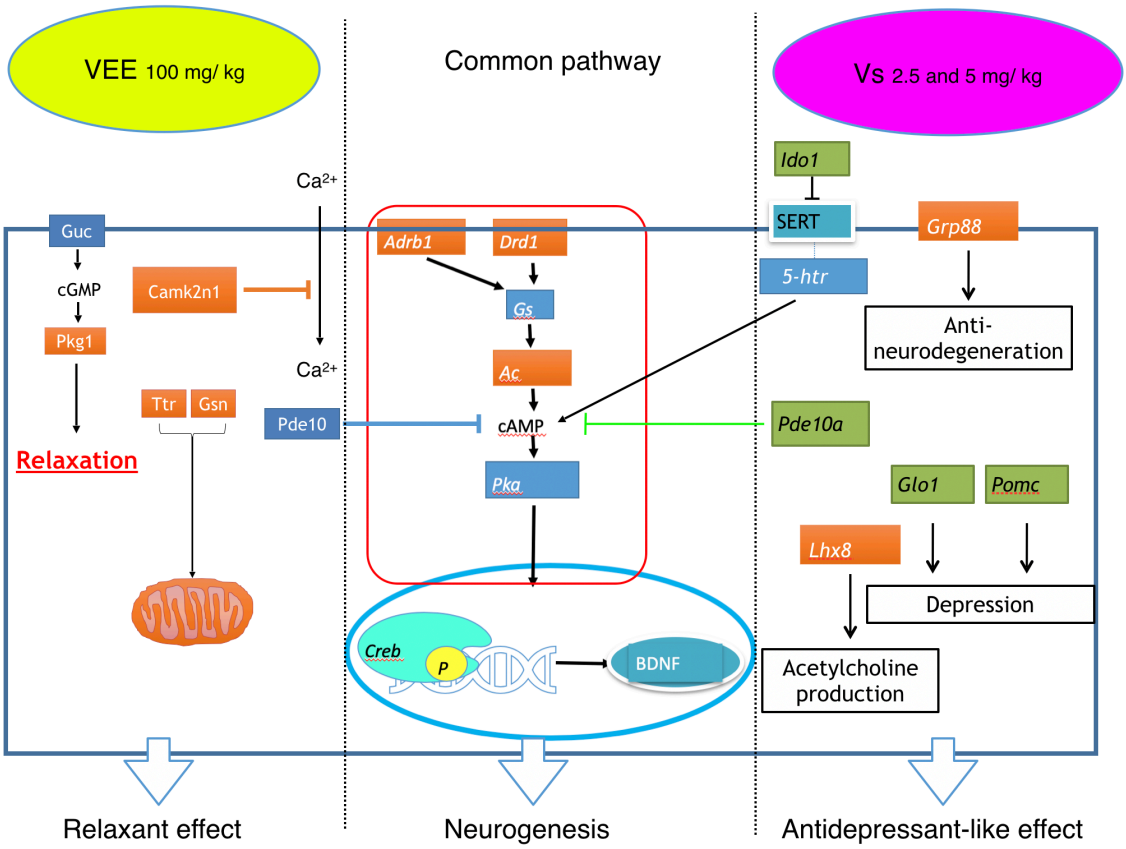
In order to potentially increase Vs bioavailability, the possibility to produce stable emulsion at different storage conditions was studied. Although VEE possessed a low interfacial tension, emulsion produced was highly unstable. Lecithin and oleic acid were used to give the emulsion more stability and dispersion, and produced stable emulsion.

This method might be useful for generation of Vs-rich products that can be stored for longtime and to increase bioavailability of active compounds [145,146].

The antidepressant-like effect of VEE-emulsion (10 mg/kg) was evaluated on mice. The treatment reduced the immobility time of mice compared to control group and results were statistically comparable to bupropion-treated group. Similar patterns were observed in low doses treated mice [97]. VEE-emulsion enhanced expressions of NA and dopamine confirming the results observed in TST. Also expressions of *Gsn* and *Ttr,* markers of mitochondrial activity, were measured and found to be up-regulated. *Itpr2* was up-regulated by VEE-emulsion suggesting the increase of intracellular Ca^{2+}. These results prove the regulation of similar pathways to VEE and Vs.

Conclusion

In the present work, VEE was found to induce a relaxant effect, while Vs contents proved to have an antidepressant-like activity. Both effects are dependent on the increase of intracellular Ca^{2+} levels and cAMP production. At *in vitro* level, lower concentration of VEE and Vs induced intracellular Ca^{2+} levels increase and mitochondrial activation, confirming the results obtained *in vivo*. In order to use Vs as a potential antidepressant-like effect, a chemical study of molecule under different conditions was conducted. Stable emulsion containing VEE can be obtained by using oleic acid- lecithin oil phase. When administered to mice, VEE-emulsion exerted antidepressant-like effect. Further study is required to compare the effect of VEE in solution and emulsion at the molecular level. Moreover, a better understanding of molecular mechanisms regulated by VEE and Vs at different doses is required to identify the key genes behind the observed effects.



Schematic conclusion. VEE 100 mg/kg and Vs 2.5 and 5 mg/kg regulated a common pathway leading to enhancement of BDNF production, a neurotrophic factor responsible of neurogenesis induction. On the other hand, VEE 100 mg/kg regulated specific pathways of relaxation, while Vs activated pathways playing key roles in antidepressant-like response.

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Dedications

First of all, I would like to dedicate this work to my precious and beloved mother and father. I would like to thank them for their support and sacrifice. They believed in me when I could not believe in myself and made be the person I am today. They are my trusted friends and advisors. I hope they are proud of me.

I dedicate this work also to my brothers, Saâd and Mehdi, that I cherish the most. They have always encouraged and helped me to overcome my fears and failures. They saw greatness in me and carried me to grasp it. I wish I became the brother they could rely on.

I dedicate this work also to my dearest friend and sister Wassila for cheering me up and being always present for me since childhood.

I would like to thank all my friends who have been a second family for me, with whom I enjoyed many priceless moments.

Finally, I dedicate this work to every person had an impact on my life.

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